IMPACT OF FREE CHLORINE ON THE FATE OF BIOCHEMICALLY ACTIVE COMPOUNDS IN DRINKING WATER

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A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Department of Environmental Sciences and Engineering.

Chapel Hill
2011

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ABSTRACT

Jacqueline Pearl Kaplan: Impact of Free Chlorine on the Fate of Biochemically Active
Compounds in Drinking Water
(Under the direction of Dr. Howard S. Weinberg)

As a result of the rapid development of the pharmaceutical and agricultural industries,

the biochemically active compounds they produce to treat human ailments, maintain

livestock health, and improve crop output are finding their way into the aquatic environment.

Surface waters supplying drinking water treatment plants may be particularly vulnerable if

the chemicals persist through upstream wastewater treatment or appear in run-off from land

application. During drinking water treatment physicochemical processes may remove some

of these chemicals or transform them into products with unknown structures and biological

activity.

In order to better understand the impacts of biochemically active compounds on water

supply and their fate during drinking water treatment source, settled, and finished waters

from plants in North Carolina were analyzed using liquid chromatography tandem mass

spectrometry. Both atrazine and the insecticide DEET were found to consistently survive

conventional treatment. Additionally, bench-scale chlorination experiments designed to

simulate disinfection were conducted to determine the extent of chlorine incorporation into

the parent chemical thereby "hiding" products from detection which may still have

biochemical activity.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
Amu	Atomic Mass Unit
APCI	Atmospheric Pressure Chemical Ionization
AR	Analytical Reagent
BACs	Biochemically Active Compounds
BSTFA	N,O-bis(trimethylsilyl) trifluoroacetamide
CCL3	Candidate Contaminant List 3
CID	Collision Induced Dissociation
DBPs	Disinfection By-Products
DEET	N,N-diethyl-meta-toluamide
DMDCS	Dimethyldichlorosilane
DOM	Dissolved Organic Matter
DPD	N,N-Diethyl-p-phenylenediamine
DWS	Drinking Water Strategy
DWTPs	Drinking Water Treatment Plants
EDCs	Endocrine Disrupting Compounds
EE2	*17-a*-ethinyl estradiol
EPA	Environmental Protection Agency
ESI	Electrospray Ionization
FAC	Free Available Chlorine
FDA	U.S. Food and Drug Administration
GAC	Granular Activated Carbon

GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
HAAs	Haloacetic Acids
HCB	Hexachlorobenzene
HLB	Hydrophilic-Lipophilic Balance
HPLC	High Performance Liquid Chromatography
IS	Internal Standard
IDL	Instrument Detection Limit
K_{ow}	Octanol Water Partition Coefficient
LC	Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LGW	Laboratory Grade Water
LOQ	Limits of Quantification
MCL	Maximum Contaminant Level
MCLG	Maximum Contaminant Level Goal
MS	Mass Spectrometry
MtBE	Methyl-tert-butyl-ether
Na_2EDTA	Disodium ethylenediamine tetraacetic acid
ND	Not Detected
NMR	Nuclear Magnetic Resonance
NOM	Natural Organic Matter
NCSWAP	North Carolina Source Water Assessment Program

NDPES	National Pollutant Discharge Elimination System
NPDWRs	National Primary Drinking Water Regulations
NSDWRs	National Secondary Drinking Water Regulations
PCPs	Personal Care Products
PCS	Potential Contaminant Sites
pK_a	Acidity Constant
PPG	Poly-(propylene glycol)
RL	Reporting Level
SDWA	Safe Drinking Water Act
S/N	Signal to Noise ratio
SPE	Solid Phase Extraction
SS	Surrogate Standard
TC	Tetracycline
TCEP	Tris (2-chloroethyl)phosphate
TMCS	Trimethylchlorosilane
TOX	Total Organic Halogen
THMs	Trihalomethanes
UC	Unquantified Concentration Estimated to Exceed the Reporting Level
UHP	Ultra High Purity
USGS	United States Geological Survey
UV	Ultra Violet
WWTPs	Wastewater Treatment Plants

1. INTRODUCTION

Areas of rapid commercial and scientific development are the pharmaceutical, personal care product and agricultural chemical industries in which compounds are synthesized to treat human ailments, improve livestock health and quality of human life, and control agricultural crop output. These compounds are synthesized to have a particular biochemical functionality that is specific to the application for which the product was designed. If the biochemically active compound (BAC) migrates away from its target it may have unintended consequences for human and environmental health. Major sources of introduction of BACs into the environment are from the release of treated wastewater effluent into receiving streams and surface water runoff with the consequence that some are found downstream in reservoirs that become drinking water sources. This means that the parent compounds as well as their metabolites are constantly released at low concentrations into the environment and drinking water sources with unknown long-term consequences. Although studies have shown the attenuation of parent BACs during drinking water treatment (Stackelberg et al., 2007), other studies have shown that the parent BAC may have undergone transformation (Glassmeyer and Shoemaker, 2005). It has been observed that the active ingredients in BACs are not limited to illicit a response in the target organism, and there are indications that aquatic organisms which are exposed to BAC residues in water may be negatively affected (Jobling et al., 1998). Determining both the fate of parent BACs and the identity of the unknown transformation products formed during drinking water treatment

remains a challenging area of environmental research, but would provide essential information to help direct toxicology and sampling research, evaluate effective treatment technologies, and develop more environmentally responsible drug and agricultural products.

1.1 Literature Review

1.1.1 Uses of Biochemically Active Compounds

Biochemically active compounds (BACs) are those manufactured to effect a biological response in a target cell, and include a broad range of compounds including human and animal use pharmaceuticals, personal care products (PCPs), endocrine disrupting compounds (EDCs), agricultural products, artificial sweeteners and stimulants. Pharmaceutical compounds are those that are available either with or without a prescription including antibiotics, lipid regulators, beta blockers, anti-epileptics, anti-cholesterols, analgesics and anti-anxiety compounds to name a few. PCPs include compounds that are in soaps and lotions such as anti-microbial agents as well as insect repellants and flame retardants. EDCs induce a change in the endocrine system and include steroid hormones and synthetic estrogens, among others. Agricultural products are those that are used for land application and include herbicides and insecticides. Other compounds that are biochemically active are stimulants such as caffeine, artificial sweeteners such as sucralose, and illegal drugs.

In 2009, human-use pharmaceutical sales accounted for a $300.3 billion dollar industry in the United States (Gatyas and Savage, 2008), and this figure does not take into

account the illegal purchase of prescription or recreational drug use. The top six most commonly prescribed pharmaceuticals in the U.S. were lipid regulators, analgesics, thyroid regulators, blood pressure regulators and antibiotics (rxlist.com). Antibiotics and steroid hormones are the most commonly used veterinary pharmaceuticals and account for a significant portion of the pharmaceuticals used.

In the U.S. PCPs and agricultural chemicals including flame retardants, antimicrobials, detergents, pesticides and herbicides are highly used. For example, the average annual estimate of the use of the insect repellant N,N-diethyl-meta-toluamide (DEET) in 1990 was approximately 4 million pounds of the active ingredient (U.S. EPA, 1998). The herbicide, atrazine, is one of the two most highly used agricultural pesticides, and is used for the majority of the corn, sugarcane, and sorghum production (U.S.EPA, 2006). The estimated annual use of atrazine is 64 to 76 million pounds and prohibiting its use would result in a significant loss in crop yield and revenue for the industry (U.S.EPA, 2006). Although these figures are not up to date it may be assumed that the current use of human and veterinary pharmaceuticals, PCPs, and agricultural chemicals is at least as high as the reported values.

1.1.2 Routes of BAC Exposure into the Environment

Due to their variety of uses, there are many pathways of BACs into the environment (Figure 1.1). Veterinary pharmaceuticals such as antibiotics and steroid hormones are incompletely metabolized and the pharmaceutical residues in animal waste can contaminate surface waters (Table 1.1). Incomplete metabolism of human-use pharmaceuticals in the

body also results in their excretion and subsequent introduction into wastewater treatment plants (WWTPs). Limited options for unused prescription drug disposal results in BACs being flushed down the toilet or thrown out in the trash allowing them to enter WWTPs or landfills, respectively. PCPs such as sunscreens, bug sprays, lotions, etc. may wash off the user when either washing hands, showering, or swimming in lakes/rivers.
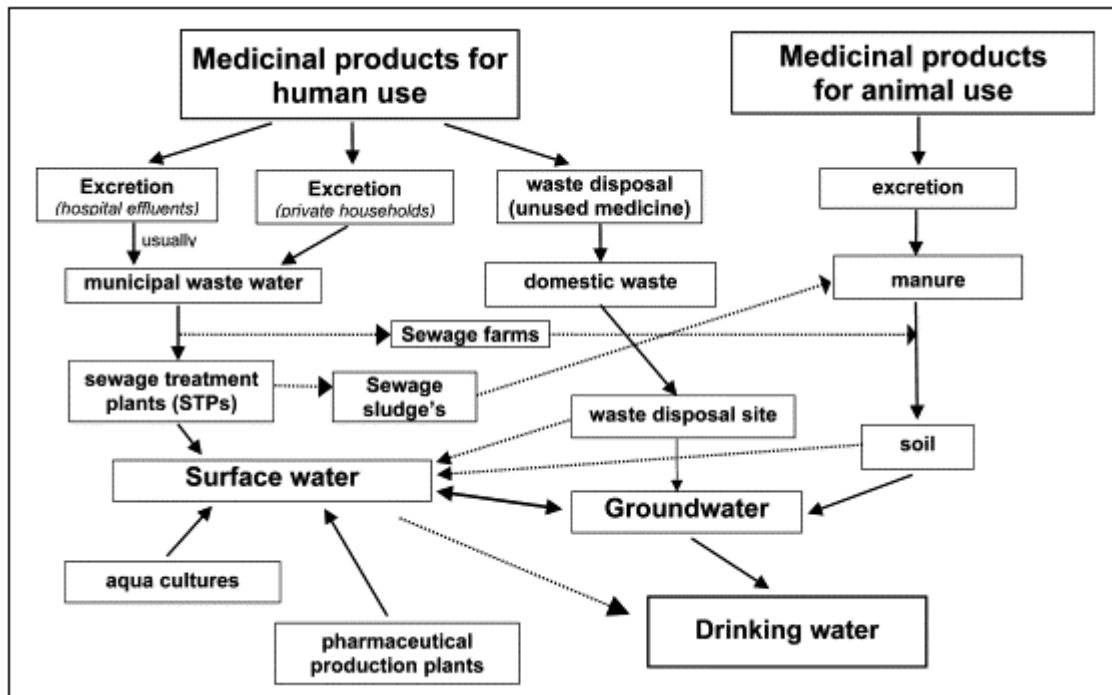
Figure 1.1 Pathways of BACs into Drinking Water (Heberer, 2002)

Table 1.1 Percent Excretion of BACs from the Body Unchanged

Compound	Excretion as unchanged (%)	Reference
Atenolol	50	Kasprzyk-Hordern et al., 2009
Bezafibrate	50	Kasprzyk-Hordern et al., 2009
Carbamazepine	3	Kasprzyk-Hordern et al., 2009
Diclofenac	5-10	Kasprzyk-Hordern et al., 2009
Erythromycin-H_2O	>60	Hirsch et al., 1999
Ibuprofen	1	Kasprzyk-Hordern et al., 2009
Sulfamethoxazole	30	Kasprzyk-Hordern et al., 2009
Tetracycline	80-90	Hirsch et al., 1999
Trimethoprim	80	Kasprzyk-Hordern et al., 2009

Wastewater treatment does not achieve effective removal for many BACs (Table 1.2), and this may be due to the high polarities of the compounds and their preference for the aqueous phase as well as their biological properties. Treated wastewater effluent is released into receiving streams that may become downstream sources of drinking water. In fact, compounds resistant to removal during wastewater treatment are used as indicator compounds to determine if surface or drinking waters have been impacted by wastewater treatment plant effluent (Ferreira et al., 2005). These multiple routes of exposure into the environment including incomplete removal during wastewater treatment results in BACs being constantly introduced into surface waters and increases the potential for contaminating drinking water sources.

Table 1.2 Removal of BACs During Wastewater Treatment (Heberer, et al., 2002)

Compound	C_{ave} influent(μg/L)	C_{ave} effluent(μg/L)	Removal (%)
Carbamazepine	1.78	1.63	8
Clofibric acid	0.46	0.48	0
Diclofenac	3.02	2.51	17
Caffeine	230	0.18	<99.9

There are also many ways in which BACs may contaminate water resources as the result of land use. The application of herbicides and insecticides to agricultural land is usually completed by spraying fields. Due to this practice, herbicide aerosols may be directly introduced into the atmosphere or may sorb onto particulate matter in the air where it is then transported long distances via wind. A study of air samples collected in 1995 showed that in agricultural areas during the application of the widely used herbicide atrazine, it was detected in 67% (n = 21) of particulate samples (C_{max}= 0.42ng/m^3) and in 42% (n = 21) of air samples (C_{max}=2.6ng/m^3) (Coupe et al., 2000). The same study also showed that atrazine was detected in urban air in 29% (n = 24) (C_{max}= 0.019ng/m^3) on particulate and was not detected in the gas phase (Coupe et al., 2000).

Atrazine that reaches land can be transported during rain events to surface water due to storm water runoff from agricultural fields (Thurman et al., 1992). Additionally, atrazine may be transported to ground water by migrating through soil and has been detected in wells at concentrations up to 2.09μg/L (Burkart and Kolpin, 1993). Atrazine that has been deposited onto fields may volatilize into the atmosphere, although the rate was found to be relatively low in comparison to other herbicides most likely due to its low vapor pressure (Glotfelty et al., 1989).

Whether atrazine is directly released into the atmosphere as an aerosol, bound to particulate matter in the air, or evaporates from soil, rain events may scrub the atmosphere, with particulate matter being more effectively removed (Goolsby et al., 1997). A two year study showed that atrazine was always present in rainwater samples with the highest detected concentration of 2.19 µg/L during the early spring (May), which correlated to the spraying of the fields with the herbicide around that time (Wu, 1981). Rainwater can incorporate aerosols and particulate matter and one study by the U.S. Geological Survey (USGS) revealed that atrazine was detected in 30.2% of all rainwater samples tested from the midwestern and northeastern U.S. (n=2,085) and was present in rainfall at concentrations up to 10.9 µg/L (Goolsby et al., 1997). As the result of atrazine being transported via wind and scrubbed from the atmosphere by rain it is potentially deposited into surface water.

The aforementioned discussion is just a brief overview of the complex nature of the fate of atrazine after its introduction to the environment. As with many anthropogenic compounds, its fate is extremely complex and not well understood. Therefore, extreme caution should be undertaken when highly persistent and ubiquitous chemicals are being constantly introduced into the environment. A summary of some of the routes of exposure of BACs into the environment are shown in Figure 1.1.

1.1.3 Fate of BACs in the Environment

Physicochemical Properties

Pharmaceuticals and their metabolites that persist through both the human body and wastewater treatment, enter receiving streams where they are subjected to dilution, photolysis, biodegradation, hydrolysis, and sorption onto soils and sediments (Lam et al.,

2004; Lam and Mabury, 2005; Löffler et al., 2005). PCPs are either introduced directly into surface waters as the result of recreational use or may be subjected to wastewater treatment and eventual release into the environment. Agricultural chemicals and animal use pharmaceuticals are subjected to dilution, photolysis, and hydrolysis in surface water, mobility through soils, and transport via evaporation or sorption onto particulate matter in the atmosphere. Important physicochemical properties that govern a compound's behavior and ultimate fate in the environment are the octanol-water distribution coefficient (K_{ow}) and the acidity constant(s) (pK_a).

The K_{ow} describes a compounds affinity for either the organic or aqueous phase of a solution and is helpful in understanding if a compound will be more likely to remain in water or migrate into soils/sediments. If a chemical has a high log K_{ow} value which indicates hydrophobicity such as for the steroid hormones compounds (estrone and *17-α*-ethinyl estradiol (EE2)), this means that the chemical is more likely to sorb onto soils and not be detected in the aqueous phase. As a result BACs that migrate into soils may be subjected to biodegradation or uptake into plants. BACs with low log K_{ow} values are expected to be more polar and hydrophilic and are more likely to remain in the aqueous phase.

The pK_a, or acidity constant, indicates the speciation (protonated or deprotonated) of a compound based on the pH of the solution. When the pK_a of a compound is higher than the pH of water the BAC will exist in its protonated state. As an example, acetaminophen with a pK_a of 9.7 will exist as a neutral species in natural waters where the pH is typically in the range 6-9. In summary, compounds with low values of log K_{ow} and pK_a are more likely to persist in the aquatic environment as opposed to the sediment system (Löffler et al., 2005). Table 1.3 shows log K_{ow} and pK_a values for a range of BACs and Figure 1.2 the pH

dependent dissociation in water for three BACs. Table 1.3 also shows, in parenthesis, the pH dependent transitions of each BAC.

Photolysis and Sorption onto Soils

Organic compounds are able to undergo direct (i.e. light directly from the sun) or indirect (i.e. from photolysis radicals) photolysis, and the mechanism of action varies between BACs (Lam and Mabury, 2005). A study on the persistence of eight pharmaceuticals in controlled outdoor field microcosms concluded that indirect photolysis reactions due to the presence of dissolved organic matter (DOM) in water are more likely to degrade pharmaceuticals in the natural environment than hydrolysis or biodegradation (Lam et al., 2004). A compound in the tetracycline family of antibiotics was found to degrade rapidly under sunlight irradiation and photolysis was proposed to contribute more significantly to the attenuation than hydrolysis in shallow non-turbid water (Xuan et al., 2010). The chemical structure of BACs including heteroatoms, aromatic rings, phenol, and nitro groups explain their reactivity due to their ability to absorb solar radiation (Boreen et al., 2003).

Interactions of BACs between the water/sediment systems can also impact their fate. Hydrophobic chemicals have a higher affinity towards sediment systems (Löffler et al., 2005). Of the BACs listed in Table 1.3 those with the highest log K_{ow} values are *17-α-ethinyl estradiol, estrone, bezafibrate,* and *gemfibrozil*. The log K_{ow} values of these compounds correlate to their intended biochemical endpoints in which all are lipids and hydrophobic. Compounds with the lowest log K_{ow} values include acetaminophen, atenolol and caffeine,

and these polar and hydrophilic compounds have been found to remain in the aqueous phase and are not detected in sediments (Wu et al., 2009). The persistence of BACs that are applied directly to land are of particular interest, and the adsorption of pesticides including atrazine is largely dependent on soil composition and pH (Gao et al., 1998).

Hydrolysis

The results of a microcosm study in water revealed that attenuation of the studied BACs (acetaminophen, caffeine, carbamazepine, sulfamethoxazole, and trimethoprim) was minimal and therefore hydrolysis was not considered to have a large impact on the degradation of the studied compounds (Lam et al., 2004). Also, a compound in the tetracycline family of antibiotics was found to undergo hydrolysis fastest at neutral pH, but was not the primary method of attenuation as degradation was found to be minimal in the dark (Xuan et al., 2010).

Table 1.3 Physical Properties of BACs

BAC	Log K_{ow}	pK_a
17-α-ethynylestradiol	3.67[b]	~10.5[b] (0/-)
Acetaminophen	0.46[b]	9.7[b] (0/-)
Atenolol	0.16[c]	9.6[e] (0/-)
Atrazine	2.61[b]	<2[b] (+/0)
Bezafibrate	4.25[f]	3.6[e] (0/-)
Caffeine	<0[b]	6.1[b] (+/0)
Carbamazepine	2.45[b]	<2[b] (+/0)
Clofibric Acid	2.57[f]	3.2[g] (0/-)
DEET= N,N-Diethyl-meta-toluamide	2.18[b]	<2[b] (+/0)
Diazepam	2.82[b]	2.4/1.5[b] (2+/+/0)
Diclofenac	0.7[b]	4.15[e] (0/-)
Estrone	3.13[c]	10.3[b] (0/-)
Erythromycin-hydrate	3.06[b]	8.2[h] (0/-)
Fenoprop	3.86[m]	3.2[a] (0/-)
Gemfibrozil	4.77[b]	4.7[b] (0/-)
Ibuprofen	3.97[b]	4.5[b] (0/-)
Meprobamate	0.7[b]	<2[b](+/0)
Sucralose	-0.5[i]	Not Applicable
Sulfamethoxazole	0.89[b]	1.7/5.6[n] (0/-/2-)
TCEP= tris(2-carboxyethyl)phosphine	1.44[d]	Not Found
Tetracycline	-1.19[j]	3.30/7.68/9.69[k] (+/0/-/2-)
Trimethoprim	0.91[b]	1.3[o]/7.5[l] (2+/+/0)

Legend 0 = neutral; - = negatively charged species; + = positively charged species
(a)Garrison et al., 1994; (b)Westerhoff et al., 2005; (c) Hansch et al., 1995; (d) Trenholm et al., 2006; (e) SRC, 2011; (f) Kujawa-Roeleveld et al., 2008; (g) Bhandari et al., 2009; (h) Qiang and Adams, 2004; (i) Neset et al., 2010; (j) Wollenberger et al., 2000; (k) Stephens et al., 1956; (l) Cocco et al., 1983; (m) Isnard and Lambert, 1989; (n) Lucida et al., 2000; (o) Cao and Cross, 2000
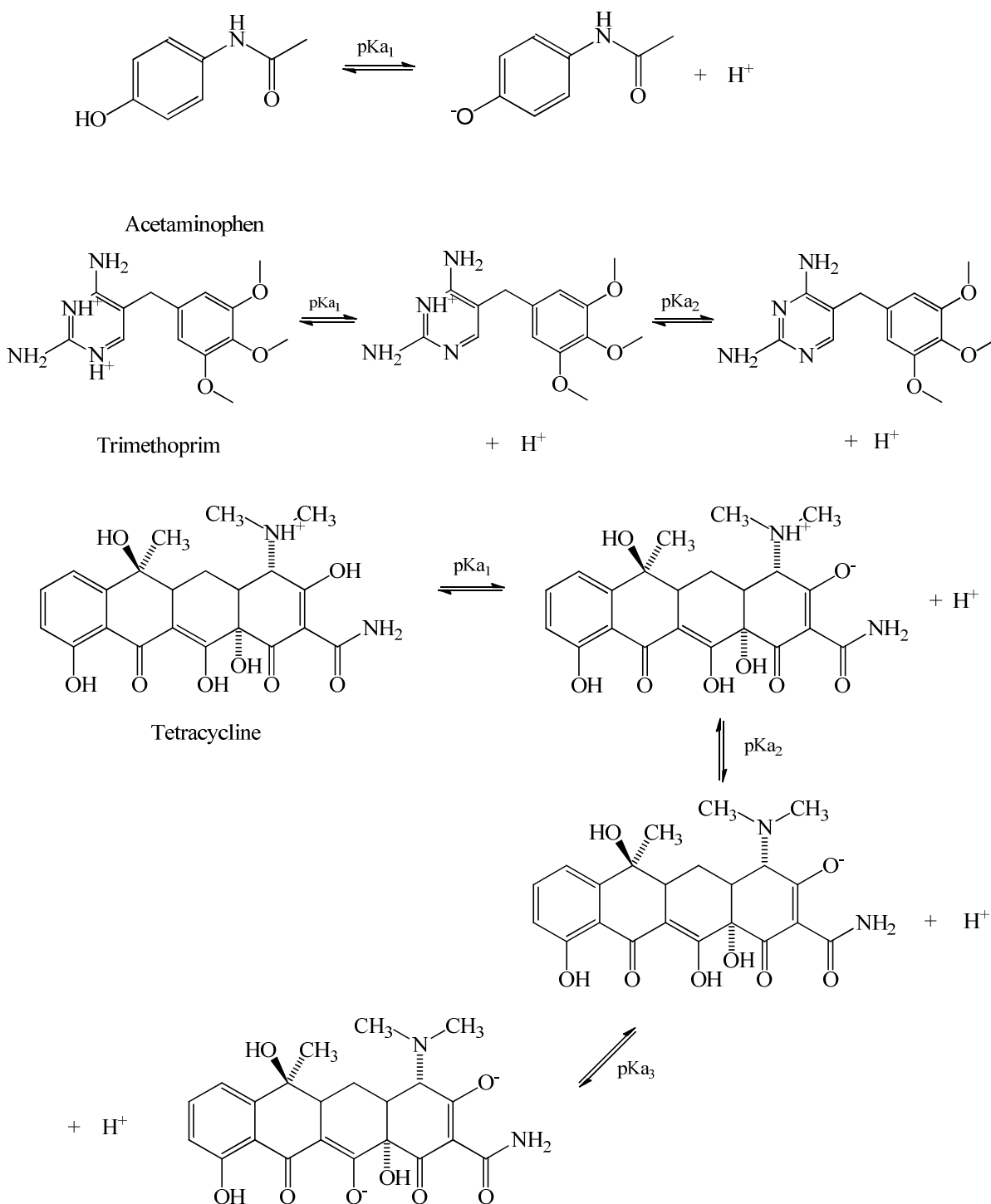
Figure 1.2 pH Dependent Speciation of BACs: (a) Acetaminophen, (b) Trimethoprim, and (c) Tetracycline

1.1.4 Analytical Methods

The development of sensitive methods amenable to the extraction and analysis of BACs is based on pairing knowledge of physicochemical properties of target BACs with available analytical instrumentation. This is not an easy task as BACs have a wide range of polarities and exist as acidic, neutral, or basic species. An effective goal is to have a comprehensive method that can process a variety of BACs with the minimal amount of sample preparation which usually includes sample collection, preservation, filtration, extraction, elution, concentration, and instrumental analysis.

Due to their high sensitivity and selectivity, the analytical instruments of choice are mass spectrometers (MS). While it is possible to scan for a broad range of masses (full scan analysis) this practice drastically reduces sensitivity. Therefore, one limitation of mass spectrometry is that you can only detect compounds with a high level of sensitivity that are directly targeted in the method. This is achieved by selecting specific ions and/or ion transitions associated with a particular molecule to reduce any noise relating to non-target compounds. Mass spectrometry has been paired with liquid chromatography (LC) or gas chromatography (GC) for the separation and analysis of more polar and more volatile compounds, respectively.

Preservation of drinking water samples for analysis is achieved by quenching any residual disinfectant to stop reactions that may be transforming BACs. Methods have been developed for the analysis of surface and drinking water that utilize pH adjustment with sample clean-up and concentration using solid phase extraction (SPE) to extract a broad range of BACs by adsorption onto a hydrophilic-lipophilic balance (HLB) cartridge (Vanderford et al.,2003; Trenholm et al., 2006). BAC analysis can be completed after

sample extraction and concentration using both gas chromatography-mass spectrometry (GC-MS) and liquid chromatography (tandem) mass spectrometry LC-MS/(MS) methods with reporting limits between 1-10ng/L (Trenholm et al., 2006). Sample preparation is minimized through the use of different analytical methods that use LC-MS/MS in both positive and negative electrospray ionization (+/-ESI) and positive atmospheric pressure chemical ionization (+APCI) with reporting limits of 1.0ng/L (Vanderford et al., 2003). One major analytical challenge is maintaining up to date methods with the production of new pharmaceuticals. Occurrence data for environmental samples depends on targeting known chemicals since LC-MS methods are, for the most part, unable to screen for unknowns at the low levels expected. Identifying effective methods to screen drugs for environmental toxicity screening or persistence may help guide environmental sampling efforts.

1.1.5 Presence of BACs in Natural Waters

Occurrence studies have revealed that BACs are ubiquitous environmental contaminants being detected in both U.S. streams (Kolpin et al., 2002) and drinking water sources (Focazio et al., 2008). This is a cause for public health concern because the effects of long-term exposure to low level contaminants are unknown. Additionally, the impact of chronic low level exposure to BACs on aquatic life is not well understood, although some negative effects have been observed (Chambers and Leiker, 2006). Many occurrence studies have been completed to determine the concentration of BACs in surface waters. The U.S. Geological Survey (USGS) has completed nationwide occurrence studies on organic wastewater contaminants in U.S. streams (Kolpin et al., 2002) and data from this study is

summarized in Table 1.4. It is important to note that in this study the selection of the 139 sampling sites were biased towards those that were more likely to be impacted by both urbanization and livestock production.

Table 1.4 Occurrence Levels of BACs in U.S. Streams 1999-2000 (Kolpin et al., 2002)

Compound	$C_{max}(\mu g/L)$	Frequency (%)
17-α-ethinyl estradiol (n=70)	0.831	15.7
17β-estradiol (n=70)	0.093	10
Acetaminophen (n=84)	10	23.8
Caffeine (n=84)	6	61.9
DEET (n=54)	1.1	74.1
Erythromycin-H_2O (n=104)	1.7	21.5
Estriol (n=70)	0.051	21.4
Estrone (n=70)	0.112	7.1
Gemfibrozil (n=84)	0.79	3.6
Ibuprofen (n=84)	1	9.5
Mestranol (n=70)	0.407	10
Sulfamethoxazole (n=104)	1.9	12.5
Tetracycline (n=84)	0.11	1.2
TCEP (n=85)	0.54	57.6
Triclosan (n=85)	2.3	57.6
Trimethoprim (n=104)	0.71	12.5

The most frequently detected BACs in Table 1.5 were the PCPs caffeine, DEET, triclosan, and tris(2-carboxyethyl)phosphine (TCEP), and this is most likely a direct result of their high use (do not need a prescription to buy) and preference for the aqueous phase (see log K_{ow} values in Table 1.4). The non-prescription pharmaceuticals (acetaminophen and ibuprofen) were detected less frequently than the PCPs but acetaminophen was detected at

high concentrations. Ibuprofen, due to its high log K_{ow} value, likely migrates out of the aqueous phase and eludes detection. Conversely, acetaminophen has a very strong affinity for the aqueous phase and is present at very high concentrations. More hydrophobic BACs (compounds with higher log K_{ow} values) including gemfibrozil and the steroid hormones were detected less frequently and this may be due to their ability to sorb onto soils.

The antibiotics (erythryomycin-H_2O, sulfamethoxazole, tetracycline, and trimethoprim) were detected at very low concentrations which may be the result of their sensitivity to light (Lam et al., 2004; Xuan et al., 2010). It is interesting to note that the antibiotics sulfamethoxazole and trimethoprim, which are usually prescribed together, were both detected with the same frequency. The least frequently detected BAC was tetracycline and this may be due to several factors. It is known that tetracycline enters the environment as it is minimally metabolized in the body (80-90% unchanged) and, therefore, enters wastewater treatment plants as the result of human use or streams due to runoff as the result of animal use. Therefore, the fate of tetracycline may be determined by physicochemical treatment including disinfection at WWTPs, chelation with metal ions in the environment (Halling-Sørensen et al., 2002), or photolysis (Xuan et al., 2010). In the case of photolysis or chemical treatment such as disinfection the transformation of tetracycline into by-products not targeted by analysis would lead to an underestimation of their environmental impact.

1.1.6 Occurrence in Drinking Water Sources

The result of high BAC use, their persistence through WWTPs and in the environment is that they are continuously being introduced into waters that may become sources for drinking water. Many occurrence studies have been completed to determine the concentration of BACs in drinking water sources, and data from three different sampling studies are shown in Table 1.5. One study completed by the U.S.G.S. sampled from only one DWTP source during a three week time period. The source was located in a heavily populated, highly urbanized area with significant WWTP effluent entering the source water (Stackelberg et al., 2007). Since this sampling event took place at only one source it could be expected that the same BACs would be detected due to usage patterns, and this information is reflected in Table 1.5 with the bias shown in relative high frequency of those compounds detected (42% or greater). While this information does not provide representative information for a wide range of drinking water sources it does reveal that a particular drinking water source with upstream wastewater influences is constantly receiving detectable levels of particular chemical loads.

Another study completed by the U.S.G.S. sampled from a wide range of drinking water sources (25 ground and 49 surface waters) of varying sizes of utilities and potential contaminant sources across the U.S. It is important to note that sites were chosen that were known to have at least some upstream human and or animal wastewater inputs (Focazio et al., 2008). The relatively low frequencies of detection shown in Table 1.5 from this study may be the result of sampling from a broad range of drinking water sources and/or sampling from many ground water sites.

The third study shown in Table 1.5 was completed by the Southern Nevada Water Authority and represents information gathered from an occurrence study on 19 US utilities. Source waters were selectively chosen based on their uses and upstream influences. In this study one ground water site was sampled, one lake with upstream wastewater influences, four reservoirs with WWTPs upstream, six rivers with no WWTP influences, four reservoirs with no WWTP inputs but with recreational use, and three reservoirs with no WWTP inputs or recreational use (Benotti et al., 2009). Correlations were observed between detecting BACs in drinking water sources and the direct input of wastewater and recreational use (Benotti et al., 2009). This study represented a wide range of drinking water sources and the frequencies of detection in Table 1.5 from this study reflect site selection choice.

Several of the BACs were targeted in all three discussed occurrence studies (carbamazepine, DEET, gemfibrozil, TCEP, triclosan, and trimethoprim) and trends based on the selection of sampling locations can be seen in these compounds. For example, when the compounds were detected by Stackelberg et al. (2007) the frequencies of detection were always the highest (DEET, carbamazepine, and TCEP), and the opposite is true for those other compounds which were not detected (gemfibrozil, triclosan, and trimethoprim). This suggests that a particular contaminant in a source water will most likely be chronically introduced due to usage patterns, but these may be subjected to seasonal variations.

Table 1.5 Occurrence Levels of BACs in Drinking Water Sources

Compound	C_{max}(ng/L)	n	Frequency (%)	Reference
17-α-ethinyl estradiol	1.4	19	5.3	Benotti et al., 2009
Acetaminophen	160	74	8.1	Focazio et al., 2008
Acetaminophen	120	12	75	Stackelberg et al., 2007
Atenolol	36	19	63	Benotti et al., 2009
Atrazine	870	19	79	Benotti et al., 2009
Caffeine	270	74	7.5	Focazio et al., 2008
Caffeine	100	12	42	Stackelberg et al., 2007
Carbamazepine	51	19	79	Benotti et al., 2009
Carbamazepine	190	74	21.6	Focazio et al., 2008
Carbamazepine	600	12	92	Stackelberg et al., 2007
DEET	<500	73	14	Focazio et al., 2008
DEET	200	12	92	Stackelberg et al., 2007
DEET	110	19	32	Benotti et al., 2009
Diazepam	0.47	19	11	Benotti et al., 2009
Diclofenac	1.2	19	21	Benotti et al., 2009
Erythromycin-hydrate	300	73	8.1	Focazio et al., 2008
Erythromycin-hydrate	10	12	58	Stackelberg et al., 2007
Estrone	0.9	19	79	Benotti et al., 2009
Gemfibrozil	ND	74	0	Focazio et al., 2008
Gemfibrozil	ND	12	0	Stackelberg et al., 2007
Gemfibrozil	24	19	58	Benotti et al., 2009
Ibuprofen	270	74	1.4	Focazio et al., 2008
Ibuprofen	ND	12	0	Stackelberg et al., 2007
Meprobamate	73	19	84	Benotti et al., 2009
Sulfamethoxazole	60	12	83	Stackelberg et al., 2007
Sulfamethoxazole	110	19	89	Benotti et al., 2009
TCEP	<500	73	20.3	Focazio et al., 2008
TCEP	120	12	100	Stackelberg et al., 2007
TCEP	530	19	53	Benotti et al., 2009
Tetracycline	ND	73	0	Focazio et al., 2008
Tetracycline	ND	12	0	Stackelberg et al., 2007
Triclosan	ND	12	0	Stackelberg et al., 2007
Triclosan	<100	73	8.1	Focazio et al., 2008
Triclosan	6.4	19	32	Benotti et al., 2009
Trimethoprim	20	71	6.8	Focazio et al., 2008
Trimethoprim	11	19	58	Benotti et al., 2009
Trimethoprim	ND	12	0	Stackelberg et al., 2007

ND=not detected

Of the six compounds targeted in all three studies the results from Benotti et al. (2009) have the second highest frequency of detection (carbamazepine, DEET, TCEP, triclosan, and trimethoprim) and this is most likely a result of site selection and sampling from mainly surface water sources. The lowest frequencies of detection are from the study by Focazio et al. (2008) for these six compounds, and this may be the result of sampling from many ground water sites or selecting source waters with less anthropogenic impacts.

The concentrations of all BACs in drinking water sources (Table 1.5) are lower than those in U.S. streams (Table 1.4). This attenuation from streams to drinking water sources is most likely due to a combination of hydrolysis, photolysis, sorption onto soils, and biodegradation. Another possible explanation is dilution of streams in larger reservoirs or degradation due to the half-lives of the BACs. Also it should be noted that the sampling completed by Kolpin et al. (2002) had a greater bias towards impacted sites which may provide another explanation for this observation.

The concentrations of the PCPs targeted (DEET and TCEP) were relatively high as were the concentrations of the non-prescription BACs (acetaminophen, ibuprofen, and caffeine), and this result is most likely due to high use and availability without a prescription. The compounds with the lowest concentrations or not detected were the steroid hormones, *17-α-ethinyl estradiol* and estrone, and this may be due to their use in lower quantities, affinity towards soils (see their high log K_{ow} values in Table 1.3), and degradation by organisms in the water.

The compound that was detected with the highest concentration and very high frequency was the agricultural compound, atrazine. The concentration of atrazine detected in 79% of the U.S. drinking water sources sampled is approximately one third of the Maximum

Contaminant Level Goal (MCLG)/Maximum Contaminant Level (MCL) for atrazine in drinking water which is 0.003mg/L (U.S. EPA, 2009a). One particularly troubling result from occurrence studies is the ubiquitous nature of certain anthropogenic compounds. Atrazine, a widely used herbicide, is frequently detected in source waters close to and even those that are far removed from agricultural land (Benotti et al., 2009). The widespread occurrence of atrazine in water poses a troubling public health concern and highlights the challenges associated with effectively managing persistent environmental contaminants. These occurrence studies are helpful to gather current information and guide future research studies and policy decisions.

1.1.7 Fate of BACs during Drinking Water Treatment

Understanding the fate of BACs during drinking water treatment is important in order to effectively manage the risks associated with anthropogenic wastewater contaminants in drinking water supplies. When discussing the fate of BACs during drinking water treatment careful attention should be placed on stating whether a particular compound is removed as some have been shown to transform during drinking water treatment (Gould and Richards, 1984; Miyamoto et al., 1997; Dodd and Huang, 2004; Moriyama et al., 2004; Pinkston and Sedlak, 2004; Glassmeyer and Shoemaker, 2005; Ye, 2005; Bedner and MacCrehan, 2006; Nakamura et al., 2006; Dodd and Huang, 2007; Brix et al., 2008; Kotcharaksa, 2008; DellaGreca et al., 2009; Lee and Gunten, 2009; Quintana et al., 2010; Wulfeck-Kleier et al., 2010; Krkošek et al., 2011; Soh et al., 2011; Wang et al., 2011) as is described in greater detail in Section 1.1.8.

The fate of BACs during drinking water treatment could be:

1. Complete removal of the intact chemical by physical treatment processes (e.g., coagulation, powdered or granular activated carbon)

2. Complete removal by chemical processes (e.g., mineralization due to oxidation)

3. Incomplete removal by both physical and chemical treatment processes

4. Incomplete removal and/or transformation due to both physical (e.g., ultraviolet) and chemical disinfectants (e.g., chlorination)

5. Transformation due to chemical treatments.

The fate of BACs during drinking water treatment is not well understood although several studies have been completed including laboratory simulations (Ternes et al., 2002; Westerhoff et al., 2005; Simazaki et al., 2008), pilot scale studies (Ternes et al., 2002; Vieno et al., 2007), and studies at full-scale plants (Ternes et al., 2002; Stackelberg et al., 2007). Conventional drinking water treatment consists of various physical and chemical processes such as screening, coagulation/flocculation, sedimentation, filtration, and disinfection using chlorine or chloramines. Table 1.6 shows the removal of BACs in a conventional drinking water treatment plant.

Table 1.6 Average concentration (C_{ave}) of BACs during Conventional Drinking Water Treatment (n=12) (Stackelberg et al., 2007)

Compound	Source (ng/L)	Clarified FeCl$_3$ (ng/L)	Disinfected NaOCl (ng/L)	Filtered Sand/GAC (ng/L)	Finished ~1.2mg/L Cl$_2$ residual (ng/L)
Acetaminophen	15	6	ND	1	0.3
Caffeine	126	126	116	4	15
Carbamazepine	191	186	149	4	29
DEET	120	130	125	71	78
Erythromycin-H$_2$O	10	5.3	0.4	ND	ND
Gemfibrozil	ND	ND	ND	ND	ND
Ibuprofen	ND	ND	ND	ND	ND
Sulfamethoxazole	30	20	ND	ND	ND
TCEP	95	94	92	ND	4
Tetracycline	ND	ND	ND	ND	ND
Trimethoprim	ND	ND	ND	ND	ND

ND = not detected

It is interesting to note in Table 1.6 that two of the most non-polar compounds (gemfibrozil and ibuprofen) were not detected in source water. This may be due to their sorption onto soils or other particulate matter as a result of their high log K_{ow} values or presence as anionic species (see pK_a and log K_{ow} values in Table 1.3) in which case, they would elude detection in the aqueous phase. Also it is interesting to note that the antibiotics tetracycline and trimethoprim are both not present in source water (the other antibiotics sulfamethoxazole and erythromycin-H$_2$O are present at lower concentrations), and this may be due to either photolysis, microbial degradation, sorption onto soils, transformation during wastewater treatment, or a combination of the above. Table 1.6 shows that for those BACs that were detected in the source water minimal removal is observed for most of the

compounds with the addition of the coagulant, iron chloride, (FeCl$_3$), which may be the result of both high solubility of these compounds in water and low log K_{ow} values. FeCl$_3$ removed erythromycin-H$_2$O and acetaminophen most effectively which may be the result of sorption of BACs onto natural organic matter (NOM) that is removed during coagulation. The removal of erythromycin-H$_2$O can be explained due to its relatively high log K_{ow} value and its low solubility in water. The removal of acetaminophen due to coagulation is not well understood because it is a very hydrophilic compound, but may be due to the base or acid hydrolysis that results from the addition of FeCl$_3$, but this remains to be studied (Stackelberg et al., 2007). Disinfection using sodium hypochlorite (NaOCl) resulted in minimal removal for caffeine, DEET, and TCEP, while sulfamethoxazole, erythromycin-H$_2$O, carbamazepine, and acetaminophen had measureable levels of removal. These results are due to the molecular structure of the particular BACs and will be explained in greater detail in Section 1.1.8. Table 1.6 reveals that the most effective treatment step was the sand/granular activated carbon (GAC) filtration in which most compounds remaining the water were effectively removed (except DEET). The low removal of DEET may be due to the chemical structure or it is outcompeted for adsorption sites on the GAC relative to other compounds. The concentration of the detected compounds in finished water are lower than then source water, but some are higher than the levels observed in the sand/GAC filtered samples. This may be due to sampling at only one of the eight GAC filter banks in operation and variability may exist in the plant between the filter banks (Stackelberg et al., 2007). Another possible reason for this discrepancy is that although 24 hour continuous flow composite samples were obtained in this study the same plug of water was not monitored throughout its duration in

the water treatment plant and any change in influent levels would have contributed to variable levels of removal during treatment.

Coagulation/Flocculation

Coagulation/flocculation involves the destabilization of suspended solid particles (<0.45μm) in source water with the use of either iron hydroxide or aluminum hydroxide. With the addition of these coagulants the suspended solids clump together to form flocs, which are removed from the water during sedimentation and filtration. Settled solids have been analyzed for their concentration of BACs, and it has been found that hydrophobic compounds are removed during coagulation while hydrophilic compounds are not (Stackelberg et al., 2007). Therefore, the octanol-water partition coefficient, K_{ow}, can predict whether a compound will be effectively removed during coagulation/flocculation (Table 1.3), and BACs that associate with NOM will likely be more effectively removed. Additionally, it has been observed in laboratory batch studies that compounds with relatively high polarity are not removed during coagulation using $FeCl_3$ (Ternes et al., 2002). Both simulated and plant sampling studies have shown that due to the chemical nature of many BACs (hydrophilic, polar, high solubility, ionic) coagulation is not a reliable treatment option for their removal (Ternes et al., 2002; Westerhoff et al., 2005; Stackelberg et al., 2007; Simazaki et al., 2008). This can be seen in Table 1.6 with the low removal of most BACs except the most hydrophobic compound, erythromycin-H_2O.

Adsorption onto Activated Carbon

Activated carbon is used during water treatment because many organic compounds can adsorb onto the carbon via van der Waals forces. This makes activated carbon an attractive option for removal of compounds but lab-scale studies have shown that competition for active sites between BACs minimizes the removal of the more hydrophilic compounds (Simazaki et al., 2008). It is hypothesized that DOM in raw water samples outcompetes with BACs during adsorption and minimizes BAC removal (Simazaki et al., 2008). Compounds more amenable to removal using activated carbon are those that are hydrophobic or neutral (Simazaki et al., 2008). Simulated drinking water treatment studies have shown that increasing the powdered activated carbon (PAC) dose (Westerhoff et al., 2005) or contact time (Simazaki et al., 2008) will increase the removal efficiency of BACs. Plant sampling studies have shown that adsorption onto PAC is a very effective strategy employed by conventional drinking water treatment plants to remove hydrophobic compounds (Ternes et al., 2002; Stackelberg et al., 2007), but the materials for this treatment option are very expensive relative to the use of ozone, UV/H_2O_2, or membranes. The results in Table 1.7 show that PAC is most effective for the removal of the relatively hydrophobic steroid hormone compounds (*17-α-ethinyl estradiol* and estrone) and protonated bases (acetaminophen, trimethoprim, and caffeine (Westerhoff et al., 2005). Conversely, Table 1.7 shows that compounds with either low log K_{ow} values or deprotonated acid functional groups are more difficult to remove (e.g., ibuprofen, diclofenac, and gemfibrozil) (Westerhoff et al., 2005).

Table 1.7 Percent Removal of BACs using PAC (Westerhoff et al., 2005)

Compound	Percent Removal (5mg/L PAC, 4-hour contact time)
17-α-ethinyl estradiol	77
Acetaminophen	72
Atrazine	60
Caffeine	70
Carbamazepine	74
DEET	49
Diazepam	67
Diclofenac	39
Erythromycin-hydrate	54
Estrone	76
Gemfibrozil	37
Ibuprofen	16
Meprobamate	33
Sulfamethoxazole	36
Trimethoprim	83
TCEP	52

Disinfection Using Chlorine

Chlorine has been used as a disinfectant for water since the early 20$^{\text{th}}$ century in the United States. Before that time there were no safeguards against the pathogens in water, the cause of many disease outbreaks, illnesses, and death. The use of chlorine as a drinking water disinfectant has increased life expectancy and improved overall public health due to the removal of pathogens and other organic contaminants.

The most commonly used disinfectants in the United States are sodium hypochlorite (NaOCl) and chloramines (NH$_2$Cl). To disinfect water chlorine is either added as chlorine gas (Cl$_2$) or as sodium hypochlorite solution (NaOCl). The reactive species of free available chlorine (FAC) are Cl$_2$, HOCl, and OCl$^-$, with HOCl being the most reactive species. The

equilibrium of HOCl in water is dependent on whether the reaction is carried out above or below the pK$_a$ of HOCl (pK$_a$ = 7.54). For example, when the pH of the water is lower than the pK$_a$ of HOCl, the acid is in its more reactive protonated state and as a result will react more with species in the water. The equilibrium of HOCl in water is:

$$NaOCl \; + \; H_2O \; \rightleftharpoons \; HOCl \; + \; NaOH$$

$$HOCl + H_2O \; \rightleftharpoons \; OCl^- + H_3O^+$$
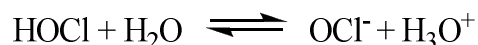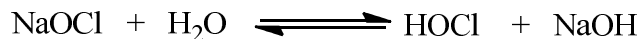
Chlorine is a strong oxidant and it acts as an electrophile, attacking areas on a molecule that are rich in electron density. Many studies have also shown that when chlorine reacts with dissolved organic matter or other organic compounds in drinking water disinfection by-products (DBPs) can be formed. Dissolved organic matter, microbiological communities and anthropogenic compounds contribute to the chlorine demand of a particular water. Chlorine demand is the dose of chlorine that can be applied to completely react with substances in the water before a residual will be present (AHPA, 1999).

The effectiveness of chlorine on the removal of parent BACs has been studied in both simulated treatment scenarios and in plant scale studies (Table 1.8 shows results from a lab scale simulation). Different parent BACs have removal efficiencies with chlorine based on their chemical structures (Section 1.1.8) and physicochemical properties (Table 1.3) which complicates managing the most effective strategies for their control (removals ranging from <10% to >90%) (Ternes et al., 2002; Westerhoff et al., 2005; Stackelberg et al., 2007; Simazaki et al., 2008). There are several trends in reactivity observed under the conditions shown in Table 1.8. For example, for parent BACs that are both weak acids (pK$_a\geq$ 9.7) and

have electron donating functional groups on the aromatic ring, such as acetaminophen, *17-α-*ethinyl estradiol, and estrone, high reactivity with free chlorine was observed. This is the result of the BAC being in its neutral form and having the electron-donating phenol functional group on the aromatic ring, and most likely ring chlorination had occurred. Conversely, for compounds with aliphatic regions and/or very low pK_a values such as meprobamate, DEET, TCEP, and atrazine, very low reactivity was observed.

Table 1.8 Approximate Percent of Parent BAC Removed using 3.5 mg/L NaOCl as Cl$_2$ at pH 5.5, Contact time 24 hours (Westerhoff et al., 2005)

Compound	Approximate Percentage Parent BAC Removed
17-α-ethinyl estradiol	100
Acetaminophen	95
Atrazine	5
Caffeine	60
Carbamazepine	95
DEET	5
Diazepam	75
Diclofenac	100
Erythromycin-hydrate	100
Estrone	100
Gemfibrozil	100
Ibuprofen	25
Meprobamate	10
Sulfamethoxazole	100
TCEP	0
Trimethoprim	100

NT= Not tested

Disinfection using Chloramines

When hypochlorite is used as a disinfectant, natural organic matter (NOM) in the water has been found to be a precursor for disinfection by product (DBP) formation including trihalomethanes (THMs) (Rook, 1977) and haloacetic acids (HAAs) (Christman et al., 1983). Many utilities are switching to use a weaker disinfectant, chloramines, to meet DBP regulations for drinking water, under the U.S. Environmental Protection Agency Stage 1 Disinfectants and Disinfection Byproducts Rule (U.S EPA, 2001). Chloramines can exist as monochloramine or dichloramine, with the former being the primary disinfectant chosen by utilities.

$$NaOCl \; + \; H_2O \; \rightleftharpoons \; HOCl \; + \; NaOH$$

$$HOCl \; + \; NH_3 \; \rightleftharpoons \; NH_2Cl \; + \; H_2O$$
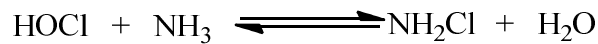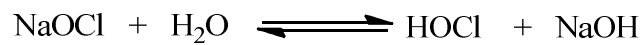
Chloramines are formed during drinking water treatment by utilities first adding chlorine to the water as a primary disinfectant and then adding ammonia as a secondary disinfectant later in the treatment train. Since chloramine is a weaker disinfectant than hypochlorite it has been found to react slower with BACs and remove and/or transform them to a lesser degree (Pinkston and Sedlak, 2004).

1.1.8 Reactive sites on BACs with free chlorine

One result of BACs being exposed to free chlorine is the formation of by-products (Gould and Richards, 1984; Miyamoto et al., 1997; Dodd and Huang, 2004; Moriyama et al.,

2004; Pinkston and Sedlak, 2004; Glassmeyer and Shoemaker, 2005; Ye, 2005; Bedner and MacCrehan, 2006; Nakamura et al., 2006; Dodd and Huang, 2007; Brix et al., 2008; Kotcharaksa, 2008; DellaGreca et al., 2009; Lee and Gunten, 2009; Quintana et al., 2010; Wulfeck-Kleier et al., 2010; Krkošek et al., 2011; Soh et al., 2011; Wang et al., 2011). As described in section 1.1.7 free chlorine is an oxidant and reacts non-selectively with regions of electron density and/or regions of extended π conjugation, including anthropogenic contaminants such as BACs. Aromatic regions are more likely to react with chlorine than aliphatic regions due to the electron density on the aromatic rings. Another important factor to predict the reactivity of chlorine with BACs is steric hinderence; bulkier molecules, such as atrazine and caffeine, are expected to be less reactive. In order to make predictions of the fate of BACs during disinfection, chlorination chemistry in aqueous solution will be briefly reviewed.

Substituted Benzene Rings

Substituted benzene rings act as a nucleophile and react with chlorine via electrophilic aromatic substitution. The electron density on the aromatic ring (π electrons above and below the ring) makes it available to react with an electrophile, such as chlorine (McMurry, 2004). Substituents on the benzene ring affect the reactivity of the aromatic structure, either activating or deactivating the ring for electrophilic attack and directing the electrophile to a particular position on the substituted benzene ring (Table 1.9)

Table 1.9 Electrophilic Aromatic Substitution-Substituent Effects (McMurry, 2004)

Substituent	Reactivity	Orientation	Inductive Effect
-CH$_3$	Activating	Ortho, para	Weak; electron-donating
-OH, -NH$_2$	Activating	Ortho, para	Weak; electron-withdrawing
-Cl,	Deactivating	Ortho, para	Strong; electron-withdrawing
-N$^+$(CH$_3$)$_3$	Deactivating	Meta	Strong; electron-withdrawing
-NO$_2$, -CN, CHO, -CO$_2$CH$_3$, -COCH$_3$, -CO$_2$H	Deactivating	Meta	Strong; electron-withdrawing

Table 1.9 helps in the understanding of the reactions between ring-substituted BACs and an electrophile such as chlorine. For example, in a compound containing the ring-activating phenol group (-OH) it can be expected that the electrophile will substitute on the aromatic ring and this explains the high reactivity of phenol containing compounds acetaminophen, estrone, and *17-α*-ethinyl estradiol in Table 1.8. This is the result of the phenol group donating electron density to the aromatic ring and making it more susceptible to electrophilic attack.

Phenols

The reaction between free chlorine, Cl$_2$/HOCl/OCl$^-$, and phenols under aqueous conditions proceeds via electrophilic aromatic substitutions and this reaction has been studied in the aqueous chlorination of *17-α*-ethinyl estradiol (EE2) (Moriyama et al., 2004) (Figure 1.3). The mono-chlorinated transformation product was found to have similar estrogenic

activity as EE2, and the dichlorinated product was found to be less estrogenic (Moriyama et al., 2004). In addition to estrogenic impacts of chlorination by-products of EE2 other biological endpoints such as toxicity remain unknown. As indicated in Table 1.9, chlorine will substitute at the *ortho-* and/or *para-* positions to the OH- group on the benzene ring. Other BACs studied containing aromatic phenols which are expected to react in a similar manner are estrone, acetaminophen, and tetracycline. The aqueous chlorination of acetaminophen has been shown to produce both mono- and di-chlorinated transformation products (Glassmeyer and Shoemaker, 2005) in addition to non-chlorinated by-products (Bedner and MacCrehan, 2006). A kinetic study determining the rate constant for the reaction between the phenol group on acetaminophen and HOCl revealed that the reaction will proceed faster via the phenolate form of the compound due to the increased electron density on the aromatic ring (Pinkston and Sedlak, 2004).
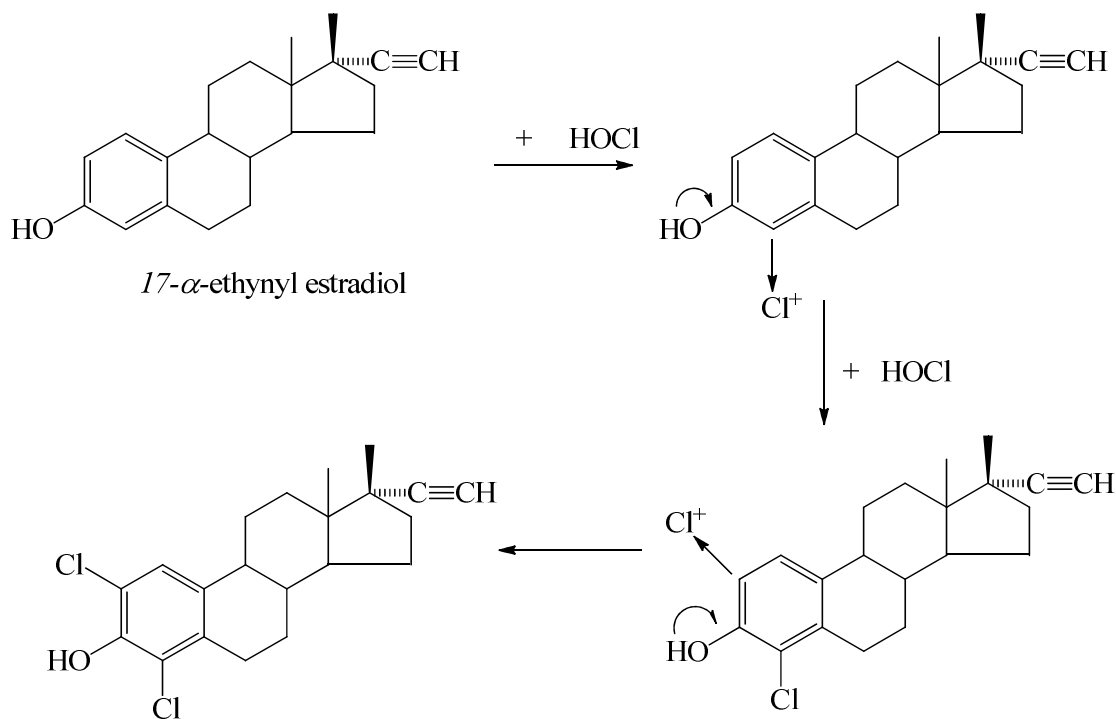
Figure 1.3 Chlorination of *17-α*-Ethynylestradiol (EE2), an Aromatic Phenol Compound

Sulfonamides

The chlorination of a sulfonamide pharmaceutical, sulfamethoxazole, under aqueous conditions showed that when the ratio of the initial concentration of chlorine to sulfamethoxazole is less than one, the free chlorine preferentially reacts with the aniline nitrogen to form a halogenated aniline ring product and an *N*-chlorinated product, as shown in Figure 1.4. Conversely, when the initial concentration of chlorine to sulfamethoxazole is greater than one, the free chlorine ruptures the sulfonamide functional group as shown in Figure 1.5 (Dodd and Huang, 2004). During the chlorination of sulfamethoxazole the structural moiety responsible for antibiotic activity is transformed and, therefore, changes in antibiotic activity or other biological endpoints of the chemical might be expected, although

this has not yet been confirmed. In occurrence studies where only the parent BAC is targeted, the transformation products will not be detected, and the environmental and human health impact of the products may be underestimated.
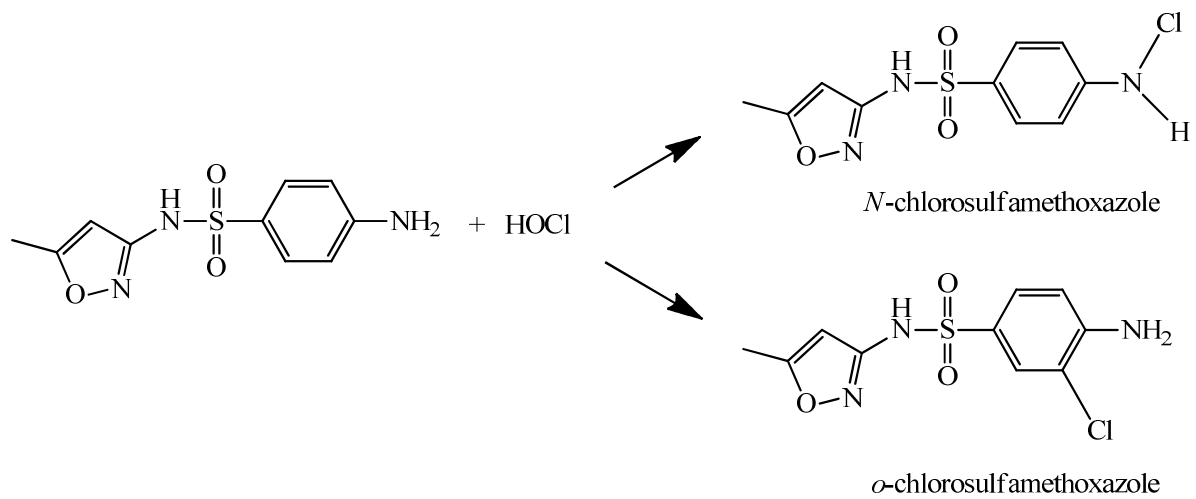
Figure 1.4 Reaction of the Sulfonamide Antibiotic, Sulfamethoxazole, with Free Chlorine. Molar Ratio of Sulfamethoxazole to FAC is Less Than One (Extracted from Dodd and Huang, 2004)
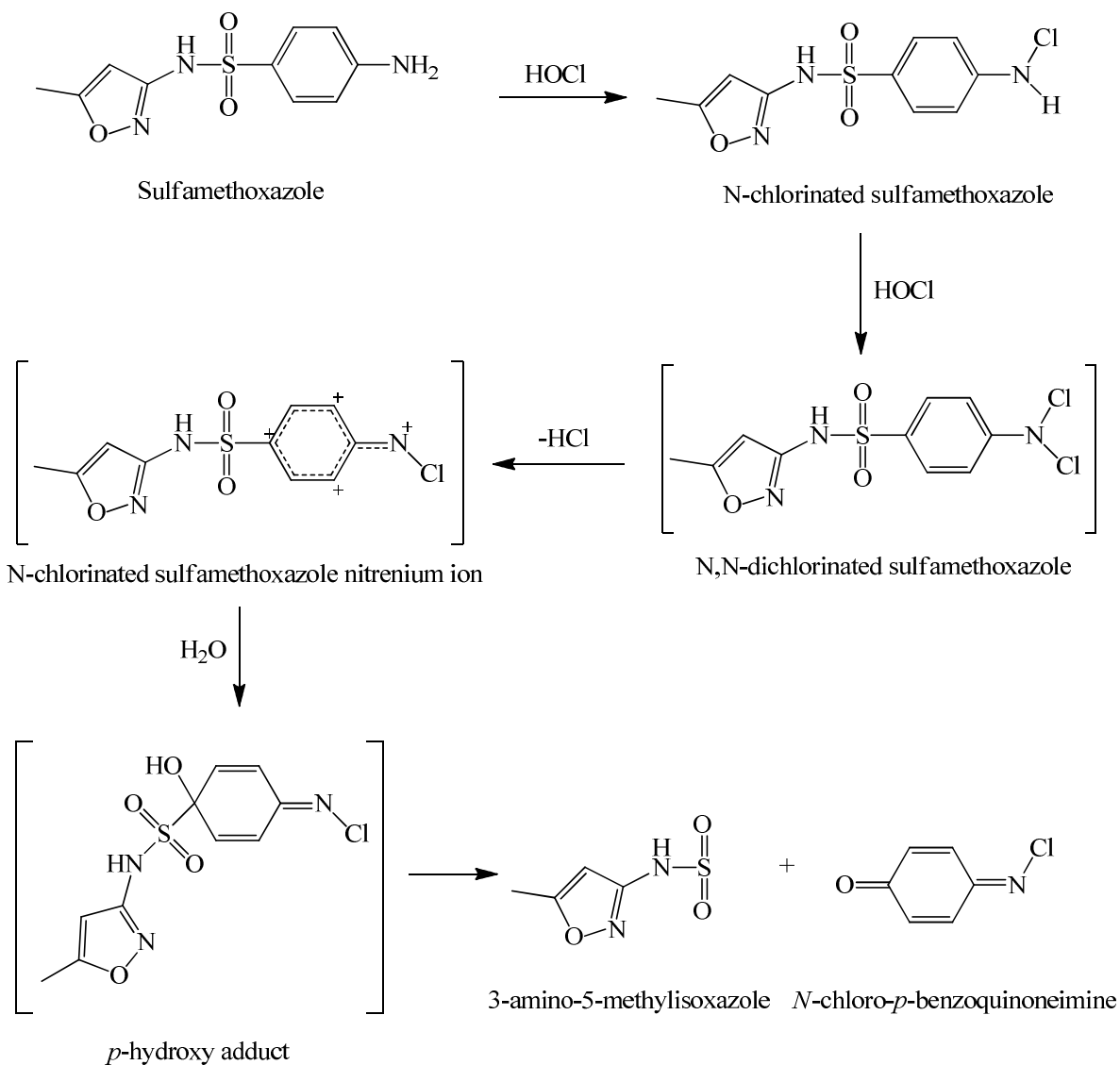
Figure 1.5 Reaction of Sulfonamide Antibiotic, Sulfamethoxazole, with a Molar Excess of Free Chlorine. Species in Brackets Indicate Proposed Intermediates (Extracted from Dodd and Huang, 2004)

Amines

When amines are chlorinated in aqueous solution, the reaction takes place via chlorine addition between the deprotonated amine and HOCl. Therefore, the reactions are dependent on the pH of the solution, and the reaction will proceed fastest between the pKa values of HOCl and the amine (Pinkston and Sedlak, 2004). Aliphatic amine groups (R_2-NH_2) react with free chlorine in aqueous solutions through the transfer of chlorine from HOCl to the amine nitrogen as is shown in Figure 1.6 (Abia et al., 1998). Chlorination of primary and secondary amines proceeds more quickly than chlorination of tertiary amines.
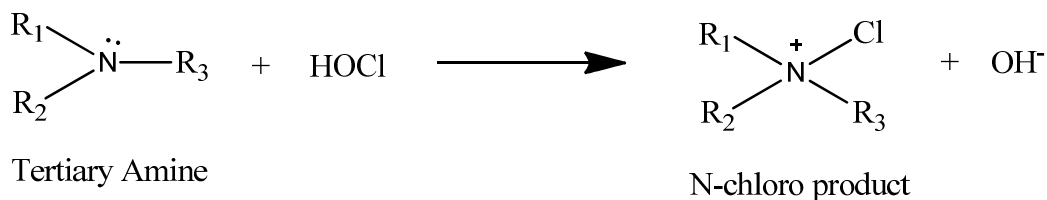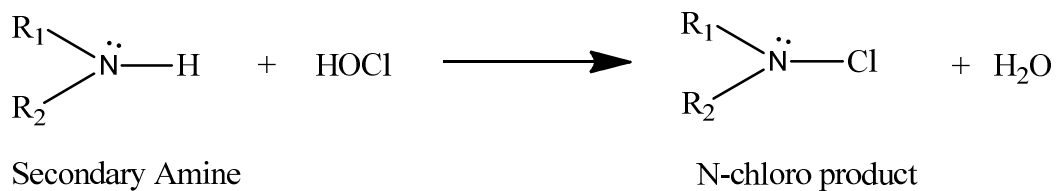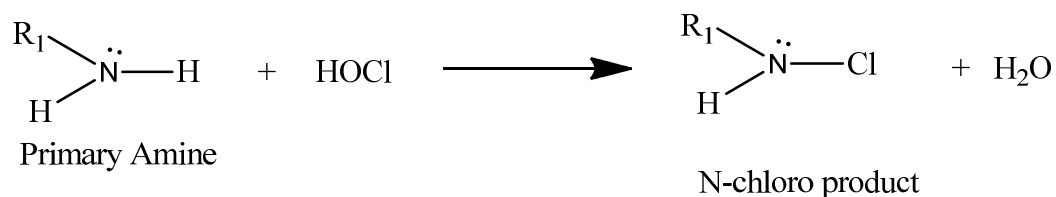
Figure 1.6 Reactions of Amines with Free Chlorine. (Extracted from Abia et al., 1998).

1.1.9 Identification of transformation products of BACs due to chlorination

Wastewater and drinking water treatment have been simulated in laboratory studies to determine whether BACs are removed or transformed into by-products during chlorination (Gould and Richards, 1984; Miyamoto et al., 1997; Dodd and Huang, 2004;Moriyama et al., 2004; Pinkston and Sedlak, 2004; Glassmeyer and Shoemaker, 2005; Ye, 2005; Bedner and MacCrehan, 2006; Nakamura et al., 2006; Dodd and Huang, 2007; Brix et al., 2008; Kotcharaksa, 2008; DellaGreca et al., 2009; Lee and Gunten, 2009; Quintana et al., 2010; Wulfeck-Kleier et al., 2010; Krkošek et al., 2011; Soh et al., 2011; Wang et al., 2011). Determining if BACs are removed or transformed is important to accurately assess the risk that these compounds pose to environmental and human health. BACs that are not removed, but transformed into new products may have unknown health outcomes, and it is important to gain a greater understanding of these potential risks. These reactions are usually conducted in aqueous media and use an initial molar excess of chlorine to BAC in order to simulate treatment conditions. Determining the fate of BACs during drinking water treatment is a challenging task that requires the use sophisticated analytical instruments such as liquid chromatography or gas chromatography paired with detection such as: ultraviolet (UV), mass spectrometry (MS), nuclear magnetic resonance (NMR), or post-column reaction/reductive electrochemistry.

Preparative or semi-preparative high pressure liquid chromatography (HPLC) has been utilized to isolate transformation products. Both chlorinated samples and non-chlorinated control samples are prepared to determine both the formation of new products and to calculate the decrease in parent compound concentration, respectively. The formation of new chromatographic peaks using liquid chromatography paired with UV detection is used

initially to determine product formation and then followed by a separate LC-MS analysis to determine the identity of transformation products in many experiments. A limitation of LC is that transformation products may not elute off the analytical column and the products will remain undetected, and a limitation of using MS analysis is that the transformation products may not ionize to a great degree. Another technique used to determine the structure of transformation products and mechanism of reaction is to perform sub-structure analysis where sub-structures of the parent compound are reacted with free chlorine to determine the reactive sites (Dodd and Huang, 2004; Dodd and Huang, 2007). Commercially available standards have been used to compare to potential transformation products of BACs with chlorine (Bedner and MacCrehan, 2006).

A mass spectrometer, an extremely sensitive analytical instrument, can be used to determine the identity of transformation products. Ionization sources that have been used are both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Techniques include full scan mass spectrometry to gather information on all ions that are ionized in the MS. Additionally chlorine isotope ratios can be used to determine the number of chlorine substituents that are on a molecule.

One limitation of analytical techniques that are highly selective is that only specific analytes can be targeted, and a significant challenge is determining the concentration and identity of unknown species in a sample. Total organic halogen (TOX) analysis is a useful tool that quantifies the concentration of organo-chlorine species in a sample. Although TOX analysis has not yet been used to quantify unknown halogenated by-products of BACs, it has been used to quantify the unknown percentage of DBPs in drinking water (Krasner, et al., 2006). A literature summary focusing on the detection of transformation by-products of

some BACs due to aqueous chlorination and the impact of these by-products on biological

effect is shown in Table 1.10.

Table 1.10 Transformation of BACs in Reactions with Free Chlorine and Impact on Biological Effect

Compound	Reference	Proposed by-products/Location of Cl- Addition	Analytical Instrumentation Used	Impact to Biochemical Activity
17-α-ethinyl estradiol	Nakamura et al., 2006[k]; Moriyama et al., 2004[l]	Three mono- and di-chlorinated derivatives isolated (carried out in organic solvent)[k]; Mono- and di-chlorinated products formed (major) and four other minor products[l]	FAB-MS, ^{1}H NMR, ^{13}C NMR, HPLC-UV, preparative HPLC[k]; Preparative HPLC-UV, LC-(+/-)-APCI-MS, ^{1}H NMR, ^{13}C NMR[l]	Yeast assays revealed chlorinated products less estrogenic than parent (more than BPA)[k]; ELISA test kit used for estrogen receptor and showed that mono-Cl product had similar activity and di-Cl was less estrogenic than parent[l]

Table 1.10 (continued) Transformation of BACs in Reactions with Free Chlorine and Impact on Biological Effect

Compound	Reference	Proposed by-products/Location of Cl- Addition	Analytical Instrumentation Used	Impact to Biochemical Activity
Acetaminophen	Glassmeyer and Shoemaker, 2005[b] Bedner and MacCrehan, 2006[i]	Mono- and di- Cl-addition to aromatic ring, 1,4-benzoquinone and *N*-acetyl-*p*-benzoquinone; transform back to acetaminophen with sodium sulfite[i]; mono- and di-chlorinated ring products[b]	LC-UV, LC-UV-(+)-ESI-MS, LC-UV-EC[i]; LC-MS[b]	LD_{50} toxicity testing in mouse reveals transformation products much more toxic[i]
Atenolol	DellaGreca et al., 2009[f]	*N*-chlorination; de-chlorinated upon addition of sodium thiosulfate[f]	HPLC-UV, HPLC-MS, ^{1}H NMR, TLC, (+)-ESI-LC-MS[f]	Chlorinated products more phytotoxic than atenolol[f]
Atrazine	Wulfeck-Kleier et. al., 2010[m]; Brix et al., 2008[n]	*N*-chloro atrazine, transforms back to Atrazine with sodium sulfite and ascorbic acid (ammonium chloride does not reform Atrazine)[m]; No reaction observed[n]	GC-MS, HPLC-(+)-ESI-MS, HPLC-UV[m]; UPLC-Q-ToF-MS/MS using Masslynx software (Waters Corp)[n]	Chlorinated product retains s-chlorotriazine moiety[m] which is the cause of toxicity (EPA, 2006) not yet studied; Toxicity of other chlorinated triazines studied using *V.fischeri* and found to be more toxic than parent compound

Table 1.10 (continued) Transformation of BACs in Reactions with Free Chlorine and Impact on Biological Effect

Compound	Reference	Proposed by-products/Location of Cl- Addition	Analytical Instrumentation Used	Impact to Biochemical Activity
Bezafibrate	Quintana et al., 2010[e]	No reaction observed[e]	LC-MS, LC-MS/MS[e]	N/A
Caffeine	Glassmeyer and Shoemaker, 2005[b]; Gould and Richards, 1984[q]	No change observed[b]; Many products formed-ring cleavage and rearrangement forming non-aromatic nitrogen heterocycles. (8-chlorocaffeine formed, but in small amount). No organochloramines detected[q]	LC-PB-MS[b]; GC-MS, TLC, UV[q]	Unknown
Carbamazepine	Kotcharaksa, 2008[r]; Lee and Gunten, 2009[s]	Four unchlorinated intermediates[r]; Minimal reactivity observed[s]	HPLC-UV, LC-ITMS[r]; HPLC-UV[s]	Unknown
Clofibric acid	Quintana et al., 2010[e]	No reaction observed[e]	LC-MS, LC-MS/MS[e]	N/A
DEET	None	N/A	NA	NA
Diazepam	None	N/A	N/A	NA

Table 1.10 (continued) Transformation of BACs in Reactions with Free Chlorine and Impact on Biological Effect

Compound	Reference	Proposed by-products/Location of Cl- Addition	Analytical Instrumentation Used	Impact to Biochemical Activity
Diclofenac	Miyamoto et al., 1997[h]; Quintana et al., 2010[e]	Cl- addition to aromatic ring and/or loss of CO from ring[e]; Cl- addition to aromatic ring among other non-chlorinated products[h]	LC-MS, LC-MS/MS[e]; HPLC-UV, LC-MS, preparative HPLC, MS/MS, ^{1}H NMR[h]	Unknown ecological impact of chlorination by-products, suspected hepatoxicity or bone marrow toxicity[h]
Erythromycin-hydrate	Ye, 2005[o]	*N*-chlorinated product and *N*-demethylation product[o]	ESI-MS, LC-MS/MS, TOX[o]	Unknown
Estrone	Nakamura et al., 2006[k]	Five chlorinated derivatives isolated (carried out in organic solvent)[k]	FAB-MS, ^{1}H NMR, ^{13}C NMR, HPLC-UV, preparative HPLC[k]	Yeast assays revealed chlorinated products less estrogenic than parent (but more than BPA)
Fenoprop	None	N/A	N/A	NA
Gemfibrozil	Krkošek et al., 2011[c]; Glassmeyer and Shoemaker, 2005[b]	Cl- addition to aromatic ring[c]; Mono-chlorinated product[b]	GC-MS, ^{1}H NMR, (-)-ESI-MS/MS, (-)-ESI-MS[c]; LC-PB-MS[b]	Reduction in lipophilic character hypothesized to reduce activity[c]

Table 1.10 (continued) Transformation of BACs in Reactions with Free Chlorine and Impact on Biological Effect

Compound	Reference	Proposed by-products/Location of Cl- Addition	Analytical Instrumenta-tion Used	Impact to Biochemical Activity
Ibuprofen	Pinkston and Sedlak, 2004[d]; Quintana et al., 2010[e]	No reaction observed[d,e]	LC-MS, LC-MS/MS[e]; HPLC-UV[d]	NA
Meprobamate	None	N/A	N/A	NA
Sucralose	Soh et al., 2011[g]	No reaction observed[g]	LC-MS[g]	NA
Sulfamethoxazole	Dodd and Huang, 2004[j]	Degradation of sulfonamide moiety with excess of FAC to form 3-amino-5-methylisoxazoleand N-chloro-*p*-benzoquinoneimine[j]	LC-MS; ^{1}H NMR, GC-EI-MS[j]	Reduction of antibiotic activity hypothesized due to alteration of antibiotic moiety[j]
TCEP	None	N/A	N/A	NA
Tetracycline	Wang et al., 2011[p]; Ye, 2005[o]	Cl- and OH-substituted products observed, no products identified[p]; New peaks observed in chromatogram due to transformation products, none identified[o]	LC-(+)-ESI-MS[p]; LC-(+)-ESI-MS[o]	Structural change to antibiotic moieties: activity unknown[p]

Table 1.10 (continued) Transformation of BACs in Reactions with Free Chlorine and Impact on Biological Effect

Compound	Reference	Proposed by-products/Location of Cl- Addition	Analytical Instrumenta-tion Used	Impact to Biochemical Activity
Trimethoprim	Dodd and Huang, 2007[a];Glass meyer and Shoemaker, 2005[b]	Cl- and OH-substituted products[a]; Non-chlorinated product[b]	LC-(+)-ESI-MS[a]; LC-PB-MS[b]	Reduction of antibiotic activity hypothesized due to alteration of antibiotic moiety[a]

(a) Dodd and Huang, 2007; (b) Glassmeyer and Shoemaker, 2005; (c) Krkošek et al., 2011; (d) Pinkston and Sedlak, 2004; (e) Quintana et al., 2010; (f) DellaGreca et al., 2009; (g) Soh et al., 2011; (h) Miyamoto et al., 1997; (i) Bedner and MacCrehan, 2006; (j) Dodd and Huang, 2004; (k) Nakamura et al., 2006; (l) Moriyama et al., 2004; (m) Wulfeck-Kleier et. al., 2010; (n) Brix et al., 2008; (o) Ye, 2005; (p) Wang et al., 2011; (q) Gould and Richards, 1984; (r) Kotcharaksa, 2008; (s) Lee and Gunten, 2009

1.1.10 Policies

In 1974, the Safe Drinking Water Act (SDWA) was created to protect the quality of drinking water and its sources in the U.S. The U.S EPA is responsible for setting regulatory limits for contaminants in drinking water under the SDWA (P.L. 93-523, 1974). Under the SDWA, the National Primary Drinking Water Regulations (NPDWRs) contain a list of contaminants (microbial, disinfection by-products, disinfectant residuals, inorganic and organic chemicals and radionuclides) whose concentrations in drinking water are subject to legal enforcement in public drinking water systems due to their potential negative health effects (U.S. EPA, 2009a). Atrazine, a commonly used herbicide, is an example of a BAC that is regulated under the NPDWRs with a Maximum Contaminant Level Goal

(MCLG)/Maximum Contaminant Level (MCL) in drinking water of 0.003mg/L (U.S. EPA, 2009a). Also part of the SDWA are the National Secondary Drinking Water Regulations (NSDWRs) which contain a list of contaminants in water which may cause either aesthetic or taste/odor/color problems. As part of the NSDWRs these contaminants are recommended for regulation in individual states by the Federal Government.

Under the SDWA there is also a list of unregulated chemicals called the Candidate Contaminant List 3 (CCL3) (U.S. EPA, 2009b) which was created to prioritize scientific research and policy making. This daunting task is completed by screening a large set of unregulated contaminants for potential health effects and occurrence in drinking water supplies and selecting the ones with the greatest potential to pose a public health risk. The CCL3 contains a list of 116 chemical and microbial contaminants that are currently unregulated under the NPDWRs, are known to be in drinking water, and may require regulation under the SDWA. Included on this list are the steroid hormones *17-α-ethinyl estradiol* and estrone (U.S. EPA, 2009b).

In March 2010 the EPA launched a new system to help protect public health and drinking water supplies called the Drinking Water Strategy (DWS). The program's objective was to focus decision-making, increase protection of water, and promote advancement of research to determine effective treatment technologies for public water supplies (U.S. EPA, 2010). The four goals of the DWS are to: 1) address contaminants as groups 2) encourage new drinking water treatment technologies 3) link authority of other statutes to apply to drinking water, and 4) work with states to share monitoring results (U.S. EPA, 2010).

Additional Federal Government agencies that have programs set up to regulate the exposure of BACs into the environment are the U.S. Food and Drug Administration (FDA)

and the USGS. The FDA is responsible for the regulation of food, medicines cosmetics and other products. The USGS conducts scientific research including national occurrence sampling studies of U.S. drinking water sources (Kolpin et al., 2002; Focazio et al., 2008). Despite agency efforts to highlight the presence of BACs in the environment, opposition from industry makes regulation difficult as, for example, the regulation of spray drifts from the application of atrazine to agricultural lands. Although environmental regulators desire to set stricter regulations to protect water resources, the agricultural industry prioritizes maintaining high product yields and protecting job security (Erickson, 2011).

1.1.11 Future management strategies

It is clear that the impact of BAC residues in the environment is not an issue that can be ignored. Recent media reports, (e.g., Shah, 2010), have highlighted dramatic events linked to BAC use such as mass die-offs of vultures in Asia due to ingestion of the anti-arthritic drug diclofenac (Oaks et al., 2004), antibiotic-resistant pathogens (Khachatourians, 1998), and the feminization of fish due to estrogenic compounds (Jobling et al., 1998). Additionally, the herbicide atrazine has also been in the media spotlight due to the re-evaluation of its toxicity in order to set regulatory limits.

Since the long term effects of BACs in the environment are unknown it is imperative that scientists, regulators and pharmaceutical companies agree on management strategies that allow for the safe use of BACs and protection of the environment. In order to evaluate possible management strategies to control the release of BACs into the environment stakeholders from the government, academia, pharmaceutical industry, scientific community,

and policy and management experts from the US, Canada and Europe evaluated possible options (Doerr-MacEwen and Haight, 2006). The opinions from the stakeholders were that the most effective management strategies to minimize the release of BACs were advanced wastewater treatment processes, education to reduce over prescription, pharmaceutical take-back programs, and public education (Doerr-MacEwen and Haight, 2006). The stakeholders also indicated that some of the gaps in scientific research were the uncertainties associated with mixture effects, low level chronic exposure, and risk assessment methods (Doerr-MacEwen and Haight, 2006). Additional management strategies that may reduce the release of BACs into the environment are regulation of prescription drug sales over the internet, careful monitoring of sewage biosolids used for land application, and development of more effective WWTP technologies to remove BACs (Daughton and Ternes, 1999).

With countless BACs being introduced to the market there remains many research gaps in the understanding of the fate and impact of these anthropogenic compounds in the environment. Careful planning of research is necessary in order to maximize time and cost of resources and to guide future regulations. Several areas of research that need expansion in order to guide risk assessment and policy making include the improvement of sample pre-concentration and analytical analysis, evaluation of chemical toxicity on aquatic organisms, assessment of mixture effects of BACs, and monitoring the fate of BACs including degradation products and metabolites (Daughton and Ternes, 1999).

1.2 Research Questions

In summary, effectively managing BAC use and protecting water supplies is a complicated task due to widespread use, unknown fate in the environment, and transformation during physicochemical drinking water treatment. Maintaining sensitive and current analytical methods is one challenging area of research because new BACs are constantly being introduced into the market. Additionally, one area of challenging work is studying the fate of BACs and how to identify unknown degradation products. Primary research questions remain to determine the occurrence of BACs in drinking water supplies, evaluate reactivity of BACs, and identify transformation products. A greater understanding of the reactivity and fate of these chemicals will provide invaluable information for future occurrence sampling and toxicity testing.

1.3 Research Objectives

The hypotheses that validate the completion of this study are the following:

1. Treatment processes employed at conventional drinking water treatment plants may not be effective in completely removing BACs.

2. The degree of chlorine reactivity with BACs can be measured using TOX analysis.

3. Chlorine reacts with BACs to form transformation products.

4. BACs and/or their chlorination by-products may be present in treated drinking water.

These hypotheses are tested by the following experimental objectives in the same order as presented above:

1. Determine the occurrence and fate of BACs in North Carolina drinking water treatment plants.

2. Study the incorporation of chlorine on the parent BAC molecule using total organic halogen (TOX) analysis and infusion mass spectrometry (MS).

3. Assess the removal of BACs as the result of chlorination.

4. Relate the reactivity of BACs with chlorine to their molecular structure and presence in drinking water.

2. MATERIALS AND METHODS

2.1 Materials

BAC Standards

Standards purchased from Sigma-Aldrich (St. Louis, MO) were tetracycline (≥98%), tris(2-chloroethyl)phosphate (TCEP) (97%), 4-acetaminophenol (98%), diazepam, bezafibrate, gemfibrozil, diclofenac, erythryomycin-hydrate (96%), *17-α*-ethinyl estradiol (98%), and estrone (U.S. Pharmacopeia grade). Standards purchased from MP Biomedicals, LLC (Solon, OH) were atenolol, trimethoprim, and sulfamethoxazole. Clofibric acid was purchased from Tocris Bioscience (Ellisville, MO). Atrazine (97.5%) and anhydrous caffeine (≥99%) were purchased from Fluka Analytical (Buchs, Switzerland). N,N-diethyl-meta-toluamide (DEET) (97.3%) and 2-(2,4,5-trichlorophenoxy)propionic acid (fenoprop) (99%) were purchased from Riedel-de-Haën (Seelze, Germany). Meprobamate (98%) was purchased from Cerilliant (Round Rock, TX). Sucralose (98%) was purchased from Toronto Research Chemicals (TRC), Inc. (North York, Ontario). Ibuprofen (99%) and carbamazepine (99%) were purchased from Acros Organics (Geel, Belgium). HPLC-grade methanol was purchased from either Acros Organics (Geel, Belgium) or Fisher Scientific (Pittsburgh, PA). Due to the countless number of BACs available on the market not every synthesized compound could be included in this study. The BACs evaluated are shown in Table 2.1 and were chosen based on high usage, occurrence in the environment, persistence through drinking water treatment, and suspected toxicological importance.

Table 2.1 Studied BACs and Intended Uses

Compound	Molecular Weight (g/mol)	Use
Acetaminophen	151.2	Analgesic
Atenolol	266.3	Beta-blocker
Atrazine	215.7	Herbicide
Bezafibrate	361.8	Lipid Regulator
Caffeine	194.1	Stimulant
Carbamazepine	236.3	Antiepileptic
Clofibric acid	214.6	Lipid regulator
N,N-Diethyl-meta-toluamide (DEET)	191.3	Insect Repellent
Diazepam	284.7	Anti-anxiety
Diclofenac	296.2	Analgesic
17-α-ethinyl estradiol	296.4	Synthetic Estrogen
Estrone	270.4	Steroid
Erythyromycin	733.9	Antibiotic
Fenoprop	269.5	Herbicide
Gemfibrozil	250.3	Anti-cholesterol
Ibuprofen	206.3	Analgesic
Meprobamate	218.3	Anti-anxiety
Sucralose	397.6	Artificial Sweetener
Sulfamethoxazole	253.3	Antibiotic
Tetracycline	444.4	Antibiotic
tris(2-carboxyethyl)phosphine (TCEP)	285.7	Flame Retardant
Trimethoprim	290.3	Antibiotic

Chlorination

Laboratory grade water (LGW) was prepared in the University of North Carolina laboratory using an in-house Dracor (Durham, NC) water purification system which pre-filters inlet 7 MΩ house deionized water to 1 μm, removes residual disinfectants, reduces total organic carbon to less than 0.2 mg C/L with an activated carbon resin, and removes ions to 18 MΩ with mixed bed ion-exchange resins. Sodium hypochlorite stock solution (NaOCl)

as 5.65-6% in water was purchased from Fisher Scientific (Pittsburgh, PA) and the concentration of the sodium hypochlorite stock solution prepared after diluting (1:50) was measured monthly using the Iodometric Titration 1 Procedure following Standard Method 408 A (APHA, 1999). Chlorine residuals were measured using a HACH test kit pocket colorimeter and HACH permachem DPD free/total chlorine reagents (HACH, Loveland, CO). Excess chlorine in samples prepared for TOX analysis was quenched using analytical reagent (AR) grade anhydrous sodium sulfite (98.9%) (Mallinckrodt, Phillipsburg, NJ). Excess chlorine in samples prepared for MS/MS analysis was quenched with L-ascorbic acid (Sigma, St. Louis, MO).

TOX Analysis

TOX analysis used glacial acetic acid (99.8% Certified ACS Grade) and Certified ACS plus grade concentrated sulfuric acid both purchased from Fisher (Fair Lawn, NJ). Potassium nitrate (Certified ACS grade) for the nitrate rinse was also purchased from Fisher (Fair Lawn, NJ). Sodium chloride (~80%) for cell performance check was purchased from Fluka Analytical (Buchs, Switzerland). Sodium bicarbonate (99.9%) was purchased from Mallinckrodt AR (Paris, Kentucky). Purified silver acetate was purchased from Fisher (Fair Lawn, NJ). The performance check standard, 2,4,6-trichlorophenol (98%), was purchased from Sigma Aldrich (St. Louis, MO).

Mass Spectrometry

HPLC-grade methanol and HPLC-grade acetonitrile (0.2μm filtered) were purchased for tandem mass spectrometry (MS/MS) from either Acros Organics (Geel, Belgium) or Fisher Scientific (Pittsburgh, PA). The polypropylene glycol (PPG) tuning solution was purchased from Varian, Inc. (Santa Clara, CA). High pressure liquid nitrogen, zero grade air, ultra high purity (UHP) nitrogen and UHP argon were purchased from National Welders Supply, Inc. (Morrisville, NC). The isotopically labeled surrogate standard, caffeine-d_3, was purchased from C/D/N Isotopes (Pointe-Claire, QC, Canada) 99.8% pure. The internal standard, simeton, was purchased from AccuStandard (New Haven, CT) at 100μg/mL.

Sample Collection and Preservation

The tetradentate chelating agent, disodium ethylenediamine tetraacetic acid, (Na_2EDTA) (99+%) was purchased from Sigma-Aldrich (St. Louis, MO). The glassware silanizing agent, 5% dimethyldichlorosilane (DMDCS) in toluene (99% and 99.9% pure respectively), was purchased from Supelco Analytical (Bellefonte, PA). Chlorine residuals were measured using a HACH test kit pocket colorimeter and HACH permachem DPD free/total chlorine reagents purchased from HACH (Loveland, CO). pH adjustment of the aqueous samples for extraction used formic acid (98+%) which was purchased from Acros Organics (Geel, Belguim).

2.2 Methods

2.2.1 Bench Scale Chlorination Experiments

Preparation of Chlorination Samples

Stock solutions of studied BACs (Table 2.1) at a target concentration of 1000mg/L of each were prepared in HPLC grade methanol from neat analytical standards stored in a freezer at approximately -20°C, and used within five months in HPLC grade methanol. On the day of sample preparation, 100μL of the stock solution was spiked into 100mL of LGW to achieve a target concentration of 1mg/L for subsequent dosing with chlorine. In order to facilitate analysis of reaction products without excessive sample processing, the target initial concentration of each BAC was 1mg/L and a chlorine dose of about 25mg/L as Cl_2 was used. Although this chlorine dose is larger than what is typically employed at a DWTP, the objective of the experiment was to simulate disinfection where an excess of chlorine is added relative to the concentration of organic microcontaminants, and using high enough doses of BACs that are well above limits of instrument detection. The 100mL sample reactions took place in sealed 125mL amber glass jars (no mixing) with headspace in order to more accurately simulate DWTP conditions over 24 hours at 20°C in the dark.

The pH of the reaction was not controlled during the course of the experiment in order to avoid adding interfering ions that would cause ion suppression during MS analysis but was in the range of 6-8. After the 24 hour contact time, a 1mL aliquot of the chlorinated samples (diluted 1:10 in LGW) was removed for measurement of free chlorine residual using a HACH test kit pocket colorimeter based on Standard Method 4500-Cl G. (APHA, 1999), and the remaining sample quenched with a 40mg/mL sodium sulfite solution in LGW for TOX analysis or a 40mg/mL ascorbic acid solution in LGW for MS/MS analysis. The

solutions mentioned above used for quenching the chlorine reaction were prepared immediately before addition into the sample. Separate sets of samples were prepared for the different analyses because each analysis was completed on a different day.

The control samples included LGW blanks, free chlorine solutions prepared in LGW at a target concentration of 25mg/L and quenched with sodium sulfite, and BAC solutions in LGW at the target initial concentration of 1mg/L (same as the chlorinated samples). All control samples were stored for the same reaction time and under the same conditions as the chlorinated BAC samples. After the 24 hour contact time, a 10mL aliquot of the control samples was removed for measurement of free chlorine residual (typically a 1:10 or 1:20 dilution) using a HACH test kit pocket colorimeter based on Standard Method 4500-Cl G. (APHA, 1999), and quenching agent was added to the remaining sample as described previously to ensure uniform conditions between the chlorinated and non-chlorinated samples. Chlorine demand was calculated by subtracting the free chlorine residual from the concentration of the chorine dose applied (APHA, 1999), and since chlorine demand indicates that chlorine has reacted with species in the water, this value provides insight into the extent of the reaction that occurred. The chlorine demand of the LGW control sample was determined to be negligible during the experiment. Chlorine demand was used to determine the relative reactivity of the BACs with chlorine, TOX analysis was used to study the incorporation of chlorine onto the parent BAC molecule during the reaction, and MS/MS analysis was used to confirm transformation of select BACs. The net chlorine demand was calculated by subtracting the chlorine demand of the relevant controls from the chlorine demand of the chlorinated samples.

Chlorine Demand Determination

The chlorine residual was determined for all chlorinated samples using a HACH test kit pocket colorimeter based on Standard Method 4500-Cl G (APHA, 1999) and N,N-Diethyl-p-Phenylenediamine (DPD) packets. If needed, samples were diluted to ensure the free chlorine residual was in the range of the colorimeter. The free chlorine residual was then subtracted from the initial chlorine dose to calculate the chlorine demand of the sample.

Total Organic Halide (TOX) Analysis

TOX analysis was used to determine the extent to which chlorine had become incorporated into the parent BAC molecule during reaction. After quenching the residual chlorine as described above, the samples were analyzed for TOX using a procedure adapted from Standard Method 5320 (APHA, 1999). 100mL samples were acidified using 20 drops of concentrated sulfuric acid to ensure optimal adsorption onto one glass-packed activated carbon (GAC) column using a Tekmar-Dohrmann AD-2000 Adsorption Module (Cincinnati, OH). While the standard method calls for two carbon columns, preliminary experimental results showed that the amount of breakthrough of the analytes onto the second column was less than 5% and, therefore, in order to increase productivity, only one glass-packed carbon column was used (see Table 3.3). The sample adsorption rate was 3mL/min and the sample volume adsorbed was 50mL. Removal of inorganic halides adsorbed on the carbon was achieved by flowing 2mL of a ~5g/L NO_3-N solution prepared from potassium nitrate at a rate of 0.5mL/min through the column after the sample.

After sample adsorption and removal of halides, the carbon was pyrolyzed using a Rosemount Dohrmann DX-2000 Organic Halide Analyzer (Cincinnati, OH). The carbon columns were completely combusted at 850°C and the organohalogen component of the molecule converted to hydrogen halide which was then transported with the carrier gas, helium, to the coulometric cell containing silver electrodes. As the current in the cell increases due to the presence of the halide ions the change in voltage is recorded. The average TOX recovery of the performance check standard, 500ng Cl/μL trichlorophenol, was 88.5% (n = 5; standard deviation: 18%). The net increase in concentration of organic halogen was calculated by taking the difference between the unchlorinated BAC and chlorinated BAC samples.

Ultraviolet-Visible (UV-Vis) Analysis

The fate of the aromaticity of the reacted tetracycline was monitored using a Hitachi U-3300 Spectrophotometer. First the wavelength of maximum absorption of tetracycline was determined and then the parent compound and products in solution were scanned from 200-750nm to provide some additional characterization of the reaction products. Additionally, for a chlorinated sample a wider wavelength range was scanned, 200-750nm, to determine if new λ_{max} associated with transformation products were formed.

Tandem Mass Spectrometry Analysis (MS/MS)

A separate aliquot was prepared for MS/MS analysis by quenching residual chlorine in a 100mL sample using a 40mg/mL solution of ascorbic acid in LGW and then infusing directly into a Varian 1200L mass spectrometer (Palo Alto, CA) using a Harvard Apparatus syringe pump (Holliston, MA) at a flow rate of 20µL/min. Liquid nitrogen was used as the drying gas, argon was the collision induced dissociation (CID) gas in positive electrospray ionization mode (+ESI), while zero grade air was used as the CID gas in negative electrospray ionization mode (-ESI). The API housing was at 50°C, and the drying gas was at 300°C. The detector was set at 1300V, the shield at 600V, and the capillary between 40-60V. In order to increase analyte ionization in the electrospray interface, the quenched chlorinated samples was mixed with HPLC grade methanol at a volume ratio of sample:methanol of 9:1. All samples were filtered through 0.45µm Laboratory Supply Distributors (Millville, NJ) syringe tip filters 0.45µm prior to MS/MS analysis. All control samples were analyzed first followed by the chlorinated and unchlorinated BAC samples. While infusing the sample into the mass spectrometer, data was collected using Varian MSWS Software Version 6.8., and the full scan spectra ranging from *m/z* 50-700 was observed in real time in quadrupole 1 (Q1). The full scan spectra were scanned in order to locate prominent ions. The chlorinated tetracycline samples were compared to the unchlorinated tetracycline samples in order to identify new ions that may be associated with transformation products. During infusion experiments it is possible to distinguish characteristic chlorine isotope ratios. These chlorine isotope ratios are helpful searching for unknown chlorination by-products of BACs because they show, through characteristic patterns, the number of chlorine atoms on a molecule. Prominent peaks observed in Q1 were

optimized and daughter ions were scanned in quadrupole 2 (Q2) by applying argon, the collision gas, at 2.00mTorr and increasing the collision energy from 5-50eV.

2.2.2 Occurrence Sampling

Sample Sites and Collection

Aqueous samples were collected in silanized (5% DMDCS in toluene) amber glass jars ranging from 0.5L to 4L based on size of sample. Silanization of the glassware was completed by allowing the silanizing agent to sit in the glassware for approximately 10 minutes, rinsed three times with toluene, rinsed three times with methanol, rinsed three times with LGW, and then dried in an oven at 150°C overnight and capped immediately after cooling. Na$_2$EDTA was added to sample bottles before sample collection for sample preservation. Either 24-hour composite samples or grab samples were collected headspace-free, based on the needs of the study. For 24-hour composite samples, approximately ¼ of the sample vessel was filled every six hours for a total of four sample collection events. Sample collection was completed by the operators at each plant following sample collection instructions (Appendix 1). Samples were stored at ~4°C in a refrigerator at the DWTPs and transported back to the laboratory in a cooler with ice-packs.

Samples were transported via automobile within one day of sample collection to the environmental chemistry laboratory in the Michael Hooker Research Center at the University of North Carolina at Chapel Hill in a cooler containing ice-packs, and immediately refrigerated at 4°C (temperature was evaluated weekly for quality control) and extracted within one week of receipt.

Samples were first filtered to 0.45µm, to remove suspended solids that would interfere with subsequent chlorine residual testing and pH analysis, using 47mm Whatman nylon filters (Fisher Scientific Pittsburgh, PA), and placed back into the rinsed sample collection bottles. The suspended solids that were removed during filtration were not analyzed for BACs that may have associated with the NOM. After filtration, aliquots of each sample were tested for free and total chlorine residuals with a HACH test kit pocket colorimeter using HACH permachem DPD free and total chlorine reagents. The residual chlorine in the samples was then quenched with excess ascorbic acid, 25mg/L, regardless of the residual to ensure uniform conditions between samples (125g/L stock solution of ascorbic acid in LGW prepared fresh for each sample set) to stabilize samples. Additionally, to ensure uniform extraction conditions, the pH was adjusted to approximately pH 6 using 2% formic acid in LGW (Ye, 2005) as described in the standard operating procedure (Appendix 2). Samples were processed and extracted within one week of sample collection.

Solid Phase Extraction (SPE)

Neat pharmaceutical standards were used to prepare stock solutions at a target concentration of 1000mg/L in HPLC grade methanol. Stock pharmaceutical standards were stored in a freezer and used within five months of preparation. Table 2.2 shows the concentration of the pharmaceutical stocks that were used for the duration of the entire occurrence study. A 22 BAC mixture (1° dilution) was prepared at a target concentration of 20mg/L in HPLC grade methanol by combining 200µL of each pharmaceutical stock and diluting to 10mL (Table 2.2). This 22 BAC 1° dilution was stored in the freezer for use

within five months, and was used to prepare a 2° dilution on the date of sample extraction at a target concentration of 0.5mg/L by spiking 250µL of the pharmaceutical mixture into 10mL of HPLC grade methanol (Table 2.2). This 2° dilution BAC mixture was used to spike into the 250mL sample aliquots to create a standard addition curve for quantifying the BACs in samples. The concentrations of each BAC in the various standard solutions is shown in Table 2.3

All standards and extracting solutions used for sample extraction were prepared one day in advance including a 0.25g/L Na_2EDTA stock solution in LGW and a 0.1% formic acid in methanol solution used for sample elution off the solid phase employed for extraction. On the date of extraction a dilution of the internal standard, simeton, was prepared at 1.25mg/L in HPLC grade methanol. A standard containing both surrogate standards, meclocycline and d_3-caffeine, was prepared at a target concentration of 2mg/L in HPLC grade methanol.

Extraction of the BAC analytes from pH-adjusted and 0.45µm-filtered aqueous samples was achieved through the use of solid phase extraction (SPE) using a Supelco visiprep (Bellefonte, PA) extraction manifold. The Waters (Milford, MA) Oasis hydrophilic-lipophilic balance (HLB) 3cc (60mg) SPE cartridges (Lot:W3156J4) were pre-conditioned with 6mL methanol followed by 1mL 0.1%formic acid in methanol and then 8mL LGW (Ye, 2005).In order to quantify the concentration of the target analytes using the method of standard addition, collected water samples were split into six 250mL sample aliquots so as to present two unspiked samples (US) and four calibration samples (Cal) (Table 2.3).

Table 2.2 BAC Solutions used for Analytical Calibration

BAC	Stock (mg/L)	(1° dilution) (mg/L)	(2° dilution) (mg/L)
17-α-ethinyl estradiol	1046	21	0.52
Acetaminophen	1016	20	0.51
Atenolol	1034	21	0.52
Atrazine	1055	21	0.53
Bezafibrate	1023	20	0.51
Caffeine	1020	20	0.51
Carbamazepine	1034	21	0.52
Clofibric acid	1006	20	0.50
DEET	971	19	0.49
Diazepam	1000	20	0.50
Diclofenac	1026	21	0.51
Erythromycin-H_2O	1020	20	0.51
Estrone	1028	21	0.51
Fenoprop	1008	20	0.50
Gemfibrozil	1017	20	0.51
Ibuprofen	1020	20	0.51
Meprobamate	1000	20	0.50
Sucralose	1435	29	0.72
Sulfamethoxazole	1033	21	0.52
TCEP	1079	22	0.54
Tetracycline	1008	20	0.50
Trimethoprim	1022	20	0.51

Table 2.3 Concentration of Standard Addition Levels used for Quantification

BAC	Raw Water				Finished Water			
	Cal 1	Cal 2	Cal 3	Cal 4	Cal 1	Cal 2	Cal 3	Cal 4
		(ng/L)				(ng/L)		
17-α-ethinyl estradiol	5.2	10	52	105	2.1	5.2	10	21
Acetaminophen	5.1	10	51	102	2.0	5.1	10	20
Atenolol	5.2	10	52	103	2.1	5.2	10	21
Atrazine	5.3	11	53	106	2.1	5.3	11	21
Bezafibrate	5.1	10	51	102	2.0	5.1	10	20
Caffeine	5.1	10	51	102	2.0	5.1	10	20
Carbamazepine	5.2	10	52	103	2.1	5.2	10	21
Clofibric acid	5.0	10	50	101	2.0	5.0	10	20
DEET	4.9	10	49	97	1.9	4.9	10	19
Diclofenac	5.1	10	51	103	2.1	5.1	10	21
Erythromycin-H_2O	5.1	10	51	102	2.0	5.1	10	20
Diazepam	5.0	10	50	100	2.0	5.0	10	20
Estrone	5.1	10	51	103	2.1	5.1	10	21
Fenoprop	5.0	10	50	101	2.0	5.0	10	20
Gemfibrozil	5.1	10	51	102	2.0	5.1	10	20
Ibuprofen	5.1	10	51	102	2.0	5.1	10	20
Sulfamethoxazole	5.2	10	52	103	2.1	5.2	10	21
Tetracycline	5.0	10	50	101	2.0	5.0	10	20
Trimethoprim	5.1	10	51	102	2.0	5.1	10	20
Meprobamate	5.0	10	50	100	2.0	5.0	10	20
Sucralose	7.2	14	72	144	2.9	7.2	14	29
TCEP	5.4	11	54	108	2.2	5.4	11	22

In order to chelate the metal ions in the samples and prevent them from binding to active sites on the solid phase cartridge which would lower analyte retention and recovery, 1mL of the Na_2EDTA stock solution was added to each sample aliqout. In order to assess percent recovery of the extraction, 25µL of the surrogate standard mixture (meclocycline and *d₃*-caffeine) was added using a micropipette under the surface of the liquid to each 250mL graduated cylinder-measured sample aliquot which was then capped and inverted three times

to mix. After connecting the sample aliquots contained in 250mL amber glass bottles to the SPE cartridges through Teflon tubing, the samples were extracted at a flow rate of approximately 5mL/min. After rinsing the sample aliquot bottles, lines, and cartridges with LGW just before the last part of the sample was pulled through the Teflon tubing, the cartridges were dried under vacuum (18 Hg) for five minutes to remove excess water. Sample elution was performed using 8mL of 0.1% formic acid in methanol by gravity in approximately 20 minutes, and sample extracts were collected in 10mL silanized clear glass conical vials. Samples were then concentrated under ultra high purity (UHP) nitrogen (National Welders, Morrisville, NC) using a Pierce (Rockford, IL) Reacti-Vap Model 18770 to a volume of approximately 50μL on a VWR Scientifics Products (West Chester, PA) Standards Heatblock, at ~45°C for approximately two hours. Samples were then reconstituted to a target concentration of 250μL using 9/1 LGW/methanol and a glass syringe was used to add 10μL of the 1.25mg/L simeton internal standard solution. The internal standard, which was chosen because it is not found in the environment, accounts for variability between sample volume injection during instrumental analysis, and the area of each analyte is normalized to this standard. Sample extracts were then vortexed using a Thermolyne type 16700 mixer (Dubuque, IA), and then filtered through 0.45μm Laboratory Supply Distributors (Millville, NJ) syringe tip filters into 250μL glass inserts in liquid chromatography (LC) autosampler vials. Sample extracts were stored in the freezer up to a week until LC-MS/MS analysis.

Analytical Methods

The MS was tuned with a Varian, Inc. (Santa Clara, CA) PPG tuning solution prior to occurrence sampling. The PPG solution was infused into the MS using a Harvard Apparatus (Holliston, MA) Syringe Pump at 20µg/L. First, the detector was optimized using the instrument software followed by tuning of both quadrupole 1 (Q1) and quandrupole 3 (Q3) in both (+/-) electrospray ionization (ESI). This process ensures that the detector is operating at its optimum voltage for signal detection and that the masses detected are accurate. Analysis of sample extracts was completed using a Varian (Walnut Creek, CA) ProStar solvent delivery module (Model 210) which used a gradient binary mobile phase system comprised of 100% acetonitrile (B), and 0.1% formic acid in LGW (A). Table 2.4 shows the gradient program used for both positive electrospray ionization (+ESI) and negative electrospray ionization (-ESI) (Ye, 2005). Both mobile phases were filtered through 0.2µm Whatman filters from Fisher Scientific (Pittsburgh, PA) before use. The mobile phases were pumped through a Metachem Technologies, Inc. Degassit Unit (Torrance, CA), in order to remove any dissolved gases. The flow rate of the mobile phase was 0.2mL/min. A Varian ProStar autosampler (Model 430) (Walnut Creek, CA) injected 20µL onto a Varian C18 guard column (3cm x 2mm, 3µm) (Walnut Creek, CA) and a Varian (Torrance, CA) Pursuit C-18 analytical column (15cm x 2mm, 3µm) was used interfaced to a Varian (Walnut Creek, CA) 1200L triple quandrupole mass spectrometer. Varian MSWS Software Version 6.8 was used for data analysis. Liquid nitrogen (99.99%) was used as the drying gas, UHP argon the collision gas (CID) in positive ionization mode (+ESI), and zero grade air was used as the CID gas in negative ionization mode (-ESI). Table 2.5 shows the mass spectrometer parameters used in the analytical methods (Ye, 2005).

Table 2.4 Mobile Phase Gradient used for LC-(+/-)-ESI-MS/MS (flow rate=0.2mL/min)

Time (min)	%A (0.1% Formic Acid in LGW)	%B (100% Acetonitrile)
0	90	10
2	90	10
25	10	90
27	10	90
28	0	100
38	0	100
47	90	10
55	90	10

Table 2.5 Source Dependent Mass Spectrometer Parameters

Parameter	+ESI	-ESI
CID gas Pressure (mTorr)	1.5	1.5
Detector (V)	1320	-1320
Drying gas Temperature (°C)	300	200
Spray Chamber Housing (°C)	50	50
Spray Shield (V)	600	-600
Nebulizing gas Pressure (psi)	55	55
Drying gas Pressure (psi)	18	18
Needle (V)	5000	4500

Mass spectrometry acquisition data for target analytes used in the analysis is shown in Tables 2.6-2.8. The ion transitions and collision energies shown in Tables 2.6-2.8 were determined by infusing individual 1mg/L standards of each target BAC in 9/1 LGW/MeOH in the Varian 1200L MS (Palo Alto, CA) using a Harvard Apparatus syringe pump (Holliston, MA). The molecular ion of the BAC was targeted in Q2 and as the collision gas increased from 0-50V the characteristic breakdown ions were determined. The target analytes were broken up into several groups of compounds that were analyzed in separate sample injections in order to maximize sensitivity and increase analyte resolution. Although

the same chromatography is used the MS is programmed using instrument software to target different ion transitions in each method. To this end, target analytes will not co-elute or lose sensitivity due to increasing the target analyte transitions. As a result, in order to analyze all BACs a total of three injections for each sample extract were made. It can be seen in these tables that the presence of both a major and minor ion were used in the quantification and confirmation of an analyte, respectively. These major and minor ions are characteristic fragments (or daughter ions) of the parent compound under the utilized analytical conditions and confirmation is achieved by calculating the ratio of the daughter ions in a sample. Table 2.9 summarizes the acquisition data for the internal standard and surrogate standard. Optimized parameters for each compound were obtained through analysis of individual standards by directly infusing the standards into the mass spectrometer using a Harvard Apparatus syringe pump (Holliston, MA) and optimizing instrumental conditions.   The standard operating procedure (SOP) is presented in Appendix 2.

Before the analysis of extracted drinking water samples several quality control measures were taken. Several injections of the solvent, 9/1 LGW/methanol, were analyzed to ensure that the solvent and instrument were free of contamination. In addition, the sensitivity of the instrument was verified using a BAC mixture standard and caffeine. Finally, the method for each group was run with the BAC mixture at 50µg/L before sample analysis in order to evaluate retention times and analytical sensitivity.

Percent recovery of the target BAC analytes in the samples was evaluated throughout the extraction procedure by determining the instrument response of a surrogate standard. That is, the area of the surrogate standard was determined in each sample to determine the variability between sample extracts.

Table 2.6 Optimized Acquisition Data for Group 1 (+ESI) Compounds

BAC	Retention Time major (minor) minutes	Parent Ion m/z	Major Ion (Collision Energy) m/z(volts)	Minor Ion (Collision Energy) m/z(volts)
Acetaminophen	5.3 (N/A)	151.9	110.0 (11.5)	N/A
Caffeine	7.9 (7.97)	195.1	137.9 (11.5)	109.9 (16.5)
Tetracycline	10.5 (10.95)	445.3	427.0.3 (8)	410.0 (16)
Meprobamate	13.4 (N/A)	219.1	158.0 (6.5)	N/A
TCEP	18.2 (18.27)	285.0	160.8 (7.5)	222.8 (6.0)
Erythromycin-H_2O	17.3 (17.42)	734.6	158.0 (19)	82.9 (27)

Table 2.7 Optimized Acquisition Data for Group 2 (+ESI) Compounds

BAC	Retention Time major (minor) minutes	Parent Ion m/z	Major Ion (Collision Energy) m/z (volts)	Minor Ion (Collision Energy) m/z (volts)
Atenolol	4.3 (4.2)	267.2	144.9 (17)	189.9 (10.5)
Trimethoprim	9.2 (9.2)	291	229.9 (20.5)	122.9 (20.5)
Sulfamethoxazole	13.6 (13.6)	253.9	155.9 (14)	107.9 (18.5)
Atrazine	18.3 (18.7)	216	173.9 (10.5)	95.8 (21)
Carbamazepine	16.6 (16.6)	237	193.9 (12)	192.0 (18.5)
DEET	16.6 (18.6)	192.2	118.9 (14)	90.8 (26)
Diazepam	20.4 (20.4)	285	192.9 (27)	153.9 (24)
Bezafibrate	20.8 (20.8)	362.2	138.9 (17)	120.9 (19.5)

Table 2.8 Optimized Acquisition Data for Group 3 (–ESI) Compounds

BAC	Retention Time major (minor) minutes	Parent Ion *m/z*	Major Ion (Collision Energy) *m/z*(volts)	Minor Ion (Collision Energy) *m/z*(volts)
Ibuprofen	24.30/Not Seen	205.0	160.7 (6)	N/A
Clofibric Acid	19.90/Not Seen	213.1	126.7 (10.5)	N/A
Gemfibrozil	26.4 (26.5)	249.3	120.8 (8.5)	121.8 (10)
Fenoprop	23. 0 (23.1)	268.7	196.6 (8)	160.4 (26.5)
Diclofenac	23.7(23.6)	294.0	249.8 (8)	250.8 (8)

Table 2.9 Optimized Acquisition Data for Internal and Surrogate Standards

Standard	Retention Time major (minor) minutes	Parent Ion *m/z*	Major Ion (Collision Energy) *m/z*(volts)	Minor Ion (Collision Energy) *m/z*(volts)
Simeton (Internal)	9.8 (N/A)	198.0	123.9 (16)	Not observed
Caffeine-d^3 (Surrogate)	7.9 (8.0)	198.1	140.8 (12)	112.8 (16.5)
Meclocycline (Surrogate)	9.3 (9.8)	477.0	459.9 (14.5)	234.4 (34.5)

The instrument detection limit (IDL) is the lowest concentration that the instrument can detect for a particular analyte with a defined level of confidence above noise. For this study, this limit was set so that the detector signal (S) compared to noise (N) was at least 30. By analyzing a 50µg/L BAC standard mixture in 9/1 LGW/MeOH using the appropriate method, the S:N value was determined and then, assuming linearity from zero concentration to 50µg/ L, the concentration that would give a S:N of 30 could then be calculated (Table 2.10). The IDL was used to determine the lowest concentration of each analyte that could be

detected in the water samples using the analytical method which employs a concentration factor of 1,000 (and assuming 100% recovery). Therefore, this information was used to generate the calibration points used for sample quantification (Table 2.3).

Table 2.10 Instrument Detection Limit for Targeted BACs

BAC	Instrument Calculated S:N at 50µg/L in 9/1 LGW/MeOH	Predicted Concentration (µg/L) giving S:N=30
Acetaminophen	117	13
Atenolol	242	6.2
Atrazine	1431	1.1
Bezafibrate	476	3.2
Caffeine	107	14
Carbamazepine	1153	1.3
Clofibric acid	38	40
DEET	447	3.4
Diazepam	354	4.2
Diclofenac	256	5.9
Erythyromycin	7130	0.21
Fenoprop	147	10
Gemfibrozil	18	83
Ibuprofen	1	1500
Meprobamate	150	10
Sulfamethoxazole	923	1.6
Tetracycline	95	16
TCEP	223	6.7
Trimethoprim	472	3.2

The limit of quantification (LOQ) is reported as the lowest detectable non-zero response from the extracted samples analyzed in the calibration curves shown in Table 2.3. The values are reported as the concentration in the appropriate calibration sample. The LOQs for the BACs are shown in Table 2.11. Although data was obtained for the BACs

analyzed in (-)-ESI in a 50µg/L standard in 9/1 LGW/MeOH, instrument response was limited in sample extracts due to lack of instrument sensitivity in this mode of ionization. Therefore, the analytes requiring ionization in (-)-ESI were not searched for in subsequent occurrence sampling. It was later determined that instrument sensitivity in (-)-ESI could be increased by using higher grade methanol for sample extraction.

Table 2.11 Limit of Quantification for Targeted BACs in North Carolina Drinking Water

BAC	In raw water (ng/L)	In finished water (ng/L)
Acetaminophen	5.1	5.1
Atenolol	5.2	2.1
Atrazine	5.3	2.1
Bezafibrate	5.1	5.1
Caffeine	5.1	2.0
Carbamazepine	5.2	2.1
Clofibric acid	Not seen	Not seen
DEET	5.0	1.9
Diazepam	5.0	5.0
Diclofenac	Not seen	Not seen
Erythyromycin	Not seen	Not seen
Fenoprop	Not seen	Not seen
Gemfibrozil	Not seen	Not seen
Ibuprofen	Not seen	Not seen
Meprobamate	Not seen	Not seen
Sulfamethoxazole	5.2	5.2
Tetracycline	5.2	5.2
TCEP	54	5.4
Trimethoprim	5.1	2.0

3. CHLORINATION OF BACs - INCORPORATION OF CHLORINE ONTO PARENT MOLECULE

3.1 Introduction

Incomplete metabolism of BACs in the body, incomplete removal through wastewater treatment plants, and subsequent persistence in the environment allow for BACs to enter drinking water treatment plants. What happens to these chemicals during drinking water treatment is yet another challenge for which little information is available. During drinking water treatment, chemical processes such as disinfection using hypochlorite can transform the structure of the parent BAC molecule. Several studies of the chlorination of BACs have shown that incorporation of chlorine onto regions with electron density are the most likely mechanism of reaction (Gould and Richards, 1984; Miyamoto et al., 1997; Dodd and Huang, 2004; Moriyama et al., 2004; Pinkston and Sedlak, 2004; Glassmeyer and Shoemaker, 2005; Ye, 2005; Bedner and MacCrehan, 2006; Nakamura et al., 2006; Dodd and Huang, 2007; Brix et al., 2008; Kotcharaksa, 2008; DellaGreca et al., 2009; Lee and Gunten, 2009; Quintana et al., 2010; Wulfeck-Kleier et al., 2010; Krkošek et al., 2011; Soh et al., 2011; Wang et al., 2011), as opposed to complete removal (or mineralization). Understanding the fate of BACs during drinking treatment is important in order to evaluate whether biochemically active species remain undetected in consumers' drinking water. In the experiments that follow the extent to which chlorine can affect the structure of targeted

BACs was determined using measurements of TOX and the fate of the parent compound determined using tandem mass spectrometry.

3.2 Chlorination Experiments

TOX Analysis

Previous studies have shown the incorporation of chlorine onto BACs without significant degradation of the parent molecule and, therefore, measuring the concentration of incorporated chlorine is useful to determine the extent of substitution. This can be achieved using TOX analysis where the carbon which has adsorbed the organic halogens in a sample is pyrolyzed and the halogens measured using a coulometric detector. Therefore, the objective of these experiments was to measure the increase in organic halogens in the sample due to chlorination and relate this to the consumption of both free chlorine and parent BAC. The molar ratio of chlorine to BAC is shown in Table 3.1. Preliminary experiments on all targeted BACs (n = 1) were designed to determine relative reactivity. Subsequent targeted reactions of four BACs that were highly reactive with free chlorine used triplicate analysis to allow for statistical analysis and verification of results.

Table 3.1 Studied BACs and Experimental Conditions (n = 1 unless noted)

BAC	Molar Ratio of Chlorine to BAC
17-α-ethinyl estradiol	69.4
Acetaminophen (n=3)	45
Atenolol	69.7
Atrazine	69.9
Bezafibrate	110
Caffeine	62.2
Carbamazepine	66.5
Clofibric Acid	38
DEET	64.9
Diazepam	93.8
Diclofenac	54.4
Erythromycin-hydrate	209
Estrone	77.1
Fenoprop (n=3)	75.2
Gemfibrozil	74.4
Ibuprofen	51.5
Meprobamate	71.9
Sucralose	136
Sulfamethoxazole	80.8
TCEP	61.2
Tetracycline (n=3)	114
Trimethoprim (n=3)	89.1

Case Study on the Reactivity of Tetracycline

The reactivity between tetracycline and free chlorine was studied in greater detail due to its high incorporation of chlorine during TOX experiments and the limited information available about its fate during chlorination. Aqueous solutions of tetracycline were prepared in LGW at a target concentration of 22μM and reacted with free chlorine at a molar ratio of free chlorine to tetracycline ([HOCl]:[TC]) ranging from 1:1 to 20:1 in order to study the transformation of tetracycline and detect transformation products.   A high initial

concentration of tetracycline was used in order to aid in the detection of transformation products. The higher doses of [HOCl]:[TC] were sufficient to ensure that the reaction between free chlorine and tetracycline went to completion. Previous studies have shown that tetracycline compounds are very reactive with free chlorine and have an estimated half life of 3 s at pH 7 (Ye, 2005).

3.3 Results and Discussion

3.3.1 Chlorine Demand

The results of chlorine demand are shown in Table 3.2. The net chlorine demand is the chlorine demand of the BAC solutions with chlorine minus the average chlorine demand of the chlorinated LGW (control sample). The net chlorine demand for fenoprop is negative indicating that the free chlorine residual for the sample is higher than the dose of chlorine that was applied to the sample. This may be the result of fenoprop, a tri-chlorinated compound, losing some of its chlorine substituents during the reaction or may be due to the limitations of accuracy of the HACH test kits. This would be subsequently confirmed by TOX analysis. The results of multiple chlorinations of LGW reveal that the average value for chlorine demand by LGW was 3.8 ± 1.58 mg/L as Cl_2 with a coefficient of variation of 0.42 (n = 9). This high value is the result of the chlorine demand of the glassware used in the experiments due to organics that might have remained on the glass surface. For the qualitative nature of the TOX experiments this chlorine demand of the glassware did not interfere with results, and the chlorine dose was applied in excess to ensure a residual was present. Chlorine demand of the BAC solution shows quantitatively the reactivity of each

BAC in contact with chlorine under the experimental conditions described above and can be used to calculate the number of moles of chlorine that have reacted during chlorination experiments.

Table 3.2 Net Free Chlorine Demand of Chlorinated$^\wedge$ BACs (n = 1 unless noted)

BAC		Net Free Chlorine Demand*	
Name	Initial Concentration (µmoles)	(mg/L as Cl_2)	(µmoles Cl_2)
17-α-ethinyl estradiol	0.475	1.5	2.1
Acetaminophen (n=3)	0.851	3.8 ± 1.5	3.4 ± 1.4
Atenolol	0.473	1.7	2.4
Atrazine	0.472	<0.2	NA
Bezafibrate	0.3	2.8	3.9
Caffeine	0.53	3.9	5.5
Carbamazepine	0.496	0.9	1.3
Clofibric Acid	0.867	1.5	2.1
Diazepam	0.351	1.3	1.8
Diclofenac	0.606	2.4	3.4
Erythromycin-hydrate	0.158	1.7	2.4
Estrone	0.428	1.9	2.7
Fenoprop (n=3)	0.51	-0.2	-0.2
Gemfibrozil	0.443	1.2	1.7
Ibuprofen	0.649	2.4	3.4
Meprobamate	0.458	<0.2	NA
Sucralose	0.242	0.4	0.56
Sulfamethoxazole	0.408	4.3	6.1
TCEP	0.539	<0.2	NA
Tetracycline (n=3)	0.245	0.9 ± 0.6	1.3 ± 0.78
Trimethoprim (n=3)	0.589	2.4 ± 1.0	3.4 ± 2.1

* Net chlorine demand=chlorine demand of chlorinated BAC sample minus chlorine demand of chlorinated LGW sample
^ Chlorine dose was ~20mg/L NaOCl as Cl_2
Free chlorine demand of LGW 3.8 ± 1.58mg/L as Cl_2
DEET=*N,N*-diethyl-*meta*-toluamide
TCEP=tris(2-carboxyethyl)phosphine
NA=not applicable

3.3.2 Reactivity of Parent BACs with Free Chlorine

TOX Analysis

The results from analysis of breakthrough of analytes onto a second column are shown in Table 3.3. As described in Chapter 2 although the Standard Method 5320 (APHA, 1999) calls for the use of two carbon columns, the percentage breakthrough was determined to be less than 5% and therefore only one carbon column was used in subsequent experiments to increase productivity.

Table 3.3 Breakthrough Determination of Select Chlorinated BACs during TOX Analysis

Chlorinated Sample[*]	Raw Data[#] (µg Cl)	Net Data[@] (µg Cl)	TOX Concentration[^] (µg Cl/L)	Breakthrough (%)
Tetracycline Column 1	17.005	16.744	3.4×10^2	NA
Tetracycline Column 2	1.047	0.816	16	4.6
Acetaminophen Column 1	10.243	10.196	2.0×10^2	NA
Acetaminophen Column 2	0.435	0.388	7.8	3.7
Fenoprop Column 1	22.172	22.124	440	NA
Fenoprop Column 2	0.486	0.439	8.6	1.9
Trimethoprim Column 1	3.292	3.244	65	NA
Trimethoprim Column 2	0.192	0.145	2.9	4.3

*Target chlorine dose was 20mg/L as Cl_2 and target BAC dose was 1mg/L
#For a 50mL sample
@After subtraction of LGW/NO_3^- carbon column rinse control sample
^Net data/sample volume

The results from the TOX analysis of the chlorinated BACs are shown in Table 3.4 and the TOX generated, which is a measure of the amount of chlorine incorporation into the parent BAC molecule during the chlorine reaction, is given on a weight and molar basis. The molar ratio of the generated TOX to starting BAC indicates the extent to which a BAC molecule was chlorinated. For example, in the case of tetracycline a molar ratio of 3 indicates that three atoms of chlorine may have been added to each molecule of tetracycline. For trimethoprim the molar ratio of 0.4 may indicate that one atom of chlorine may be incorporated onto approximately every two molecules of trimethoprim.

The results from TOX analysis shown in Table 3.4 and chlorine demand shown in Table 3.2 can be compared to determine the extent to which loss of chlorine in solution is accounted for by incorporation into the BAC molecule. Among the most reactive BACs, relatively high values of net chlorine demand were recorded. Conversely, results for a BAC that did not produce measureable TOX, such as atrazine, and where the net chlorine demand is very low confirm that no chlorine reaction had occurred. For chemicals such as caffeine and bezafibrate, where there is a chlorine demand and no TOX generated it is possible that a chlorine reaction involving hydrolysis or oxidation rather than chlorine substitution, occurred. Additionally, the net chlorine demand of BAC reactions is compared to the chlorine incorporation into the chemical to help evaluate the extent to which chlorine consumption is accounted for by formation of organo-chlorine products.

Table 3.4 Results from TOX Analysis of Chlorinated BACs (n = 1 unless noted)

BAC	TOX generated (μg/L Cl)	(μmoles Cl)[*]	Molar ratio of TOX to BAC[**]
17-α-ethinyl estradiol	77	0.22	0.5
Acetaminophen (n=3)	181 ± 18	0.511 ± 0.051	0.7 ± 0.1
Atenolol	33	0.092	0.2
Atrazine	<10	NA	NA
Bezafibrate	<10	NA	NA
Caffeine	<10	NA	NA
Carbamazepine	43	0.12	0.2
Clofibric Acid	<10	NA	NA
DEET	<10	NA	NA
Diazepam	33	0.094	0.3
Diclofenac	<10	NA	NA
Erythromycin-hydrate	40	0.11	0.7
Estrone	74	0.21	0.5
Fenoprop (n=3)	8 ± 7	0.021 ± 0.019	0.05 ± 0.1
Gemfibrozil	26	0.073	0.2
Ibuprofen	<10	NA	NA
Meprobamate	<10	NA	NA
Sucralose	36	0.1	0.4
Sulfamethoxazole	69	0.19	0.5
TCEP	<10	NA	NA
Tetracycline (n=3)	334 ± 25	0.940 ± 0.071	3 ± 0.3
Trimethoprim (n=3)	53 ± 10	0.149 ± 0.29	0.4 ± 0.1

*Based on a 100mL reaction solution
** See Table 2.2 for initial molar concentration of BACs; calculations assume that 100% of the parent BAC was consumed during the reaction
DEET=*N,N*-diethyl-*meta*-toluamide
TCEP=tris(2-carboxyethyl)phosphine
NA=not applicable

BACs Highly Reactive with Free Chlorine

From the results shown in Table 3.4 of the TOX generated during chlorination experiments it can be seen that tetracycline, trimethoprim, acetaminophen, *17-α-ethinyl estradiol*, sulfamethoxazole, and estrone (Figure 3.1) were the most reactive BAC species with chlorine. These BACs have several characteristics in common: aromatic regions (extended π-conjugation), ring activating substituents (-OH,-NH$_2$), and available positions for electrophilic attack on the aromatic ring. In the literature, the reactivity of these compounds towards chlorine has been reported to be due to ring substitution with no significant degradation of the parent BAC molecule backbone. For example, the aqueous chlorination of acetaminophen (Bedner and MacCrehan, 2006) and *17-α-ethinyl estradiol* (Moriyama et al., 2004) have been studied and both mono- and di-chlorinated transformation products attributed to ring chlorination in the position *ortho-* to the phenol group were observed using mass spectrometry. Due to its similar structure, the chlorination of estrone is expected to occur on the aromatic ring in the *ortho-* position of the phenol substituent. Results from the current study (Table 3.4) show that acetaminophen, 17-α-ethinyl estradiol, and estrone incorporated 0.7, 0.4, and 0.4 moles of chlorine per mole of parent BAC, respectively. These compounds contain a phenol group and it can be concluded that mono-chlorination occurred to approximately half of the starting BAC material under the conditions used during this experiment. The incorporation of chlorine onto acetaminophen was slightly higher than that of estrone and 17-α-ethinyl estradiol and this may be due to the higher solubility of acetaminophen in water (Table 1.3) or due to the aliphatic portions on the steroid hormone structures. Studies have shown that the aqueous mono- and di- chlorination of trimethoprim is expected to occur at the 2,4-diamino-5-methylpyrimidine moiety (Dodd and Huang, 2007),

and the results from the current study show that for every two moles of trimethoprim one mole of chlorine was incorporated onto the molecule which agrees with Dodd and Huang (2007). Trimethoprim was observed to incorporate less chlorine than any of the phenol-containing compounds and this is most likely the result of the slower reaction rate between free chlorine and the parent compound which contains two amine groups and a highly substituted ring system. The reactivity of sulfamethoxazole has also been previously investigated and yields *N*-chloro-*p*-benzoquinoneimine (Dodd and Huang, 2004). The results from the current study show that for every two moles of sulfamethoxazole one mole of chlorine is incorporated which agrees with the work of Dodd and Huang (2004).

Tetracycline

Acetaminophen

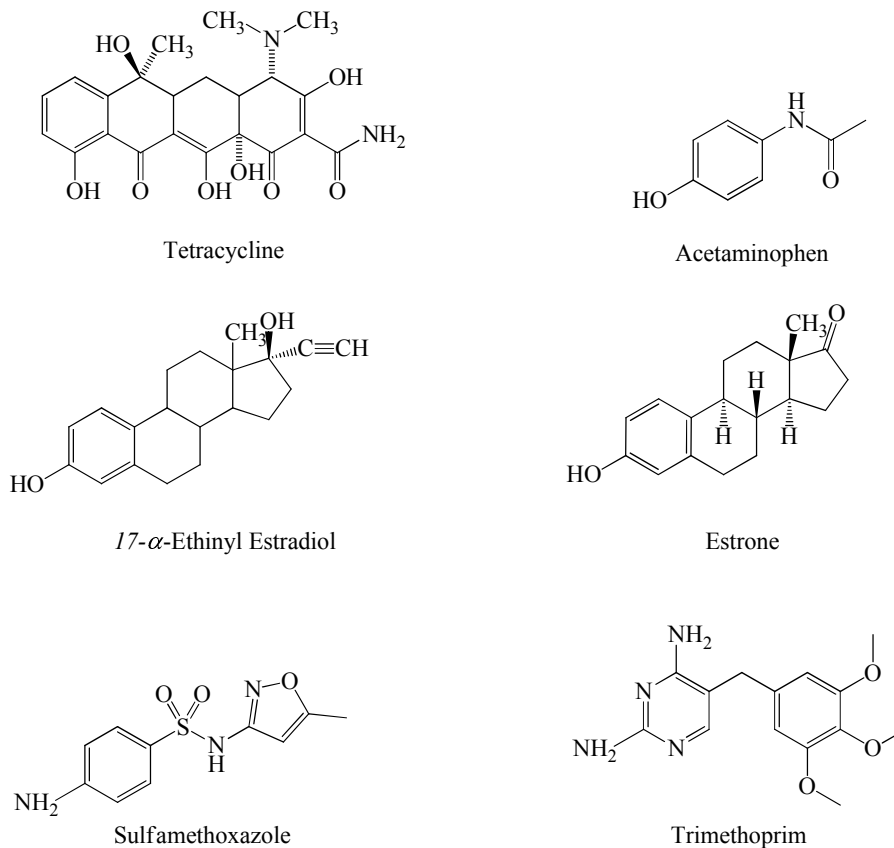*17-α*-Ethinyl Estradiol

Estrone

Sulfamethoxazole

Trimethoprim

Figure 3.1 Structures of BACs Studied which Showed the Greatest Degree of Chlorine Incorporation during Reaction with Free Chlorine

The most reactive BAC based on TOX analysis in this current study was tetracycline and limited information is available in the literature concerning its reactivity with free chlorine. One preliminary study on the aqueous chlorination of tetracycline showed the appearance of transformation products in the chromatogram using LC-MS in full scan mode, but no chlorinated products were identified (Ye, 2005). In another study tetracycline was found to react very rapidly with free chlorine, and LC-MS analysis showed that transformation products with Cl- and OH- substituents were formed, but no product structures were proposed (Wang et al., 2011).

The results from the current study show that for every mole of tetracycline three moles of chlorine are incorporated. This level of incorporation is significantly higher than that of any of the previously mentioned BACs, and based on the structure of tetracycline it can be anticipated this is due to its extended π-conjugation, phenol, and tertiary amine groups. The chlorination of tetracycline will be discussed in greater detail in a case study.

The high reactivity of these compounds is a cause for concern because the exposure to these compounds and/or their transformation products may be underestimated. These compounds may transform into chlorination by-products in WWTPs that use chlorine as a disinfectant, and these transformed BACs may enter receiving streams and elude detection. Another possible fate of these BACs is that they may transform during drinking water treatment and will not be detected using analytical methods that only target parent BACs. The result is that the reaction of chlorine with the parent molecule could be generating undetected biochemically active by-products leaving the potential impact on water quality significantly underestimated. An additional implication is that that the biological activity of

these chlorinated transformation products of these compounds remains not well understood. Additional studies should be undertaken to determine the bioactivity of these compounds after chlorination to determine their risk to the environment, aquatic organisms, and humans.

BACs Unreactive with Free Chlorine

The results from TOX analysis reveal that many of the BACs studied have very limited reactivity with free chlorine under the experimental conditions employed. The results from the current study agree with the available literature where caffeine (Glassmeyer and Shoemaker, 2005), ibuprofen (Pinkston and Sedlak, 2004; Quintana et al., 2010), clofibric acid, and bezafibrate (Quintana et al., 2010) were all observed not to react with chlorine. The chlorination of atrazine has been observed in the literature and it was observed that the use of the quenching agent, sodium sulfite, dechlorinates transformation products back into atrazine (Wulfeck-Kleier et al., 2010). During this study the quenching agent used was sodium sulfite and in future studies a different chemical should be used when evaluating reactivity. The pK_a values of the compounds with low reactivity towards free chlorine are typically <6.1 (see Table 1.3) and therefore these compounds exist in their deprotonated forms. The deprotonation of these compounds increases resonance across the BACs and stabilizes the molecular structure, minimizing reaction. While additional studies are needed to confirm the persistence of the parent BACs shown in Figure 3.2 during chlorination, there are several structural characteristics of the compounds that may help drive hypotheses on reactivity and guide future studies. Among all of the BACs studied, the two compounds without any regions of aromaticity (TCEP and meprobamate) were not reactive with free chlorine, which

confirms that chlorine reacts most easily in aqueous solutions with organics through electrophilic attack. Compounds with aliphatic regions lack sufficient electron density and, therefore, react less with free chlorine. Another characteristic prevalent among the least reactive compounds is the presence of chlorine on the parent BAC (fenoprop, atrazine, diclofenac, bezafibrate, and clofibric acid) which as a highly electronegative species, withdraws electron density from the aromatic ring and deactivates it from reacting with an electrophile. Additionally, another common characteristic in this group is highly substituted aromatic rings (fenoprop, caffeine, and atrazine) in which steric hindrance may prevent chlorine substitution from occurring. Another example of steric hindrance is seen in ibuprofen where the bulky aliphatic chain on the aromatic ring inhibits the substitution of chlorine.

The low reactivity of these compounds with free chlorine highlights a problem in the effective management of anthropogenic compounds. Many of these compounds have been found to be ubiquitous environmental contaminants and, therefore, more effective treatment technologies are needed in order to protect both drinking water consumers and the environment from chronic low level exposure. If these compounds are not effectively removed during drinking water treatment then their use should become more regulated in order to prevent consumers from being exposed to persistent contaminants.
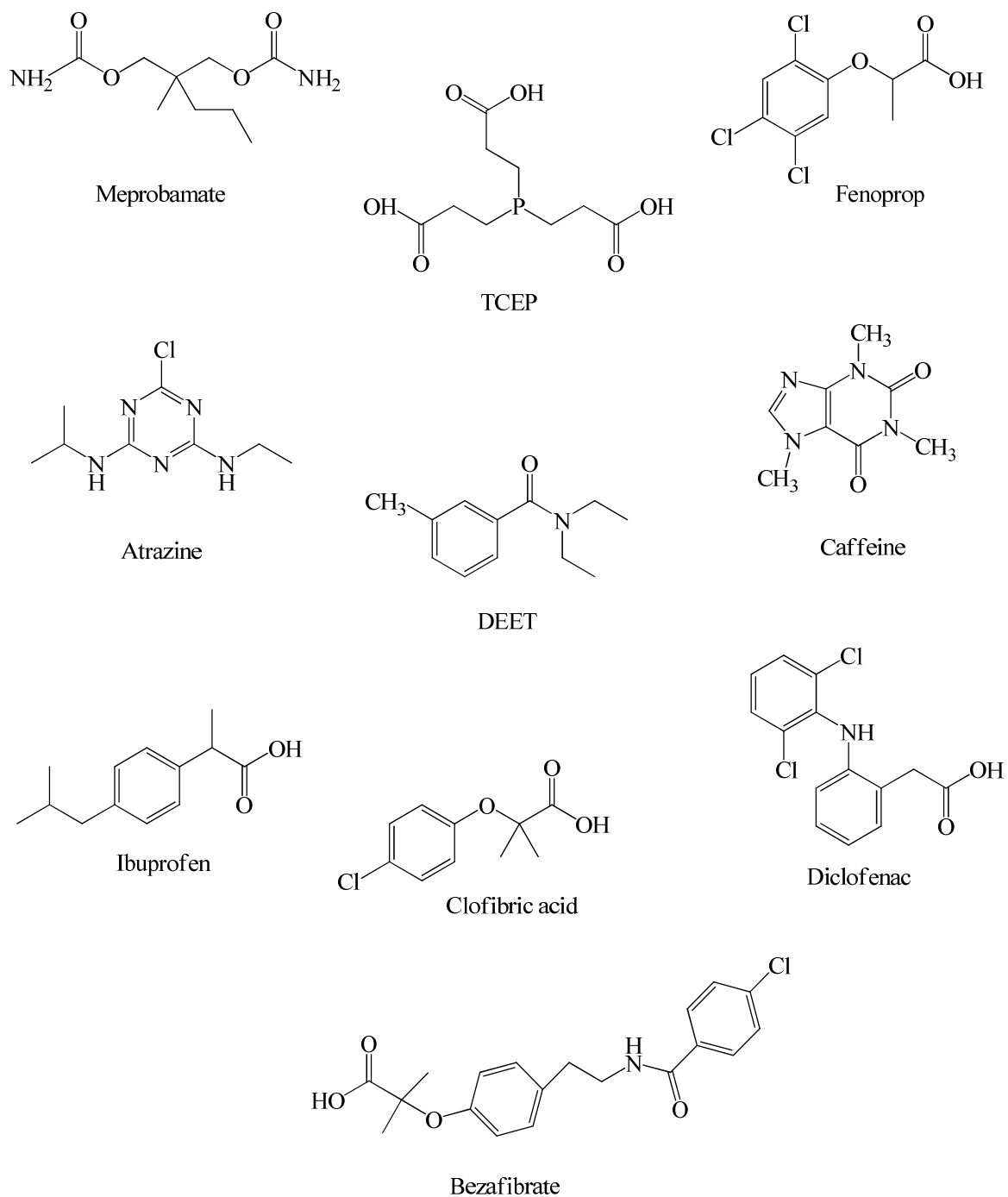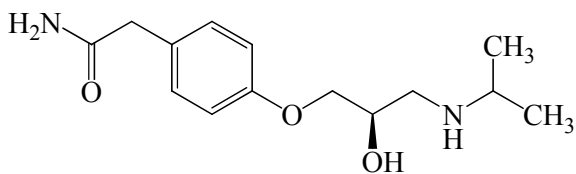
Figure 3.2 Structures of BACs Studied with the Lowest Degree of Chlorine Incorporation During Reaction with Free Chlorine
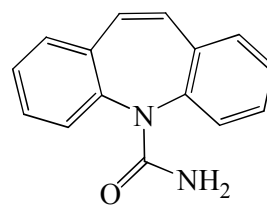
BACs Moderately Reactive with Free Chlorine

The results from TOX analysis reveal that several compounds have moderate reactivity with free chlorine (Figure 3.3) in reference to the least and most reactive BACs. Both erythromycin-hydrate and sucralose lack aromaticity, in which the lack of electron rich aromatic rings decreases the possibility for reaction with chlorine. While electrophilic aromatic substitution reactions are the most significant to occur between free chlorine and organic structures in water, other reactions such as addition and oxidation are also possible. In the case of erythromycin-hydrate it is possible that the tertiary amine group may react with chlorine. The TOX results from Table 3.4 show that if all of the erythromycin-hydrate was consumed during the reaction with free chlorine approximately 1 (0.7) mole of chlorine would be incorporated into each parent molecule. Therefore, it is possible that the chlorine is added to the tertiary amine group forming an *N*-chloro transformation product of erythromycin and because reactions with tertiary amines are less kinetically favorable (Abia et al., 1998) not all of the erythromycin-hydrate is chlorinated after the 2 hour contact time. The reaction kinetics of the chlorination of erythromycin-hydrate was observed to have a strong dependency on pH, with reaction proceeding slower when the molecule was in its protonated form (Ye, 2005) as it would be under the conditions employed in this experiment. Mono-chlorination of the amine of erythromycin-hydrate has been observed previously (Ye, 2005) and agrees with the current TOX study.

Both diazepam and carbamazepine contain several fused aromatic rings and although these regions of electron density are possible locations for electrophilic aromatic substitution of chlorine, electron withdrawing substituents (Cl- and amides) reduce the reactivity of these BACs. Carbamazepine has been observed to form un-chlorinated transformation by-products

when chlorinated (Kotcharaksa, 2008), and the minimal reactivity observed with TOX (Table 3.4) experiments may indicate that other products are also forming. Atenolol and gemfibrozil also contain aromatic rings where substitution of chlorine is the most likely mechanism of reaction with chlorine, but the reactivity of this ring is deactivated due to electronegative species (i.e. oxygen) in the alpha position to the aromatic ring. Additionally, the long aliphatic chains increase steric hindrance and minimize the availability of the ring for reaction. Mono- ring chlorination of gemfibrozil has been observed in reactions with free chlorine (Glassmeyer and Shoemaker, 2005; Krkošek et al., 2011) and an N-chlorinated product of atenolol has also been reported (DellaGreca et al., 2009). The results from the current study show that while chlorination of atenolol and gemfibrozil occurs, it is only to a small degree. In summary, although these BACs contain structures and functional groups that may react with chlorine, the moderate reactivity of these BACs is most likely the result of electron withdrawing functional groups and the result is slow reaction rates and only moderate reactivity with free chlorine.

Atenolol

Carbamazepine
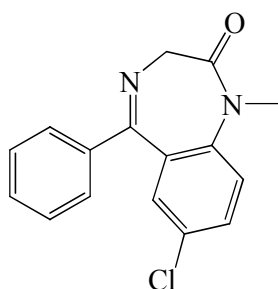
Diazepam

Sucralose

Erythromycin-hydrate
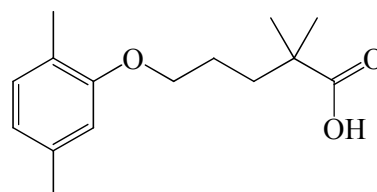
Gemfibrozil

Figure 3.3 Structures of BACs Studied with Moderate Chlorine Incorporation During Reaction with Free Chlorine

3.3.3 Removal of Parent BAC

MS/MS Analysis

In order to confirm the reactivity of the parent BAC during chlorination, infusion based tandem mass spectrometry (MS/MS) experiments were performed to monitor that parent BAC concentration. Ba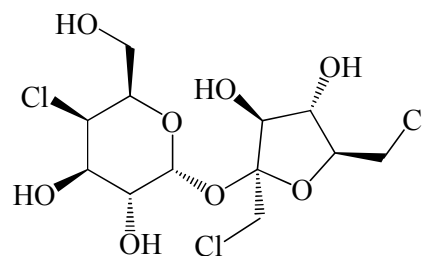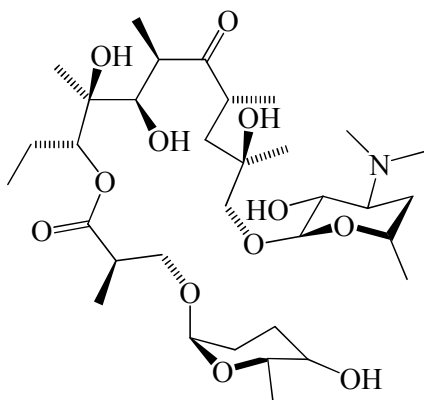sed on their observed reactivity with chlorine (see Table 3.4), two BACs were chosen to confirm the extent to which the parent compound had reacted using infusion-based tandem MS/MS. Table 3.5 shows the results of targeting the parent molecule and breakdown of the ions as well as their daughter ions in Q2 of the mass spectrometer before and after chlorination of the compound. The parent ion counts are a measure of the concentration of the parent molecule while the daughter ions are the fragments of the corresponding parent molecule used to confirm the identity of the parent compound. Each chemical has a characteristic fragmentation pattern based on its chemical properties and applied experimental conditions. Therefore, this "fingerprint" is often used to confirm the identity of a parent compound. This structural confirmation is completed by selecting two prominent daughter fragments. These two daughter ions are shown in Table 3.5 for the atenolol and tetracycline that have not undergone a chlorination reaction. The presence of the daughter ions confirms the identity of the parent compound. It can be seen from Table 3.5 that in both the chlorinated and unchlorinated atenolol samples the same two daughter fragments were detected. Additionally, the parent ion counts for atenolol show that in the unchlorinated and chlorinated samples, the concentration of atenolol did not change significantly. These results indicate that chlorination did not transform atenolol to a significant degree and these results are consistent with the findings from TOX and chlorine demand experiments.

Table 3.5 (+)-ESI-MS/MS Results of Targeting Parent BAC Before/After Chlorination

Sample Name	Concentration[*] (mg/L)	Parent ion counts	Daughter 1 *m/z*	Daughter 2 *m/z*
Atenolol unchlorinated	1.0	6679	145	190
Atenolol chlorinated A	0.97	6248	145	190
Atenolol chlorinated B	0.98	6330	145	190
Sample Name	**Concentration[*] (mg/L)**	**Parent ion counts**	**Daughter 1 *m/z***	**Daughter 2 *m/z***
Tetracycline unchlorinated	1.0	1276	428	410
Tetracycline chlorinated A	0.23	297	not seen	not seen
Tetracycline chlorinated B	0.12	153	not seen	not seen

*Concentration is based on the number of parent BAC ion counts

Table 3.5 also shows the results of analysis of the tetracycline after chlorination. The results for the unchlorinated tetracycline sample show the ion counts and characteristic daughter fragments of the parent molecule which were absent in the chlorinated samples. The absence of the characteristic daughter ions of tetracycline and the low ion counts for tetracycline indicates that the concentration of tetracycline is significantly reduced in the chlorinated samples, approximately 82% consumed. Hence the formation of TOX can be assumed to be as a result of complete chlorination if matched by the chlorine demand and these results are shown in Table 3.7. This result is consistent with the findings from the TOX and chlorine demand experiments which showed a high degree of incorporation of chlorine into the tetracycline molecule. The results show the benefit of combining TOX and MS/MS measurements for determining the extent of chemical reactions with chlorine.

3.3.4 Case Study of Tetracycline Reactivity with Free Chlorine

TOX analysis and preliminary MS/MS infusion analysis revealed that tetracycline (Figure 3.1) was highly reactive with free chlorine. In the case of TOX, a high level of incorporation of chlorine into the parent tetracycline molecule was revealed (36% of the applied chlorine dose incorporated). In summary, this was indicated by a significant incorporation of chlorine into the parent molecule (3 chlorine atoms per tetracycline molecule), an 82% decrease in tetracycline concentration, and the absence of the daughter ions of tetracycline during collision induced dissociation (CID) experiments in quadrupole 2 (Q2). Confirmation of these preliminary results and elucidation of reaction products is now described.

Impact of Free Chlorine Dose

Figure 3.4 shows results from the reactivity study between free chlorine and tetracycline where a single contact time was used for varying doses of chlorine (shown in Table 3.6) to determine the impact on the transformation of tetracycline. In this figure the free chlorine residual in the chlorinated samples containing tetracycline is superimposed with the tetracycline ion counts from MS/MS direct infusion analysis.

Table 3.6 Concentration of HOCl Used during Experiment and Corresponding Molar Ratio

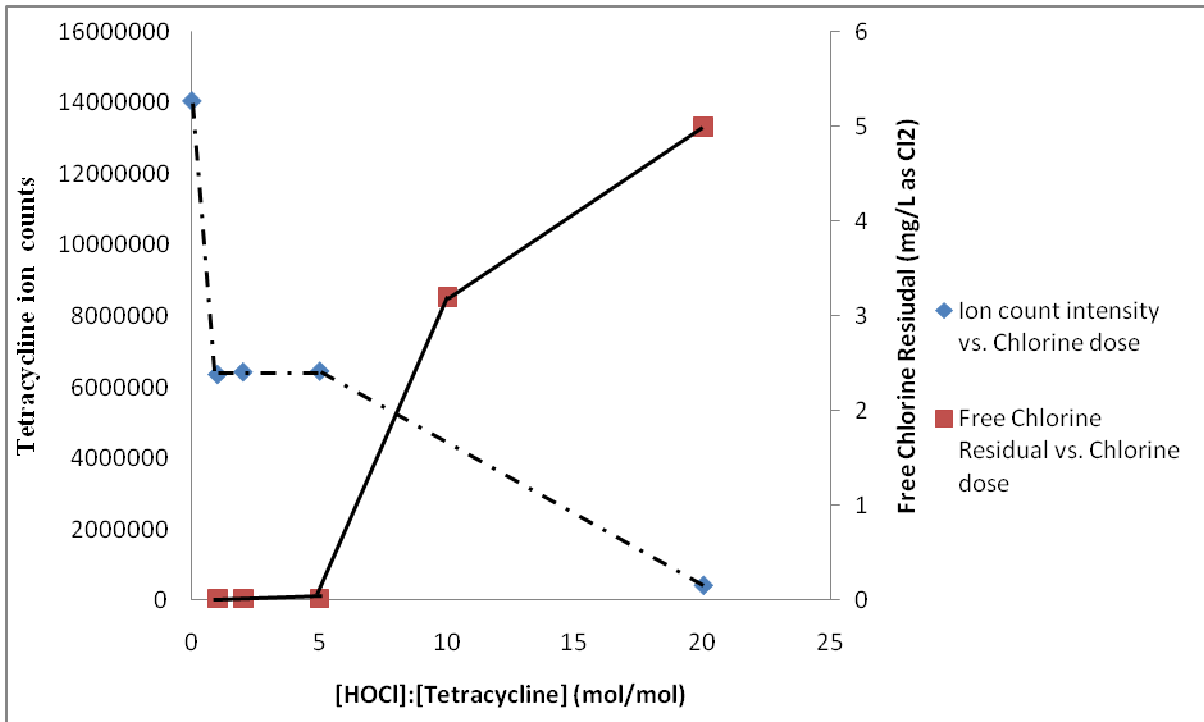| Concentration of HOCl (μM as Cl_2)	[TC]:[HOCl]
22.5	1:1
22.5	1:1
45	1:2
45	1:2
112	1:5
112	1:5
450	1:20
450	1:20

Figure 3.4 Reactivity of Tetracycline (C_0 = 22.5μM) as a Function of Free Chlorine Dose and Measured by Chlorine Residual (Right-Hand y-axis) and MS/MS Ion Intensity (Left Hand y-axis)

As the free chlorine dose increases, the tetracycline (TC) ion counts as measured by MS/MS analysis decreases. At a molar ratio of [HOCl]:[TC] = 1 there is a large drop in the concentration of tetracycline as measured by ion counts. The concentration of TC remains constant up to a [HOCl]:[TC] ratio of 5 only decreasing further when a chlorine residual is measured at [HOCl]:[TC] = 20. This is an indication that although tetracycline is reactive with chlorine at lower doses the reaction may not be complete until a large excess of chlorine is added which may demonstrate either a two-step reaction or simply that insufficient chlorine was available to react with all the tetracycline at the lower doses. In the former case, the initial chlorination of the molecule could be providing stabilization or steric hindrance could be inhibiting the further substitution of the molecule until a large excess of chlorine is applied. Table 3.7 shows the chlorine dose that would be required to completely mono-, di-, and tri-chlorinate the parent tetracycline molecule in LGW. Comparing these calculated doses to the applied doses used in the experiment (Table 3.6) and the results shown in Figure 3.4 it can be seen that at the molar ratio [HOCl]:[TC] = 5 the chlorine dose is sufficient to add three chlorine substituents onto parent TC, but the reaction is not complete as evidenced by the absence of a chlorine residual. The TOX experiments described previously utilized a very large molar ratio excess of chlorine, [HOCl]:[TC] of 135, which is well above the dose demonstrated here to have completed the reaction. Chlorine doses employed to disinfect wastewater and drinking water (mg/L) are likely to be in significant molar excess of the concentration of tetracycline that may be present (sub-μg/L or ng/L), and, therefore, it can be expected that the reaction between tetracycline and chlorine at full-scale plants will effectively transform the tetracycline molecule into chlorinated by-products.

Table 3.7 Chlorine Dose (mg/L as Cl$_2$) Required for Mono-, Di-, and Tri- Substitution of Tetracycline (C$_0$ = 22μM) in LGW Assuming All Chlorine is Consumed

Chlorine substitution (μmoles Cl/L) in 100mL sample		
Mono-	Di-	Tri-
22.5	45	67.5

Chlorine substitution (μmoles Cl$_2$/L) in 100mL sample		
Mono-	Di-	Tri-
11.3	22.5	33.8

Chlorine Dose Required (mg/L as Cl$_2$)		
Mono-	Di-	Tri-
0.788	1.58	2.36

Figure 3.4 shows that a residual is not observed until the highest chlorine dose when the parent tetracycline molecule remaining is only 3% of its initial concentration. These results indicate that the reaction between free chlorine and tetracycline is not complete until a chlorine residual is detected. An interesting observation during the experiment was that when samples of tetracycline in LGW were chlorinated at the highest dose ([HOCl]:[TC] =20:1) a rapid color change from clear to light yellow occurred immediately upon addition of free chlorine to the sample. This color change was not observed for any of the samples with lower concentration doses of free chlorine. This observation led to an evaluation of the reaction of tetracycline with free chlorine using UV analysis, to confirm the transformation of the molecule (Ahuja et al., 2001).

Change in Aromatic Structure of Tetracycline

An ~100μM tetracycline solution in LGW was scanned in a UV-Vis spectrophotometer in the range of 200-400nm to provide a baseline against which chlorinated solutions were compared. Energy absorbed in the UV spectrum by the chemical corresponds to the nature of the conjugated π electron systems, and as conjugation increases less energy is needed to promote an electron (McMurry, 2004). As can be seen in the spectrum of tetracycline in LGW in Figure 3.5 there are two wavelengths at which maximum absorbance occurs, 274nm and 356nm. It can be concluded from the spectrum that the absorption at 274nm corresponds to the tricarbonyl-amide side of the molecule while the absorption at 356nm corresponds to the conjugation on the phenolic-diketone side of the molecule (Figure 3.1). The aqueous solution containing the highest chlorine dose used in reactions with tetracycline (450μM HOCl), was scanned from 200-700nm to ensure that the presence of a chlorine residual did not impact absorbance. Figure 3.6 of this sample shows absorbance at a λ_{max} of 290nm. This absorbance would interfere with the analysis of chlorinated tetracycline only if a residual was present which was not the case for the [TC]:[HOCl] = 1:1 and 1:5 in LGW samples shown in Table 3.4. For the samples where a chlorine residual was present, the absorbance at 356nm was monitored to confirm the disappearance of tetracycline. When scanning for transformation products of tetracycline the residual chlorine absorbance will not interfere with analysis as chlorinated by-products will absorb at higher wavelengths. Additionally a lower residual of chlorine was analyzed in LGW (22μM) and no absorption was observed.
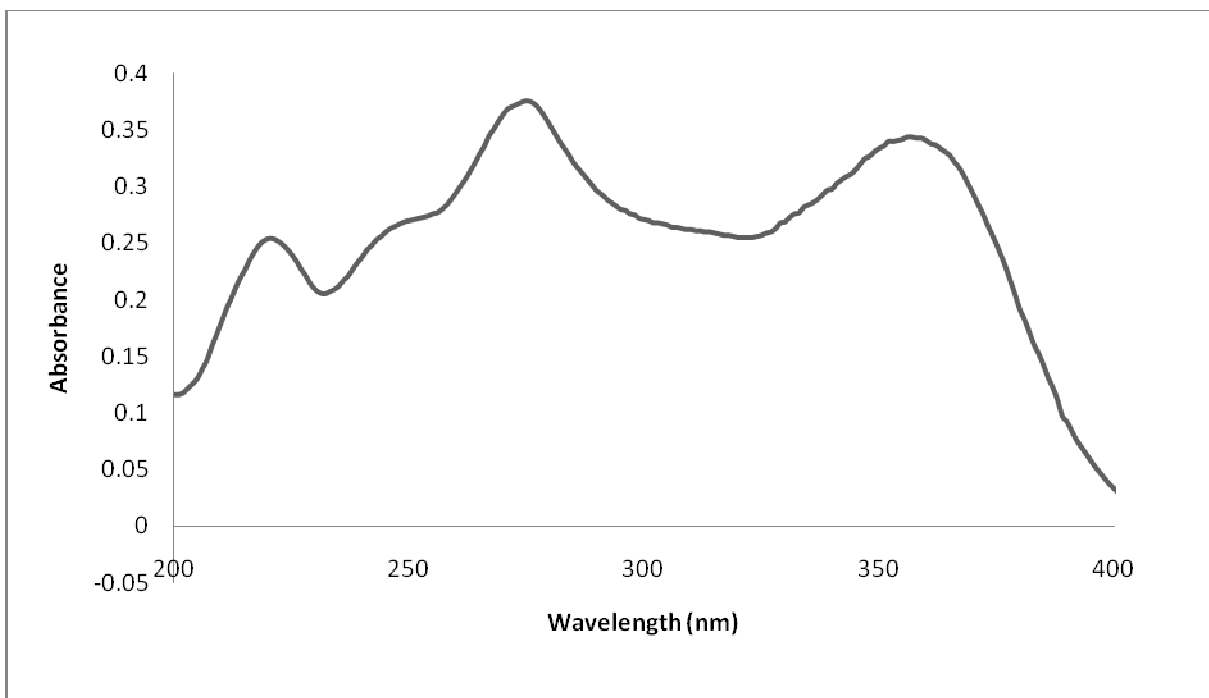
Figure 3.5 UV-Vis Spectrum of Tetracycline in LGW
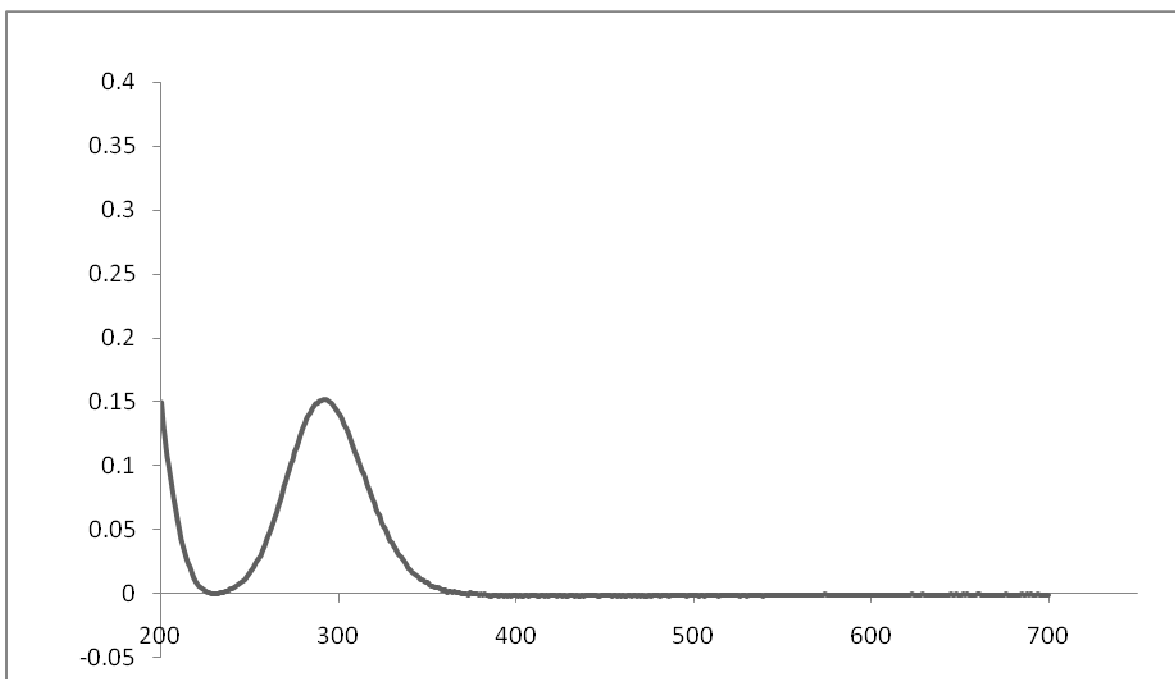
Figure 3.6 UV-Vis Spectrum of 450μM Free Chlorine in LGW

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When tetracycline in LGW is treated with a chlorine dose at a molar ratio of 1:1, only the absorption at 356nm decreases while that of 274nm remains the same as shown in Table 3.8. This is an indication that the first site of chlorination is on the phenolic-diketone side of the molecule. This is most likely due to the chlorination of the ring via electrophilic aromatic substitution. When the chlorine dose is increased to a molar ratio of 5:1 (chlorine to TC) the absorption at both wavelengths decreases, with the amount of decrease at 356nm the same as observed in the [TC]:[HOCl] 1:1 sample. This is an indication that after chlorination of the ring the site of subsequent chlorination of the molecule is on the tricarbonyl-amide side of the molecule. The incorporation of chlorine may either be on the amide group or on the tertiary amine group. At the highest chlorine doses there is no absorption at either 356nm or 274nm which indicates that the conjugation of tetracycline has changed and the parent tetracycline molecule has been completely transformed.

Table 3.8 Effect of Chlorination on the UV Absorbance of Tetracycline at Different Chlorine Doses with Contact Time of 24 Hours

Sample	Tetracycline Concentration (mg/L)	Absorbance at 356nm	at 274nm	Free Chlorine Residual (mg/L as Cl_2)
LGW control		0	0	0
22µM HOCl in LGW control	NA	0	0	0
22µM TC in LGW control	10mg/L	0.134	0.163	0
[TC]:[HOCl] 1:1 in LGW	8mg/L	0.117	0.163	0
[TC]:[HOCl] 1:5 in LGW	8mg/L	0.117	0.145	0
[TC]:[HOCl] 1:10 in LGW	0mg/L	0	0	3
[TC]:[HOCl] 1:20 in LGW	0mg/L	0	0	5

This disappearance of tetracycline correlates to the appearance of a free chlorine residual which indicates that the reaction between tetracycline and free chlorine is nearing completion, as described previously. In summary, three different transformations of tetracycline were observed with the increases in chlorine doses. This supports the TOX data (Table 3.2) in which tetracycline was found to incorporate three chlorine atoms per molecule. A wider wavelength range, 200-750nm, was scanned for the highest chlorine dose in order to evaluate the yellow product formation described earlier, but no additional absorption was observed (Figure 3.7). This sample was quenched with sodium sulfite prior to analysis and the control sample of quenched chlorine is shown in Figure 3.8. It can be seen that no interference would result from the quenched chlorine in solution. This indicates that the transformation products are tetracycline either lack aromatic character or that the concentration of the transformation products was too low to be detected during UV analysis. Future studies using the double-beam UV-Vis Spectrometer to study the transformation of tetracycline due to aqueous chlorination should use a quenched chlorine solution in the reference cell in order to discern any transformation products in the sample from the background.

The decrease in characteristic UV peaks at 275 and 350 nm associated with tetracycline due to reaction with ozone has also been reported in the literature, and it was concluded that the chemical structures of the products retained similar aromatic character to tetracycline (Dalmázio et al., 2007). HPLC-UV studies using detection at 275nm showed the disappearance of the tetracycline peak during the course of the reaction and the formation of more polar transformation products (Dalmázio et al., 2007). Future studies on the fate of

tetracycline with chlorine should be monitored using HPLC-UV in order to separate potential
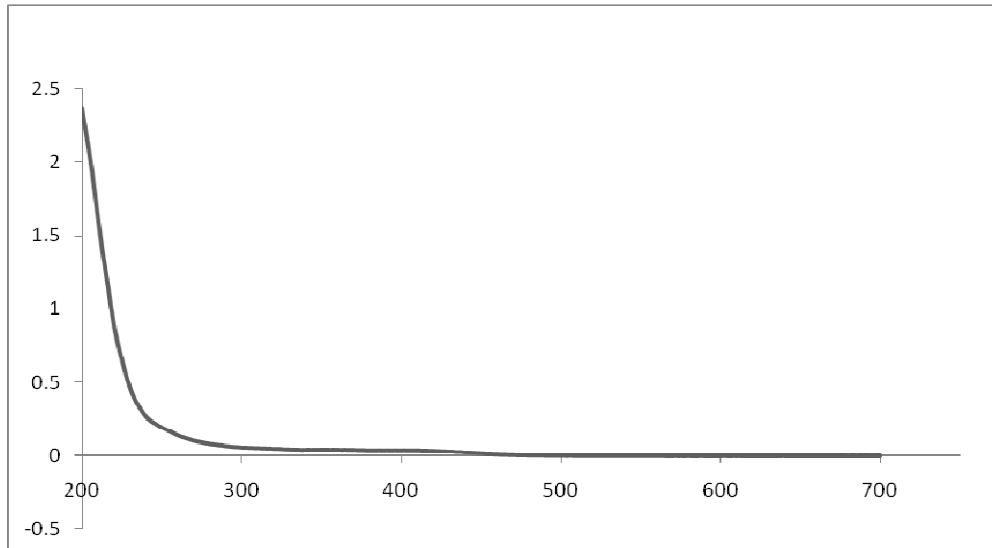
transformation products from tetracycline.

Figure 3.7 UV-Vis Spectrum of Chlorinated Tetracycline [HOCl]:[TC] = 20 in LGW after
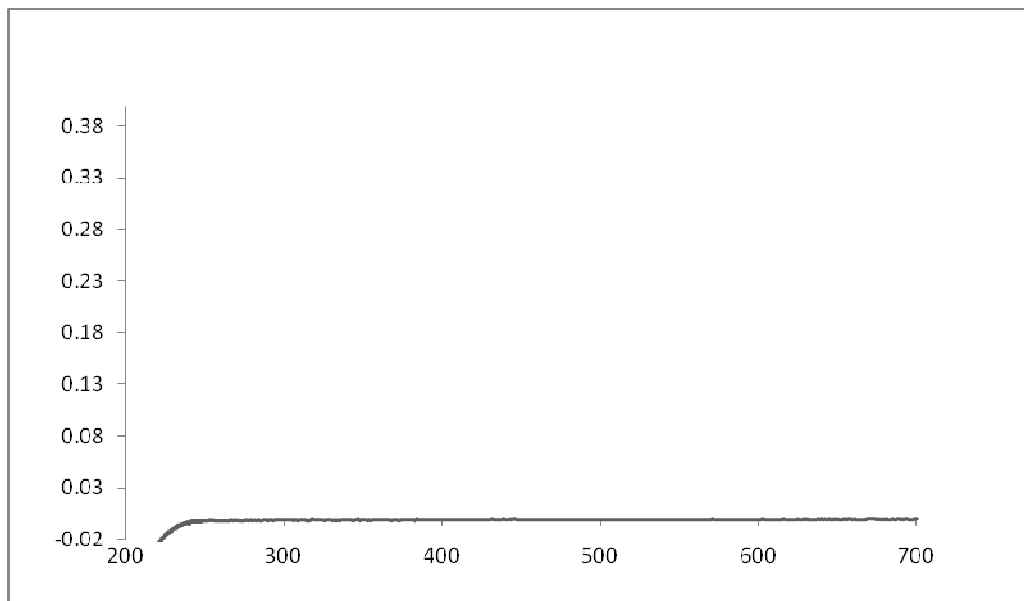Quenching with Sodium Sulfite

Figure 3.8 UV-Vis Spectrum of Sodium-Sulfite Quenched Chlorine (450µM) in LGW

Reaction Kinetics

The rate of reaction of a chlorinated tetracycline sample in which all of the chlorine is observed to be consumed ([HOCl]:[TC] 5:1) was monitored at t = 0, 10, 100, and 1420 minutes. This sample was chosen for analysis because the chlorine is completely consumed and, therefore, the entire reaction could be monitored. The samples were stored in amber glass vials with headspace in the dark at 20°C. An aliquot of the sample was removed at the specified time for chlorine residual analysis. Figure 3.9 shows the measured free chlorine residual (mole/L as Cl_2) in the sample as a function of time where it can be seen that the reaction of tetracycline with free chlorine is initially very fast and then slows down. This rapid reaction has also been reported in the literature (Wang et al., 2011).

An integrated rate law analysis was completed to determine the order of the reaction between tetracycline and free chlorine with respect to free chlorine by determining the mathematical expression that accurately describes the reaction. A zero-order reaction is linear in a plot of residual vs. time. A first-order reaction is linear in a plot of ln[residual] vs. time, and a second-order reaction is linear is a plot of the inverse of residual vs. time. The results of this analysis showed that the reaction is second order, as is shown by the linear relationship between the inverse of free chlorine residual and time in Figure 3.10. The mathematical expression that describes a second order reaction is shown below where [A] is the concentration of the free chlorine residual, *k* is the rate constant, and *t* is time.

$$[A] = \frac{[A]_0}{1 + kt\,[A]_0}$$
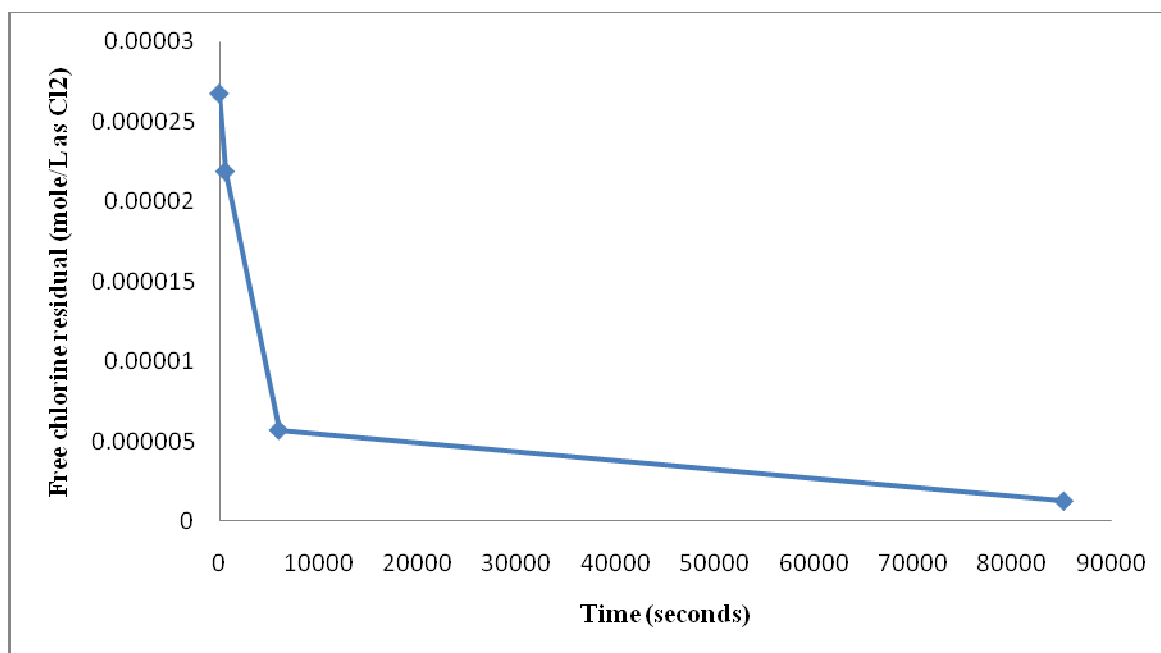
Figure 3.9 Free Chlorine Residual (mole/L as Cl$_2$) as a Function of Reaction Time for a 5:1 Molar Ratio of Free Chlorine to Tetracycline

Although these results monitor the depletion of chlorine in contact with tetracycline and not the depletion of tetracycline itself the reaction is estimated to be second order, and second order rate constants for this reaction have been previously reported (Wang et al., 2011). The rate constant, k, is the slope of Figure 3.10, which is 8.397 M^{-1}s^{-1}. Wang et al., 2011 have reported rate constants for reactions between tetracycline and free chlorine to be much larger, 2.83 x 10^6 M^{-1} s^{-1}. This is an indication that the tetracycline is consumed at a much faster rate than chlorine and the tetracycline will be the limiting reagent. These results indicate that the length of time used for the TOX experiment (24hours) is sufficient to ensure that the reaction has gone to completion, and that the reaction is dependent on both the concentration of tetracycline and free chlorine.
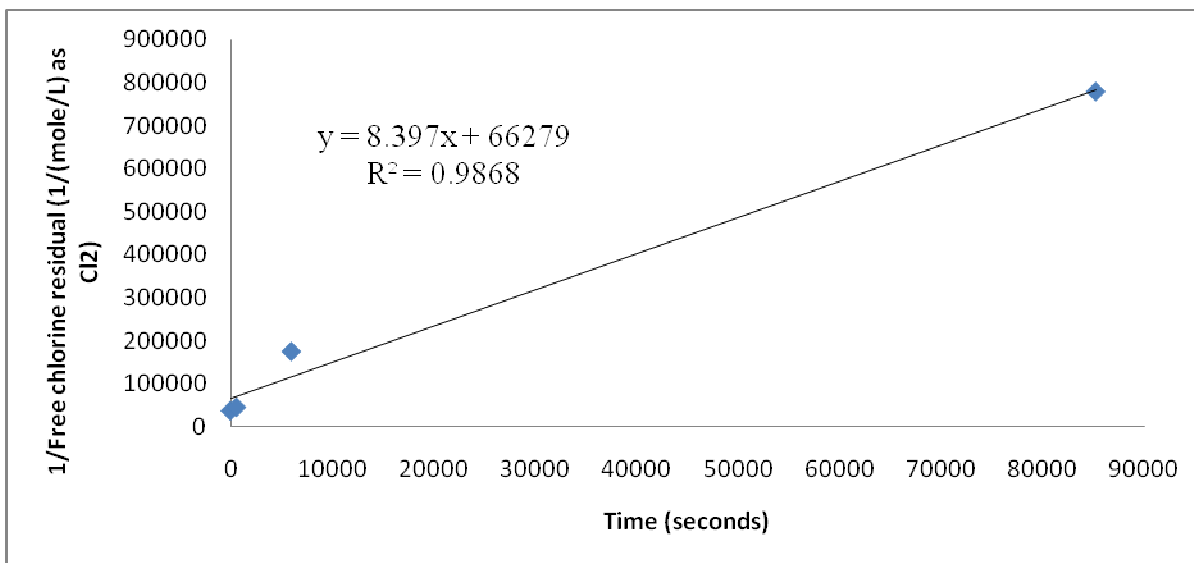
Figure 3.10 Integrated Rate Law Analysis of the Reaction between Free Chlorine and Tetracycline

Analysis of an Unquenched Chlorinated Sample with No Free Chlorine Residual

One complexity in using mass spectrometry as a tool for the analysis of transformation by-products involving free chlorine is the interference between quenching agents (and/or chlorine residual) and chemical ionization in the source of the mass spectrometer. Signal suppression from the presence of residual chlorine in the sample was avoided by adding the quenching agent (ascorbic acid), and ions associated with quenched chlorine, *m/z* 214.8, 406.8, and 475.4, were observed in the mass spectra, as shown in Figures 3.12-5.13. Figure 3.11 shows the full scan mass spectra of the unchlorinated tetracycline sample (22μM) where only the molecular ion of tetracycline [M+1]$^+$ at *m/z* 445.1 of tetracycline is observed. Figure 3.12 shows the mass spectra of the quenched chlorinated tetracycline sample ([TC]:[HOCl] = 1:20); a new mass spectra is observed and the molecular ion for tetracycline is absent. Figure 3.13 shows a similar mass spectrum with prominent ions at *m/z* 215 and 407, when a quenched chlorinated control sample is analyzed. Therefore,

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in order to identify transformation products without the presence of quenching agent ions a chlorinated sample which left no free chlorine residual after 24 hours was analyzed which meant that the reaction most likely did not proceed to completion, as described earlier. In the experimental results that follow the chlorinated sample that was analyzed by (+)-ESI was that from Table 3.8 where the molar ratio of tetracycline to free chlorine was 1:5.
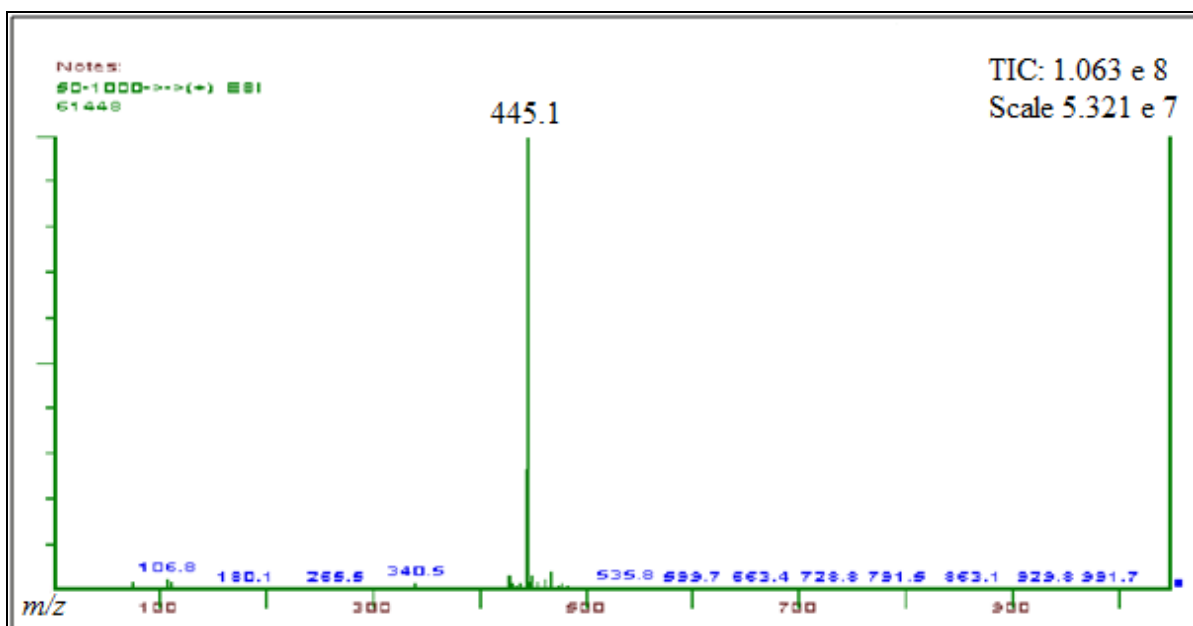
Figure 3.11 Full Scan Spectra of 22μM Tetracycline in LGW. Prominent Ion at *m/z* 445.1 is that of [M+H]$^{+}$for Tetracycline
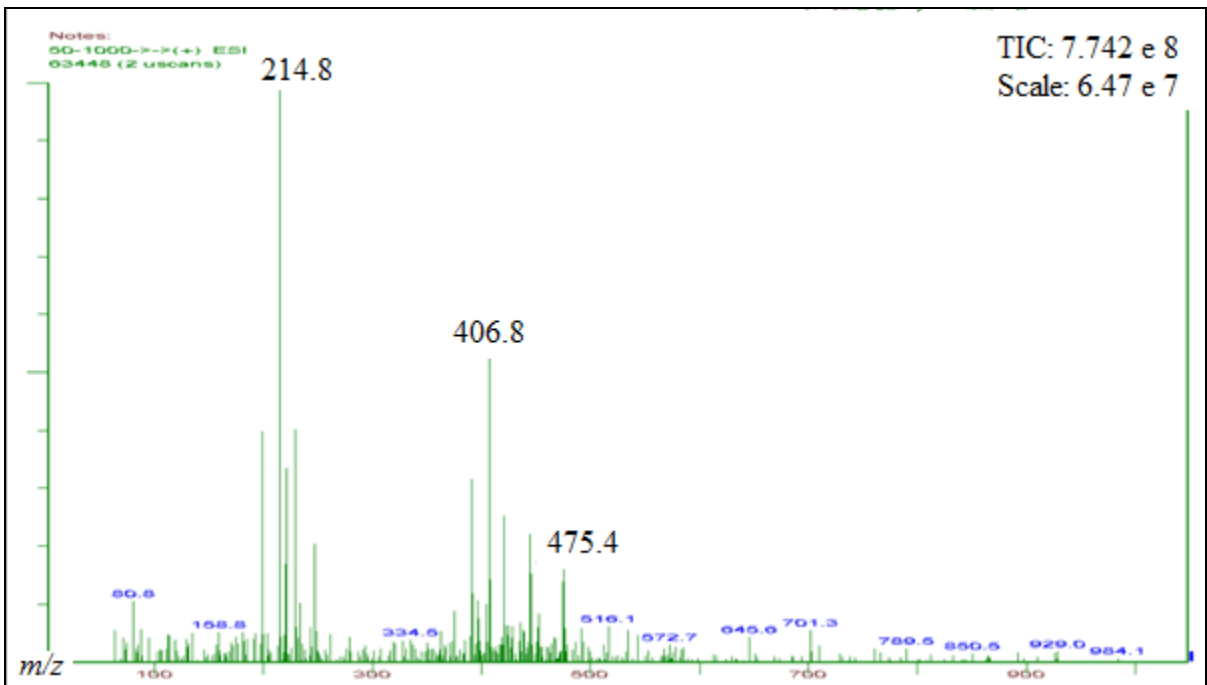
Figure 3.12 Full Scan Spectra of a Quenched Chlorinated Tetracycline Sample [TC]:[HOCl] = 1:20. Prominent Tetracycline Ion (*m/z* 445.1) is Missing and a New Spectra is Observed.
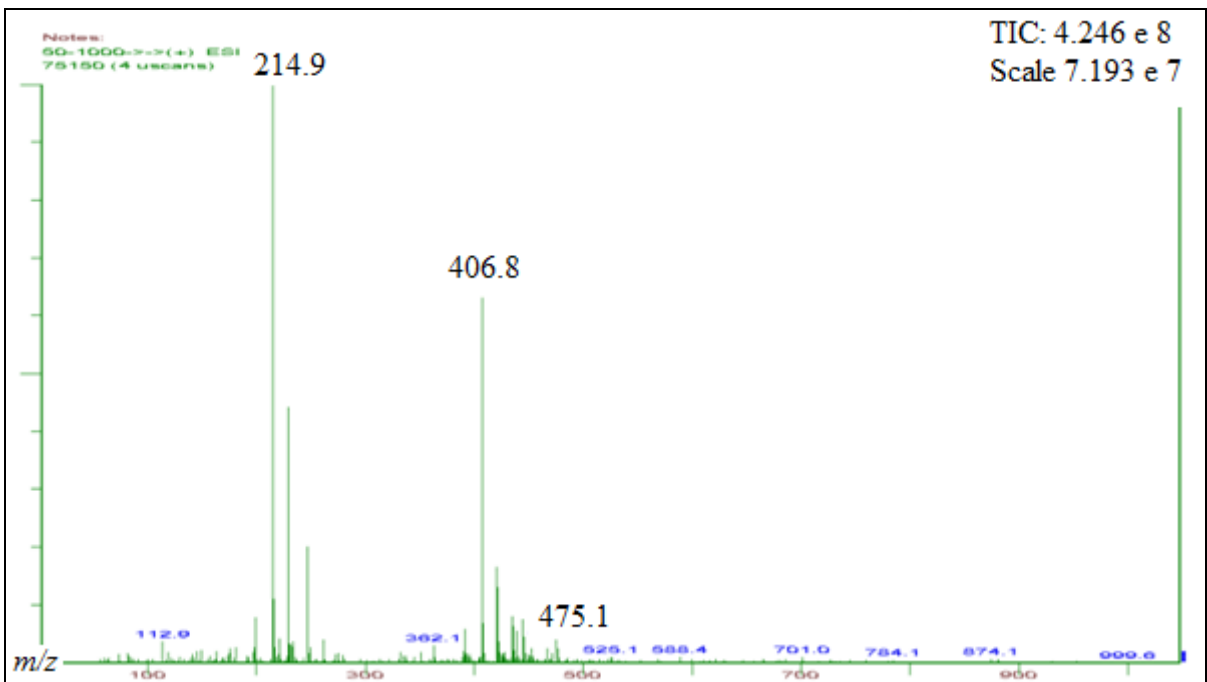
Figure 3.13 Full Scan Spectra of HOCl in LGW Quenched with Ascorbic Acid. Prominent Ions *m/z* 215 and 407 are Seen in Both Chlorinated Tetracycline Sample (Figure 3.11) and Chlorinated Lab Grade Water Control Sample.
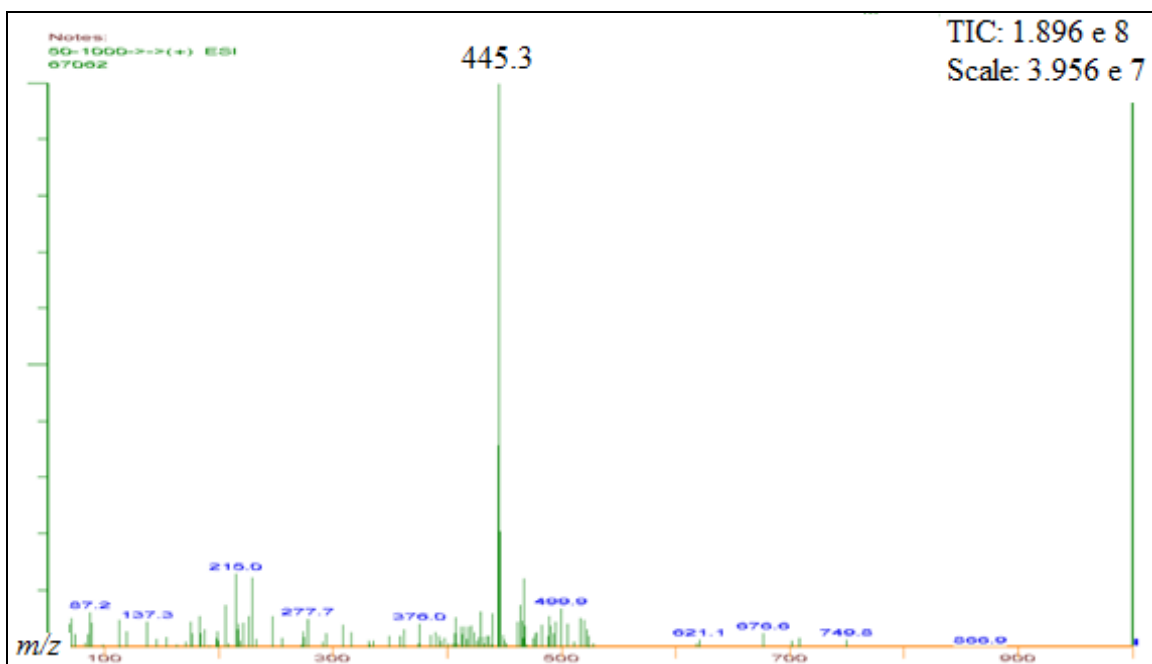
Figure 3.14 Full Scan Mass Spectra (50-1000 *m/z*) of Chlorinated Tetracycline ([HOCl]:[TC] = 1:5) in LGW After 24 Hours Contact Time Leaving No Cl_2 Residual.

The possible chlorination by-products of tetracycline were identified in real time using direct infusion-based tandem mass spectroscopy by comparing the full scan mass spectra of the unchlorinated and chlorinated tetracycline samples. Figures 3.11 and 3.14 show the differences in the mass spectra observed between an unchlorinated and chlorinated tetracycline sample, respectively. The mass spectrum for the chlorinated tetracycline sample (Figure 3.14) contains many more ion peaks which may be due to the presence of transformation products of chlorinated tetracycline. In both samples the molecular ion of tetracycline (*m/z* 445) was targeted in Q2 of the mass spectrometer and dissociated using argon gas to determine the change in ion counts of tetracycline due to reaction with chlorine, and the results are shown in Figures 3.15 and 3.16. The counts of tetracycline in the unchlorinated sample shown in Figure 3.15 are 1.4×10^7. Prominent daughter fragment ions are *m/z* 428, 427, and 410. In the chlorinated tetracycline sample ([HOCl]:[TC] = 1:5) shown

in Figure 3.16 the counts of tetracycline are 2.5 x 10^6; that is, a decrease in 1.2 x 10^7 counts of tetracycline is observed due to chlorination. Prominent fragment ions of *m/z* 428, 427, and 410 are still observed which indicates that the parent tetracycline remaining has not completely transformed. Comparison of these two figures shows that although the concentration of tetracycline decreases as the result of chlorination, the structure of tetracycline is not degraded because the same daughter fragments are observed. This is an indication that while a small portion of the tetracycline remains unchanged a large percentage of the tetracycline, 83%, has transformed.

The prominent ions observed in the chlorinated tetracycline sample (*m/z* 467, 477, 483) and ions found during previous work from preliminary LC-MS analysis (*m/z* 399, 456, 461, 485) (Ye, 2005) were targeted in additional infusion based tandem mass spectroscopy experiments in order to obtain more structural information about the chlorination products of tetracycline. Figures 3.17, 3.18, 3.19, and 3.20 show the results obtained from targeting potential transformation products of tetracycline in quadrupole 2 (Q2) of the mass spectrometer. Figure 3.17 shows the collision induced dissociation of prominent fragment *m/z* 483 (ion counts: 3.8 x 10^4) of a potential transformation product of tetracycline in Q2 of the mass spectrometer. A prominent daughter ion of the parent is *m/z* 466. Figure 3.18 shows the collision induced dissociation of molecular ion *m/z* 477 (ion counts: 4.0 x 10^4) of a potential transformation product of tetracycline in Q2 of the mass spectrometer. A prominent daughter ion of the parent is *m/z* 459.4. Figure 3.19 shows the collision induced dissociation of molecular ion *m/z* 467 in Q2 (ion counts: 9.2 x 10^4) of a potential transformation product of tetracycline of the mass spectrometer. A prominent daughter ion of the parent is *m/z* 449.7. Figure 3.20 shows the collision induced dissociation of molecular ion *m/z* 461 (ion

counts: 4.3 x 10^4) of a potential transformation product of tetracycline in Q2 of the mass spectrometer. A prominent daughter ion of the parent is *m/z* 444.1.

The 'smooth' lines obtained during collision induced dissociation experiments in Q2 are indicative of a molecule that is present and the numbers in parenthesis are the ion counts associated with the transition labeled on top of the curve. Table 3.9 summarizes the results of the targeted tetracycline (ion counts) and breakdown ions (ion counts) in both the unchlorinated and chlorinated tetracycline sample. Table 3.10 summarizes the results of the targeted potential transformation by products of tetracycline due to reaction with free chlorine.
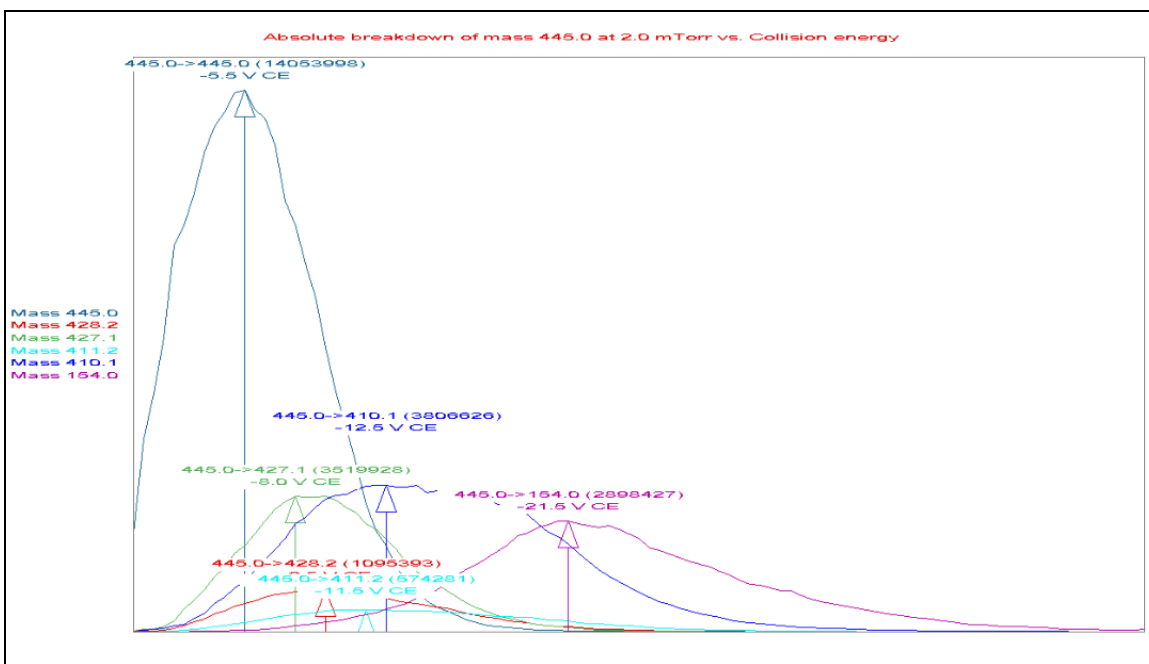
Figure 3.15 22µM Tetracycline in LGW. Collision Induced Dissociation of the Molecular Ion (*m/z* 445) of Tetracycline in Q2 of the Mass Spectrometer.
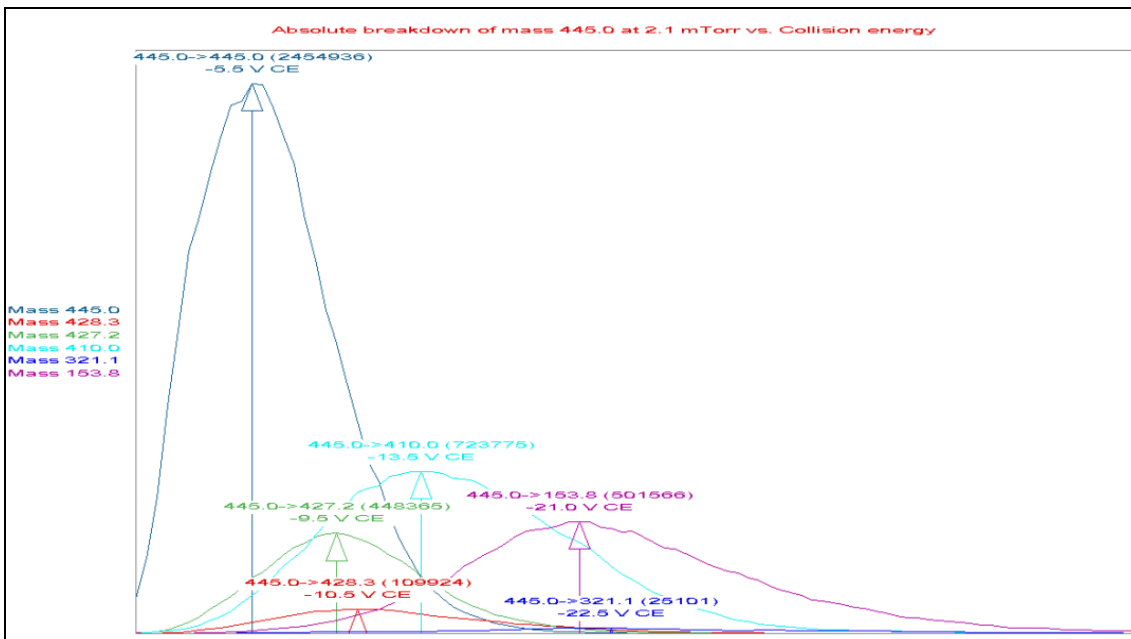
Figure 3.16 Chlorinated Tetracycline ([HOCl]:[TC] = 1:5) in LGW After 24 Hours Contact Time and 0mg/L as Cl_2 Residual. Collision Induced Dissociation of Molecular Ion (m/z 445) of Tetracycline in Q2 of the Mass Spectrometer (n = 1).
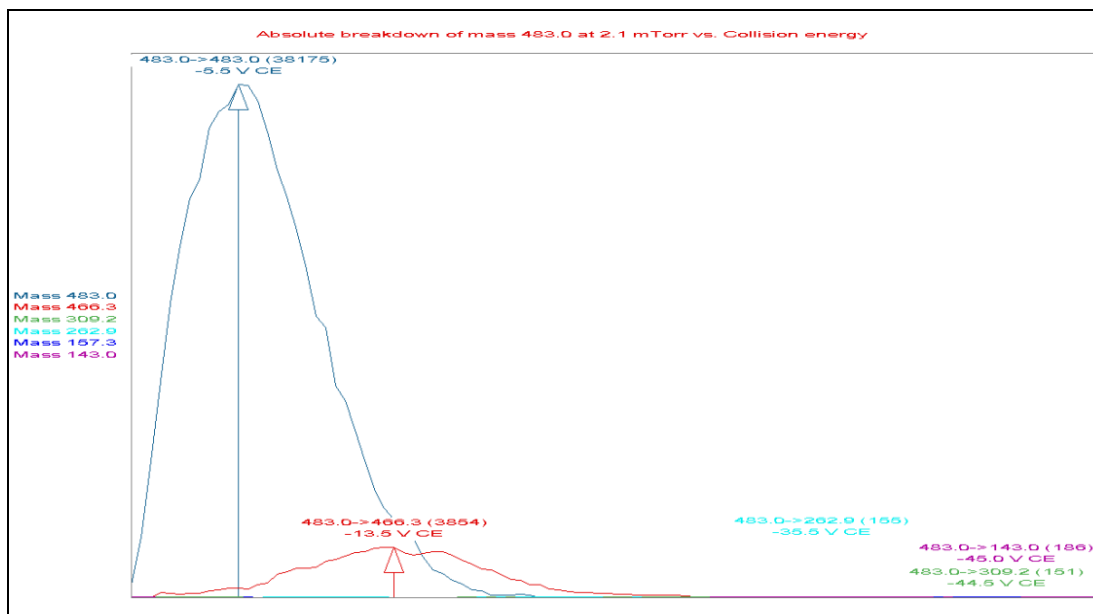
Figure 3.17 Chlorinated Tetracycline ([HOCl]:[TC] = 1:5) in LGW After 24 Hours Contact time and 0mg/L as Cl_2 Residual. Collision Induced Dissociation of Prominent Fragment m/z 483 of a Potential Transformation Product of Tetracycline in Q2 of the Mass Spectrometer (n = 1).
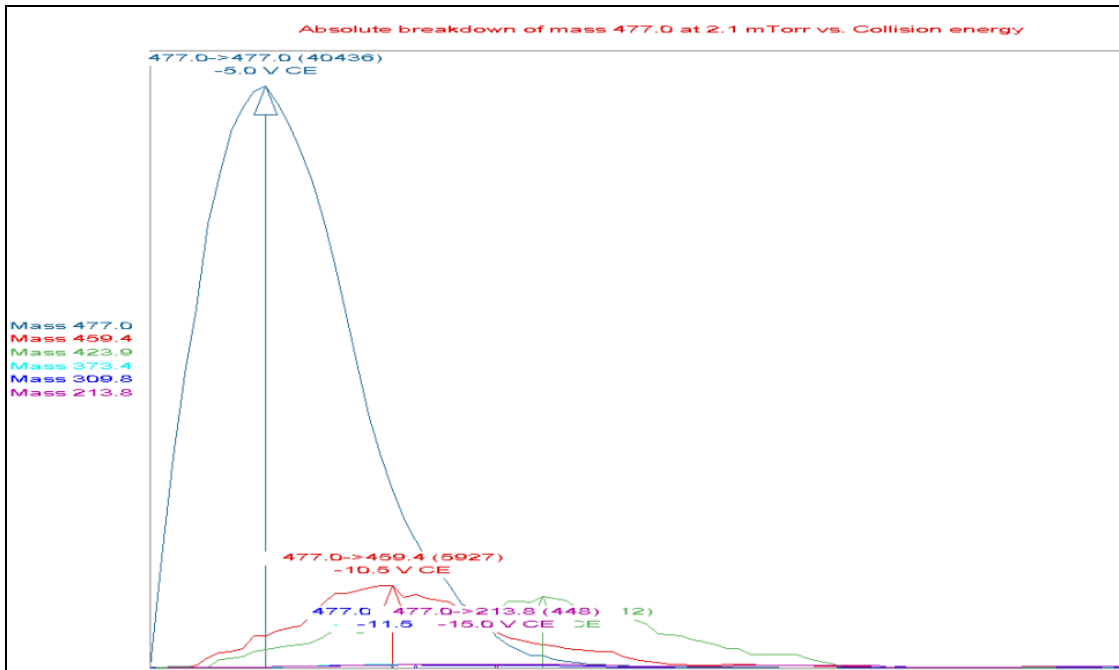
Figure 3.18 Chlorinated Tetracycline ([HOCl]:[TC] = 1:5) in LGW After 24 Hours Contact Time and 0mg/L as Cl_2 Residual. Collision Induced Dissociation of Molecular Ion *m/z* 477 of a Potential Transformation Product of Tetracycline in Q2 of the Mass Spectrometer (n = 1).
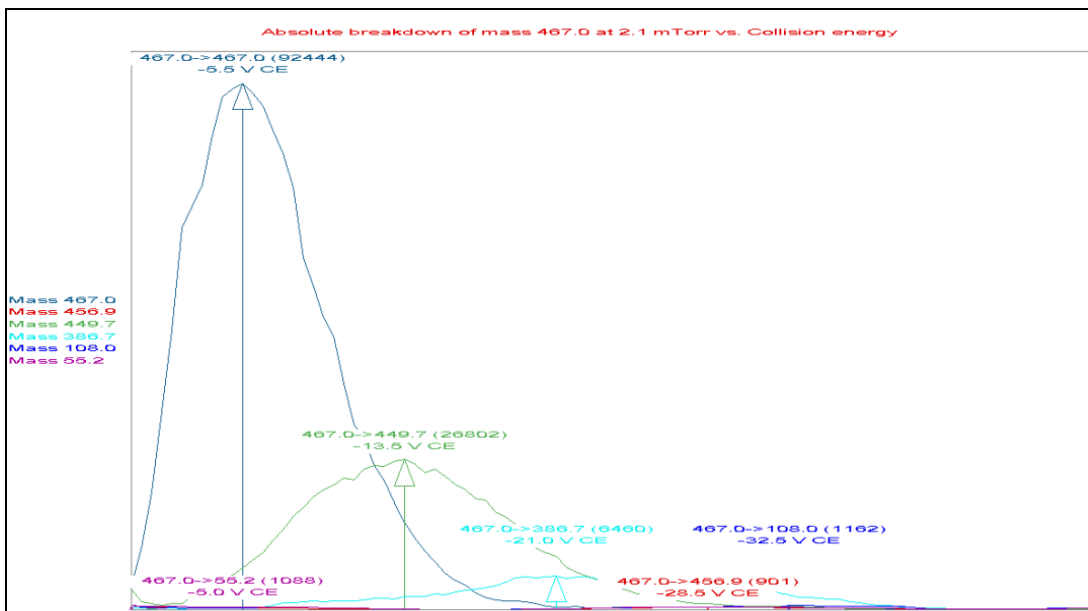
Figure 3.19 Chlorinated Tetracycline ([HOCl]:[TC] = 1:5) in LGW After 24 Hours Contact Time and 0mg/L as Cl_2 Residual. Collision Induced Dissociation of Molecular Ion *m/z* 467 in Q2 of a Potential Transformation Product of Tetracycline of the Mass Spectrometer (n = 1).
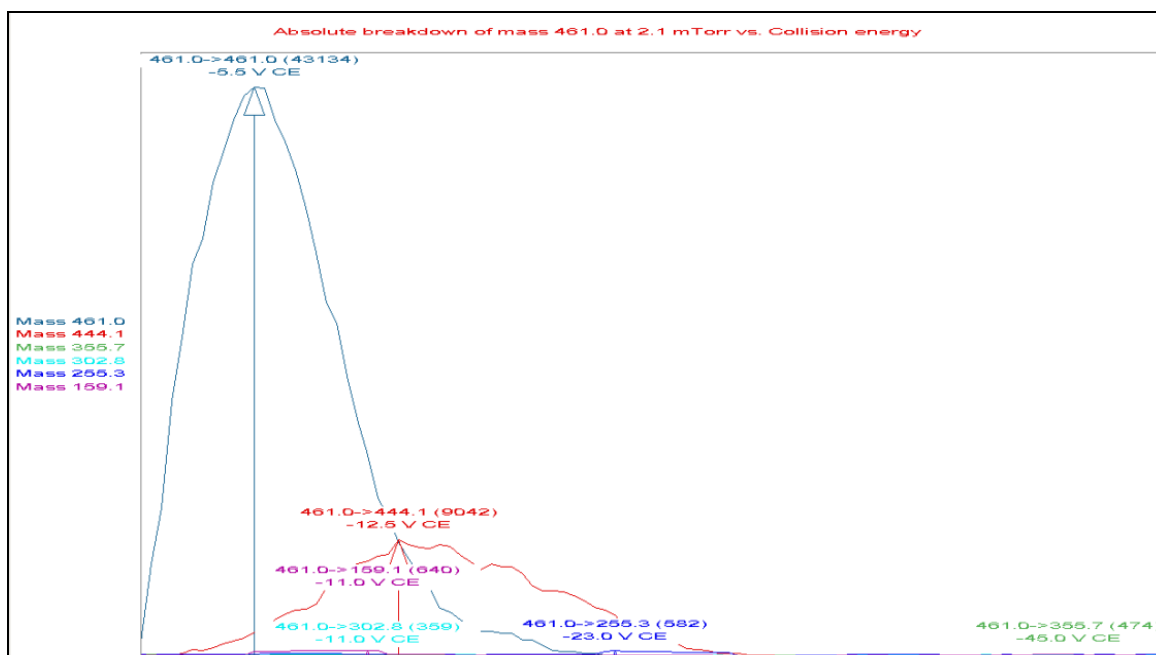
Figure 3.20 Chlorinated Tetracycline ([HOCl]:[TC] = 1:5) in LGW After 24 Hours Contact Time and 0mg/L as Cl_2 Residual. Collision Induced Dissociation of Molecular Ion *m/z* 461 of a Potential Transformation Product of Tetracycline in Q2 of the Mass Spectrometer (n = 1).

Table 3.9 Direct Infusion MS/MS Results of Tetracycline Before and After Reaction with Chlorine [HOCl]:[TC] = 1:5 in LGW 24 hours after Reaction of Tetracycline with Free Chlorine with a Residual of 0mg/L as Cl_2

Fragment ion mass loss	Unchlorinated Tetracycline		Chlorinated Tetracycline	
	m/z	ion counts	*m/z*	ion counts
	445	1.4×10^7	445	2.5×10^7
-17 [-NH$_2$]	428	1.1×10^7	428	1.1×10^5
-18 [-H$_2$O]	427	3.5×10^7	427	4.5×10^5
-35 [-NH$_2$-H$_2$O]	410	3.8×10^7	410	7.2×10^5

The results in Table 3.10 show the characteristic breakdown data for the transformation products of tetracycline due to reactions with free chlorine. In the literature both Cl- and OH- substituted products were observed in the reaction between tetracycline

and free chlorine (Wang et al., 2011). While not enough structural information was obtained to identify the transformation products in Table 3.10, several hypotheses can be made. The loss an ion fragment mass of 17 corresponds to the loss of NH_3 from the amide group. Therefore, if this loss is not observed in a transformation product (such as for TC+32) this suggests the transformation of the amino functional group, possibly by loss of NH_2 and the substitution of Cl. Conversely, if the loss of mass fragment 17 is still observed (TC+38, TC+22, and TC+16) it can be assumed that the amino group is intact and the transformation (Cl- and/or OH- substitution) of the molecule was elsewhere.

Table 3.10 Direct Infusion MS/MS Results of Targeted Potential Transformation Products from [HOCl]:[TC] = 1:5 in LGW 24 hours after Reaction of Tetracycline with Free Chlorine with a Residual of 0mg/L as Cl₂

Fragment ion mass loss	Chlorinated Tetracycline (TC)							
	TC+38		TC+22		TC+32		TC+16	
	m/z	ion counts	m/z	ion counts	m/z	ion counts	m/z	ion counts
	483	3.8×10^4	467	9.2×10^4	477	4.0×10^4	461	4.3×10^4
-17	466	3.8×10^3	450	2.7×10^4	-	-	444	9.0×10^3
-18	-	-	-	-	459	5.9×10^3	-	-
-35	-	-	-	-	-	-	-	-
-53	-	-	-	-	424	-	-	-
-80	-	-	387	6.5×10^3	-	-	-	-
-105	-	-	-	-	-	-	3.6×10^2	4.7×10^2

Analysis of Quenched Chlorinated Sample-Reaction to Completion

While the previous experiment was completed without the use of a quenching agent, it can be seen from Table 3.9 that the reaction between tetracycline and chlorine was not complete. Therefore, to target potential transformation products of a reaction that was driven to completion the chlorinated tetracycline sample with the highest dose the [TC]:[HOCl] = 1:20 in LGW (mol/mol) was analyzed. The full scan mass spectra for the unchlorinated tetracycline (22μM) sample can be seen in Figure 3.11. A control sample containing the quenched chlorine with the same excess of ascorbic acid as used in the chlorinated samples is shown in Figure 3.13., and prominent peaks *m/z* 215 and 407 are observed due to the quenching agents (or quenched chlorine). Figure 3.21 shows the full scan mass spectra of the [TC]:[HOCl] = 1:20 in LGW sample in which the prominent peaks at *m/z* 215 and 407 are observed in addition to many prominent peaks which are transformation products due to the chlorination of tetracycline. It can be seen that the prominent ion at *m/z* 445 for tetracycline is no longer observed in the mass spectrum, and at values of *m/z* larger than 445 many ions are observed, which may be potential transformation products of tetracycline. Tetracycline was targeted in both samples to determine the relative ion counts, and the results of the collision induced dissociation of the molecular ion in Q2 of the mass spectrometer are shown in Figures 3.9 and 3.22 for unchlorinated and chlorinated tetracycline, respectively. The counts of tetracycline in Figure 5.16 are 4.2×10^5. That is, a decrease in 1.4×10^7 counts of tetracycline is observed due to chlorination. A summary of these results are shown in Table 3.11.

Prominent ions observed in the mass spectra of the [TC]:[HOCl] = 1:20 in LGW sample (*m/z* 477 and 451) were targeted in Q2 of the mass spectrometer as well as potential

transformation products from previous work; *m/z* 461, 399, 485, and 456 (Ye, 2005). The results from targeting these potential transformation ions in Q2 revealed that *m/z* 456, 399, 461, 477, and 451 (no data was obtained for *m/z* 485) were detected in Q2, and the breakdown curves are shown in Figures 3.23-3.27, respectively. The sum of the targeted molecular ions shown in Figures 3.23-3.27 add up to 1.3×10^6 which represents only a small fraction of the total amount of tetracycline that was transformed (1.4×10^7) (only 9% of the transformed tetracycline is accounted for from this analysis). Therefore, many other transformation products of tetracycline remain undetected. One explanation is that they may be below the limits of detection of the instrument and sample concentration techniques may be necessary so that the product can be observed.
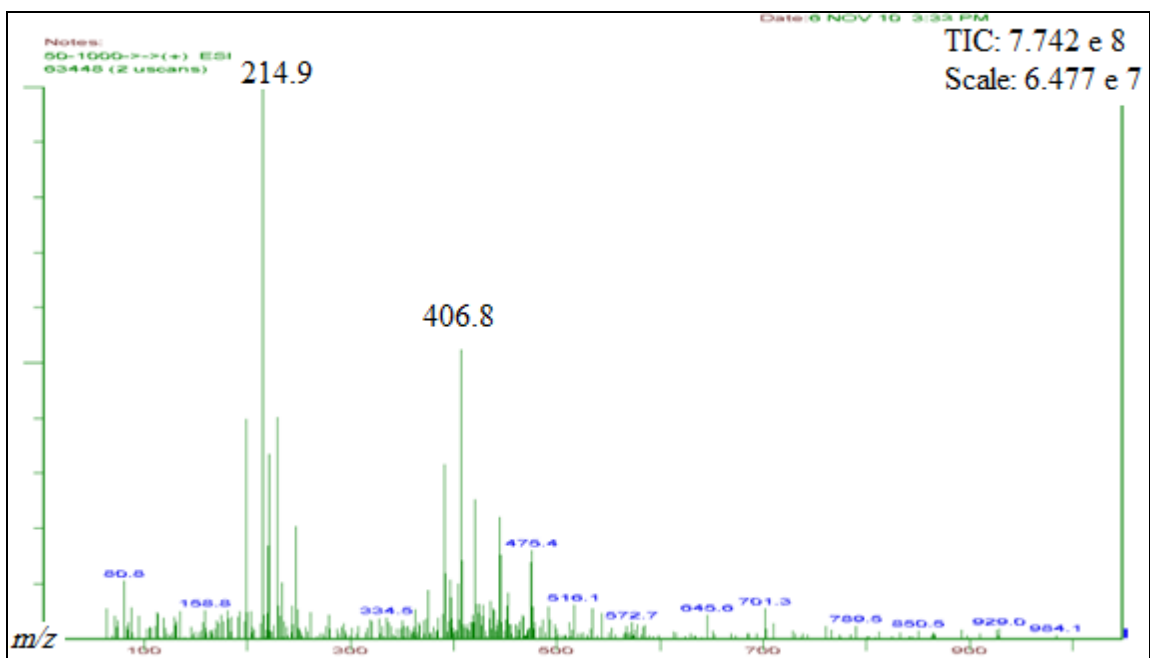
Figure 3.21 Full Scan Mass Spectra (50-1000 *m/z*) of Chlorinated Tetracycline ([HOCl]:[TC] = 1:20) in LGW After 24 Hours Contact Time and Quenched Chlorine Residual.
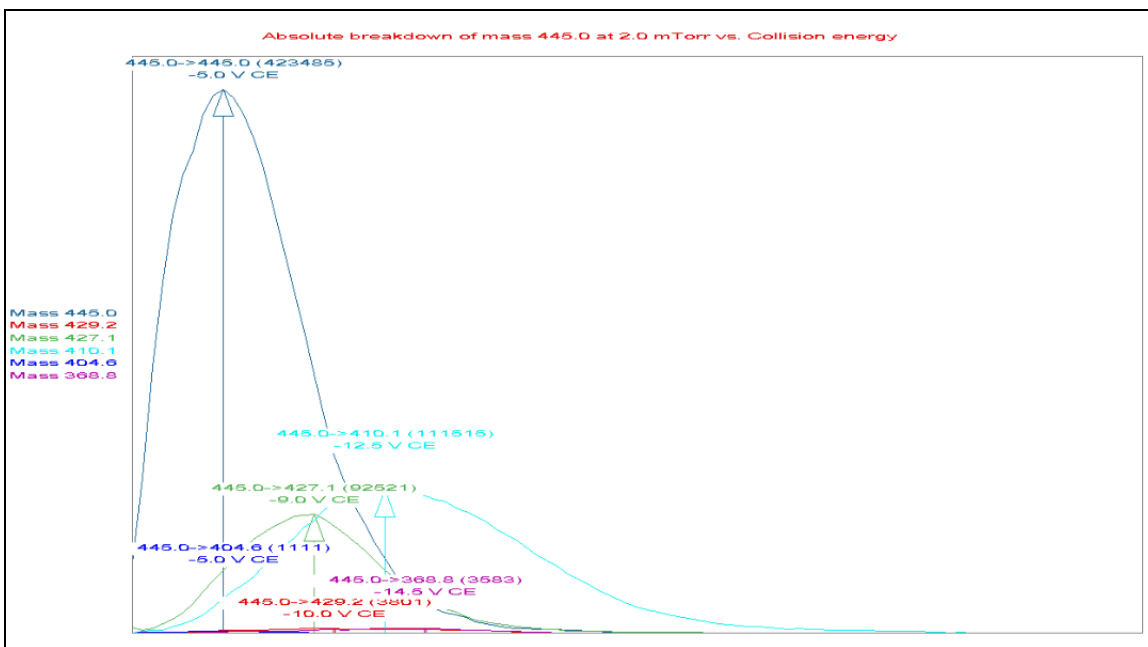
Figure 3.22 [TC]:[HOCl] = 1:20 in LGW. Collision Induced Dissociation of Molecular Ion (*m/z* 445) of Tetracycline in Q2 of the Mass Spectrometer.

Figure 3.23 Chlorinated Tetracycline ([TC]:[HOCl] = 1:20) in LGW After 24 Hours Contact Time and Quenched Chlorine. Collision Induced Dissociation of Molecular Ion *m/z* 456 of a Potential Transformation Product of Tetracycline in Q2 of the Mass Spectrometer (n =1).

Figure 3.24 Chlorinated Tetracycline ([TC]:[HOCl] = 1:20) in LGW 24 Hours Contact Time and Quenched Chlorine. Collision Induced Dissociation of Molecular Ion *m/z* 399 of a Potential Transformation Product of Tetracycline in Q2 of the Mass Spectrometer.
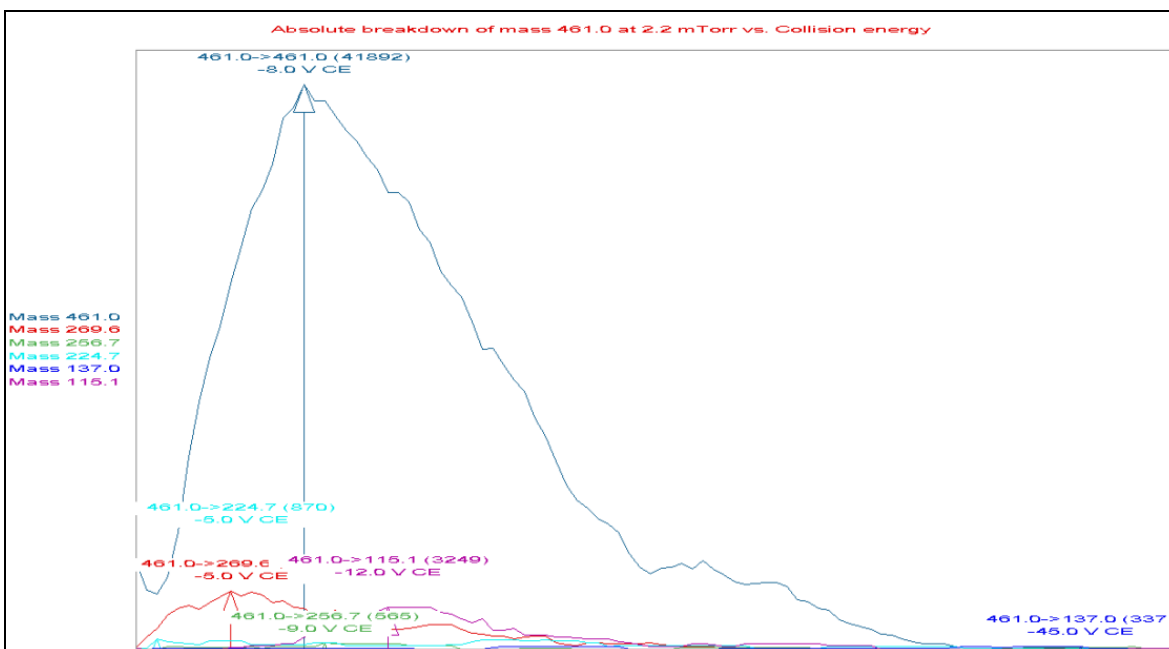
Figure 3.25 Chlorinated Tetracycline ([TC]:[HOCl] = 1:20) in LGW 24 Hours Contact Time and Quenched Chlorine. Collision Induced Dissociation of Molecular Ion *m/z* 461 of a Potential Transformation Product of Tetracycline in Q2 of the Mass Spectrometer.
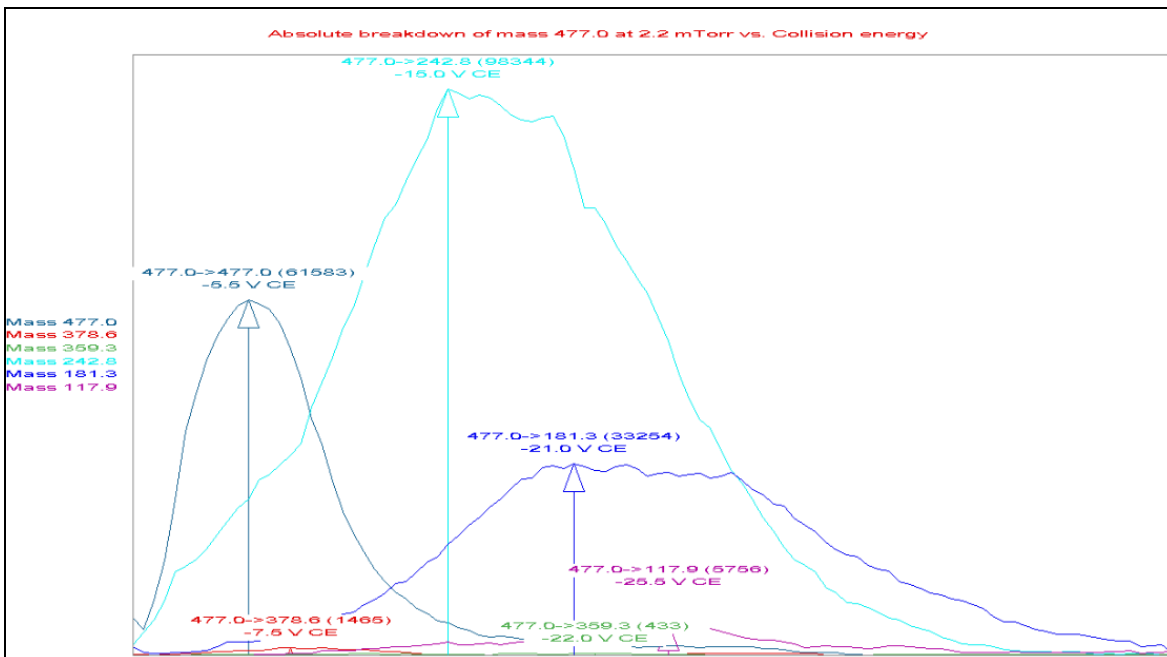
Figure 3.26 Chlorinated Tetracycline ([TC]:[HOCl] = 1:20) in LGW 24 Hours Contact Time and Quenched Chlorine. Collision Induced Dissociation of Molecular Ion *m/z* 477 of a Potential Transformation Product of Tetracycline in Q2 of the Mass Spectrometer.
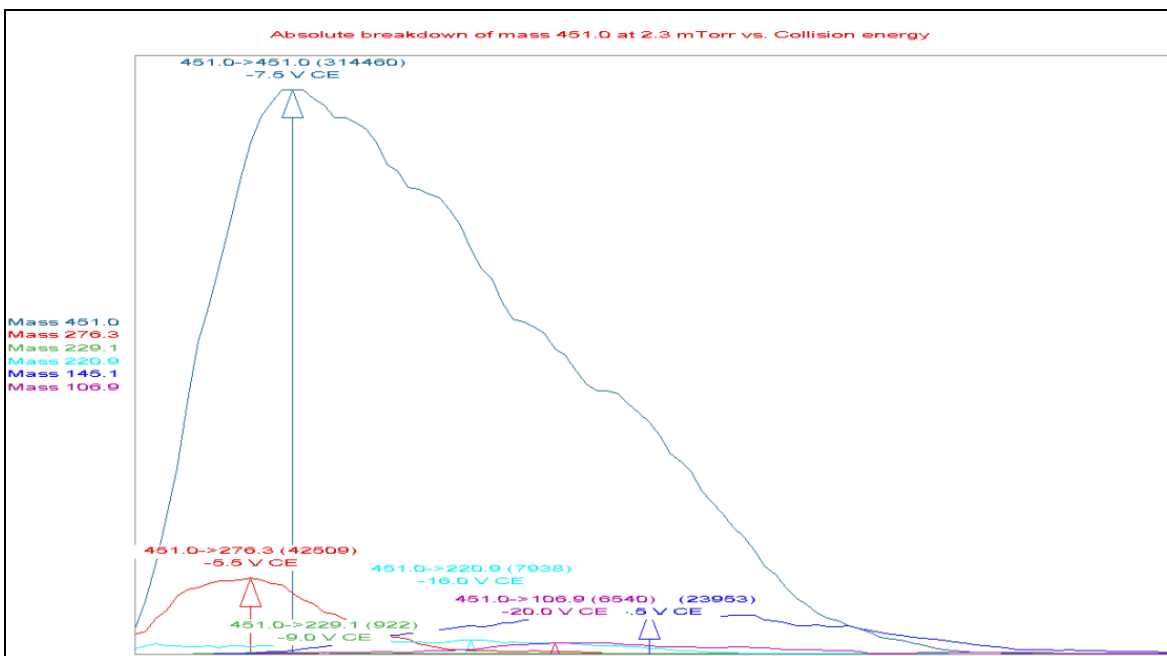
Figure 3.27 Chlorinated Tetracycline ([TC]:[HOCl] = 1:20) in LGW 24 Hours Contact Time and Quenched Chlorine. Collision Induce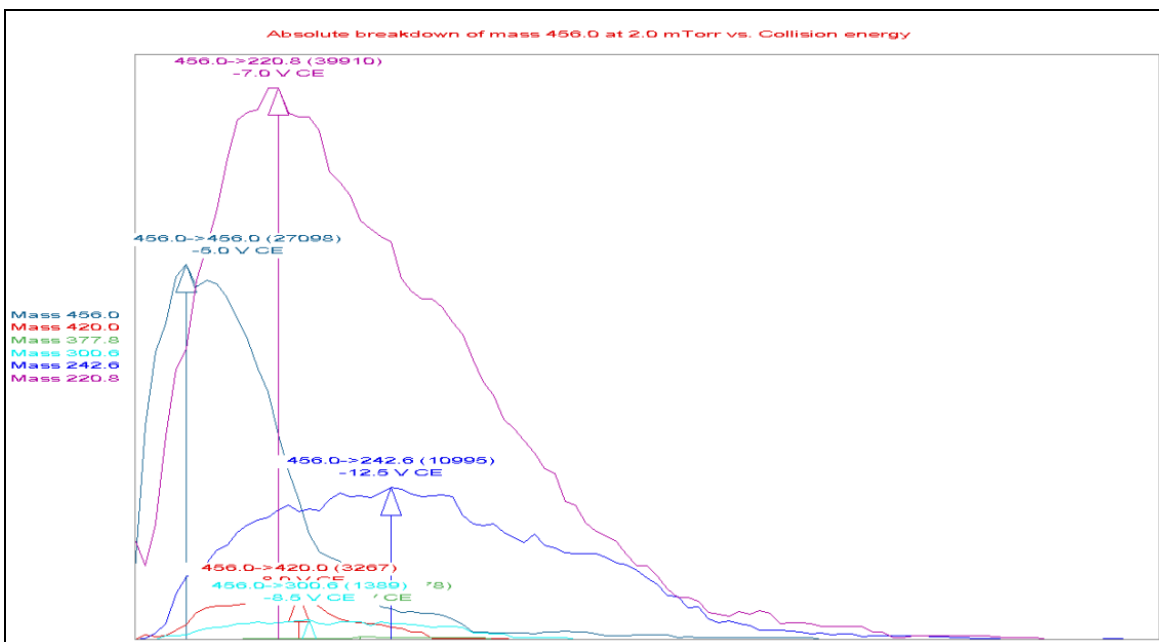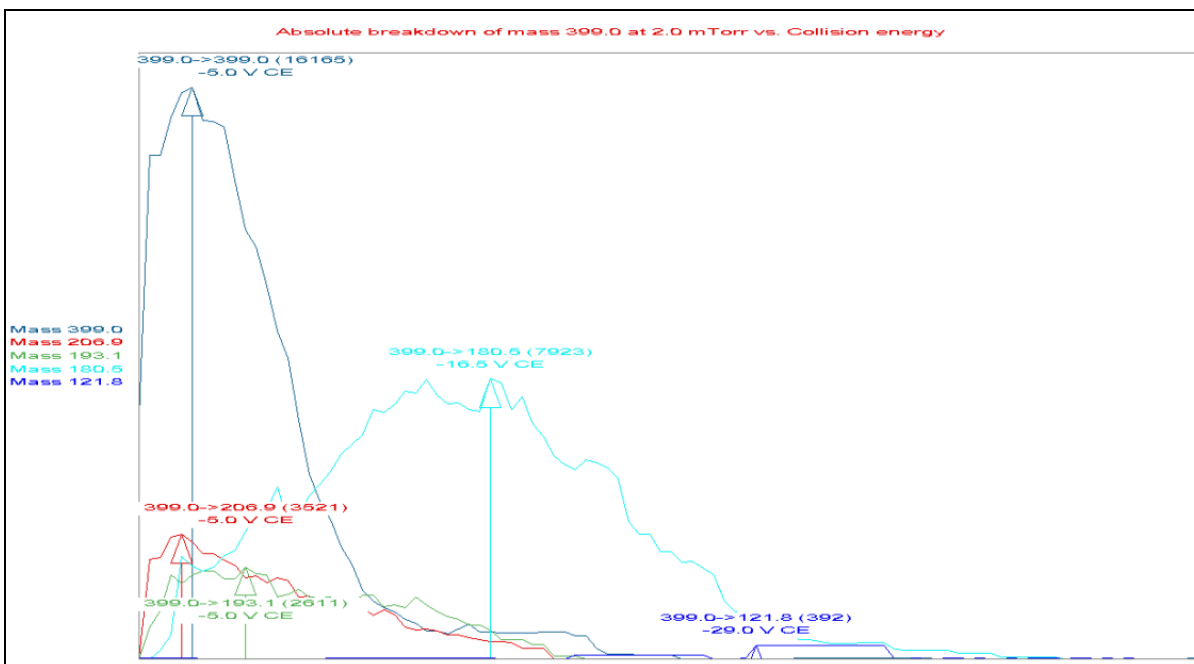d Dissociation of Molecular Ion *m/z* 451 of a Potential Transformation Product of Tetracycline in Q2 of the Mass Spectrometer.

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Table 3.11 Direct Infusion MS/MS Results of Tetracycline in the Unchlorinated Sample and the [HOCl]:[TC] 1:20 in LGW 24 hours after Reaction of Tetracycline with Free Chlorine with a Residual of 0mg/L as Cl$_2$ due to the addition of quenching agent

Fragment ion mass loss	Unchlorinated Tetracycline		Chlorinated Tetracycline	
	m/z	ion counts	*m/z*	ion counts
	445	14,053,998	445	423,485
-17	428	1,095,393	428	Not seen
-18	427	3,519,928	427	92,521
-35	410	3,806,626	410	111,515

Chlorine Isotope Ratios

Chlorine isotope ratios in the [TC]:[HOCl] = 1:20 sample were targeted to obtain structural information to enhance the results from TOX analysis (Table 3.4). Results from TOX analysis showed the incorporation of three chlorine substituents onto the parent tetracycline molecule. Based on the experiments described above several potential transformation products of tetracycline (*m/z* 461, 467, 477, 483) were targeted in quadrupole 1 of the mass spectrometer as defined by the instrument software to determine the extent to which chlorine was incorporated onto the parent molecule. These ions [M] and the chlorine isotopes ([M+2], [M+4], and [M+6]) were targeted in the [HOCl]:[TC] 1:20 in LGW sample to determine the degree of chlorine substitution based on the presence of peaks corresponding to ^{35}Cl, ^{37}Cl, ^{39}Cl, and ^{41}Cl, respectively. Preliminary experiments on chlorinated BACs revealed the characteristic chlorine isotope ratios that can be expected for a mono-, di-, and tri- chlorinated compound (Appendix 4). A mono-chlorinated compound has an isotope ratio of ^{35}Cl:^{37}Cl of 3:1. A di-chlorinated compound has an isotope ratio of ^{35}Cl:^{37}Cl:^{39}Cl of 9:6:1 while a tri-chlorinated compound has an isotope ratio of

$^{35}Cl:^{37}Cl:^{39}Cl:^{41}Cl$ of 27:27:9:1. These characteristic chlorine isotope ratios were not observed for the targeted molecular ions as shown in Figures 3.28 and 3.29 of *m/z* 477 and *m/z* 467. Therefore, if the chlorinated organics are formed as indicated from the TOX results and higher molecular weight species correlating to chlorinated tetracycline are not observed then it can be concluded that the parent tetracycline has broken apart into smaller chlorinated species that lack aromaticity which was suggested by the absence of absorbance during UV analysis. Although LC-MS analysis can be used to target specific species it is very difficult to analyze every ion that the MS detects. Additionally while UV analysis is helpful to determine the aromatic character of a species it cannot provide information when aromaticity is lost. In conclusion, other tests are needed in order to evaluate the fate of tetracycline after chlorination. Most useful would be the evaluation of biochemical activity of the product species in order to assess the risk from the transformation of tetracycline.
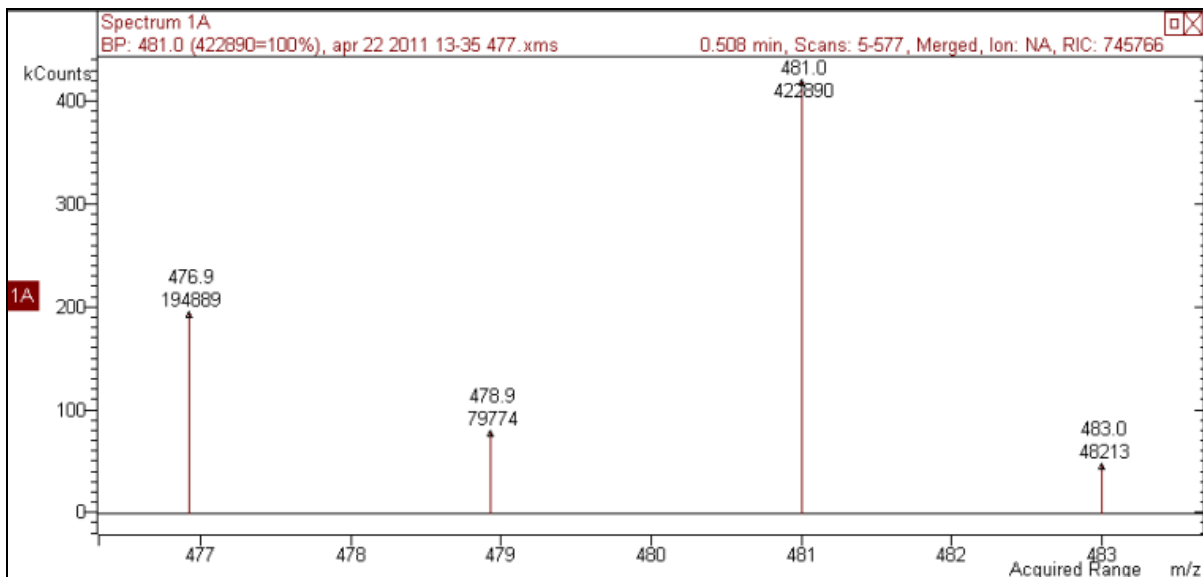
Figure 3.28 Chlorinated Tetracycline [TC]:[HOCl] = 1:20 in LGW After 24 Hours Contact Time with Quenched Chlorine. Chlorine Isotopes [M], [M+2], [M+4], and [M+6] of Potential Transformation Product *m/z* 477 Targeted in Q1 of the Mass Spectrometer. The average ion counts are shown below each ion. The ratio of 476.9 : 478.9 is 2.5:1.
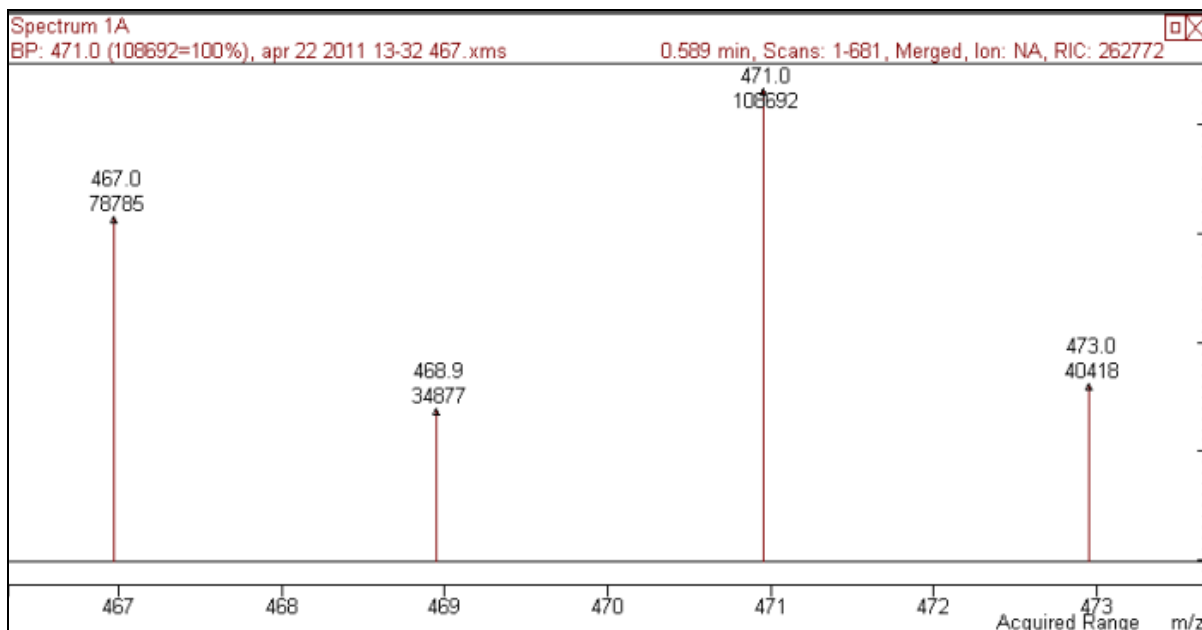
Figure 3.29 Chlorinated Tetracycline [TC]:[HOCl] = 1:20 in LGW After 24 Hours Contact Time and Quenched Chlorine. Chlorine Isotopes [M], [M+2], [M+4], and [M+6] of Potential Transformation Product *m/z* 467 Targeted in Q1 of the Mass Spectrometer. The average ion counts are shown below each ion. The ratio of 467.0 : 468.9 is 2.3 : 1.

3.4 Conclusions

Summary of Tetracycline Case Study

The results from the analysis of the reaction between the antibiotic tetracycline and free chlorine revealed that a rapid reaction occurs that can significantly transform the parent tetracycline molecule into by-products at high chlorine doses (mol/mol). Monitoring the fate of the parent molecule revealed that tetracycline undergoes a multistep reaction that is dependent of the dose of chlorine applied. Additionally UV analysis showed three separate stages in the transformation of tetracycline that was dependent on chlorine dose. TOX results showed the incorporation of three chlorine substituents into the parent molecule, and future experiments using TOX analysis could be used to evaluate incorporation of chlorine as a function of chlorine dose applied.

Results from a previous LC-MS study show the formation of transformation products of tetracycline due to reactions with free chlorine (Ye, 2005), and the current MS analysis confirmed the presence of these ions and others (*m/z* 467, 477, 483), but the identity of these products remain unknown. Chlorine isotope ratios of potential molecular ions of transformation products were targeted, and transformation products at *m/z* 477 and 467 were found to have isotope ratio of [M] : [M+2] of 2.5:1 and 2.3:1, respectively. Although mass spectrometers are highly sensitive analytical tools for the detection of known and optimized target compounds, sensitivity is significantly reduced when searching for unknown compounds. A major analytical challenge is determining how to scan for chemical substances for which the structure is not known. Completing product isolation and/or concentration prior to MS analysis may aid in the analysis of transformation products.

The results from this study show that if tetracycline is present at WWTPs or DWTPs it can be expected to react with free chlorine and transform to a high degree under conditions employed since tetracycline is present at nano- to submicro-gram/L levels and, therefore, can be expected to face a large molar excess of chlorine (mg/L). The implications are that tetracycline will undergo transformation into unknown chlorination by-products that elude detection during occurrence studies and have unknown biochemical properties. This rapid and high degree of transformation may be one reason (in addition to chelation with metals (Halling-Sørensen et al., 2002)) why tetracycline is infrequently found in surface waters (Kolpin et al., 2002), source drinking waters (Stackelberg et al., 2007; Focazio et al., 2008) and drinking waters (Stackelberg et al., 2007) during occurrence studies.

As a widely used antibiotic that is highly reactive with free chlorine, understanding the environmental fate of tetracycline is very important to guide future occurrence and

toxicity studies. Since the tetracycline molecule undergoes a rapid and rather complex transformation, understanding the resulting biochemical properties of the transformation by-products is very important in order to determine risk. Additionally, potential transformation products should be scanned during occurrence studies in order to determine if waters are impacted by these chemicals.

General Conclusions

The results from TOX analysis show that the reactivity of BACs with free chlorine depends on the structure of the compound. High reactivity was observed for compounds with extended regions of π-conjugation and ring activating functional groups on the aromatic ring. Conversely, low reactivity was observed for aliphatic compounds and compounds that already contained chlorine. The varying degrees of reactivity highlight the cumbersome task of effectively managing countless anthropogenic contaminants in drinking water. How can those compounds that persist through treatment with free chlorine be removed? One area of research is in the evaluation of different treatment technologies for their effectiveness in the removal of BACs. Also, how can the fate of BACs highly reactive with free chlorine be monitored during water treatment? Is it possible that the environmental and human health impacts of these BACs are being underestimated as their transformation into by-products is not accounted for by current monitoring? Additional areas of research to answer these questions require a study of the biological activity of BACs after chlorination as well as the targeting of chlorination transformation by-products in future occurrence studies. Chapter 4

describes an occurrence study completed at several North Carolina drinking water plants, and

the presence of BACs detected is related to their reactivity observed with free chlorine.

4. OCCURRENCE OF BIOCHEMICALLY ACTIVE COMPOUNDS DURING DRINKING WATER TREATMENT

4.1 Introduction

High use of biochemically active compounds (BACs) paired with their incomplete metabolism in the body (Table 1.1), incomplete removal during wastewater treatment (Table 1.2), and transport through the environment results in BACs impacting drinking water sources (Table 1.5). While full-scale occurrence studies have been completed (Stackelberg et al., 2007), limited information is known about the occurrence and fate of BACs in the State of North Carolina. North Carolina is a rapidly growing state and it is imperative to water utilities that their treatment technologies can withstand the impact of a growing population. There are many origins of BACs in the State's surface waters from point (wastewater treatment plant, processing waste from medical, health-care and manufacturing industries) and non-point sources (concentrated animal feeding operations, landfill leachate, failed septic tanks, and land runoff). Providing up-to-date information on sources and causes of impaired water quality for water treatment utilities is essential to evaluate current treatment performance, identify areas to improve, and determine the most effective management strategies. In order to provide a snapshot of BAC fate and occurrence in North Carolina drinking water treatment plans an occurrence study was undertaken whose objectives were to: (1) complete an initial broad screen of various BACs to determine their fate and occurrence in North Carolina Drinking Water Treatment Plants (2) to relate the occurrence of

BACs to their fate during final disinfection at these plants, and (3) to relate the presence of BACs to potential upstream water quality impacts.

4.2 Sample Collection Site Information

Sampling was completed between July 2010 to October 2010, from three different drinking water treatment plants; DWTP1: Orange Water and Sewer Authority (OWASA) (Carrboro, NC), DWTP2: J.D. Mackintosh Drinking Water Treatment Plant (Burlington, NC), and DWTP3: Williams Drinking Water Treatment Plant (Durham NC). Each plant was studied once during this time period.

Each DWTP obtains source water from surface water reservoirs and employs treatment processes including screening, powdered activated carbon (PAC) (DWTP1), potassium permanganate (DWTP1), coagulation/flocculation, sedimentation, granular media filtration, chlorine primary disinfection, chlorine secondary disinfection (DWTP2), and treatment with chloramines for secondary disinfection (DWTP1, DWTP3). After treatment, water travels through the distribution system to consumers. Figures 4.1 to 4.3 show the schematics of the treatment plant processes employed at each DWTP and include chemical doses and detention times used on the dates of sample collection as provided by plant personnel.

During the current study 24 hour composite samples (four sample fractions every six hours) were collected with the only exception being the settled water from DWTP3 where grab samples were collected from each settling basin due to lack of feasibility of composite sample collection. Since temporal variability can occur, composite sampling provides a more

accurate measure of occurrence. Sampling points were untreated source water, conventional settled water, and finished water. Additionally, one distributed water sample was collected from a residence that received water from DWTP1. Table 4.1 provides water quality data on the dates of sample collection, which was provided by utility personnel.

Samples were collected from DWTP1 between 7/20/2010 and 7/21/2010. The first set of chemicals including aluminum sulfate, powdered activated carbon, and potassium permanganate were flash mixed and added to the screened raw water. The water was subsequently mixed for approximately 30 minutes in the flocculation basin before entering the conventional sedimentation basin. The detention time of the pre-disinfectant (sodium hypochlorite), which was added on top of the filters, varied from 15-25 minutes depending on the filter column. Post-chlorine was added after filtration and prior transport to the clearwell which took approximately 150 minutes. The ammonia was added at the end of the clearwell to form chloramines prior to release into the distribution system.

Samples were collected from DWTP2 between 8/31/2010 and 9/1/2010. The aluminum sulfate added to the screened raw water for coagulation/flocculation had an approximate contact time of 73 minutes. Samples were collected from DWTP3 between 9/16/2010 and 9/17/2010. The coagulant, ferric sulfate, is added to screened raw water for a contact time of 2.2 minutes. The detention time of flocculation is 15 minutes and the detention time in the settling basin in 2.2 hours. Sodium hypochlorite is added both before and after filtration and the contact time is 2.6 hours. Ammonia, to form chloramines, is added after the clearwell and has a contact time of 2.6 hours.

Table 4.1 Water Quality Data of Source and Finished Waters of DWTPs 1-3 Provided by the Utility

	DWTP1	DWTP2	DWTP3
Water Source	Cane Creek Reservoir	Lake Mackintosh	Lake Michie/Little River Reservoir
Source Location	Carrboro, NC	Burlington, NC	Durham, NC
Source Type	Surface Water	Surface Water	Surface Water
Raw Water			
pH (average)	6.7	NT	6.7
TOC (mg/L as C)	5.2	6.9	6.28
Average flow rate through plant (MGD)	8.43	7.14	10
Temperature (°C)	25.5	30	25
Finished Water			
pH (average)	8.4	NT	7.7
TOC (mg/L as C)	0.95	2.9	1.9
Residual Free Chlorine (mg/L as Cl_2)	NT	1.3*	0.1
Residual Total Chlorine (mg/L as Cl_2)	3.46	NA	3.1
Collection date	July 20/21, 2010	August 31/September, 2010	September 16/17,2010
Sample Type	24 hour composite samples	24 hour composite samples except settled (grab)	24 hour composite samples

*Data from analysis at UNC
NA=Not Applicable
NT=Not Tested
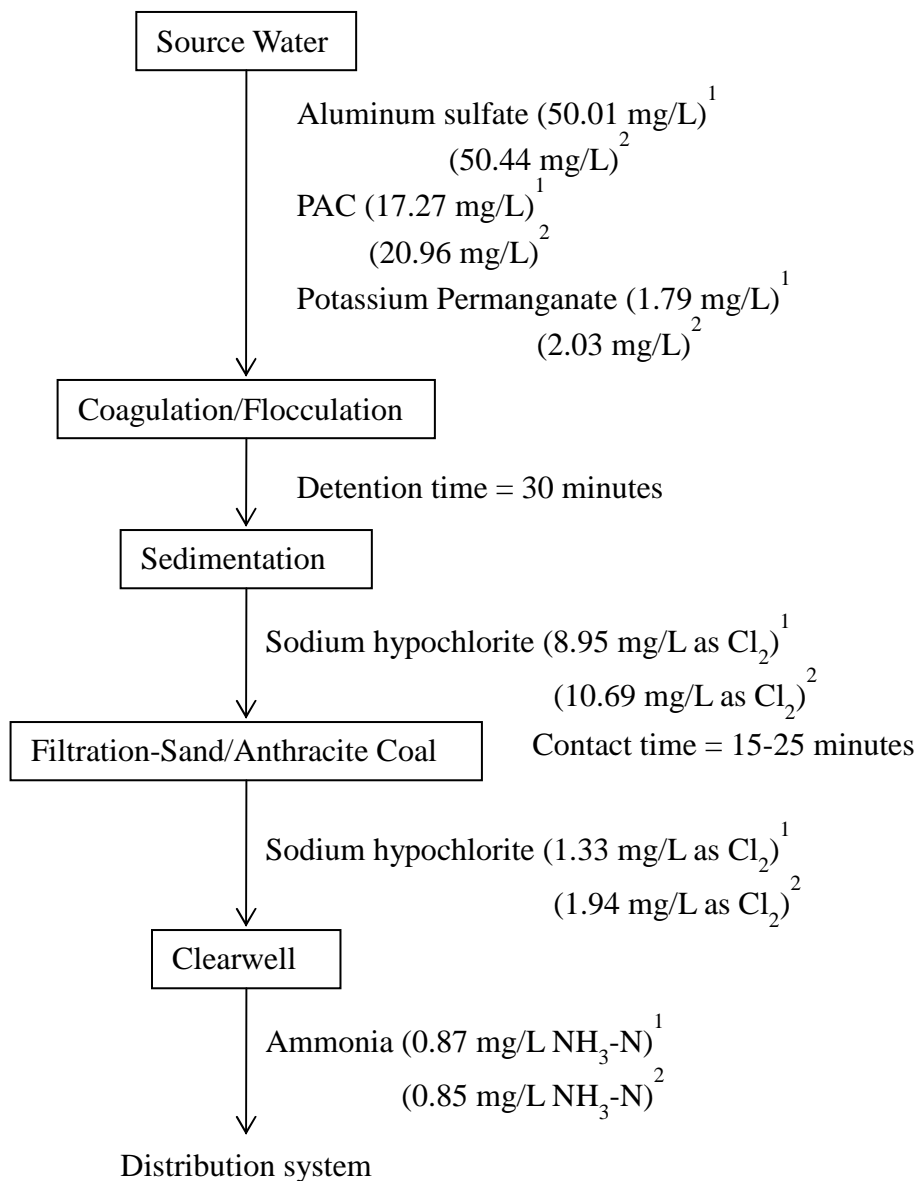
Figure 4.1 Plant Schematic of DWTP1 including Chemical Doses on [1]7/20/2010 and [2]7/21/2010. (PAC: Powdered Activated Carbon)

Source Water

Aluminum sulfate (~50mg/L)

Coagulation/Flocculation   Contact time 1.13 hours

Sedimentation

Sodium hypochlorite (~4.7 mg/L as Cl_2)

Filtration-Sand/Anthracite Coal

Sodium hypochlorite (~1.6mg/L as Cl_2)

Clearwell

Distribution system
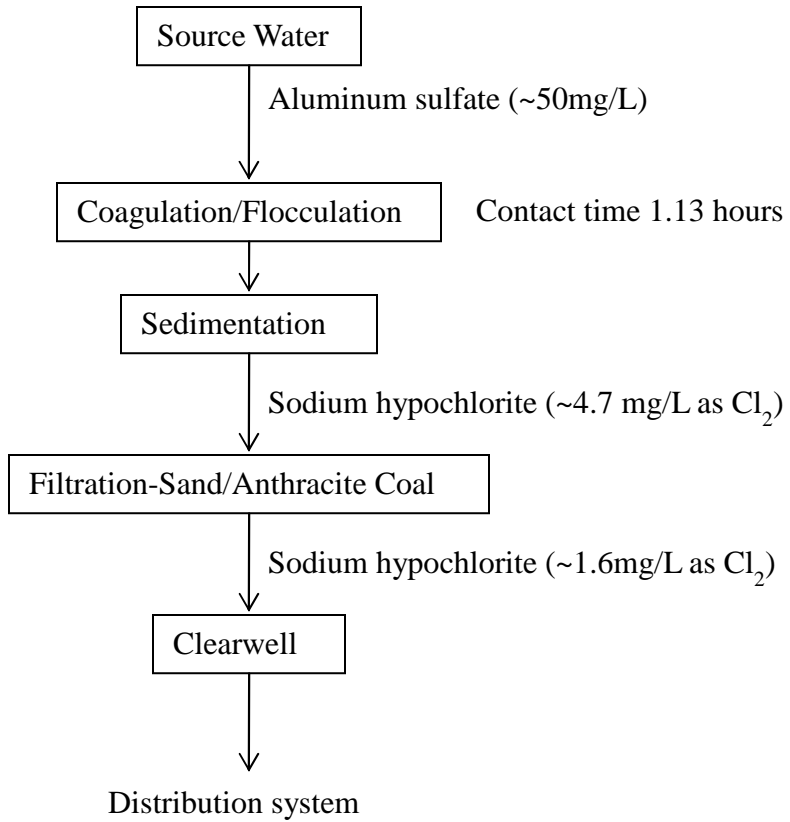
Figure 4.2 Plant Schematic of DWTP2 including Chemical Doses. Values are approximate because annual average doses were obtained from the City of Burlington, NC 2009 Water Treatment Annual Operations Report.
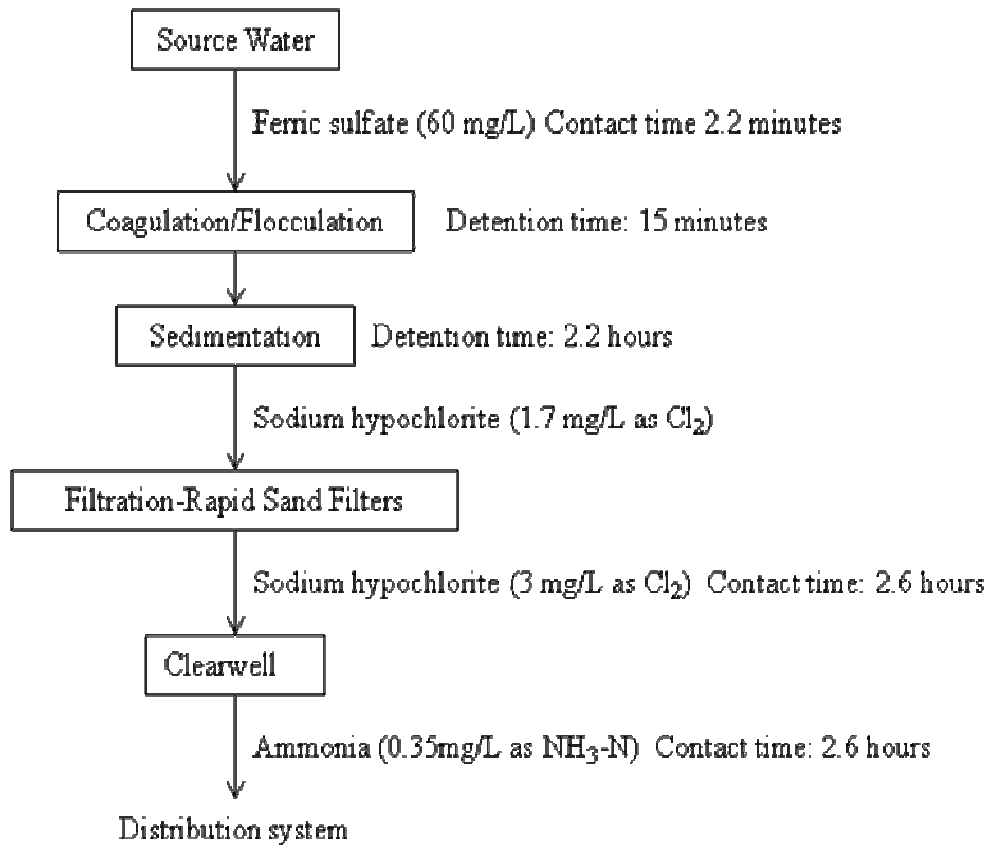
Figure 4.3 Plant Schematic of DWTP3 including chemical doses, contact time of chemicals and detention times.

4.3 Results and Discussion

4.3.1 Fate of BACs during Drinking Water Treatment

Table 4.2 shows the concentration of the BACs detected in the sampled source waters during the occurrence study. The underlined values indicate that both daughter fragment ions of the parent BAC were detected which means a higher level of confidence is associated in reporting the detection of these compounds. BACs that are not listed were not detected.

Table 4.2 Concentrations (ng/L) of BACs Detected in Drinking Water Sources in North Carolina

BAC	DWTP1	DWTP2	DWTP3
		Concentration (ng/L)	
Date	July 20/21, 2010	August 31-September 1, 2010	September 16/17, 2010
Atrazine	<u>190</u>	<u>260</u>	<u>110</u>
Caffeine	<5.1	11	<5.1
DEET	<u>2.5</u>	<u>8.2</u>	<u>10</u>
TCEP	<45	18	<45

"_" indicates that the detection of the analyte is confirmed by the ratio of the two targeted MS/MS daughter fragment ions
Analytes not detected are reported as <LOQ

Both DEET and atrazine were detected in the source waters of all three DWTPs sampled. This occurrence may be the direct result of their high use during the sampling period (summer months). DEET has been reported as among the most prevalent contaminants in Germany (Quednow and Püttman, 2009) and atrazine in Canada (Byer et

al., 2011).   Insect-repellents containing the active ingredient DEET are commonly used during the summer and may wash off the user in the shower or excreted in urine after absorption through the skin and has been found to be in urine 5.6% unchanged (Selim et al., 1995).   In either case, DEET will be transported to WWTPs where incomplete removal has been reported (Sui et al., 2010).   Occurrence studies have shown that DEET is present in wastewater effluents at average levels of 27ng/L (5 detections; n = 7) (Kim et al., 2007) and 98ng/L (11 detections; n = 11) (Ryu et al., 2011) that are subsequently released into the environment.   Another possible route of exposure of DEET into surface water is direct deposition from persons using DEET-containing insect-repellants while boating and fishing.

Atrazine, a widely used herbicide, may have contaminated these source waters either as the result of surface water runoff from agricultural land (Thurman et al., 1992), or from aerial deposition of atrazine that had transported through the atmosphere during rain events (Goolsby et al., 1997).   While the land directly surrounding the source waters is not extensively used for agriculture (see Figures in Appendix 3.1, 3.3, and 3.5), land use in the watersheds of the sampled source waters includes row crops (NC SWAP, 2010a; NCSWAPb, 2010; NCSWAPc, 2010).   Other potential contaminant sites (PCS) in the watersheds include animal operations, isolated pollution incidents, non-discharge permits, minor national pollutant discharge elimination system (NPDES) (DWTP2 and WWTP3), airports (DWTP2 only) (see Figures in Appendix 3.2, 3.4, 3.6). Table 4.2 also shows that caffeine and TCEP were detected in the source water used by DWTP2.   Caffeine can be used as an indicator for WWTP effluent (Ferreira et al., 2005), and the source water used by DWTP2 is impacted by an upstream discharging WWTP while DWTP1 and DWTP2 are not (NC SWAP, 2010a; NCSWAP, 2010b; NCSWAP, 2010c).

Table 4.3 shows the concentration of detected BACs in settled water as well as the percent removal from raw water. The most dramatic decrease in concentration is observed for the detected analytes in DWTP1, which is the only plant of the three to use both powdered activated carbon (PAC) and potassium permanganate (for taste and odor control) in addition to the chemical coagulants. Organic compounds have been found to adsorb very effectively onto PAC due to its high surface area, and studies have shown effective removal of BACs with PAC (Ternes et al., 2002; Westerhoff et al., 2005; Stackelberg et al., 2007)including DEET and atrazine at, 49% and 60%, respectively (Westerhoff et al., 2005). The results from this current study are consistent with these published values. The use of PAC more effectively removed atrazine relative to DEET which is the result of its greater hydrophobic character (Table 1.3), and this correlation has been studied in the literature (Simazaki et al., 2008). In contrast it can be seen that a significantly smaller reduction in parent BAC is observed in settled water for DWTP2 and DWTP3, where only coagulation is used. The removal of atrazine during coagulation in all DWTPs was greater than DEET which can be explained by the greater hydrophobic character of atrazine (Table 1.3), and this has been observed previously (Stackelberg et al., 2007).

Table 4.3 Concentrations (ng/L) of BACs Detected in Settled Drinking Water in North Carolina (and percentage decrease from surface water)

BAC	DWTP1	DWTP2	DWTP3
		Concentration (ng/L)	
Atrazine	<u>36</u> (81%)	<u>240</u> (9.7%)	<u>98</u> (8.1%)
Caffeine	<5.1	9.1 (14%)	<5.1
DEET	<u>1.6</u> (38%)	<u>8.2</u> (0.22%)	<u>11</u> (0%)
TCEP	<54	11 (36%)	<54

"_" indicates that the detection of the analyte is confirmed by the ratio of the two targeted MS/MS fragment ions
Analytes not detected are reported as <LOQ

Table 4.4 shows the concentration of the detected BACs in finished drinking water, as well as the percent removal between settled and finished water, which can be attributed to filtration and the addition of chemical disinfectants. The removal of the detected BACs is variable, and this may be due to temporal variability within the plant, the age of the filters being used, and the contact time of chemical disinfectants. Since caffeine, DEET, atrazine, and TCEP were observed to incorporate minimal amounts of chlorine onto the parent BAC molecule as described in Chapter 3 and minimal reactivity has been reported in the literature (Glassmeyer and Shoemaker, 2005; Brix et al., 2008), it can be concluded that any decrease in the concentration of these BACs may be due to filtration, hydrolysis, biological degradation, or a combination of these factors.

Atrazine was found to have the largest percent removal and this may be due to transformation by reaction with free chlorine. Although the results from Chapter 3 indicated minimal incorporation of chlorine onto the parent molecule, a study by Wulfeck-Kleier et.

al., (2010) showed that upon the addition of the quenching agent (sodium sulfite) the chlorinated product of atrazine, *N*-chloro atrazine, can transform back into its original form, atrazine. Since this quenching agent was not added at DWTP1 but was added during the TOX experiments in Chapter 3, this finding may explain the larger percent removal of atrazine during disinfection than would be hypothesized based on TOX results.

Table 4.4 Concentrations (ng/L) of BACs Detected in Finished Drinking Water in North Carolina (and percentage decrease from settled water)

BAC	DWTP1	DWTP2	DWTP3
		Concentration (ng/L)	
Atrazine	13 (64%)	230 (1.9%)	84 (15%)
Caffeine	<2.0	5.8 (36%)	<2.0
DEET	1.3 (15%)	8.3 (0%)	6.4 (40%)
TCEP	<5.4	<6.7 (100%)	<4.5

"_" indicates that the detection of the analyte is confirmed by the ratio of the two targeted MS/MS fragment ions
Analytes not detected are reported as <LOQ

Table 4.5 shows the concentration of the detected BACs in a distributed water sample from DWTP1, as well as the percent removal between finished and drinking water, which can be attributed to the reactions with residual chemical disinfectants. The attenuation of DEET was observed to be minimal which is supported by its minimal reactivity with free chlorine as described in Chapter 3. The removal of atrazine observed in Table 4.5 can be attributed to reactions with residual disinfectants and transformation of the parent molecule to form *N*-chloro atrazine (Wulfeck-Kleier et. al., 2010).

Table 4.5 Concentrations (ng/L) of BACs Detected in Distributed Drinking Water in North Carolina (and percentage decrease from finished water)

BAC	DWTP1	DWTP2	DWTP3
		Concentration (ng/L)	
Atrazine	<u>5</u> (58%)	NT	NT
Caffeine	<2.0	NT	NT
DEET	<u>1.3</u> (0.36%)	NT	NT
TCEP	<5.4	NT	NT

"_" indicates that the detection of the analyte is confirmed by the ratio of the two targeted MS/MS fragment ions

NT=not tested

Analytes not detected are reported as <LOQ

In summary, Table 4.6 shows the total percent removal of the detected BACs from source water to finished drinking water. The largest percent removal for DEET and atrazine was observed for DWTP1 where PAC is used, and this efficient removal has been observed in previous laboratory studies (Westerhoff et al., 2005). For the BACs in DWTP2 and DWTP3 the observed removal shown in Table 4.6 is the result of the chemical properties of the analytes, the characteristics of the source water, temporal and special variability within the plant, and the interactions with the physico-chemical treatment processes employed. The graphical representations of the removal of the detected BACs at each plant are shown in Figures 4.4-4.6.

Table 4.6 Percent Removal of BACs from Source to Finished Water (%)

BAC	DWTP1[*]	DWTP2	DWTP3
Atrazine	97	11	22
Caffeine	NA	45	NA
DEET	47	0	36
TCEP	NA	NA	NA

NA= not applicable

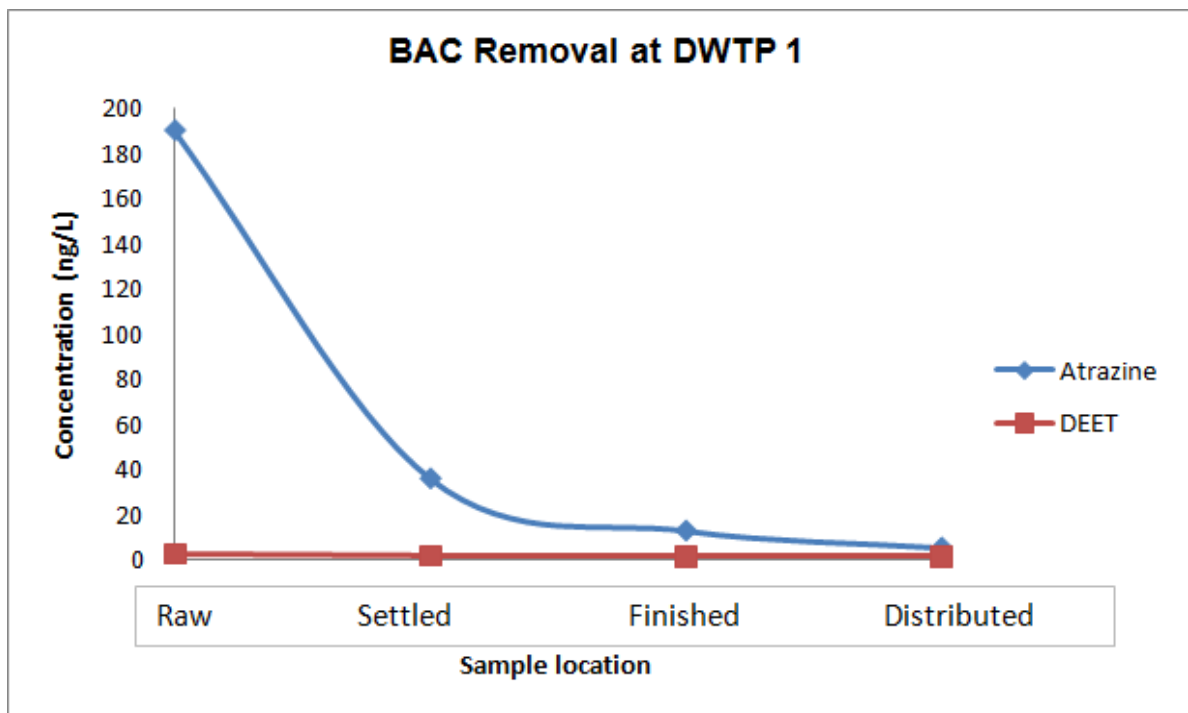[*]Removal is from source to distributed water

Figure 4.4 Removal of Detected BACs from DWTP1
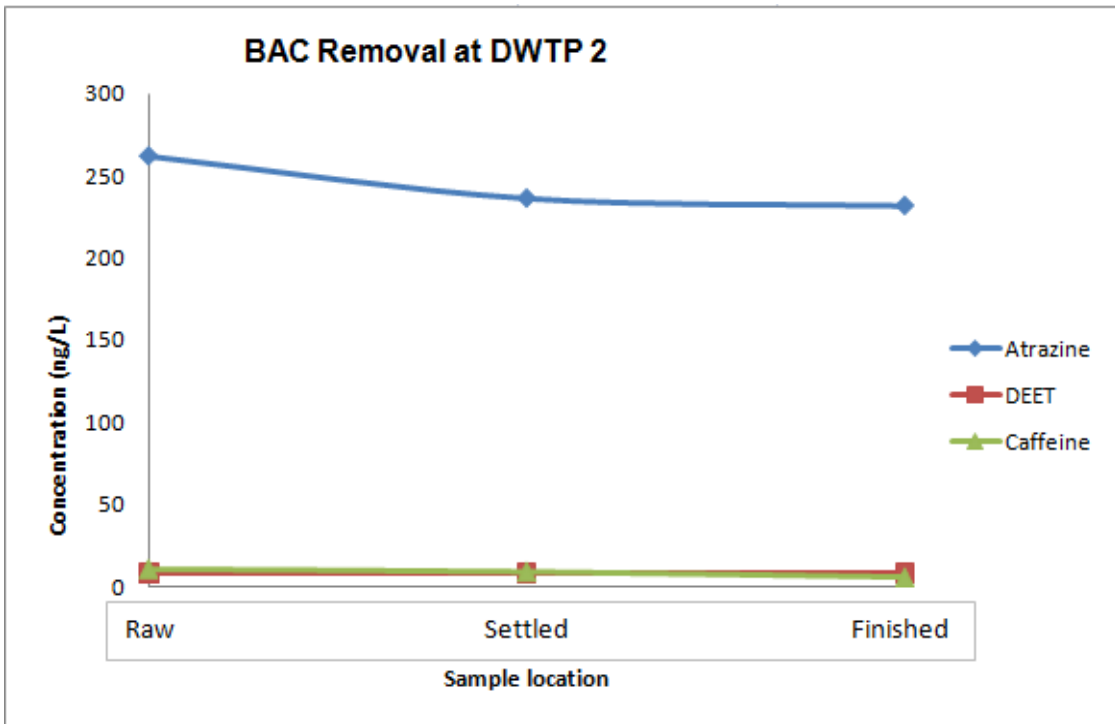
Figure 4.5 Removal of Detected BACs from DWTP2
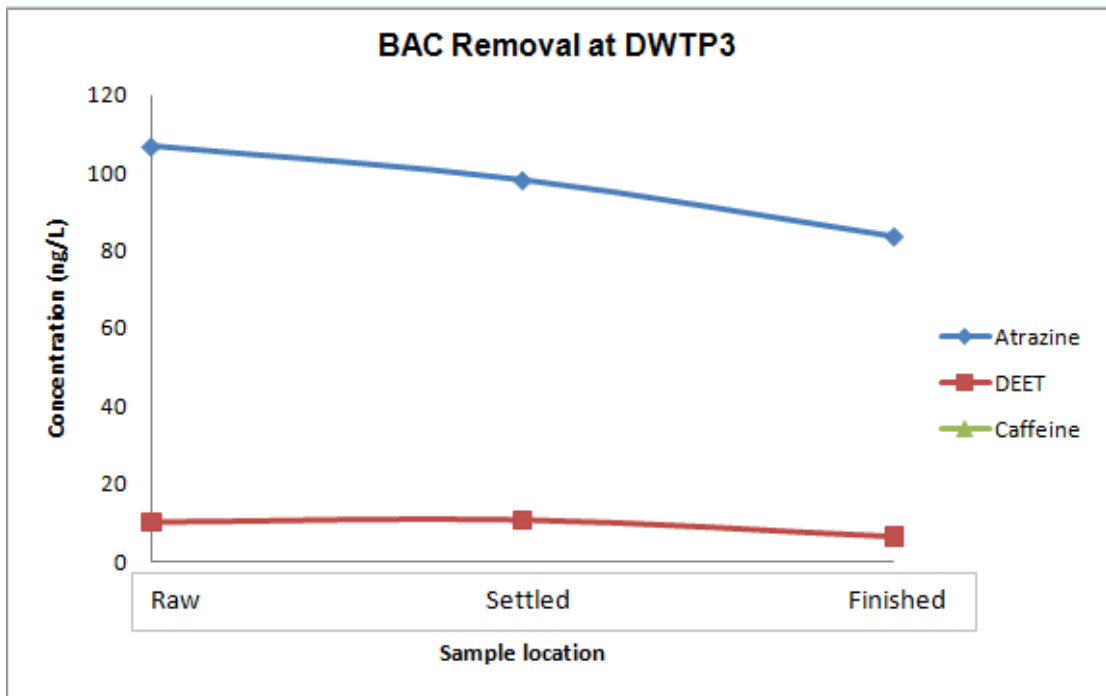
Figure 4.6 Removal of Detected BACs from DWTP3

4.4 Conclusions

The results from the occurrence study of three DWTPs in North Carolina reveal that DEET and atrazine were present in all study sites, that their removal during treatment is dependent on their chemical properties, and that they persist into consumers' drinking water. The presence of DEET and atrazine in all source waters may be the result of the sampling being conducted during the summer months when these chemicals are more widely used. Further investigation would be required in order to determine if they are present throughout the year.

Atrazine and DEET have been detected in many surface waters in the U.S., (Kolpin et al., 2002; Benotti et al., 2009). Atrazine, a member of the triazine pesticide group and containing an s-chlorotriazine moiety is under review by the U.S. EPA for toxicity (U.S. EPA, 2006). Additionally, chlorinated transformation products of atrazine have been detected which still contain the s-chlorotriazine moiety (Wulfeck-Kleier et. al., 2010), and the toxicity of these species remains unknown. DEET has been observed to have acute toxicity in birds and aquatic organisms (U.S. EPA, 1998). Therefore stricter regulations should be placed on the use of these persistent anthropogenic contaminants to minimize their release into the environment.

Removal of the detected compounds, DEET and atrazine, was observed to the highest extent when PAC was used, but this still did not eliminate them completely from drinking water. PAC, usually employed for taste and odor control, was observed to be the most effective treatment option in this current study for the removal of BACs, and this has been observed in previous studies (Ternes et al., 2002; Stackelberg et al., 2007). Incentives should be given to utilities in order to expand the use of PAC for BAC removal such as the

production of information leaflets in monthly bills to raise awareness with consumers about the importance of this treatment technology.

The results from the occurrence study can be explained by the reactivity of the studied BACs with chlorine (Chapter 3). For those compounds with low reactivity towards free chlorine (i.e. caffeine, atrazine, and DEET) it can be expected that their removal during disinfection using chlorine will be low. As a result, these compounds persist into drinking water and the long-term impacts are not well understood. On the other hand, compounds highly reactive with chlorine were not detected in any of the sampled source waters. One possibility is that these compounds may be transforming during wastewater treatment into chlorinated by-products that elude detection when only the parent compounds are targeted in analytical methods. For example, the wastewater treatment plant upstream of DWTP2 uses chlorine as a terminal disinfectant and only those compounds with low reactivity towards chlorine were detected in the source water at DWPT2. On the other hand, the wastewater contaminant, sulfamethoxazole, which is commonly found in surface waters (Kolpin et al., 2002) and drinking water sources (Stackelberg et al., 2007; Benotti et al., 2009), has been observed to react with free chlorine to produce chlorinated by-products in both the literature (Dodd and Huang, 2004) and in the current study (Chapter 3). An implication of chlorinated BACs eluding detection is that the environmental and health impacts of BACs may be underestimated and the risk associated with exposure to these transformation by-products should be evaluated during toxicity studies. Additionally, in future occurrence studies scanning samples using full scan mass spectrometry for chlorination by-products of BACs may provide insight onto the occurrence of these compounds into drinking water.

5. CONCLUSIONS

The impact of chlorine on the structure of parent BACs was evaluated in controlled laboratory experiments using TOX analysis, which has been used only to a limited extent for studying the transformation of antibiotics (Ye, 2005). TOX analysis was used to determine the extent to which chlorine was incorporated onto the parent BAC, and the variability was determined to be a direct result of the chemical structure of the BACs. Those with aliphatic regions (TCEP and meprobamate), electronegative substituents (fenoprop, atrazine, diclofenac, bezafibrate, and clofibric acid), bulky side chains (ibuprofen) or highly substituted aromatic structure (fenoprop, caffeine, and atrazine) were found to have minimal reactivity with chlorine. On the other hand those with aromatic regions (extended π-conjugation), ring activating substituents (-OH, -NH$_2$), and available positions for electrophilic attack on the aromatic ring (estrone, 17-α-ethinyl estradiol, acetaminophen, tetracycline, sulfamethoxazole, and trimethoprim) were found to have high reactivity with chlorine which was observed by high levels of chlorine incorporation. The high reactivity of tetracycline was investigated in detail and its disappearance was observed using UV and MS/MS analysis. Although the parent tetracycline molecule was not observed after chlorination no transformation products were identified. Future UV-Vis experiments should employ a different control sample in the double-beam spectrophotometer which may allow for transformation by-products to be distinguished from the background signal associated

with the control sample. Additionally, future MS/MS experiments should aim to target lower molecular weight fragments of the tetracycline molecule since the UV results point to a loss of aromaticity. While both UV-Vis and MS/MS experiments are helpful in monitoring a known chemical compound there are limitations encountered when looking for unknowns. Directing risk-assessment of transformation products and guiding sampling efforts would be aided by determining the biochemical activity of the transformation by-products, and in future studies the antibiotic activity of tetracycline should be monitored. TOX analysis revealed that several compounds have moderate reactivity with free chlorine (erythromycin-hydrate, sucralose, gemfibrozil, atenolol, and carbamazepine) which is attributed to a combination of chemical structure and slow reaction rates.

An occurrence study was completed in the state of North Carolina to determine the presence of targeted BACs in three source, settled, and finished (and one disturbed) water samples. The results of the study revealed that DEET and atrazine were persistent contaminants that were detected in the source waters of each DWTP. This high occurrence is most likely the result of sampling during the summer months when these compounds are used in higher quantities. TCEP and caffeine were only detected at one of the DWTPs, which is impacted by upstream WWTP effluent. Although the upstream WWTP uses chlorine as a disinfectant, TCEP and caffeine were not removed or transformed and this can be expected since they were observed to have low levels of reactivity with free chlorine during TOX experiments.

The removal of the detected BACs in each sampled DWTP was found to be dependent of the treatment processes employed. The most effective treatment for the removal of the DEET and atrazine was found to be PAC, but even with this treatment

technology the compounds were not completely removed from consumers' drinking water. This highlights the importance of determining the most effective treatment options for the removal of persistent contaminants or the enforcement of stricter regulations on the usage of these contaminants. Even more troubling are the unknown impacts of the transformed BACs that are not detected when only the parent BAC is targeted. Only those BACs with low reactivity towards chlorine were detected in drinking water sources and it is possible that those BACs with high reactivity have transformed into unknown products that elude detection using the employed analytical method. Challenging future work would be to develop ways to screen waters for the presence of unknown contaminants and transformation products. Additionally, the transformation of those BACs with high reactivity towards chlorine should be studied in greater detail in order to provide more information for both occurrence studies and toxicity studies.

Appendix 1:

Composite Sample Collection Procedure

Points of Collection: Raw, conventional settled and finished water.

Procedure to collect composite samples: The glass amber bottles contain a solid preservative (ethylenediaminetetraacetic acid).

Composite Samples: In order to collect the composite samples, approximately 1/4 of the sample bottle will be filled every 6 hours for a total time of 24 hours. The total sample volume of the larger bottles are 4L, the medium is 2.5L and the small bottles are 1L.

Sample collection procedure:

1. The bottles do not need to be rinsed before sample collection. Choose the correct bottle labeled for the specific tap in the lab for sample collection.

2. To collect the sample fraction at each time, hold the bottle at an angle close to the sample tap to prevent dust from the air to enter the bottle and fill approximately to the premarked line. Cap the bottle, mark the time of each fraction's collection on the bottle label and place in a fridge until the next time of sample collection. At that time repeat the procedure until 4 samples have been collected in each bottle. DO NOT use sharpies, pencil is best to use.

3. DO NOT mix the sample until the last sample fraction has been collected.

4. For the final sample collection fill the bottle headspace free, replace the cap, invert the bottle three times, and place immediately in the fridge.

5. The bottles will be collected as per initial discussion.

Appendix 2:

Standard Operating Procedure (SOP)

Title: Analysis of Antibiotics in Surface and Drinking Water Samples

Purpose: This standard operating procedure (SOP) describes the analysis of 25 antibiotics including 6 tetracyclines, 7 sulfonamides, 7 (fluoro)quinolones, 3 macrolides, trimethoprim, and lincomycin in surface and drinking water samples at ng/L levels using solid phase extraction and liquid chromatography electrospray tandem mass spectrometry.

1. Preparation of solutions

1.1. Preparation of antibiotic solutions

Equipment:

- balance

- 10 and 25 mL volumetric flasks

- beakers and funnels

- 10 and 40 mL amber vials

- 250 mL amber bottles

- spatula

- weighing dishes

- pasteur pipettes

Reagents:

Antibiotic standards (Figure 1) stored in Freezer D.

Solvents:

- methanol

- LGW/methanol (1:1, 0.2% HCl, v/v)

- acetonitrile (2% NH$_4$OH, v/v)

Table A.2.1. Preparation of Stock Solutions of Antibiotics

	mol. Weight	HCl or salt form	stock conc. (mg/L)	weigh amount (g)	solvent	volume (mL)
minocycline	457.5	494.0	1000	0.0108	methanol	10
oxytetracycline	460.4	496.9	1000	0.0108	methanol	10
tetracycline	444.4	480.9	1000	0.0108	methanol	10
demeclocycline	464.9	501.4	1000	0.0108	methanol	10
chlortetracycline^ *	478.9	515.4	400	0.0135	methanol	25
doxycycline	444.4	480.9	1000	0.0108	methanol	10
meclocycline	476.9	695	1000	0.0146	methanol	10
sulfamerazine*	264.3	287.3	400	0.0109	methanol	25
sulfathiazole	255.3	278.3	1000	0.0109	methanol	10
sulfamethazine*	278.3		400	0.0100	methanol	25
sulfamethizole	270.3		1000	0.0100	methanol	10
sulfachlorpyridazine	284.7		1000	0.0100	methanol	10
sulfamethoxazole	253.3		1000	0.0100	methanol	10
sulfadimethoxine	310.3		1000	0.0100	methanol	10
trimethoprim*	290.3		400	0.0100	methanol	25
ciprofloxacin	331.3	367.8	1000	0.0111	LGW	10
Norfloxacin*	319.3		400	0.0100	LGW/methanol 1:1, 0.2% HCl, v/v	25

enrofloxacin*	359.4	395.9	400	0.0110	LGW/methanol 1:1, 0.2% HCl, v/v	25
sarafloxacin*	385.4		80	0.0080	90% LGW : 10% methanol	100
pipemidic acid*	303.3		400	0.0100	LGW/methanol 1:1, 0.2% HCl, v/v	25
oxolinic acid*	261.2		400	0.0100	Acetonitrile (2% NH_4OH, v/v)	25
Flumequine	261.2		1000	0.0100	Acetonitrile (2% NH_4OH, v/v)	10
Tylosin	916.1	1066	1000	0.0116	methanol	10
erythromycin	733.9		1000	0.0100	methanol	10
roxithromycin	837.1		1000	0.0100	methanol	10
Lincomycin	406.5	443.5	1000	0.0109	methanol	10

^: purity: 80%   *: not easily soluble

1.1.1. Stock solutions

1. For an EASILY soluble antibiotic (listed on Figure 1 without a "*"):

    - Tare the scale after placing the weighing dish on the balance

    - Close the glass door of the balance before weighing

    - Use the spatula to transfer certain amount (Figure 1) of antibiotic reagent to the

      weighing dish (listed weights are ideal – get as close as possible but not critical

      to be exact)

- Record the weight to 4 decimal digits

- Use small (5 ¾") pasteur pipettes to transfer appropriate solvent into the weighing
   dish (start with 2 pipettes of solvent)

- Use a different pipette and gently stir in dish to dissolve antibiotic

- Transfer the antibiotic solution into a volumetric flask of appropriate volume

- Transfer all remaining antibiotic in the dish to the volumetric flask by adding

   solvent and transferring a few times

- Fill up the volumetric flask up to the line with the solvent

- Cap the volumetric flask and invert 3 times

- Calculate the actual concentration of the prepared stock solution using the

   following equation:

$$\frac{W_{ideal}(g)}{W_{actual}(g)} = \frac{C_{ideal}(mg/L)}{C_{actual}(mg/L)}$$

   W: weight; C: concentration

- Transfer the solution into an amber vial and cap it

- Label the vial with antibiotic name, concentration, initials, and date of

   preparation

- Clean the spatula thoroughly (with LGW and then methanol) and dry thoroughly

   with Kimwipes

- Brush off scale gently between uses

2. For an antibiotic that is DIFFICULT to dissolve (listed on Figure 1 with a "*"):

   - Tare the scale and then weigh the volumetric flask without lid

   - Add antibiotic directly into the volumetric flask using a spatula

   - Subtract weight of the flask from the total weight of flask plus antibiotic to get

the weight of antibiotic

- Continue adding antibiotic into the flask until the desired weight is achieved

- Add appropriate solvent directly into the flask

- Can pour some solvent into the flask up to the neck and add solvent with pipette

  up to the line

- Cap the flask and invert many times until the antibiotic dissolves

- If the antibiotic still does not dissolve, cover the flask in aluminum foil (to prevent photodegradation) and let the flask sit on bench for a while, or put a small stir bar into the flask and use magnetic stir until it dissolves

- Once the antibiotic fully dissolves, transfer the solution into an amber vial (for a

  25 mL sample use 40 mL vial)

- Calculate the actual concentration (same as described in step 3 above)

- Label the vial with antibiotic name, concentration, initials, and date

3. Another technique to prepare stock solutions

   - Weigh out antibiotic in a weighing dish

   - Add some solvent into the dish

   - Transfer the antibiotic solution from the dish to the beaker by pouring carefully

  (try to get all solids in)

   - Once the solution is in the beaker, put a small stir bar into the beaker and use

     magnetic stir to help it dissolve

   - Place a funnel on top of the flask and transfer the antibiotic solution from the

     beaker to the flask (use glass rod to help control flow on the way down to the

     funnel)

Store all solutions in the freezer.   The stock solutions are stable for at least 3 months (tetracyclines, quinolones, macrolides, and lincomycin) and 1 year (sulfonamides and trimethoprim) when stored in freezer.

Notes:

- Usually takes ~ 1-2 days to prepare stock solutions of 25 antibiotics

- Antibiotic standards are stored in the little tub in freezer

- Antibiotic solutions are stored on ZY's shelf in freezer

- For ciprofloxacin ONLY, take 5 mL of the prepared 1000 mg/L stock solution to another volumetric flask and fill to 10 mL with methanol. This prevents the solution from being frozen because the solvent is LGW.

1.1.2. Mixture of each antibiotic group

Group 1: six tetracyclines (TCs) —minocycline, oxytetracycline, tetracycline, demeclocycline, chlortetracycline, doxycycline

Group 2: seven sulfonamides (SAs) and trimethoprim (TMP) —sulfamerazine, sulfathiazole, sulfamethazine, sulfamethizole, sulfachlorpyridazine, sulfamethoxazole, sulfadimethoxine, trimethoprim

Group 3: seven (fluoro)quinolones (QLs) —ciprofloxacin, norfloxacin, enrofloxacin, sarafloxacin, pipemidic acid, oxolinic acid, flumequine

Group 4: three macrolides (MAs) and lincomycin (LIN) — tylosin, erythromycin, roxithromycin, lincomycin

<u>Procedure</u>

1. Calculate the volume of antibiotic stock solution to be added to 10 mL of methanol

$$C_{stock} * V_{taken} = V_{mixture} * C_{mixture}$$

For example, to prepare a mixture at 20 mg/L for each antibiotic (typical concentration) in 10 mL methanol:

$$C_{stock} * V_{taken} = 20 \text{ mg/L} * 10 \text{ mL}$$

2. Get the antibiotic stock solutions from freezer, 10 mL volumetric flask, 50-250 μL micropipette, capillary tubes, HPLC grade methanol, waste bucket, and rinsing methanol

3. Fill the flask with some methanol (estimate total volume of solution that will be added and make sure there is more than enough room for it)

4. Pipette the stock solutions (V_{taken}) and inject to the flask (begin with ones that need the largest V_{taken})

- Adjust the micropipette volume setting to the desired value

- Clean the micropipette tip with methanol and dry with Kimwipe

- Insert capillary tube, tighten, and check

- Withdraw antibiotic solution (V_{taken})

- Wipe off solvent outside of capillary with Kimwipe

- Inject *under* liquid level and shake the pipette gently

- Remove glass capillary

- Rinse tip with methanol

- Repeat process for each injection

5. Fill the volumetric flask up to the line with methanol, cap and invert

6. Repeat for all antibiotic groups

7. Store the solutions in amber glass vials in freezer

1.1.3. Mixture of 25 antibiotics

1. Calculate volume taken from each mixture to certain volume of methanol (usually 10 mL)

$$C_{mixture} * V_{taken} = V_{mixture of\ 25} * C_{mixture of\ 25}$$

$C_{mixture}$: concentration of antibiotic in the mixture of each group (see 1.1.2)

For example, to prepare a mixture of antibiotics at 0.5 mg/L in 10 mL methanol from each 20 mg/L mixture:

$$20 \text{ mg/L} * V_{taken} = 0.5 \text{ mg/L} * 10 \text{ mL}$$

$$V_{taken} = 0.25 \text{ mL}$$

2. Prepare the mixture of 25 antibiotics using the same technique as described in step 4 above

1.2. Preparation of Surrogate and Internal Standard Solutions

1.2.1. Mixture of surrogate standards

1. Calculate the volumes of stock solutions of surrogate standards ($^{13}C_6$-sulfamethazine and meclocycline) to be taken to prepare a mixture of the two surrogates at an individual concentration of 0.5 mg/L in 5 or 10 mL of methanol.

For example, to prepare a 5 mL solution:

$$C_{stock} * V_{taken} = 0.5 \text{ mg/L} * 5 \text{ mL}$$

C_{stock} ($^{13}C_6$-sulfamethazine) = 5.9 mg/L in methanol

C_{stock} (meclocycline) = 10 mg/L in methanol

2. Prepare the mixture in methanol using the same technique as described for preparation of antibiotic solutions.

1.2.2. Internal standard (I.S.) solution

- The concentration of the internal standard simatone reagent solution is 100 µg/mL (i.e.100 mg/L)

- ALWAYS clean micropipette thoroughly with methanol to avoid contamination of the I.S. with antibiotics

- Make dilution of the simatone reagent solution to a desired I.S. concentration of 1.25 mg/L in 10 mL methanol

1.3. Preparation of other solutions

1.3.1. 0.1% Formic acid in methanol

Measure 200 mL methanol into a 250 mL amber bottle and add 200 μL formic acid with glass pipette. Cap the bottle and mix.

1.3.2. 0.25 g/L Na₂EDTA stock solution in LGW

Weigh 0.025 g Na$_2$EDTA, transfer to a volumetric flask, and dissolve in 100 mL LGW.

1.3.3. Solvent mixture of LGW and methanol (9:1)

Measure 180 mL LGW and 20 mL methanol, respectively, and mix in a 250 mL amber bottle.

2. Sample processing

2.1. Filtration

1. The 1st bench left side in MHRC 1210 is designated for filtration

2. Use filtration glassware designated for the appropriate type of water sample

3. The natural/raw water samples are filtered through glass fiber filters (0.7 μm pore size) and then nylon filters (0.45 μm). For drinking water or LGW, use nylon filter (0.45 μm) only

4. Attach the vacuum and place the filter into the system

5. Wet the filter with a small amount of LGW

6. Switch on vacuum

7. Pour water sample into the sample reservoir and let it filter through under vacuum and make sure water in flask does not exceed 1000 mL to prevent it from being sucked into the vacuum (this will damage the vacuum!)

8. Rinse the sample bottle (1 L amber) with a small amount of the filtered sample and pour the filtered water back into the bottle

9. Replace the filter with a 0.45 μm pore size filter (get from drawer, blue paper is waste, the white is the actual filter)

10. Re-run the sample through the 0.45 μm filter (this will take a longer time due to its smaller pore size)

2.2. Solid phase extraction (SPE)

2.2.1. Sample Preparation

- Samples are collected in amber glass bottles to prevent photodegradation

- Each 1 L sample is divided into four portions: two for the original unspiked samples and the other two for spiked samples (four 250 mL amber bottles are needed)

- At least two spiked levels are needed for the method of standard addition

Procedures:

1. Label 250 mL amber bottles

      For example:

            0-1 → non-spiked sample 1

            0-2 → non-spiked sample 2

            S-50 → spiked sample with 50 ng/L

            S-100 → spiked sample with 100 ng/L

2. Divide 1 L sample evenly into four 250 mL amber bottles (line near neck of bottle indicates 250 mL)

3. Addition of surrogate standards

- The surrogate standard mixture is a mixture of 2 surrogates including $^{13}C_6$-sulfamethazine and meclocycline with an individual concentration of 0.5 mg/L

1) Calculate the volume of surrogate mixture to add

Example: $\dfrac{C_{surrogate} \cdot V_{sample}}{C_{SurrogateStock}} = \dfrac{0.05\dfrac{ug}{L} \cdot 250mL}{500\dfrac{ug}{L}} = 0.025mL = 25uL$

2) Use appropriate pipette with an appropriate volume range (i.e., 10 – 50 μL) and adjust to the desired level (i.e., 25 μL)

3) Inject surrogate mixture (from nonspiked to spiked samples with increasing concentrations) using the same technique as described in section 1.1.2

4. Spike of antibiotics

Notes:

- Prepare the 25-analyte mixture (see section 1.1.3) on the day of extraction

- 0.5 mg/L is usually a good concentration for the analyte mixture, depending on how much volume you want to spike

- The spike levels for raw water are usually 10, 50, and 100 ng/L

- The spike levels for finished water are usually 2, 5, and10 or 20 ng/L

- Spike surrogate prior to analyte to avoid contamination and for surrogate to monitor the entire sample processing

1) Calculate the amount of analyte mixture to spike

Example: $\dfrac{C_{analyte} \cdot V_{sample}}{C_{mixtureof\,25}} = \dfrac{50\dfrac{ng}{L} \cdot 250mL}{0.5\dfrac{mg}{L}} = 0.025mL = 25uL$

For S-50 sample, inject 25 μL

For S-100 sample, inject 50 μL

2) Inject the analytes mixture in the same way as for the surrogate mixture

5. Addition of Na₂EDTA at 1 mg/L

Note: Na₂EDTAis added to prevent complex formation between the analytes and the metals in water samples (instead EDTA forms the complex). The surrogates and analytes are spiked into the sample before EDTA addition so that they will undergo the same procedure as the analytes present in the original sample)

1) Prepare 0.25 g/L Na₂EDTA stock solution (section 1.3.2)

2) Calculate the amount of Na₂EDTA stock solution to add:

Example: $\dfrac{C_{EDTA} \cdot V_{sample}}{C_{EDTA\_stock}} = \dfrac{1\dfrac{mg}{L} \cdot 250mL}{250\dfrac{mg}{L}} = 1mL$

2) Addition ofNa₂EDTA:

    a. Fill a 10 mL pipette and add 1 mL solution per sample on *top* of the liquid level

      of the sample

    c. Cap and invert the amber bottle once

    d. Repeat for every sample

6. pH adjustment

Notes:

- The pH meter is located in the first bench

- When not in use, store the electrode in buffer storage solution and the meter in standby mode

1) Calibration of pH meter

   a. Remove the electrode from the buffer storage solution and rinse thoroughly

      with LGW

   b. Dab electrode dry gently using Kimwipe

   c. Set "slope" on meter to 100 and "temperature" at room temperature in the lab

      (usually ~22 deg C)

   d. Insert the electrode into a pH 7 buffer

   e. Switch mode from "standby" to "pH"

   f. Shake the buffer gently to equilibrate the electrode

   g. Adjust the "standardize" knob until the reading reaches 7.00 (wait a while for

      pH to stabilize)

   h. Switch mode back to "standby"

   i. Rinse bulb with LGW and dry again with Kimwipe

   j. Use pH 4 buffer and adjust the "slope" knob only to pH 4.00 (get as close as

      possible)

   l. Switch back to "standby" mode and insert the electrode to storage solution (pH

      4 buffer)

2). Measure pH of samples from nonspiked to spiked samples with increasing concentrations

3). Adjust to pH 6.0 (range from 5.8 – 6.0 is acceptable) as described below:

- To lower the pH, use ~2% formic acid; To increase pH, use diluted NaOH solution (but this is rare)

- Due to buffer capacity of environmental water samples, it is impossible to calculate the exact amount of acid to add, so slowly add drops of diluted acid until the sample pH reaches the desired value

- It is important to adjust all samples to approximately the same pH so that the co-extracted matrix is the same for each sample from solid phase extraction

- ALWAYS adjust pH from unspiked to spiked samples, then it is not necessary to rinse the electrode each time

    a. Add ~ 5 drops of ~2% formic acid using Fisher 5 ¾" pipettes

    b. Cap, invert, and measure pH

    c. Continue adding acid until the pH reaches 6.0

     - If the pH of the nonspiked sample goes below 5.8, mix it with the other portion of the unspiked samples and readjust pH

    d. COUNT the total number of drops added into one sample to reach the desired

     pH and add the same drops to each of the other samples

    e. It is best to then check pH on each subsequent sample

    f. After pH measurement, store the electrode in the pH 4 buffer solution

2.2.2. Solid phase extraction

Preparation work

1. Equipments

    a. vacuum manifold (Supelco Visiprep 24)

b. cartridges (one 6cc HLB cartridge per sample)

2. Place cartridges on the manifold (open valves where cartridges are)

3. Close all other valves on top of the manifold

Procedure

1. Preconditioning of SPE columns

Notes:

- Start preconditioning before sample pH adjustment because it takes ~10 minutes

- Precondition using methanol which cleans and activates HLB sorbent and acidified methanol which is later used as the eluting solvent

- LGW is used to wash methanol out of the cartridge

1) Add ~ 6mL methanol into each cartridge and let it run through by gravity

2) Connect the SPE manifold to the waste container which is connected to the vacuum

3) When methanol almost goes through the cartridge, add ~1 mL of acidified methanol (0.1% formic acid) (one squeeze of 5 ¾" pasteur pipette)

4) If the sample drips through cartridge really slowly, use a 10 mL syringe filled with air to push solvent through a little bit

5) Apply 6 mL of LGW to each cartridge twice and try not to introduce any air bubbles

7) Label each cartridge according to the sample I.D.

2. Extraction

1) Insert a T adapter between each cartridge and the valve on the manifold

2) Open each amber bottle, insert one small tubing into each bottle (try to get tubings that have equal length if possible), and connect the tubing fittings to the HLB cartridge (with ~ 3 mL LGW remaining in the cartridge)

3) Apply vacuum pressure to initiate the sample extraction

5) Record start time (it takes approximately 1 hour for extraction of 250 mL sample)

6) Adjust flow rate to be at approximately 5 mL/min

- Estimate flow rate by extraction for 10 minutes and watch the water level in amber bottles (it should be down to ~50 mL) and the level should be the same in each bottle

7) Complete checks

a. tubings at very bottom of amber bottles

b. no leaks

c. each cartridge is labeled and labels match with bottles

d. equal flow through each cartridge

8) Continue to monitor and adjust flow rate using the T-connectors

9) After all samples have gone completely through the cartridges, rinse each cartridge with 6 mL LGW at least twice to remove salts remaining in cartridge

10) Let the cartridge dry for 5 minutes with a vacuum pressure of 15 ~ 18 Hg

11) Turn off vacuum and remove each cartridge from the manifold

12) Tap cartridges on bench surface to get rid of any extra water

13) Rinse methanol through each orange valve on the manifold that was used (where each cartridge was connected)

14) Wipe off water underside of the manifold cover

15) Dry inside of manifold by first applying vacuum and tilting manifold, and then wiping off remaining water with paper towels

16) Pour extraction waste into sink

3. Elution

(~ 15 minutes)

Notes:

- Use acidified methanol (0.1% formic acid) prepared no longer than 1 week prior to use because formic acid may evaporate over time

1) Collect ~10 mL test tubes with conical ends (1 per sample) and one cap for each tube

2) Label test tubes to match sample I.D.

3) Place white plastic rack inside of the manifold

4) Place cartridges on the manifold without T connectors (try to put them in the same position as for extraction)

5) Place conical test tubes into the rack according to the corresponding labels

6) Add 2 mL of acidified methanol to each cartridge using a 10 mL pipette, allow to go through the cartridge by gravity, and refill (do this 4 times for a total of 8 mL elution volume for each sample)

7) If necessary, apply some pressure with air-filled syringe or apply some low vacuum pressure to initiate the elution process (for the first 2 mL ONLY)

8) At the end of elution, apply vacuum pressure to pull through the last drops of the eluting solvent out of the cartridge

9) Place used cartridges in labeled and taped bag in freezer. Do NOT throw away cartridges until all analysis is complete

2.3. Solvent reduction

Notes:

- This process takes ~ 2 hours

- Can begin to set up blow down during elution

2.3.1. Blow down procedure

1. Place all test tubes with SPE eluent into a beaker and bring it to the blow down setup

2. Get small plastic tubes from the antibiotic drawer under the LC-MS computer monitor

3. Check the nitrogen tank in advance (to make sure there is enough nitrogen to use for blow down) by opening valve on top of the tank and checking to see if the pressure on the right pressure gauge is > 500 psi. Stop using the nitrogen tank after its pressure is below 500 psi

4. Set the heating block at "low temperature" at 4 ~ 5 (closer to 5), which means the temperature will be between 40 and 50 deg C

5. Wait ~ 5 minutes to heat up

6. Connect tubes to the top (number of tubes = number of samples, close the unused ones with metal cap)

7. Place test tubes in the heating block

8. Lower and secure the top

9. Put the plastic tubes into the test tubes and make sure that the plastic tubes are *above* the liquid surface of the SPE eluent

10. Turn on nitrogen and adjust flow rate to blow gently with minimum disturbance of the liquid surface

     - More samples necessitate higher flow rate because the pressure is distributed

      over open valves) – for example, for 4 samples the pressure was ~ 10 – 15 psi

     - Pressure is measured by left gauge and is regulated by adjusting the two valves

      on the left of the tank)

11. Pull hood down as low as possible

12. Wrap aluminum foil around sample test tubes to prevent photolysis

13. Check the liquid level in the test tubes approximately every 15 minutes, and lower the

top if necessary

14. Blow down to ~50 μL in the test tube (~ ¼") **NEVER blow down to dryness!**

15. After the desired volume is achieved, turn off heating and close the valves of nitrogen tank

2.3.2. Reconstitution

1. Estimate the residual volume of SPE eluent:

1) Add 50 μL of a solvent mixture of LGW/ methanol (9:1) to an empty conical test tube and compare its liquid level to that of the SPE eluent to estimate the remaining volume in each sample tube. If the estimated sample volume is > 50 μL, make another test tube with 70 μL of LGW/methanol solvent mixture, and try to estimate the level of the sample (it might be ~ 60 μL)

2) Record estimated volume on the label of each test tube

(Note: the sample extract after blow down often looks slightly yellow due to the presence of natural organic matter in water)

2. Reconstitute each sample to a final volume of 250 μL with the LGW/methanol solvent mixture (9:1) using a thoroughly cleaned 500 μL syringe. Add reconstituting solvent above the liquid level of the sample and avoiding touching the sides of the test tube to avoid contamination of the syringe

    - For example, if the recorded estimated sample volume was 50 μL, then add 200

      μL of reconstituting solvent to make a total volume of 250 μL

    - Syringe doesn't need to be washed each time if care is taken not to contaminate

2.3.3. Addition of internal standard simatone

1. Clean a 10 μL syringe with methanol and then rinse with internal standard solution at least 3 times by drawing in and discarding to waste.

2. Add internal standard (I.S.) solution at 1.25 mg/L (see section 1.2.2) into each sample and into internal standard blank.

      1) Calculate the volume of internal standard solution needed to add (using same

         equation) (10 μL for a spike concentration of 50 μg/L)

2) Make I.S. blank by adding same amount of I.S. to 250 μL of reconstituting

solvent (LGW/methanol). This should be done before adding I.S. into the

unspiked samples

Technique:

a. Draw I.S. slowly into the 10 μL syringe (if doing 10μL injection)

b. Inject I.S. *under* the liquid surface

c. Rinse outside of needle using methanol between every injection

d. Add I.S. from unspiked sample to spiked samples with increasing

concentrations

3. Vortex each sample

4. After vortex, turn around test tube to cover area in the test tube with the extract liquid that is not reached by vortex

5. Let each sample sit for 10-15 minutes to allow liquid settle all the way down to the bottom of the conical tube.

2.3.4. Filtration of sample extracts

1. Preparation

1) Gather a vial rack from the cabinet at the end of the second bench, vials (1 per sample + 1 for solvent blank) on left side of the second bench, 250 μL inserts (1 per sample + 1 for solvent blank), and a cap for every vial

2) Place an insert into each vial

3) Label each vial (sample identification, initials, and date)

2. Filtration of extracts

1) Get 0.45 μm pore size syringe filters from the LC-MS drawer

2) Use a 500 μL syringe to slowly draw sample from the test tube

3) Measure the total volume to verify that it is ~250 μL

4)Record the measured volume on the label on the test tube

5) With sample still in syringe, remove the needle and attach a syringe filter

6)Slowly push sample through the syringe filter directly into the insert in the vial

7) After sample is filtered as much as possible, take filter off the syringe, draw in air, re-attach the filter, and push back through to push last drops into the vial

8) Close the vial with cap

9) Flick the vial to get rid of bubbles in the insert

10) Rinse syringe twice using the same LGW/methanol mixture

11) Repeat steps 1 – 10 for each sample

Cleaning: rinse syringe 3 times with solvent, then rinse syringe with methanol in the hood, take apart and again rinse each part individually, lay syringe (still taken apart) on Kimwipe in hood to dry

Store all prepared sample extracts in vials in the freezer until LC-MS/MS analysis.

2.4. Lab cleaning

1. Collect all dishes/parts/tubes/etc in a bucket and bring to sink

2. SPE tubing: squeeze LGW through one end without connector and let LGW run through the tubing for at least 30 seconds

3. Pipettes:

1) Rinse with tap water and then with LGW, inside and outside

2) Rinse tips and the whole pipette with methanol while turning the pipette

3) Leave the pipettes to dry on paper towel in hood overnight

4) Sign and date paper towel

4. T-connectors: rinse with LGW and put back to the SPE drawer

5. Amber bottles (don't need to be acid-washed if it is cleaned right after use):

1) Use soap to clean the raw water sample bottles

2) Put a bit of soap in each bottle and fill with tap water, shake

3) Rinse the bottle with tap water at least 3 - 5 times

4) Rinse at least 3 times with LGW from black sprayer

5) Dry in oven overnight (do not change oven temp)

6. Caps: rinse with tap water and then LGW, air dry in hood

3. **LC-MS/MS analysis**

3.1. Equilibration of LC system

1. Check mobile phase levels (at least 3 inches of liquid from bottom of the bottle), and if not enough prepare more

-mobile phase A: 0.1% formic acid in LGW (2 mL formic acid into 2 L LGW, filtered to 0.45 micron with nylon filter)

-mobile phase B: acetonitrile

2. Whenever the mobile phase bottles are changed, the user needs to remove the air bubble as described below and then prime the system.

1) For mobile phase A, attach a 10 mL plastic syringe to the fitting that is facing

the user

2) Switch T valve so it is facing to the left (which is opposite of normal)

3) Slowly pull out mobile phase trying to get all bubbles out

4) Return T valve to the normal position (facing to the right)

5) Repeat for mobile phase B

Priming:

1) Loosen black B valve so that mobile phase won't go to the LC column and will

instead go to waste (this is important!)

2) Prime A pump first

3) Push "Stop" on the screen of the pump

4) Push "Prime" (max flow on pump should be 5 mL/min)

5) Prime for ~2 minutes

6) Push "Stop" again

7) Repeat process for B

8) Retighten the black B valve to return mobile phase to column (also important!)

3. For routine operation, before starting the pumps, always check the mobile phase lines to make sure there are no air bubbles. If there are air bubbles, prime the system until they are removed.

4. Need to flush the system as described below if there has been user change or the LC-MS has not been used for more than one week.

Flushing:

1) Remove the column

2) Connect red tubes directly using a union (in drawer under LCMS)

3) On the main window, open file → activate method → go to methods directory

under Varian WS → flushing → open

4) Maximize the Prostar/Dynamax window to monitor the flow rate and mobile

phase composition (should be the same as those in the method)

5) Flush for ~10 minutes

6) Maximize Prostar/Dynamax window again

7) Click "Stop Pumps" to stop flushing

8) When pressure on screen drops back down to zero, attach column (Pursuit C18)

5. Wash autosampler needle – do this every time before injecting samples

    1) Open auto sampler window 430.25

    2) Click "Wash" and do this twice

    3) Minimize window

6. Attach LC guard and analytical column: Pursuit C18 (15 cm, 2.0 mm, 3μm)

    1)Make sure the guard column is tightly connected to the analytical column

    2) Attach in the direction of flow (guard column on left)

    3) Tape column down to secure

    4) Put the caps in the little blue bag in the drawer

7. Check to see if shield is clean, if not:

    1) Use LGW and wipe shield with Kimwipe, ensuring that no water enters the MS

    2) Squirt methanol onto shield and into hold on shield

    3) Clean needle tip with methanol

3.2 LC-MS/MS analysis

Method Activation

-The 25 antibiotic analytes are separated by three different LC runs, which means that each sample extract is injected at least 3 times

-The three different LC runs are based on combining analytes of similar structure and must be independently run

-The order of analysis is sulfanomides/macrolides (SAMA) → fluoroquinolones → tetracyclines

Note: The windows control the instrument components as follows:

    - Prostar/Dynamax.24 → LC

- 430.25 → Autosampler

- 1200.42 → Mass Spec ESI

- Prostar 430 → Sequence

1. Click the view/edit method button on the top of the screen

- Open an existing method

- ZY folder → antibiotic analysis → click on a specific method

2. Make configuration adjustments to the method if necessary

- For the SAMA method:

- Click on configuration tab on the left

- Adjust needle height to avoid breaking the needle:

- For 250 μL inserts, set the needle height at 6 mm

- For 50 μL inserts, set the needle height at 10 mm

- Needle height measures the distance from the bottom of the vial

up to the tip of the needle (i.e., a needle height of 10 mm will not

go as far into   the vial as a needle height of 6 mm)

- Save the method every time you make *any* change

- May also need to change the needle tubing volume (ntv)

- Check ntv value listed on configuration tab

- Check ntv value on 430.25 (subwindow of system control) → hardware → ntv

- If the values are not equal, adjust the hardware value to be consistent with the value on the configuration tab in the method

3. Check MS settings:

- On the "Scan method" window (under Method Window) select:

- ion source: ESI

- mode: centroid

- CID gas: on

- polarity: positive

- Save method and close

4. Activate method

- System Control Window → Mass Spec subwindow → File → Activate method → open method

- Note: if unable to activate, go to Automation → Stop Automation and try again

- On the main screen (1200.42) when the quadrupole 2 (collision cell) turns

orange, it means that the collision gas is on (make sure it is) and the pressure

should be ~2 mTorr. If not, adjust the pressure by turning the CID gas

valve very carefully

- Minimize the main screen

- Open the LC Prostar screen

- Watch the pressure increase until it reaches and stabilizes at ~ 1800 -2000 psi

5. Set up sequence

- File → new sample list → Varian WS → data → Laura → create new folder using the date (e.g., 112005)

- Enter sequence parameters:

- Sample name: solvent blank, etc

- Injection mode: µL pickup

- Injection volume: 20 μL

- Well vial: begin with A1 (wherever the vials are placed in the rack)

- Click on Data File (bottom right) and save data under the user's directory

Note: the first sample should always be solvent blank. Make 2 or 3 injections of blank to equilibrate the LC column and to check the solvents for contamination. If there are any peaks on the chromatogram at the retention times of the analytes (indicating contamination), continue running solvent blank samples until there is no more contamination.

Also, if the methods have not been run in a while, make a test solution of ~10 ug/L of antibiotics to check if the retention times on the method are still correct.

6. Set up instrument parameters and turn on the ESI-MS (*before* you start the sequence)

- Select API auto

- drying gas temp at 300 deg C

- API house temp at 50 deg C

- Click OK

- Click icon with green arrow in the upper left to turn on electrospray

- Wait until drying gas temperature gets to 160 deg C, then click "turn on the

instrument" icon to turn on the detector (when the light is green, it is on). Wait

until the temperature stabilizes before starting the sequence

- Make sure the MS valve is in the "Load" mode

7. Last minute checks (*before* you start the sequence)

- Make sure samples are in the autosampler tray with the correct vial number on

the sequence

- Make sure samples have been in the tray for *at least* 30 minutes after being

taken out of freezer

- Check that the clear glass solvent vial (called transport vial) behind the rack is

full of the reconstituting solvent (90% LGW 10% methanol)

8. Start sequence

- Return to Prostar 430 sequence window → click begin on bottom left → click Ok

- Each run takes ~ 30 minutes

9. Check the chromatograms of the solvent blank to see if there is contamination

10. If there is NO contamination, add all samples to the sequence following the same scheme in step 5 above

11. After putting all samples in the sequence, activate the second method by browsing the method in the method files

Sample type: Activate method

Auto link: select method

12. Repeat the sample sequence for the second method

13. At the end of the whole sequence, put in one line saying:

Sample type: Activate method

Auto link: methods → ZY → stop run

Do one analysis of solvent to initiate the "stoprun" method.

4. Data Analysis

4.1. Integration of analyte peaks

4.1.1. Manual integration

Software: Varian WS Work station 6.4.

Notes: analytes must be integrated one by one

1. Click on the window of Review/Process MS Data

    - Find data file

    - Open chromatogram on the screen

2. Input

- Select the file names to be analyzed

- Data $\rightarrow$ Ion

- Channels $\rightarrow$ Scan $\rightarrow$ selected scan channels

3. Use ZY's method document to find certain ions

    - i.e., for sulfathiazole one of the two product ions is m/z 156 and the scan channel is m/z 256 to m/z 156. Note: the second product ion column is used for confirmation of detection of the analyte (if the ratio of the areas of the two product ions listed in the 1st and 2nd column, respectively, is within a certain range, it confirms the detection of the analyte – the ratio varies depending on individual analyte)

4. Adjust chromatograph

    - Right click $\rightarrow$ select local chromatograph plot preferences $\rightarrow$ chromatogram

    plot $\rightarrow$ under filtrating $\rightarrow$ smooth data (points 5 or 7) AND remove spikes $\rightarrow$

    OK

5, Zoom in to enlarge peak

- Adjust x axis by dragging mouse along axis

- Adjust on y axis by just clicking

6. Integrate peak

- Click on set click and drag action → integrate area

- Draw line along the base of the peak (BE consistent to where you draw the line)

- Enter data into Excel

- Apex = retention time

- Area = area of the peak (important parameter!)

4.1.2. Automated integration

Create a quantitation method

1. Create a new method (create new method → next → finish)

2. Select the file that you want to analyze (on left MS Data handling → compound table)

3. Click "add" to add the number of ions you want to integrate(two ions for one analyte), so 20 ions need to be integrated for 10 analytes

4. Double click retention time column header to get total ion chromatogram

5. Zoom out a specific peak to make it larger

6. Name the ion (e.g., Sulfamethoxazle-156)

7. On quantum ions tab select scan channels (under merged) and select the target ion

8. Under integration tab, set integration window at 3.0 min, under "filter peaks" smooth the chromatogram with a factor of either 7 or 5 and set the "remove spikes" at a factor of 5

9. If the integration peak looks weird, adjust the peak width and slope sensitivity to improve

10. Under identification tab, select retention time with search time +/- 0.5 min

11. Hit close

12. For confirmation, get peak areas of *both* fragment ions for each analyte

13. Continue for each ion, adjusting peak width and slope sensitivity if necessary

14. Save method in methods subfolder

Data processing using the quantitation method

Advantage: this method is more accurate than the manual integration method and the data files can be copied to Excel and saved

1. Open the chromatograms folder

2. Double click to open the chromatograms

3. Select one file → quantitation → process active folder

4. Under method folder, browse for the method to process the data with

5. Click "process" to analyze data file with the method

6. Click "view results"

    - For EACH peak draw a line from beginning to the end of the peak and click

     "integrate"

    - If the integration is not correct, need to edit method to adjust parameters. Save

     the method each time it is updated

    - Click "done" after adjusting each

7. Save method changes if you want (usually a good idea)

8. Save the integrated data file: Print → sample report (ASCII) → folder data file, name as sample ID and save it so it can be opened and saved by Excel

4.2. The method of standard addition

1. Integrate the peaks of a specific analyte and the internal standard (either by manual or automatic integration)

2. Set up an Excel Spreadsheet with rows or columns for spike amount (ng/L), simatone (I.S.) Area, and analyte Area

3. Calculate relative area = Area of analyte/area of I.S.

4. Build two calibrations (one with 0-1 sample and the spiked samples, the other with the duplicate nonspiked sample (0-2) and the spiked samples) – Area (y) versus concentration (x)
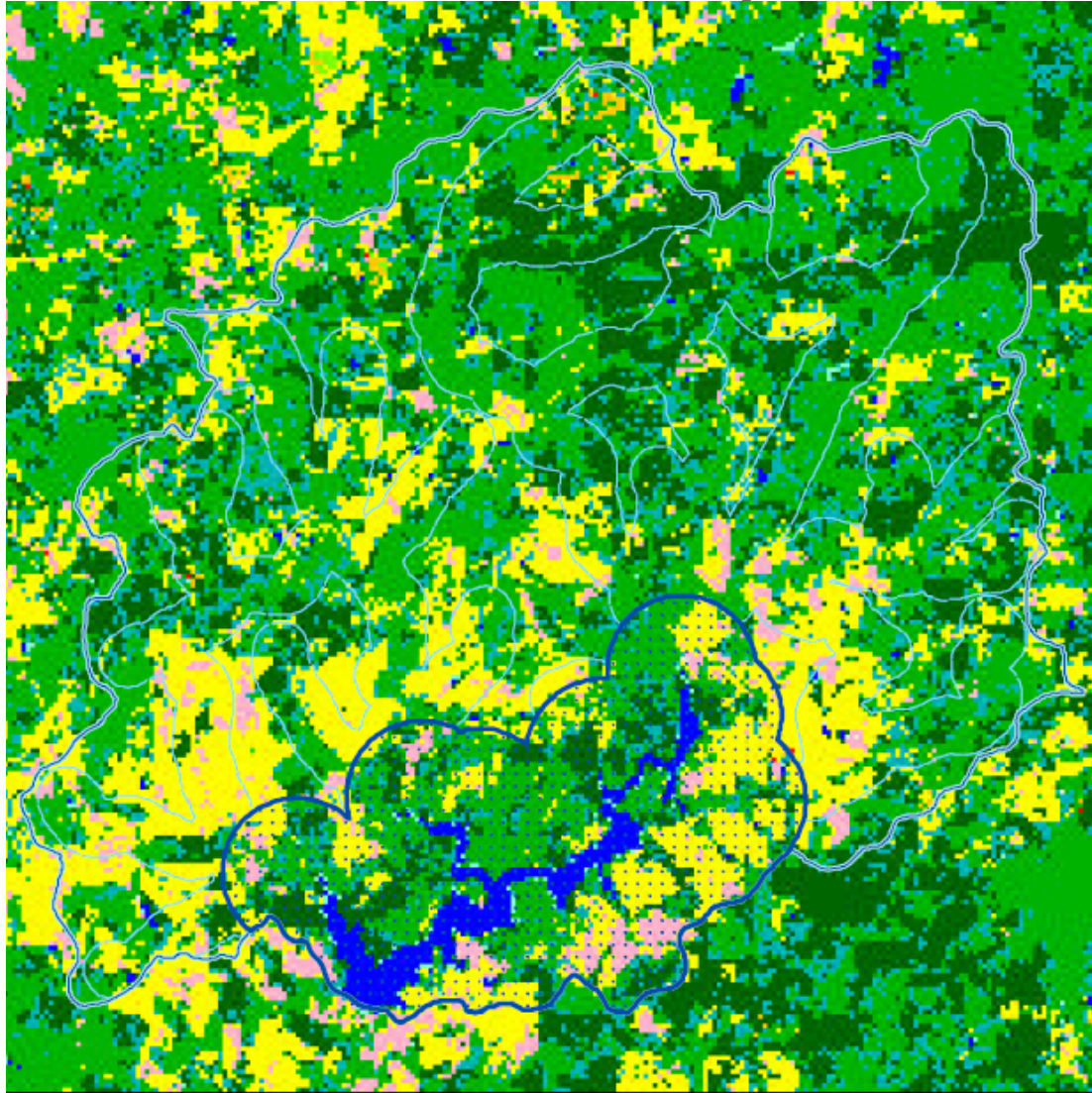
5. Extrapolate to get the absolute value of x when y = 0

6. These two values are the concentrations in the original sample from duplicate measurements – average the two values and calculate the average deviation (A.D.) and the relative percent of difference (RPD). Use two significant figures.

$$A.D. = C_{bigger} - C_{mean}$$

$$RPD(\%) = \frac{(x1 - x2)}{(x1 + x2) \cdot 2} \cdot 100$$

Appendix 3:

North Carolina Land Use Maps
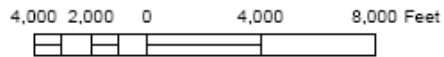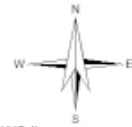
Figure A.3.1 Land uses in the Cane Creek Watershed (Location of source Water for DWTP1). The light pink color indicates the location of row crops. Yellow color indicates hay pastures, and green indicates forests. Map obtained from the NCSWAP for OWASA, 2010. (NCSWAP, 2010c)
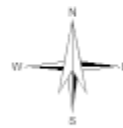
178

Figure A.3.2 Area and potential contaminant sites (PCS) for the Cane Creek Watershed (Location of source Water for DWTP1). The Cane Creek watershed protection area is outlined in light blue and the critical area in dark blue. The map shows the locations and types of PCS. A limited number of PCS are present in the watershed including; animal operations, isolated pollution incidents, and non discharge permits. Map obtained from the NCSWAP for OWASA, 2010.
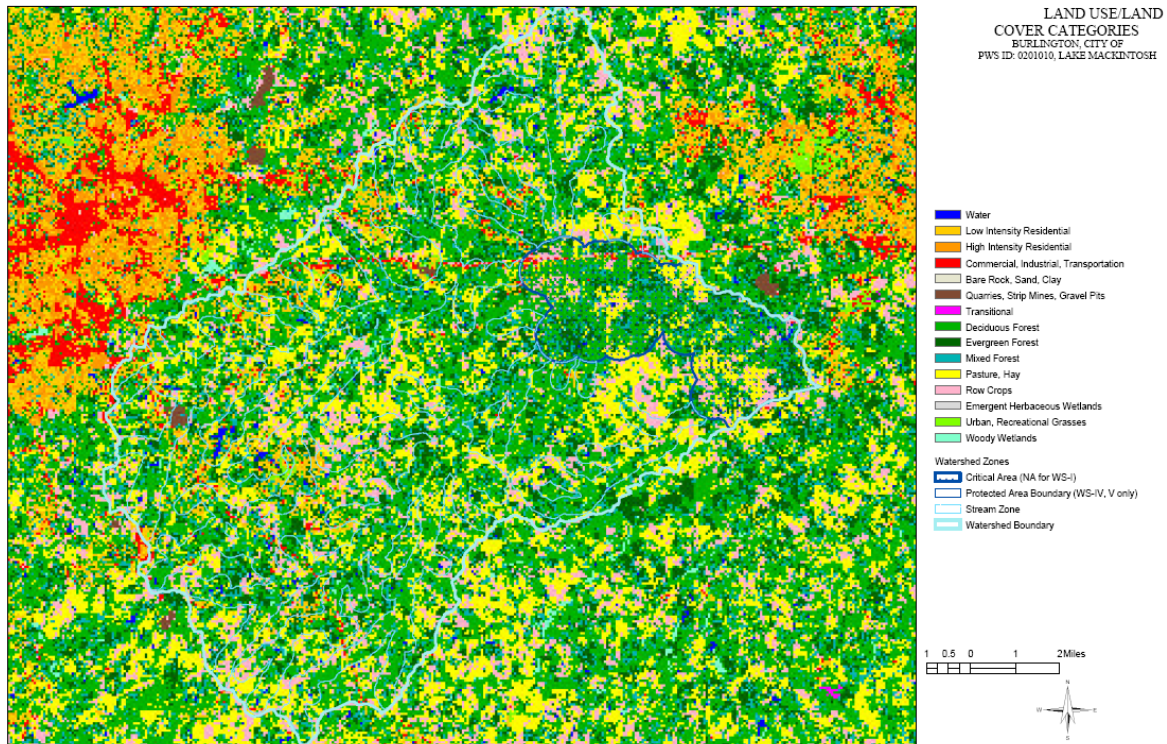
Figure A.3.3 Land uses in the Big Alamance Creek Watershed (Location of source Water for DWTP2). The red color indicates commercial, industrial, transportation uses. The orange indicates high intensity residential. The light pink color indicates the location of row crops. Yellow color indicates hay pastures, and green indicates forest. Map obtained from the NCSWAP for The City of Burlington, 2010.
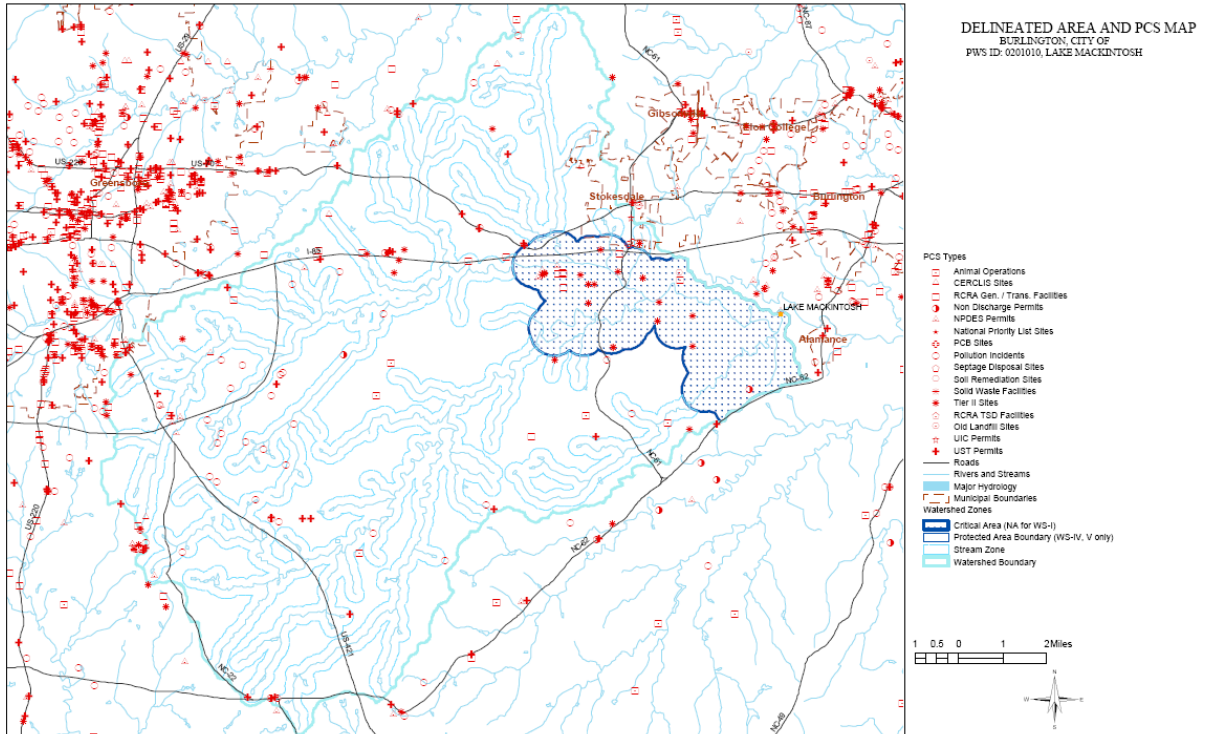
Figure A.3.4 Area and potential contaminant sites (PCS) for the Big Alamance Creek Watershed (Location of source Water for DWTP2). The Big Alamance Creek Watershed protection area is outlined in light blue and the critical area in dark blue. The map shows the locations and types of PCS. A number of PCS are present in the watershed including; animal operations, NPDES permit sites, and isolated pollution incidents among others. Map obtained from the NCSWAP for The City of Burlington, 2010.
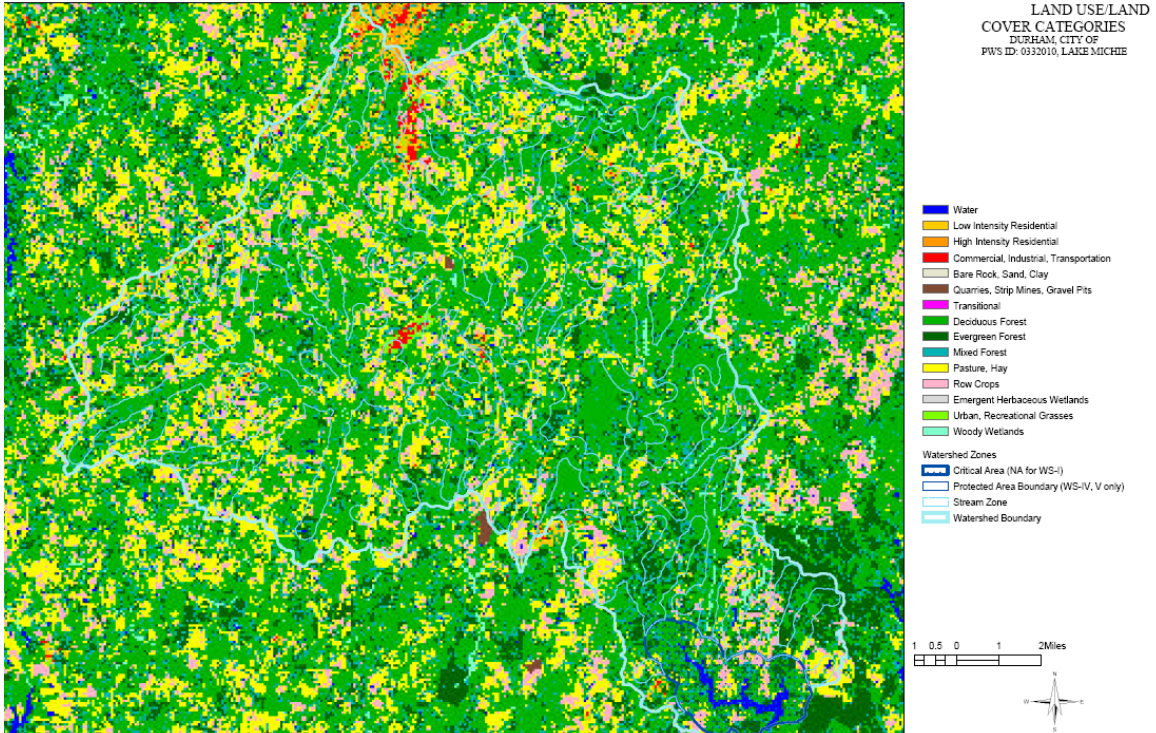
Figure A.3.5 Land uses in the Little River (Little River Reservoir) and Flat River (Lake Michie) Watershed (Locations of source water for DWTP3). The red color indicates commercial, industrial, transportation uses. The light pink color indicates the location of row crops. Yellow color indicates hay pastures, and green indicates forest. Map obtained from the NCSWAP for The City of Durham, 2010.
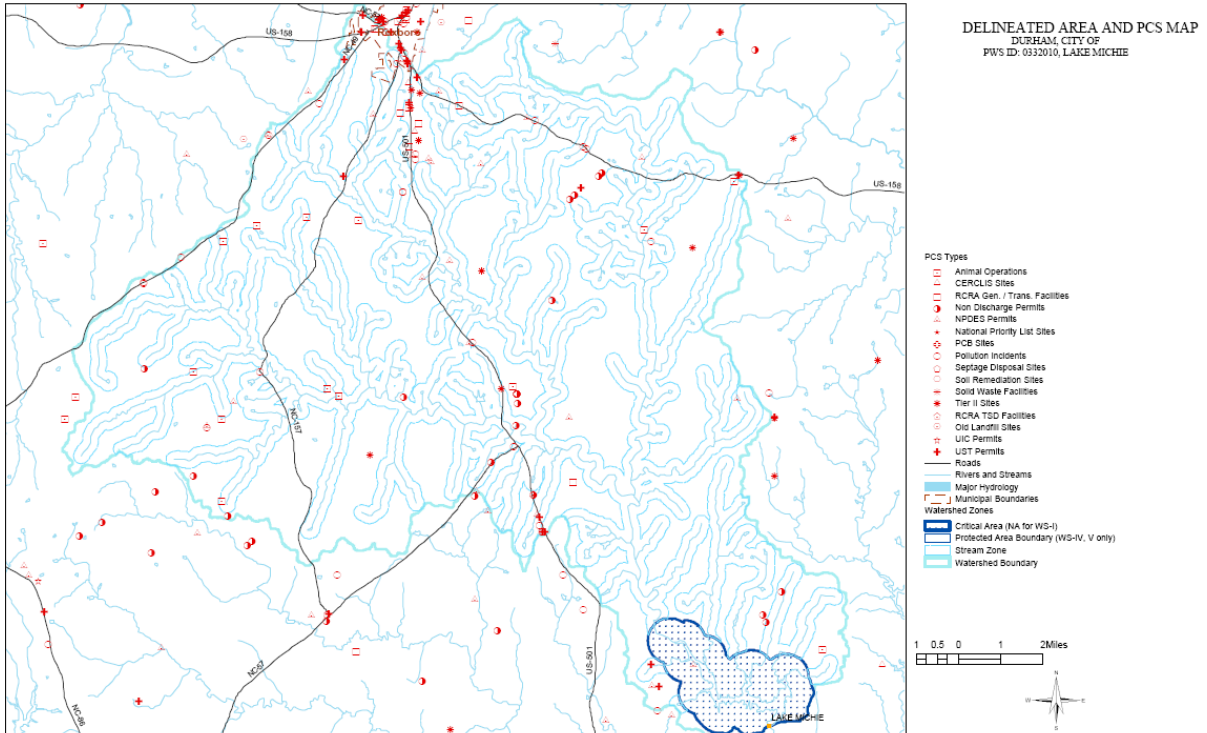
Figure A.3.6 Area and potential contaminant sites (PCS) for the Little River (Little River Reservoir) and Flat River (Lake Michie) Watershed (Locations of source water for DWTP3). The Little River (Little River Reservoir) and Flat River (Lake Michie) Watershed protection area is outlined in light blue and the critical area in dark blue. The map shows the locations and types of PCS. A number of PCS are present in the watershed including; animal operations, non discharge permit sites, NPDES permit sites, and isolated pollution incidents among others. Map obtained from the NCSWAP for The City of Durham, 2010.

Chlorine Isotope Ratios
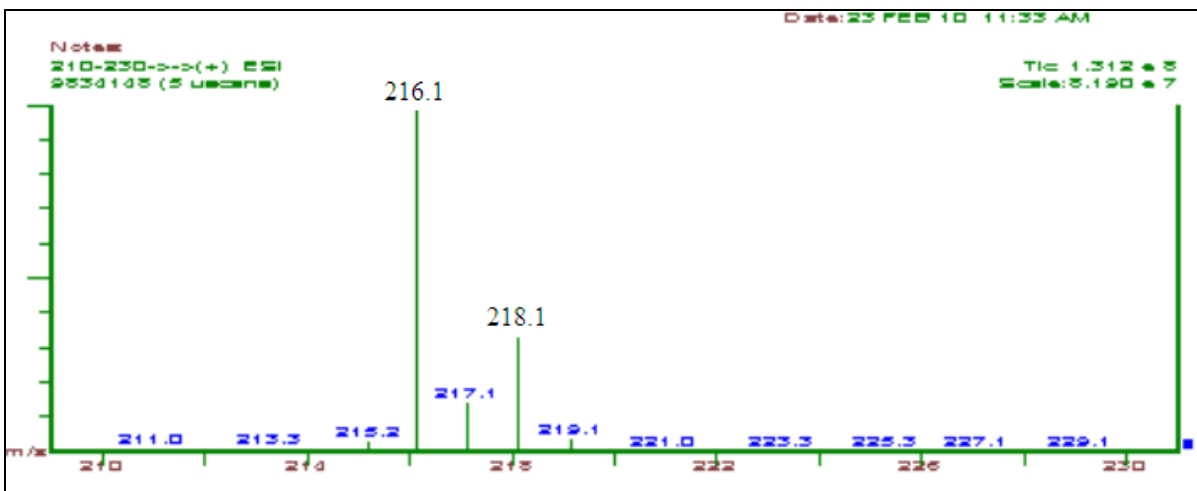

Figure A.4.1 Full Scan Mass Spectrum of Atrazine, a Mono-chlorinated BAC. The Characteristic Chlorine Ratio of [M]:[M+2] for 216.1:218.1 corresponding to ^{35}Cl:^{37}Cl is 3:1.

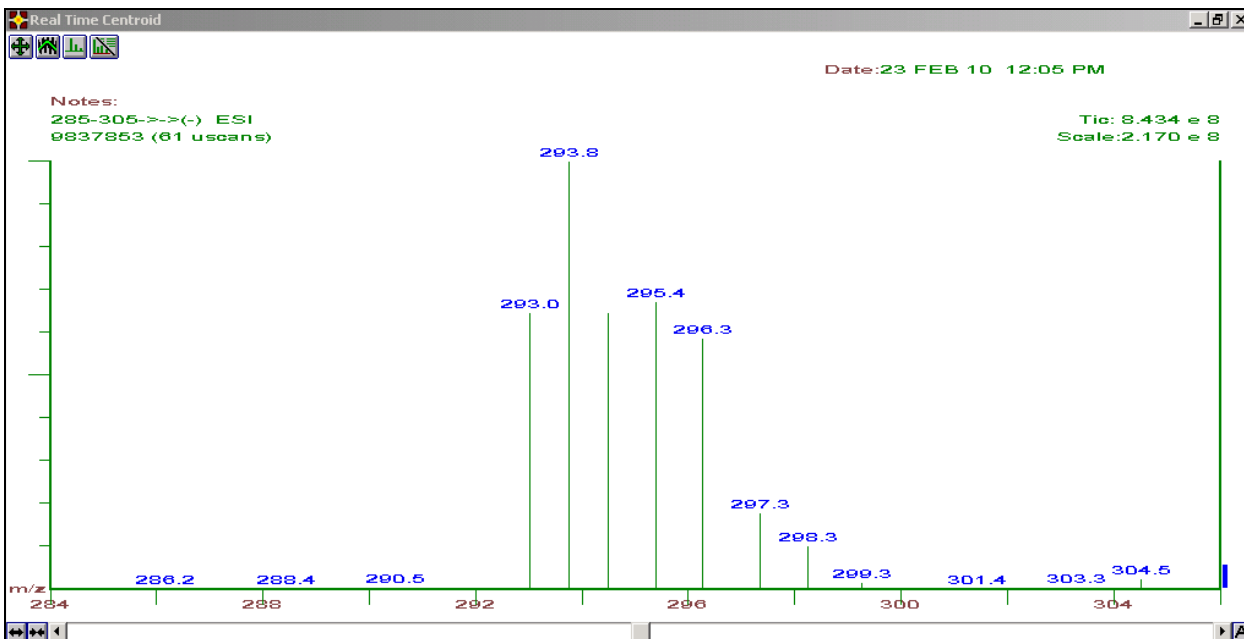Figure A.4.2 Full Scan Mass Spectrum of Diclofenac, a Di-chlorinated BAC. The Characteristic Chlorine Ratio of [M]:[M+2]:[M+4] for 293.8:296.3:298.3 corresponding to ^{35}Cl:^{37}Cl:^{39}Cl is 9:6:1.
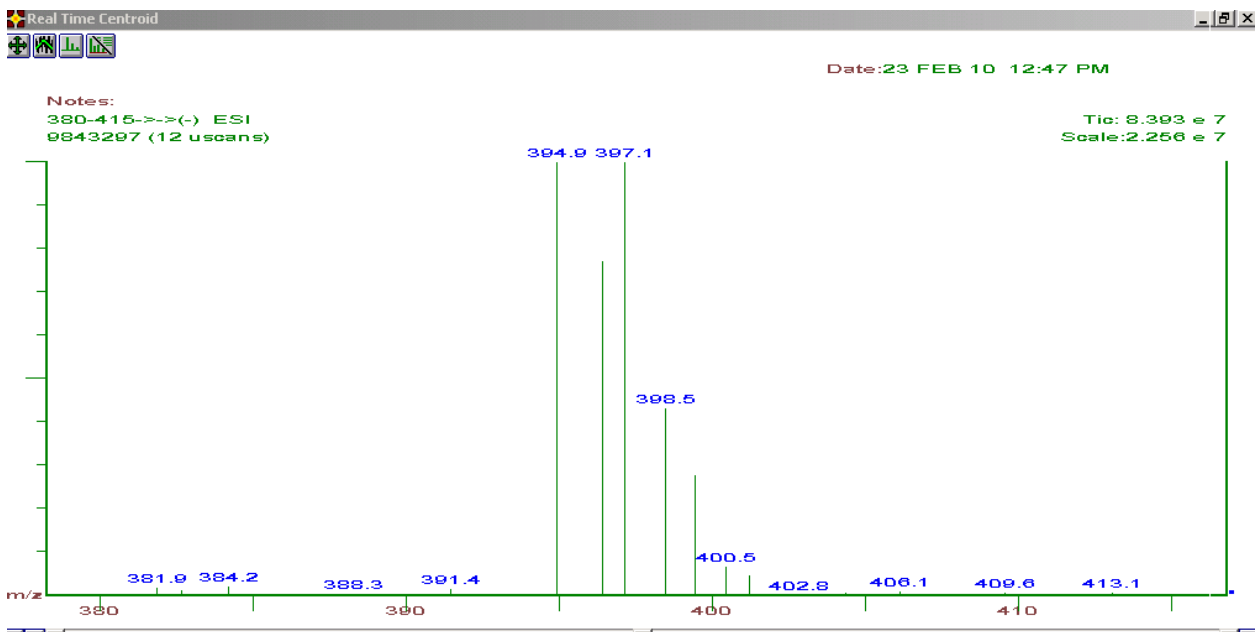
Figure A.4.3 Full Scan Mass Spectrum of Sucralose, a Tri-chlorinated BAC. The Characteristic Chlorine Ratio of [M]:[M+2]:[M+4] for 394.9:397.1:399:401 corresponding to ^{35}Cl:^{37}Cl:^{39}Cl: ^{41}Cl is 27:27:9:1.

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