Antibiotic Occurrence and Associated Environmental Hazard: A Case Study of a Drinking Water Reservoir Impacted by Wastewater Discharge

Kate Bronstein

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 Environmental Science in the Department of Environmental Sciences and Engineering, School of Public Health.

Chapel Hill
2008

Approved by:

Advisor: Howard S. Weinberg

Reader: Marc Serre

Reader: Douglas Crawford-Brown

ABSTRACT

Kate Bronstein

Antibiotic Occurrence and Associated Environmental Hazard: A Case Study of a Drinking Water Reservoir Impacted by Wastewater Discharge

(Under the direction of Howard S. Weinberg, Marc Serre, and Douglas Crawford-Brown)

The fate and transport of 26 common human and veterinary antibiotics were investigated in a small, semi-urban watershed that is impacted by wastewater discharges. The watershed ultimately links an impacted reservoir, in which twelve of the twenty-six antibiotics were detected at concentrations up to 2 μg/L, to a downstream drinking water source. A Bayesian Maximum Entropy framework with modern spatiotemporal geostatistics was used to process information about one antibiotic, sulfamethoxazole, in this watershed and this study demonstrates the practical benefit of using field measurements and model predictions to establish a more complete map of contaminant transport. Generated maps show that the areas of greatest accumulation are within the streams where antibiotics appear to follow a pseudo-first order rate of removal from the aqueous phase. An environmental hazard assessment was then performed, and among the antibiotics studied, sulfamethoxazole, ciprofloxacin, and erythromycin were found to present the greatest environmental hazard.

ACKNOWLEDGEMENTS

I would like to thank my committee members for all of their support and advice on this project and in the classroom. I am very grateful to the Weinberg lab group members during my time here for helping a non-chemist to do some chemistry and for all of the advice, support, and friendship. I would also like to thank the plant managers at the wastewater plants involved in this study for their flexibility and willingness to coordinate sampling events with me, Johnny Randall and Patricia D'Arconte for their help in site selection, Rachel Golden for the wealth of information about prescription drug disposal initiatives, Gloria Ferrell and others from the USGS, and the UNC GIS librarian Amanda Henley. Last but not least, I would like to thank my family, friends, and Kris Nordstrom for their unending support, travel experiences, and humorous encounters without which I most likely would not be who I am today.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

Abbreviation	Term Used
ACN	Acetonitrile
AF	Assessment factor
AOP	Advanced oxidation processes
APEO	Alkylphenol polyethoxalates
BCF	Bioconcentration factor
BMEGUI	Bayesian Maximum Entropy graphical user interface
CAFOs	Confined Animal Feeding Operations
CDC	Center for Disease Control
CESQG	Conditionally exempt small quantity generators
CFR	Code of Federal Regulations
CPRG	Chlorophenol-red-β-D-galactopyranoside
CWA	Clean Water Act
DEA	Drug Enforcement Agency
EE2	17α-ethyinylestradiol
E3	16α-hydroxy-17β-estradiol
E2	17β-estradiol
EC_{50}	Effective concentration that effects 50% of population
EAS	Endocrinally active substance
EA	Environmental assessment
EIS	Environmental impact statement

EPA	Environmental Protection Agency
EEQ	Estradiol equivalence
E1	Estrone
EMEA	European Medicines Agency
EU	European Union
FDA	Food and Drug Agency
F_{oc}	Fraction of organic carbon
FOIA	Freedom of Information Act
HCB	Hexachlorobenzene
IDL	Instrument detection limit
k	First order decay rate
k_{oc}	Sorption coefficient
k_d	Water distribution coefficient
k_{ow}	Octanol water distribution coefficient
LC_{50}	Lethal Concentration that effects 50% of population
LC-MS/MS	Liquid chromatography tandem mass spectrometer
LGW	Laboratory grade water
LOD	Limit of detection
LOQ	Limit of quantitation
MEC	Measured environmental concentration
MeOH	Methanol
μg/L	Micrograms per liter
m/z	Mass to charge ratio

Na_2EDTA	Disodium ethylenediamine tetraacetic acid
NEPA	National Environmental Policy Act of 1969
ng/L	Nanograms per liter
NOEC	No observed effects concentration
NPDWR	National Primary Drinking Water Regulations
ONDCP	Office of National Drug Control Policy
O_3/H_2O_2	Ozone-hydrogen peroxide
PEC	Predicted environmental concentration
PhACs	Pharmaceutically active compounds
PNEC	Predicted no effects concentration
PQL	Practical quantitation limit
QA	Quality assurance
QC	Quality control
QL	Quinolones
RCRA	Resource Conservation Recovery Act
RPD	Relative percent difference
S:N	Signal to noise ratio
SAMA	Sulfonamides
SDWA	Safe Drinking Water Act
SPE	Solid phase extraction
TC	Tetracyclines
USGS	United States Geological Survey

UV	Ultraviolet
WWTPs	Wastewater treatment plants
YES	Yeast estrogen screen

1. BACKGROUND AND LITERATURE REVIEW

1.1 Background on Pharmaceutically Active Compounds

The continued exponential growth in the human population has created an increasing demand for natural resources, specifically placing a strain on the limited supply of freshwater. Thus, protecting the integrity of drinking water sources is one of the most important issues of the 21st century. Recently, emerging contaminants, such as pharmaceuticals, biogenic hormones, and personal care products have been detected in wastewater effluents and surface waters (Karthikeyan and Meyer, 2006; Kim et al., 2007b; Yang and Carlson, 2003; Vanderford et al., 2003). Research has shown that many pharmaceutically active compounds (PhACs), such as antibiotics, can enter the environment, disperse, and persist to a greater extent than first anticipated (McArdell et al., 2003; Vanderford et al., 2003; Barber et al., 2006; Batt et al., 2006).

Pharmaceuticals are medicinal drugs that are developed with the aim to cure or prevent disease and a subset of these, antibiotics or antibacterials, are used to treat bacterial infections. There are two types of antibiotics; those that kill bacteria (bactericidal) and those that only inhibit growth of certain bacteria that will continue to grow if the drug dosage decreases (bacteriostatic). Most natural antibiotic groups, or those not obtained exclusively from chemical synthesis, were discovered by the mid-1950's, which means that antibiotics developed since then are just chemical variations of the older structures. Almost every new

generation of an antibiotic is developed to overcome a resistance to a former generation. New generations of antibiotics include a functional group on the basic nucleus structure so that the resistance mechanism developed with the original antibiotic can be overcome (Aymes, 2001).

The environmental occurrence of PhACs has been investigated in recent years (Golet et al., 2003; Kolpin et al., 2002; Batt et al., 2006; Donn et al., 2008) because of their ubiquitous usage throughout the developed world, their ability to dissolve in water, their ability to be excreted partially or fully unmetabolized, and their resistance to biodegradation in natural waters. These characteristics along with incomplete removal during wastewater treatment processes (Yang and Carlson, 2003; Gobel et al., 2004) have allowed pharmaceutical compounds to enter the aquatic environment. It has also been postulated that at environmental concentrations (low µg/L or ng/L) toxic effects may be induced in certain non-target aquatic species. Therefore, the environmental and public health risk that these compounds potentially pose should be thoroughly investigated in order to understand long-term effects and to also determine whether or not the risk is substantial enough to necessitate a change in conventional or onsite wastewater treatment, disposal practices, and the frequency with which these medications are dispensed.

1.2 Pathways into the Environment

PhACs can enter the environment through human and animal excretion and the improper disposal of unused or expired drugs (Figure 1.1). Veterinary pharmaceuticals are given to pets and used in both aquaculture and confined animal feeding operations (CAFOs). Antibiotics are given prophylactically to prevent illness and to promote growth in animals on CAFOs, which generate millions of gallons of feces and urine that contain residual levels of

these compounds (Saptoka et al., 2007). This waste is stored in lagoons and sometimes sprayed on nearby fields as fertilizer. Occasionally, the untreated wastewater can end up in nearby creeks or rivers because the lagoons may leach through the soil or breach the banks (Sapotka et al., 2007). Antibiotics are also employed in aquaculture farms where they are used as feed additives or directly added to the water to prevent disease (Lalumera et al., 2004).

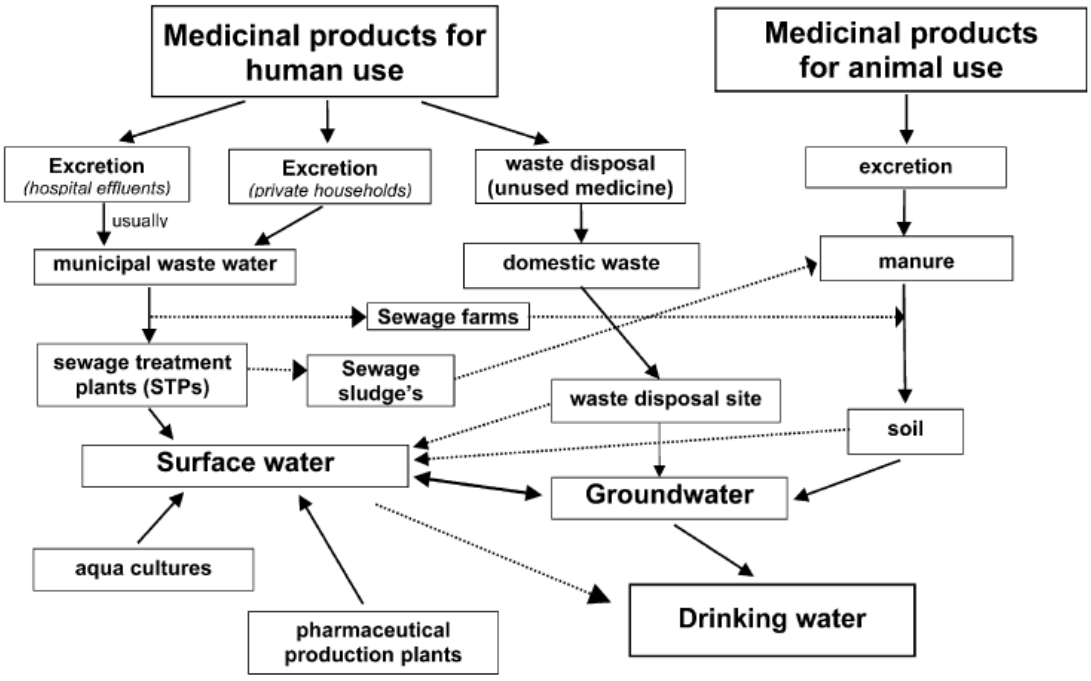


Figure 1.1: Pathways into the environment (Heberer, 2002)

PhACs administered to humans are excreted to varying degrees, or flushed down the toilet if the drugs are no longer needed or have expired. About 15 – 90% of an antibiotic or pharmaceutical can pass through the body unmetabolized (see Table 1.1). In addition to excretion from the body, effluent from pharmaceutical plants and hospitals also makes its way into surface waters. These compounds then enter the environment through point

sources, such as wastewater treatment plants (WWTPs) or non-point sources such as on-site wastewater treatment systems. Most WWTPs or domestic septic systems are designed to treat conventional pollutants such as fecal coliform, suspended solids, biochemical oxygen demanding materials, etc. and not a wide variety of modern anthropogenic chemicals, many of which are biodegradable and environmentally persistent. A large portion of antibiotics are not completely removed during WWTP processes, and therefore remain in the wastewater effluent, which is then discharged to receiving waters, or leached through the soil in the case of domestic septic system discharges. In many instances, the receiving waters flow into downstream drinking water reservoirs.

Table 1.1: Human excretion rates of commonly prescribed antibiotics

Analyte	% Excreted	Reference
	Tetracycline	
Tetracycline	80 - 90	
Doxycycline	> 70	
Oxytetracycline	> 80	Hirsch et al., 1999
Chlortetracycline	> 70	
Minocycline	~ 60	
	Quinolone	
Ciprofloxacin	65 - 85	Physician's Desk Reference,
Norfloxacin	56 - 62	2005
Enrofloxacin	65 - 80	Plumb, 2005
Roxithromycin	> 60	Hirsch et al., 1999
Levofloxacin	< 10	Ellsworth et al., 2004
	Sulfonamide	
Sulfamethoxazole	60	McEvoy, 2004
Erythromycin	> 60	Hirsch et al., 1999
	Diaminopyrimidine	
Trimethoprim	45 - 56	McEvoy, 2004

Note: Data is not readily available for all of the target analytes in this study and only values that were widely available are presented here.

Therapeutic classes of PhACs that have been investigated in the environment include antibiotics, analgesics and anti-inflammatory drugs, anti-epileptic drugs, beta-blockers, blood-lipid regulators, anti-depressants, contrast media, oral contraceptives, and cytostatic drugs (Kolpin et al., 2002; Vanderford et al., 2003). It is important to note that many pharmaceutical substances have not been investigated within each of these classes and long-term environmental effects are largely unknown.

1.3 Causes for Concern

Antibiotics and other pharmaceuticals are powerful drugs used to treat many serious and life-threatening infections and diseases. Despite their benefits and the fact that they constitute merely one of a myriad of chemical classes discharged into the environment, the evidence suggests that they are of special concern for several reasons. First, they are ubiquitous and globally distributed; they are specifically designed or serendipitously discovered to alter biological functions; they are associated with a wide range of side effects in non-target organisms that include endocrine disruption, behavioral changes, and effects on multiple levels of biological organization; they can cause chronic toxicity in aquatic species at environmentally-relevant concentrations (low μg/L to ng/L); and finally, there is the growing issue of antibiotic resistance (Halling-Sorensen et al., 1998; Kolpin et al., 2002; Ferrari et al., 2003; Enick and Moore, 2007).

Bacteria, like all other organisms, evolve over time in response to environmental situations. They are responsible for ear infections, some sinus infections, strep throat, urinary tract infections, and many wound and skin infections. On the other hand, viruses are responsible for causing influenza, colds, most sore throats, and most coughs. Antibiotics are only able to treat bacterial infections; they are ineffective in treating viruses. However, they

are commonly prescribed for viral infections and an unnecessary dose of the antibiotic is given to the patient. Because of the large use and misuse of antibiotics in modern society, many bacteria have developed resistance to antibiotics currently in use (Aymes, 2001). If large numbers of bacteria are resistant to antibiotics, it will be more difficult and more expensive to treat human bacterial infections. When antibiotics fail to work, consequences include extra visits to the doctor, hospitalization or extended hospital stays, a need for more expensive (and often more toxic) antibiotics to replace the older ineffective ones, lost workdays, and sometimes death. The Center for Disease Control and Prevention (CDC) published a report in 2007 stating that approximately 94,000 life-threatening infections and 19,000 deaths occurred in 2005 from drug-resistant *staphylococcus* bacteria in the U.S, which resulted in more deaths than from AIDS alone (CDC, 2007). Other studies have shown an increased resistance of levofloxacin and ciprofloxacin to *Streptococcus pneumoniae* (Ho et al., 2004). Only when antibiotics are used solely for bacterial infections and the whole prescribed dose is taken can the development of antibacterial resistance be somewhat controlled.

Another potential impact that has not been studied in depth due to the complexity of the issue is the potential implication to human health of consuming sub-therapeutic doses of PhACs present in treated drinking water. PhACs have been detected in the low ng/L range in treated drinking water, and it is difficult to quantify effects at such low levels because of the myriad of factors that humans are exposed to in daily life.

The case study presented in this thesis investigates the presence and persistence of PhACs in wastewater effluent on receiving surface waters and a downstream reservoir. Environmental hazard to aquatic life associated with these chemicals is estimated using

values and techniques from published literature. Case studies have been performed in the past (Golet et al., 2002; Kolpin et al., 2002; Yang and Carlson, 2003; Barber et al., 2006; Lissemore et al., 2006), but none have directly investigated the impact of PhACs in a wastewater effluent discharged to a watershed involving a drinking water reservoir. This case study is unique in that the three major water sources upstream from the reservoir each contain a wastewater treatment plant that discharges anywhere from 4 to 16 million gallons of effluent per day into the receiving stream. In times of extreme drought, as were the conditions during the period of this study, the main water source flowing into the reservoir was treated wastewater effluent. Sampling occurred upstream of the WWTP, at the point where the effluent mixes with the stream water and at two downstream locations to determine 1) the presence of any targeted compounds upstream of the point of entry, 2) the concentration of the targeted analytes in the effluent itself, 3) the persistence of PhACs downstream of the WWTP, 4) if there are any non-point sources of the targeted compounds, and 5) the concentration of the targeted compounds entering the reservoir. The benefits of this study include determining the concentrations of targeted analytes in the watershed and their persistence as they travel downstream to a drinking water source. Knowing the extent that these compounds persist can help in watershed management issues in the future that account for providing a pristine recreational area and drinking water source.

1.4 Target Analytes

Twenty-six commonly prescribed human and veterinary antibiotics have been investigated in this case study (Table 1.2) because of their widespread use in this country and around the world. Tylosin, enrofloxacin, and sarafloxacin are only used for veterinary

purposes while ciprofloxacin, roxithromycin, minocycline, and norfloxacin are only used for human purposes. Chemical structures of the targeted antibiotics are presented in Figure 1.2.

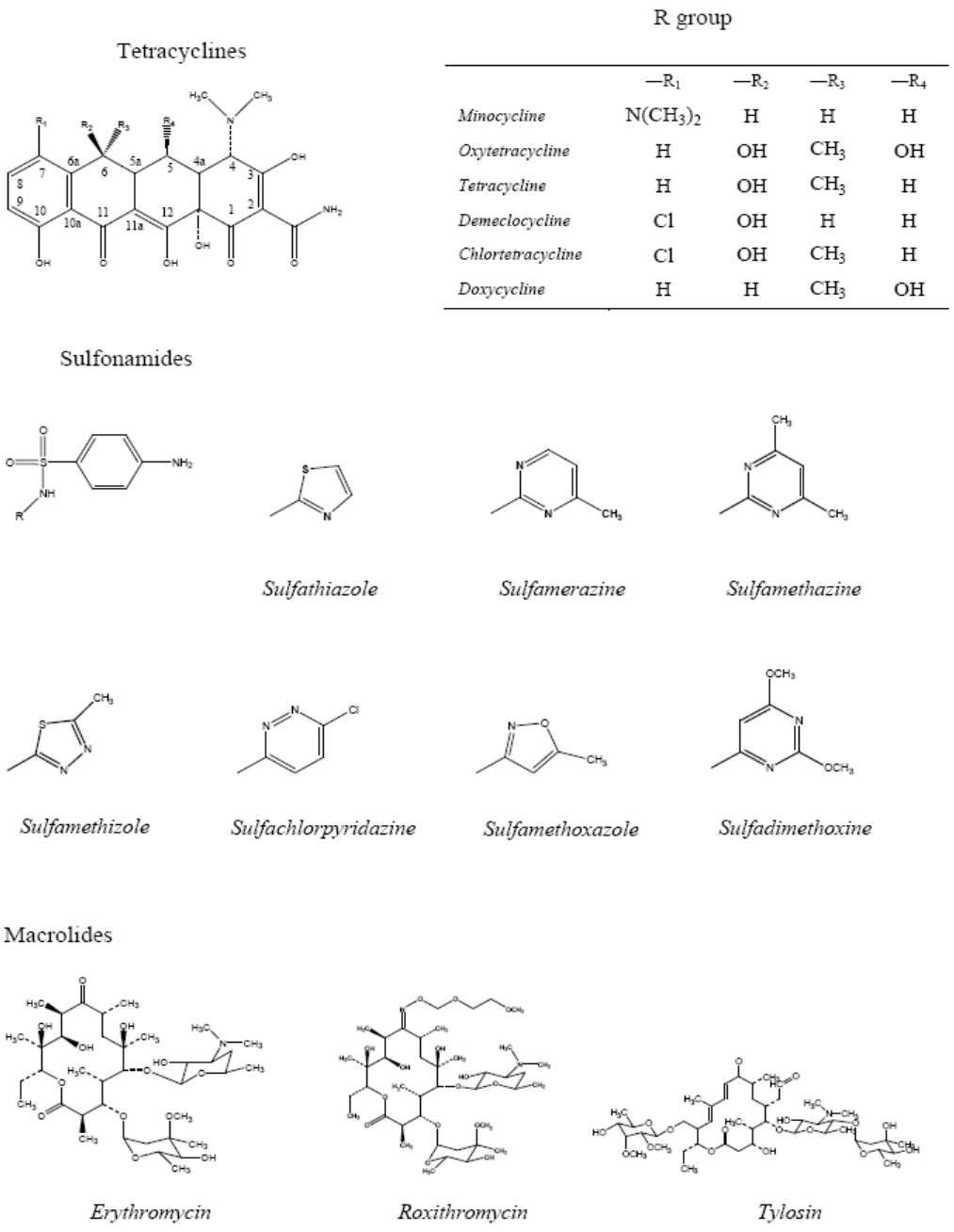
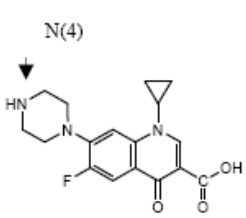


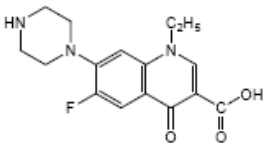
Figure 1.2: Chemical structures of target analytes (Reproduced from Ye, 2005)

Quinolones

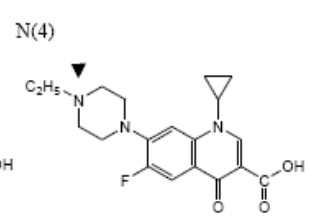
(1) Piperazinylic quinolones



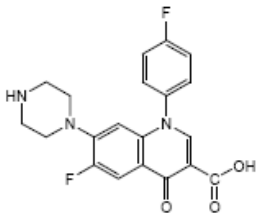
Ciprofloxacin



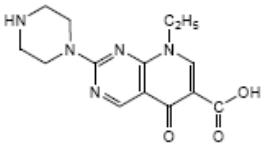
Norfloxacin



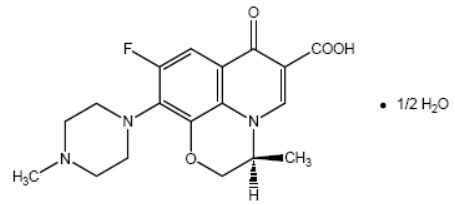
Enrofloxacin



Sarafloxacin

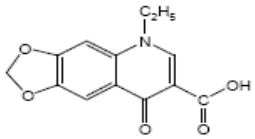


Pipemidic acid

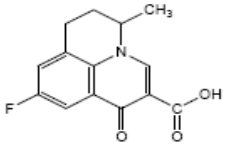


Levofloxacin

(2) Acidic quinolones

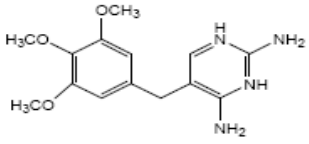


Oxolinic acid



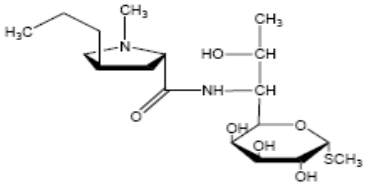
Flumequine

Diaminopyrimidine



Trimethoprim

Lincosamide



Lincomycin

Figure 1.2 (Cont.): Chemical structures of target analytes (Reproduced from Ye, 2005)

Table 1.2 presents chemical information relevant to the fate of antibiotics in the environment including molecular weight, which is used in mass spectrometric analysis to help identify the parent compound through a precursor ion. Log K_{ow} is the octanol/water partition coefficient. Values of K_{ow} represent the tendency of a chemical to partition between an organic phase and an aqueous phase. Chemicals with low K_{ow} values can be considered hydrophilic, meaning they have a higher tendency towards the aqueous phase, small soil/sediment adsorption coefficients, and small bioaccumulation factors in aquatic life (Reddy and Locke, 1994). Conversely, chemicals with high K_{ow} values tend to be hydrophobic and will be inclined to partition into the organic phase. The pKa determines the acidic or basic properties of a substance, or rather at which pH values the analytes are anionic, neutral, or cationic, which is also relevant for partitioning effects especially in the environment but also during ingestion.

Table 1.2: Physical properties of target analytes

Analyte	Molecular Weight	Log K_{ow}	pKa (20°C)
Sulfachlorpyridazine	284.7	0.31	1.4 [a]
Sulfadimethoxine	310.3	0.63	5.54 [a]
Sulfamerazine	264.3	0.14	1.58 / 6.98 [b]
Sulfamethazine	278.3	0.89	2.65 / 7.65 [d]
Sulfamethizole	270.3	0.54 [d]	5.45 [e]
Sulfamethoxazole	253.3	0.89	1.68 / 5.57 [b]
Sulfathiazole	255.3	0.05	2.30 / 7.20 [b]
Erythromycin-H_2O	733.9	3.06	8.88 [f]
Roxithromycin	837.1	2.75	8.8 [g]
Tylosin	916.1	3.5	7.1 [c]
Lincomycin	406.5	0.56	7.6 [e]
Trimethoprim	290.3	0.91	1.32 / 7.45 [f]
Ciprofloxacin	331.3	0.28	6.43 / 8.49 [f]
Enrofloxacin	359.4	1.1	6.27 / 8.30 [c]
Flumequine	261.2	1.6	6.2 [h]
Levofloxacin	370.4	not available	5.7 / 7.9 [i]
Norfloxacin	319.3	-1.03	6.34 / 8.75 [j]
Sarafloxacin	385.4	1.07	6.00 / 8.60 [c]
Oxolinic acid	261.2	0.94	6.87 [b]
Pipemidic acid	303.3	-2.15	not available [f]
Chlortetracycline	478.9	-0.62	4.50 / 9.68 [b]
Demeclocycline	464.9	-1.14	not available [f]
Doxycycline	444.4	-0.02	not available [f]
Minocycline	457.5	0.05	not available [f]
Oxytetracycline	460.4	-1.22	3.27 / 7.32 / 9.11 [c]
Tetracycline	444.4	-1.3	3.30 / 7.68 / 9.69 [c]

a.Kim et al., 2007b; b. SciFinderScholar, American Chemical Society, 2006; c. Tolls, 2001; d. Diaz-Cruz et al., 2006; e. Merck Index, 1996; f. Syracuse Research Corp., 2007; g. Huber et al., 2003; h. Delmas et al., 1997; i. Hirano et al., 2006; j. Barbosa et al., 2001.

1.5 Occurrence in the Environment

Only recently have analytical methods been developed to detect and quantify PhACs in different environmental matrices. These compounds have been investigated in wastewater

effluents and surface waters since the 1990's in the United States, Canada, Europe and more recently in other countries. A comprehensive literature review of concentrations found in the environment for the targeted analytes is presented in this section. These findings are rarely reported in the open literature as noted by the Associated Press (AP), who recently published a comprehensive nation-wide report suggesting an attempt to withhold information from the public (AP, 2008). This report states that there are several reasons as to why findings are not made publicly available. The main reason is that scientists and utilities do not want to falsely alarm the public, who may take the results out of context. The effects of PhACs on humans and the environment that are present in surface and drinking waters are not known at this time, so one can not conclusively state that there are adverse health effects associated with ingestion or exposure at the levels found in the environment. A third reason is due to research funding and utility privacy, where utilities and researchers agree ahead of time to keep the study results confidential. As more attention is focused to this issue and more research is conducted, there will hopefully be more concrete data about the exposure effects of PhACs in surface and drinking waters once valid occurrence data is obtained.

1.5.1 Wastewater Treatment Processes

Conventional wastewater treatment plants are not specifically designed to remove PhACs and because these chemicals are highly water-soluble, they can persist in the wastewater through different treatment stages (Kolpin et al., 2002). Several studies have investigated the fate of PhACs in complex matrices including dairy effluent, hospital waste, raw wastewater, and treated wastewater effluent, but as this case study focuses on the presence of these compounds in treated wastewater effluent, only these values are presented

in Table 1.3. Tables 1.3 attempts to compare and contrast levels of PhACs from many different studies around the world, and is meant to be representative of the minimum and maximum concentrations found in the literature. It is important to note that there are still antibiotic and other pharmaceutical residuals present in surface waters at detectable levels, making wastewater effluent a major source for these compounds in surface waters (Kolpin et al., 2002). There are a limited number of published studies that have investigated the levels of PhACs in the intermediate stages of wastewater treatment, hospital waste, or in effluent from CAFOs.

Table 1.3: Occurrence of target analytes in wastewater treatment plant effluent

Analyte	Conc. (µg/L)	Reference
Sulfamethazine	0.018	Gobel et al., 2004
	0.64	Yang and Carlson, 2003
Sulfamethoxazole	0.32	Yang and Carlson, 2003
	0.31	Brown et al., 2006
	1.34	Batt et al., 2006
Erythromycin-H_2O	0.3	Karthikeyan and Meyer, 2006
	0.199	McArdell et al., 2003
	2.054	Xu et al., 2007
Trimethoprim	0.180	Brown et al., 2006
	0.55	Karthikeyan and Meyer 2006
	0.180	Xu et al., 2007
Norfloxacin	0.064	Golet et al., 2003
	0.085	Xu et al., 2007
Ciprofloxacin	0.14	Karthikeyan and Meyer, 2006
	0.97	Batt et al., 2006
Roxithromycin	0.021	Gobel et al., 2004
	0.031	McArdell et al., 2003
Lincomycin	2.00	Brown et al., 2006
Enrofloxacin	< 0.034	Nakata et al., 2005

Table 1.3: Occurrence of target analytes in wastewater treatment plant effluent (continued)

Analyte	Conc. (µg/L)	Reference
Sarafloxacin	< 0.044	Nakata et al., 2005
Pipemedic Acid	< 0.031	Nakata et al., 2005
Oxytetracycline	0.66	Yang and Carlson, 2003
Tetracycline	0.16	Yang and Carlson, 2003
	0.85	Karthikeyan and Meyer, 2006
	0.56	Batt et al., 2006
Demeclocycline	0.09	Yang and Carlson, 2003
Chlortetracycline	< 0.05	Yang and Carlson, 2003

Error! Not a valid link.1.5.2 Surface Waters

The first major U.S. investigation of a wide-range of PhACs in surface waters was conducted during 1999-2000 by the United States Geological Survey (Kolpin et al., 2002). This study sampled 139 streams that were impacted by residential, industrial, or agricultural areas. Eighty percent of the 95 targeted analytes were detected; 23 of these were antibiotics and, although they were detected at lower concentrations than the other targeted analytes (ng/L vs. µg/L), this study was important because it fueled the development of many analytical techniques to more thoroughly detect this subset of pharmaceuticals at environmentally relevant concentrations.

Very few studies have investigated the presence of PhACs in groundwater which, if found, would suggest that these compounds are not adsorbing to the soil column after land application such as from agricultural runoff, incompletely sealed landfills, or waste disposal

from domestic septic systems. Results from a variety of occurrence studies in surface and ground waters are presented in Table 1.4.

Table 1.4: Occurrence data of targeted analytes in surface and ground waters

	Compound	Conc. (µg/L)	Matrix
Sulfonamides	Sulfachlorpyridazine	0.007[a]	Surface Water
	Sulfadimethoxine	0.056[a] - 0.24[b]	Surface Water
		0.046 - 0.068[c]	Groundwater
	Sulfamethazine	0.22[b] - 0.408[a]	Surface Water
		0.076 - 0.215[c]	Groundwater
	Sulfamethizole	0.13[d]	Surface Water
	Sulfamethoxazole	0.02[e] - 1.9[d]	Surface Water
		0.22[b] - 0.41[f]	Groundwater
	Sulfathiazole	0.016[a] - 0.08[b]	Surface Water
	Erythromycin - H_2O	0.051[a] - 1.7[d]	Surface Water
	Roxithromycin	0.002[a] - 0.18[d]	Surface Water
	Tylosin	0.28[d]	Surface Water
	Trimethoprim	0.015[a] - 0.71[d]	Surface Water
Quinolones, macrolides, lincosamides	Norfloxacin	0.03 - 0.12[d]	Surface Water
	Lincomycin	0.01 - 0.73[d]	Surface Water
	Ciprofloxacin	< 0.019[g] - 0.03[d]	Surface Water
	Enrofloxacin	0.01[d]	Surface Water
	Sarafloxacin	< 0.044[g]	Surface Water
	Pipemidic Acid	< 0.031[g]	Surface Water

Note: a. Lissemore et al., 2006; b. Lindsey et al., 2001; c. Batt et al., 2006; d. Kolpin et al., 2002; e. Vanderford et al., 2003; f. Sacher et al., 2001; g. Nakata et al., 2005; h. Kolpin et al., 2004.

Table 1.4: Occurrence data of targeted analytes in surface and ground waters (continued)

	Compound	Conc. (µg/L)	Matrix
Tetracyclines	Oxytetracycline	0.34[d] - 1.34[b]	Surface Water
	Tetracycline	0.11[d] - 0.3[h]	Surface Water
	Chlortetracycline	0.1[h] - 0.69[d]	Surface Water
	Doxycycline	0.02[d] - 0.073[a]	Surface Water

Note: a. Lissemore et al., 2006; b. Lindsey et al., 2001; c. Batt et al., 2006; d. Kolpin et al., 2002; e. Vanderford et al., 2003; f. Sacher et al., 2001; g. Nakata et al., 2005; h. Kolpin et al., 2004.

These occurrence studies (Table 1.3 and 1.4) suggest that many of the analytes are not completely removed during wastewater treatment processes, and their presence in wastewater effluent indicates that they are making their way into surface streams across the U.S. and Europe. In general, the sulfonamides have been found at higher concentrations in surface waters when compared to the quinolones and tetracyclines. Based on the lower K_{oc} values (Table 1.5 below), it is expected that the sulfonamides would more readily persist through wastewater treatment processes and move into surface waters.

The ability of an analyte to adsorb to an organic surface can be predicted by its solid-water distribution coefficient (K_d). K_d is the ratio of the concentration of the analyte in the sorbent and in the water at equilibrium and defined as:

$$K_d = K_{oc} * f_{oc}$$

This value can vary based on the fraction of organic carbon (f_{oc}) in the sorbent. The normalized sorption coefficient (K_{oc}) is a more accurate indicator of sorption and is, therefore, recommended to predict the sorptive change of chemicals between water and solid phase. To use K_{oc} in this relationship, the compound must be nonionic because sorption of

ionic contaminants is affected by soil pH, and the flow of the water must also be fairly calm to ensure that sorption is occurring at equilibrium (Ahel and Giger, 1993). A compound with a high K_{ow} and high K_{oc} values tends to partition into the organic phase, whereas a compound with low K_{ow} and low K_{oc} will tend to remain in the aqueous phase. Table 1.5 presents experimentally determined sorption coefficients of some antibiotics and pharmaceuticals. Based on these K_{oc} values, the sulfonamides would be expected to remain in the aqueous phase, which favors their mobility through WWTPs and into surface waters. On the other hand, the fluoroquinolones and tetracyclines will tend to strongly adsorb to soil minerals and aquatic sediments due to their high K_{oc} values.

The ability to adsorb to solid particles, both naturally occurring (e.g. clay, stream sediment) and those added to the matrix during wastewater treatment (e.g. activated carbon, coagulants) is a major factor in the removal of fluoroquinolones and tetracyclines during treatment (Boxall, 2008). These interactions facilitate their removal by physical-chemical (settling, flotation) and biological processes (biodegradation). Sorption of PhACs onto sludge particles in WWTP processes is an imperative route describing their potential fate, especially if the sludge will be used for land application.

Table 1.5: Sorption coefficients of antibiotics in soils, sediments, and slurry

Analyte	Concentration (µg/g)[a]	Sample (soil:texture/pH/%OC)	K_d (L/kg)	K_{oc} (L/kg)
Sulfachlorpyridazine	0.05 - 20	clay loam/6.5/--	1.8	--
	0.05 - 20	sandy loam/6.8/--	0.9	--
		clay loam/6.2/3.1	4[b]	129
Sulfadimethoxine	1.0 - 10	silt loam/7.0/1.6	2.3	143
		clay loam/6.2/3.1	10[b]	323
Sulfamethazine	0.2 - 25	sand/5.2/0.9	1.2	174
	0.2 - 25	loamy sand/5.6/2.3	3.1	125
	0.2 - 25	clay silt/6.9/1.1	1.0	82
	0.2 - 25	sandy loam/6.3/1.2	2.0	208
Sulfathiazole	--	clay loam/6.2/3.1	3[c]	97
Sulfamethoxazole[d]	--	digested sludge/6.0/2.1	9.6-22.9	7.85-32.1
Trimethoprim	500 µg/L	sewage sludge/6.5/37[d]	76	205
Ciprofloxacin	250 µg/L	sewage sludge/6.5/37[d]	417	1127
	2- 200	loamy sand/5.3/0.70	427	61000
Enrofloxacin	2 -200	clay/4.9/1.63	3037	186340
	2 -200	loam/5.2/0.73	5612	768740
Roxithromycin[d]	--	digested sludge/6.0/2.1	82.2-83.3	8.09-23.3
Tylosin	1.25 - 25	loamy sand/6.1/1.6	128	7990
	1.25 - 25	sand/5.6/1.4	10.8	771
	1.25 - 25	sandy loam/5.6/1.1	62.3	5660
	1.25 - 25	sand/6.3/1.5	8.3	553
Oxytetracycline	--	sewage sludge/6.5/37	3020	8160[a]
	33 - 2000 mg/g	pig manure 6h/24h[d]	83.2/77.6	195
	2.5 - 50	loamy sand/6.1/1.6	680	42500
	2.5 - 50	sand/5.6/1.4	670	47880
	2.5 - 50	sandy loam/5.6/1.1	1026	93320
	2.5 - 50	sand/6.3/1.5	417	27790
	285	organic marine sediment	2590	--
	10.9	organic marine sediment	663	--
Tetracycline[e]	--	peat/4.55/--	1620	--
Oxolinic acid[e]	--	marine sediment - mud	116	1190
		marine sediment - sandy mud	70	4510
		marine sediment	0.3	17

Note: [a] if not indicated otherwise; [b] data derived from figures (Thiele-Bruhn, 2003); [c] K_{oc} estimates for OC in dry matter from Richards, 2007; [d] adsorption time; [e] data from Tolls, 2001; "—" not available; if not indicated otherwise, all data from Thiele-Bruhn, 2003.

1.5.4 Degradation

PhACs in wastewater and surface waters can undergo several forms of natural attenuation processes such as hydrolysis, biodegradation, and photolysis. Hydrolysis is a process where organic compounds are broken down through interactions with water, and is not considered a major factor of degradation because pharmaceuticals are designed for oral ingestion and metabolism and, therefore, resistant to hydrolysis. Biodegradation is the process by which microorganisms, for instance bacteria, break down a substance into more elementary compounds. It is an important process when considering the persistence of chemicals in soils due the presence of bacteria. Because neither hydrolysis nor biodegradation are thought to readily occur in the water column, direct and indirect photolysis are thought to be major players of degradation. Direct photolysis is the process by which a substance undergoes a chemical change after absorbing light energy (i.e. sunlight), whereas indirect photolysis involves natural photosensitizers such as humic acids and nitrates (Andreozzi et al., 2003; Schmidtt-Kopplin et al., 1999). Water depth, turbidity, and humic acids that absorb sunlight can inhibit photolysis, which indicates that PhACs may be less persistent at the surface of a water body than at some depth below the surface (Andreozzi et al., 2003). Another factor that influences photolysis is the intensity of solar irradiance at a given latitude and season (Gao and Zepp, 1998). These mechanisms are not a particular focus of this study, but a brief discussion is warranted as these processes may affect the fate and transport of antibiotics and other pharmaceuticals in watersheds.

Andreozzi et al. (2003) found that the half-life of carbamazepine approached 100 days in winter at high latitutde (50°N), whereas the half-life for sulfamethoxazole, diclofenac, ofloxacin, and propanolol approach 2.4, 5.0, 10.6, and 16.8 days respectively.

This means that these compounds are remaining in the water column and may pose a significant hazard to aquatic life until they are completely broken down. The presence of nitrate ions reduced the half-life for all compounds except propanolol while humic acids acted as inner filters for carbamazepine and diclofenac, and as photosensitizers for sulfamethoxazole, clofibric acid, ofloxacin, and propanolol meaning that streams with a higher concentration of humic acids would be expected to more quickly degrade these compounds.

Verma et al. (2007) found that the half-life of tetracycline was 32, 2, and 3 days in distilled, river, and wetland waters, respectively in the presence of direct sunlight. In the same waters with no light exposure, the respective half-lives were 83, 18, and 13 days. This indicates that direct photolysis will likely decrease the persistence of tetracycline in open environmental waters and their degradation will be further enhanced due to the presence of humic materials and nitrates acting as photosensitizers.

The two most common ways that fluoroquinolone can be reduced in surface waters are via phototransformation and sorption to particles (Table 1.5, K_{oc} values > 1000 for ciprofloxacin, enrofloxacin, and tylosin); biodegradation is considered to be irrelevant (Golet et al., 2003). More than 90% of enrofloxacin adsorbed to sediment (Table 1.5; K_{oc} = 1127, 61000) (Nowara et al., 1997), and a half-life in summer months was just under 2 hours in direct sunlight. In contrast to some of the compounds mentioned earlier, the presence of humic substances has been found to decrease the rate of photodegradation of fluoroquinolones into metabolites (Schmitt-Kopplin et al., 1999). Sorption is therefore thought to be the major player due to their strong sorption properties (Table 1.5) (Nowara et al., 1997; Tolls, 2001).

1.5.5 Transport in Watersheds

Few published studies have investigated the occurrence, fate, and transport of PhACs in watersheds, and none have included a drinking water source during a period of extreme drought. Lissemore et al. (2006) conducted a watershed study with mainly agricultural inputs in Ontario, Canada. Of the 28 human and veterinary pharmaceuticals targeted, 14 were detected in surface waters, 12 of which were veterinary pharmaceuticals that were not detected in the wastewater effluent. This is evidence that agricultural areas can be a point of entry for PhACs into surface waters via land application runoff and leaching through the soil. Temporal fluctuations were also observed that correlated with manure or biosolids application in the spring and fall months (May-November).

Barber et al. (2006) conducted a watershed study in the Boulder Creek, Colorado watershed to determine if a range of chemicals including pharmaceuticals, pesticides, herbicides, surfactant-degradation products, steroids, and hormones were present. Although only two sampling events were conducted (during low-flow and spring runoff), several compounds were present, including nonylphenol and sulfamethoxazole at various locations. These were detected at higher concentrations during the low-flow event due to minimal dilution and maximum concentrations were found downstream of the WWTP discharge in the creek. Pharmaceuticals were only detected downstream of the WWTP discharge indicating that this is the major source for these contaminants.

Another Colorado study looked at five tetracyclines (oxytetracycline, chlortetracycline, demeclocycline, tetracycline, and doxycycline) and six sulfonamides (sulfathiazole, sulfamerazine, sulfamethazine, sulfachlorpyridazine, sulfamethoxazole, and

sulfadimethoxine) in the Cache la Poudre River, which is situated in a semi-arid region of the United States (Yang and Carlson, 2003). None of the targeted antibiotics were detected at the upstream pristine mountain site before anthropogenic input influences 43 miles downstream, further indicating that urban areas are sources for these compounds. Demeclocycline and tetracycline appeared in the river upstream of the influence from the urban area and then increased slightly 20 miles downstream of this point due to the addition of wastewater effluent to the river. The sulfonamides on the other hand were not detected in the river water upstream of the urban area. Demeclocycline, tetracycline, chlortetracycline, oxytetracycline, and doxycycline as well as two of the six sulfonamides (sulfamethoxazole and sulfachlorpyridazine) were found in the river 8 miles downstream of the WWTP. As the river transitioned from urban to agricultural influences 20 miles downstream of the WWTP, oxytetracycline, chlortetracycline, tetracycline, and doxycycline were observed at their highest concentration in the river. Tetracycline persisted unattenuated, while significant degradation or adsorption of sulfamethoxazole and sulfachlorpyridazine appeared to have occurred. Oxytetracycline, chlortetracycline, and sulfachlorpyridazine were not detected in the wastewater effluent, which suggests the presence of these compounds can be mainly attributed to agricultural sources, while the presence of sulfamethoxazole and doxycycline were mainly of anthropogenic input. The sulfonamides were observed to undergo natural attenuation processes such as photolysis and adsorption downstream of the WWTP, while the tetracyclines were found at similar concentrations in both the WWTP discharge and at the farthest downstream sampling location 20 miles from the WWTP.

Tetracycline sorption coefficients (Table 1.5) suggest that these compounds would be prone to adsorption but the results of this study are unclear as to whether the tetracyclines

actually persisted in the river water, or if additional non-point source pollution were responsible for the concentrations detected. This study implies that antibiotics can enter the watershed through point and nonpoint source pollution, and that inputs from agricultural areas can increase the presence of veterinary antibiotics in a watershed.

Macrolide antibiotics were investigated in the Glatt River watershed in Switzerland (McArdell et al., 2003). The river receives input from three WWTPs, is about 36 km long, and has a water residence time of 15-20 hours. Roxithromycin, erythromycin-H_2O, and clarithromycin were detected in the treated wastewater effluent, but only clarithromycin was present above detectable levels in the river. It is interesting to note that the antibiotics were present in the effluent at levels two times greater in the winter than those found during the summer months. This means that elimination during wastewater processes is lower in winter months perhaps due to a lower biological activity, or that the influx of macrolides is higher during these months. These seasonal differences must be considered when determining environmental risk to aquatic life. Daily variations were observed for clarithromycin in the effluent, but there was no pattern. This study showed that there is no significant removal of clarithromycin (> 20%) in the Glatt River, but results from a previous study within the same watershed showed substantial removal of ciprofloxacin (66%), and norfloxacin (48%) (Golet et al., 2003). These results would be expected based on the high K_{oc} values of both ciprofloxacin and norfloxacin (both are fluoroquinolones).

None of these watershed studies have examined how the fate and transport of antibiotics might impact downstream drinking water sources, which this case study intends to do. Antibiotics, other pharmaceutically active compounds, and a variety of anthropogenics are found in wastewater effluent discharged to surface waters, and many of these waters flow

downstream into drinking water sources. It is therefore important to consider the persistence and environmental hazard of these compounds when drinking water sources are nearby.

1.5.6 Drinking Water Treatment Plant Processes

There is concern that PhACs are persisting through drinking water treatment processes and into municipal drinking water due to their presence in surface waters (Kolpin et al., 2002; Gibs et al., 2007). Drinking water treatment processes include coagulation, flocculation, sedimentation, and filtration of the source water. Disinfection occurs most commonly in the U.S through the addition of chlorine or chloramines to the finished water since these disinfectants maintain a residual in the distribution network. However, use of ultraviolet light (UV) or ozonation at early stages of treatment, often for algae control or to enhance coagulation, is expanding.

Removal of PhACs during drinking water treatment can occur by their partitioning into the solid phase during coagulation or chemical transformation during reaction with disinfectants. Sorption of organics onto solids during coagulation occurs mainly with hydrophobic compounds (Snyder et al., 2003; Adams et al., 2002), but since most antibiotics tend to be hydrophilic, coagulation and flocculation are not effective in their removal (Kim et al., 2007b; Ternes et al., 2002).

Activated carbon filtration is another promising mechanism for removal, but contact time, type of carbon, contaminant solubility, and competition for adsorption sites with larger and more polar compounds such as natural organic matter may pose a problem (Kim et al., 2007b; Matsui et al., 2002). Granular activated carbon adsorption is very effective in

removing sulfamethoxazole and trimethoprim, as well as erythromycin, lincomycin, and ciprofloxacin to some extent in drinking water (Ye, 2005).

Disinfection through chlorination, chloramination, or ozonation is effective in transforming a host of micropollutants, but transformation rates depend on the compound structure and oxidant dose (Kim et al., 2007b; Zweiner and Frimmel, 2000; Adams et al., 2002; Huber et al., 2003; Ternes et al., 2002). Chlorination is practiced by dissolving chlorine gas or hypochlorite in water. Free chlorine is a strong oxidant and can react with many pollutants but it can produce disinfection by-products. Chloramines, formed by the reaction of ammonia and free chlorine, are much weaker reactants and are slow to react with pollutants. UV disinfection is a process where the most commonly used low-pressure mercury lamps emit primarily monochromatic light at 254 nm. Disinfection occurs when light photons are absorbed by the DNA nucleotides of microorganisms that absorb light around 260 nm, consequently rendering the microorganism or pollutant ineffective (Pereira, 2005). This process is growing in popularity because it is effective against a wide range of water-borne pathogens and does not produce any known disinfection by-products (DBP) (Pereira, 2005). Ozonation occurs when water is infused with unstable ozone gas that is toxic to many water-borne pathogens. Although ozonation, when used as a pre-oxidant, can remove some of the common DBP precursors, it is known to produce bromate in the presence of bromide. Zweiner and Frimmel (2000) found that ozonation is effective if the concentration is equivalent to the organic matter content of the water, but the addition of advanced oxidation processes (AOP) like ozone-hydrogen peroxide (O_3/H_2O_2) proves more effective in removing pharmaceuticals (> 90%) such as diclofenac, ibuprofen, and clofibric acid.

Ye (2005) conducted a comprehensive study of several water treatment processes and results from a sampling event at a water treatment plant in North Carolina are shown in Table 1.6. The plant added sodium hypochlorite both prior to (1.5 mg/L) and after filtration (4.2 mg/L). Ammonia was then subsequently added (1.0 mg/L) to generate chloramines for terminal disinfection. Results show that although several antibiotics were detected in finished drinking water as well as tap water, most were at or below practical quantitation limits.

Table 1.6: Occurrence of antibiotics in source, finished drinking water, and tap water samples in a North Carolina drinking water treatment plant (Ye, 2005)

Antibiotic	Mean Concentration (ng/L), (n=2)		
	Source	Finished	Tap
Sulfamethoxazole	20	(4.5)	<5.0
Tylosin	<10	(4.5)	<5.0
Erythromycin	(4.2)	(3.5)	<5.0
Roxithromycin	<10	(1.4)	<5.0
Oxolinic acid	(3.7)	(1.0)	(0.9)
Flumequine	(3.6)	(2.0)	(1.9)

Note: "__" indicates that analyte detection was confirmed by the presence of two targeted tandem mass spectrometry fragment ions and "( )" indicates that the analyte was detected and reported below the practical quantitation limit.

The decrease in concentration between the source and finished water is likely due to partitioning during coagulation or chemical transformation during disinfection. Any antibiotics present in the water could have undergone microbial degradation, hydrolysis, or adsorption onto the inner surfaces of the distribution pipes. Despite their detection below practical quantitation limits, they are still present at sub-therapeutic levels after treatment and may still pose a public health risk.

1.6 Estrogens

Thousands of compounds could potentially cause endocrine disruption or be endocrinally active in vertebrates such as fish, amphibians, reptiles, birds, and mammals. Natural endocrine activity can be altered by mimicking a natural hormone, inhibiting the binding of a natural hormone to the receptor, or changing the rate of removal of the hormone from the endocrine system (Routledge and Sumpter, 1996).

Measurable levels of endocrine active substances (EASs) in lakes, rivers, streams, and groundwater have been reported at environmentally relevant concentrations (Ternes, 1998; Sacher et al., 2001; Kolpin et al., 2002). Steroid hormones have been found in surface waters near agricultural runoff locations at concentrations up to 800 ng/L (Kolpin et al., 2002) and in conventionally treated wastewater from 10 ng/L and up to 100 ng/L (Servos et al., 2005). Several other studies have found similar low ng/L levels of estriol (E1), estradiol (E2), and estrone (E3) in, or downstream of wastewater and septic effluents (Rodriguez-Mozaz et al., 2004; D'Ascenzo et al., 2003; Wintgens and Gallenkemper, 2003). Higher levels of hormones and other EASs in the surface waters and ground waters are more commonly found closer to CAFOs than near WWTPs. Finlay-Moore et al. (2000) found concentrations of estradiol in runoff from poultry litter-fertilized fields ranging from 20 to 2500 ng/L and soil concentrations between 55 ng/kg and 675 ng/kg after land application. Kay et al. (2005) identified overland flow of land applied manure slurries as a potential source for transport of antibiotics to nearby aquatic environments.

Ethinylestradiol (EE2) has been shown to negatively impact fathead minnow population growth and reproduction at concentrations as low as 4 ng/L (Grist et al., 2003). E2 concentrations ranging from 5 to 25 ng/L can induce vitellogenin (a precursor to egg yolk

production) in adult male zebrafish, whereas this is observed in juvenile male zebrafish at concentrations up to 100 ng/L (Brion et al., 2004). Mixtures of nonylphenol and octylphenol have shown antagonistic effects with E2 and EE2 in breast cancer cell assays (solvent or aqueous based), indicating that such antagonism and/or synergism may play a significant role in total observed estrogenicity (Rajapakse et al., 2004). There are many other studies investigating the behavioral and developmental changes from exposure to EASs (Campbell et al., 2006; Choi et al., 2004; Cooper and Kavlock, 1997; Huang et al., 2003). Estrogens in treated wastewater effluent and surface waters are yet another indicator of the impact of human generated waste on the environment.

1.7 Environmental Risk

In the United States, the National Environmental Policy Act of 1969 (NEPA) requires all federal agencies to assess the environmental impact of their actions and ensure that the interested and affected public is informed of environmental analyses (U.S. EPA, 2007b). The Food and Drug Administration (FDA) is required, under NEPA, to consider the environmental impacts (use and disposal) of a drug as an integral part of its regulatory process when approving drug or feed additive applications. However, NEPA does not require that the most environmentally beneficial course of action be taken.

In order to assess the environmental impact of antibiotics and pharmaceuticals in the environment, an environmental assessment (EA) and environmental impact statement (EIS) must be completed for new drugs (FDA, 1998; U.S. EPA, 2007b). Certain drugs that will not significantly affect the quality of the human environment may be exempt from this process (FDA, 1998). Examples of this instance include approval of a drug that will not increase its use, drugs for use in nonfood animals, drugs for minor species (wildlife and endangered

species), and therapeutics under veterinarian prescription. EAs focus on environmental fate and ecotoxicology based on drug use patterns. An EIS is a statement that focuses on (i) the environmental impact of the proposed action, (ii) any adverse environmental effects which cannot be avoided, (iii) alternatives to the proposed action, (iv) the relationship between short-term uses of the environment and maintenance and enhancement of long-term productivity, and (v) any irreversible and irretrievable commitments of resources which would be involved (FDA 1998, 2001).

EA for human drugs usually focus on the aquatic environment, whereas animal drugs evaluate the fate and effects in the aquatic environment (fresh and saltwater), terrestrial environments, dung pats and direct bird exposure. All EAs are available under the Freedom of Information Act (FOIA).

1.7.1 Hazard Quotient Assessment Method

Many PhACs have been shown to induce adverse effects in microorganisms, invertebrates, fish, and even birds despite going through a strict regulatory process. Some antibacterial agents (flumequine, oxytetracycline), antidepressants and their metabolites (fluoxetine, sertraline and norfluoxetine) have been observed to bioaccumulate in living organisms such as fish and mussels (Delepee et al., 2004; Brooks et al., 2003). The anti-inflammatory, diclofenac, has been reported to have harmful effects on rainbow trout as well as vultures that have ingested deceased cows treated with this compound in India (Taggart et al., 2007; Laville et al., 2004; Schwaiger et al., 2004). There have also been PhACs such as gadobutrol, gadoxetic acid disodium, and gadofosvest trisodium that have not been found to

cause an adverse effect in the aquatic environment using predictive risk techniques discussed in this section (Farkas et al., 2008).

In order to determine the probability that an adverse effect will occur, representative species are subjected to a chemical at a certain concentration for a certain period of time until an effect or no effect is observed in a certain portion of the population. For example, an EC_{50} value or effective concentration value represents the concentration of a chemical that exerts a non-lethal effect in 50% of the test population compared to the control population. An LC_{50} is similar, but it determines a lethal effect in 50% of the population, and a no effects concentration (NOEC) is the highest observed concentration for which the effect does not significantly differ from that of the control population.

The hazard quotient method is the most widespread method used for the semi-quantitative characterization of the environmental hazard of PhACs (Emmanuel et al., 2005; EMEA, 2006; Hernando et al., 2006; Lindberg et al., 2007; Farkas et al., 2008). This involves calculating the ratio of the predicted environmental concentration (PEC) or measured environmental concentration (MEC) divided by a predicted no effects concentration (PNEC) (equation 1.1). MECs are used in the case study presented in this thesis, but when MECs are not available, PECs must be used. PECs are calculated using guidelines from the European Medicines Evaluation Agency (EMEA, 2006) using information that estimates the production volume of a pharmaceutical per year, the population size, amount of wastewater discharged per day, the removal rate during WWTP processes, and dilution effects (equation 1.2).

$$\text{Hazard Quotient} = \text{PEC or MEC} / \text{PNEC} \qquad \text{(eq. 1.1)}$$

$$PEC = A \times (100 - R) / (365 \times P \times V \times D \times 100) \quad \text{(eq. 1.2)}$$

where A = amount used per year (kg/year)

R = removal rate in %

P = the population size in the geographic area considered

V = volume of wastewater per day per capita (in m^3)

D = dilution factor of wastewater by surface water flow

The PNEC is estimated by using published, peer-reviewed NOEC, EC_{50}, or LC_{50} values for a species multiplied by an assessment factor (AF) (equation 1.3). An assessment factor is an expression of the degree of uncertainty when extrapolating from a limited number of test species to that of the actual environment. It is also used to account for inter- and intraspecies variability (EMEA, 2006; OECD, 1992). An AF is applied to the lowest NOEC if known, or and LC_{50}/EC_{50} value if a NOEC has not been determined. Table 1.7 suggests AF values for available endpoints.

$$PNEC = NOEC \text{ (or } EC_{50}, LC_{50}) / AF \quad \text{(eq. 1.3)}$$

When the hazard quotient exceeds a value of 1, a hazard is suggested. In order to interpret hazard quotients, the following breaks have been established: “low hazard” from 0.5 to 2, “medium hazard” from 2 to 10, and “high hazard” at 10 +. Other studies have used the cutoff point of 1, where a MEC/PNEC > 1 suggests an environmental hazard (EMEA, 2006; Emmanuel et al., 2005; Hernando et al., 2006; Lindberg et al., 2007; Farkas et al., 2008), but different values are used in this current research to distinguish between the comparative and

absolute environmental hazard. PNEC is a regulatory concept meant to provide a margin of safety in the face of uncertainty. It is a measure of comparative risk and should not be interpreted as a measure of absolute risk. A value above 1 does not indicate a significant value of risk; it just means that the margin of safety is being decreased (Crawford-Brown, personal communication).

Table 1.7: Suggested assessment factors for various endpoints

Endpoint	Number of Species	Assessment Factor
NOEC	3	10
NOEC	2	50
NOEC	1	100
L(E)C50	3	1000
L(E)C50	2	1000
L(E)C50	1	1000

The hazard quotient method is a relatively simple and fast calculation used to estimate potential environmental hazards. There are several weaknesses of this model as there are with all models, and of toxicological studies in general. First, there is a general absence of chronic toxicity data and there is debate about whether or not an AF can accurately be used to extrapolate from test data to the real environment. Secondly, species in toxicological experiments are exposed to high doses of pharmaceuticals for short periods of time, which is not a realistic situation. In reality, pharmaceuticals are pseudo-persistent in surface waters fed by wastewater effluent at much lower levels than used in toxicity studies. Third, there are no strict guidelines as to which species to use in these toxicological studies and comparability of LC_{50}/EC_{50} between species is difficult. A range of LC_{50}/EC_{50} values for different species is used to estimate risks, but it would be much easier to compare the risk of pollutants if one or two sensitive indicator species were used instead of the vast array

found in the literature. Fourth, many studies use a predicted exposure concentration instead of measured environmental concentrations, but there are many assumptions when predicting concentrations and the standard formula may be not be truly accurate as to what is actually occurring. Lastly, very few published toxicological studies have investigated the impact of pharmaceutical mixtures at environmentally relevant concentrations. This is important because wastewater effluent is a complex mixture of many chemicals, pharmaceuticals, etc. and it is extremely difficult to determine the risk to aquatic life by only looking at the risk of one chemical at a time. This issue is discussed in more detail in Section 1.7.2. Despite these concerns, the hazard quotient method has been used in this case study as well as other published studies (Emmanuel et al., 2005; Isidori et al., 2005; EMEA, 2006; Hernando et al., 2006; Lindberg et al., 2007; Santos et al., 2007; and Kostich and Lazorchak, 2007).

1.7.2 Complexity of Assessing Risk of Chemical Mixtures

The goal of a risk assessment is to screen for hazards by considering worst-case scenarios or to produce the best estimates of risk. Although methodologies currently exist for estimating the environmental risk or hazard of industrial chemicals, there are valid concerns about whether these environmental risk assessment tools may need to be revised or replaced to appropriately evaluate the potential environmental impact of pharmaceuticals. These concerns arise because pharmaceuticals are varied in structure, have diverse physical, chemical, and ecotoxicological characteristics, and tend to have physical and chemical properties that differ from general chemicals (Teuschler, 2007). In addition, the metabolism rate and percentage of excretion of the parent compounds and/or its metabolites is not considered in the risk assessment of general chemicals. WWTP removal efficiency must also

be considered if it is assumed that all human pharmaceuticals enter the surface waters through WWTP effluent.

Commonly, a risk assessment for chemical mixtures involves the additivity of effects based on toxicological similarities or dissimilarities among components of a mixture that lack supporting data (Teuschler, 2007). For instance, one could sum the hazard quotients of a class of antibiotics because they work in a similar fashion, but this may not be truly representative of the actual risk/hazard to an organism because very few published studies have toxicologically investigated synergistic and antagonistic effects of mixtures. The more simple additivity methods are useful in addressing potential health risks, but more research into chemical mixtures needs to be completed. A mixtures assessment is chosen to estimate the maximum potential risk and to therefore thoroughly protect the public, or to produce the most accurate risk estimate for the exposure, resulting in estimates that protect the public on average (Teuschler, 2007). Newer methods are emerging in response to the complexities of chemical mixture exposure and effects and several key questions proposed by Teuschler (2007) need to be addressed:

- When is it appropriate to use generalized approaches for chemical mixtures?
- What decision criteria can be used to determine that several chemicals share a common toxic mode of action or have similarly shaped dose-response curves?
- What statistical, chemical, or toxicological evidence is needed to ascertain that two complex chemical mixtures are sufficiently similar in nature such that known toxicity data on one mixture is useful for estimating the toxicity of the other?

- Are there ways to incorporate information on toxicological interactions into a risk assessment? At what exposure levels and mixing ratios do such data show that a simple additivity model is inappropriate to apply?
- Are there methods to evaluate a complex mixture containing a large fraction of unidentified chemicals?

Policy issues outside the realm of science play a role in whether or not it is worth investigating the risk of chemical mixtures. The Safe Drinking Water Act Amendments of 1996 (U.S. Congress, 1996) call for the EPA to "[d]evelop new approaches to the study of complex mixtures, such as mixtures found in drinking water, especially to determine the prospects for synergistic or antagonistic interactions that may affect the shape of the dose-response relationship of the individual chemicals and microbes, and to examine non-cancer endpoints and infectious diseases, and susceptible individuals and subpopulations." It is also important to determine if the amount of human health protection will be increased after an intensive look at chemical mixtures, especially when the chemicals themselves are below their no-effects concentration (Teuschler, 2007).

Additivity concepts are often used as default procedures to estimate risk or hazard from exposure to a simple mixture using data on a defined number of components under the assumption that doses are in a region where toxicological interactions are not expected to occur (Teuschler, 2007). When similar modes of action is the case, then a risk assessment based on dose addition can be used where the doses of the chemical components are scaled for relative toxicity and summed for use in estimating risk (e.g., the Hazard Index (HI)) (U.S. EPA, 2000; Teuschler, 2007). However, these simple models based on dose additivity may not hold for all toxic effects, and the relative toxic potency between chemicals may differ for

different types of toxicity or toxicity by different routes (U.S. EPA, 2000). Another problem based on dose addition is the assumption of similarly shaped dose-response curves for mixture components. However, there is evidence that even with chemicals having dissimilar modes of action, additive or near-additive interactions are common (U.S. EPA, 1998; Konemann, 1981; Hermens et al., 1984).

Toxicological interactions are complex processes that are complicated by the dose dependence of interactions, myriad chemical combinations and dose levels, and lack of data beyond experiments on binary mixtures (Teuschler, 2007). Toxicity of chemical mixtures is a concern, and more toxicological data on real world mixtures at environmentally relevant concentrations (low µg/L or ng/L) needs to be further investigated. Because there are limited published studies on chemical mixtures of antibiotics, the simple additivity approach is used to estimate the combined potential environmental hazard of the targeted analytes in this case study.

1.8 Estimating Concentrations of Antibiotics in Surface Waters with BME

A Bayesian Maximum Entropy (BME) framework with modern spatiotemporal geostatistics can be used to process information about the space/time variability of pollutants in their aquatic environment, the uncertainty and lack of monitoring data, and governing flow and transport laws in order to obtain statistically estimated concentrations of pollutants at locations within a watershed that were not sampled (Serre et al., 2004). BME consists of techniques to conduct non-linear spatial predictions of data. Variability of antibiotic concentrations in surface water over space and time is expected to be high due to daily and seasonal fluctuations in usage and wastewater discharge flow. In this instance, the BME framework is used to estimate concentrations of antibiotics in a watershed where a minimum

number of samples have been taken due to budgetary and resource constraints as well as site accessibility.

The models are useful in that they can predict analyte concentrations in areas and on days when samples were not taken. This is useful for areas that are not accessible and for budgetary constraints because less samples could be collected. However, some weaknesses should be noted. First, the model predicts concentrations over a specified area and it does not distinguish between land, air, or water so the reader must consider this when interpreting the data. Second, there can be a large amount of variance associated with the expected concentrations due to a lack of sampling points in the study area or a lack of sampling events, etc. Third, weather events are not taken into account when predicting concentrations, so if sampling data were all collected during a period of heavy rainfall, the estimations would probably differ from estimations of a dry spell, etc. Fourth, the estimations are subject to how the modeler manipulated the model to generate the estimations, and may differ from person to person. Despite these limitations, it is a useful tool that shows general trends over a whole study area during a specific time and can be useful in watershed management.

1.9 Policy and Regulatory Implications

The presence of pharmaceuticals in surface waters indicates problems with contemporary healthcare and wastewater treatment processes. From the standpoint of regulation, prescription drug disposal is one of the most important points of entry for pharmaceuticals into the environment because it is somewhat controllable with behavior modifications. The percentage of drugs that reach surface waters via disposal is somewhat high in the U.S. (35.4% in U.S. vs. 11.4% in EU (Bound and Voulvoulis, 2004), and regulating the disposal of pharmaceuticals is likely the simplest and least costly place to

begin reducing the presence of pharmaceuticals in surface waters. Community education and take-back programs are also relatively easy to implement at a low cost (Ph:ARM Team, 2007).

In the United States, regulation of pharmaceutical waste and disposal is managed at different stages by various government agencies with distinctive agendas (FDA, 1998; Erikson et al., 2005). Regulations by these agencies are often overlapping or poorly defined and may not be enforced. Disposal by end-users is currently not regulated by any agency; there are only suggestions for disposal that are not widely advertised. The FDA, EPA and DEA are the three government agencies that have the most authority to regulate the disposal of pharmaceuticals.

1.9.1 The FDA

The FDA regulates the safety of medicinal compounds for human use and, therefore, has the potential to take the lead in addressing the issue of their presence in surface waters. As mentioned earlier, NEPA specifies that before the FDA can approve any new drugs, an EA of the drug must be completed. However, the FDA takes a more lenient approach than would be assumed based on NEPA, as the agency categorically excludes the majority of actions regarding drugs from the requirement of an EA (Erikson et al., 2005; Vincent, 1993). A categorical exclusion is granted by the FDA for a "category of actions which do not individually or cumulatively have a significant effect on the human environment and which have been found to have no such effect" (CFR, 2006). Instead of strengthening its requirements, the FDA has been making categorical exclusions easier to obtain.

In the 1990s, the FDA established "additional categorical exclusions" and "reevaluated and revised its environmental regulations to reduce the number of EAs required to be submitted by industry" (DHHS, 1998). The FDA only requires that pharmaceutical companies perform an environmental assessment of a new product if their stated anticipated production of the drug is more than 40,000 kilograms per year, ignoring the possibility that multiple companies might all be making the same drug (Thacker, 2005).

1.9.2 The EPA

There are no EPA programs that specifically regulate the presence of pharmaceuticals in ground or surface waters, and it is unclear if there will be any in the near future due to the complexity of accurately quantifying potential environmental and human health risks. The presence and potential effects of pharmaceutical compounds may be addressed under EPA programs such as the Safe Drinking Water Act (SDWA). This act requires EPA's Office of Water to set maximum contaminant levels in public drinking water based on the best available peer-reviewed literature and the protection of the "at risk" portion of the population. There are currently no existing regulations for pharmaceuticals (Conerly, 2005). However, specific pharmaceuticals could be included in the Contaminant Candidate List (CCL) under the SDWA, which identifies and lists contaminants unregulated by existing regulations "known or anticipated to occur in public water systems" (U.S. EPA, 2007a). The SDWA and other regulations may become important in situations of groundwater recharge or the intentional reuse of wastewater. Though not intended to address the safety of wastewater reuse, the SDWA and the National Primary Drinking Water Regulations (NPDWR) have

served as starting points for developing water quality standards for reclaimed wastewater (CDM, 2004).

Criteria for pharmaceuticals as toxins could be also developed under the Clean Water Act (CWA) if adequate supporting data becomes available (Conerly, 2005). Other CWA approaches that could help control the presence of pharmaceuticals in ambient waters include the Effluent Guidelines program for the regulation of point sources (e.g., the pharmaceutical manufacturing industry and the aquaculture industry), the Combined Animal feeding Operations Rule, and the Fish Advisory Program (Conerly, 2005). The EPA requires more research before any decision can be made as to which individual types of pharmaceuticals (if any) might necessitate further attention in surface and drinking waters (Daughton, 2000).

The EPA also has the ability to regulate pharmaceutical compounds in the solid waste stream. Pharmaceutical waste generated by end-users, households and certain small, non-household generators are known as Conditionally Exempt Small Quantity Generators (CESQGs), and are not regulated as hazardous waste (DTSC, 2003). Some pharmaceutical waste is classified as hazardous waste under the Resource Conservation and Recovery Act (RCRA) and the Code of Federal Regulations (CFR) (40 CFR Part 261) enforced by the EPA and authorized states. Hazardous waste management involves specific management practices including permits, special transportation manifests, and specific bans against land disposal without treatment. Hospitals, nursing homes, pharmacies, and reverse distributors are required to follow guidelines regarding the destruction of drugs that are considered hazardous waste, but many of these institutions are either unaware of their RCRA obligations or choose to ignore them (Oliver and Chapman, 2003). RCRA regulations have not been significantly updated since 1976 and have not kept up with drug development (Smith, 2005). Compliance

with RCRA regulations has proven difficult due to the difficulties of implementation and enforcement within a health care setting, as well as a lack of interpretive guidance from the EPA (Smith, 2005). Some wastes that are not regulated as hazardous under RCRA are identified as hazardous in states with stricter regulations, such as California.

A pharmaceutical can be considered a hazardous waste if it is listed as a hazardous or characteristic waste as per the CFR Title 40 Part 261 Subpart D. A characteristic waste meets the characteristics of ignitability, corrosivity, reactivity, or toxicity. A number of common drugs meet the definition of hazardous waste, including epinephrine, nitroglycerin, nicotine, and many chemotherapy agents (Smith, 2002). Only 5% of pharmaceutical products on the market would be regulated as RCRA hazardous waste if discarded by an entity other than a CESQG (Smith, 2005).

Many consumers and businesses are not aware of the CFR regulations and have not been informed of how to properly dispose of their unused or expired drugs. The Office of National Drug Control and Policy currently suggests mixing unused or expired drugs with an undesirable substance such as coffee grounds or cat litter, placing them in a sealable, nondescript container and disposing of them in the garbage as opposed to flushing the drugs down the drain. However, the FDA still suggests that several highly toxic compounds be flushed due to their addictive properties (ONDCP, 2006) and these include;

- Actiq (fentanyl citrate)
- Daytrana Transdermal Patch (methylphenidate)
- Duragesic Transdermal System (fentanyl)
- OxyContin Tablets (oxycodone)
- Avinza Capsules (morphine sulfate)

- Baraclude Tablets (entecavir)
- Reyataz Capsules (atazanavir sulfate)
- Tequin Tablets (gatifloxacin)
- Zerit for Oral Solution (stavudine)
- Meperidine HCl Tablets
- Percocet (Oxycodone and Acetaminophen)
- Xyrem (Sodium Oxybate)
- Fentora (fentanyl buccal tablet)

1.9.3 The DEA

The DEA limits its regulation of pharmaceuticals to those that are "controlled substances" or their precursors. Controlled substances include legal and illegal drugs meeting certain guidelines regarding potential for abuse, accepted medicinal use, and safety (FDA, 2002). The DEA maintains a yearly registration program of individuals, known as DEA registrants, within organizations or institutions that are legally able to handle controlled substances in specific capacities (ODC, 2007b). DEA registrants include individuals that fall into, or are employed in one of the DEA approved categories: pharmacy, hospital, clinic, practitioner, teaching institution, mid-level practitioner, manufacturer, distributor, researcher, analytical laboratory, importer, exporter, domestic chemicals, and narcotic treatment programs (ODC, 2007b). Disposal of controlled substances by DEA registrants is carefully regulated to ensure that the substance is rendered destroyed or unrecoverable (i.e. incineration). The agency accepts several methods of disposal, including flushing them down the drain, as viable means of destruction for controlled substances (RDWG, 2003;

FDA, 2002). The DEA forbids the return of controlled substances from the end-user to any DEA registrant, or transfer to anyone, except in certain cases (i.e. a law-enforcement agent) (ODC, 2007a). The agency provides no guidance or recommendations regarding disposal at the level of the end-user or patient (ODC, 2007a).

Other countries have also implemented take-back programs, such as Australia (Return Unwanted Medicines www.returnmed.com.au project initiated in July 1998), British Columbia, Canada (The Medications Return Program www.medicationsreturn.ca started in November 1996) as well as Prince Edward Island and Ottawa, both in Canada, Sweden, and 11 European countries (Belgium, France, Luxembourg, Portugal, Spain, Denmark, Finland, Germany, Italy, Sweden, and the U.K.). These programs range from a small-scale involving only a few territories to countrywide pharmaceutical company involvement (Ph:ARM Team, 2007).

1.9.4 Previous Surveys and Findings

Institutional surveys

Several institutional surveys have been conducted across the U.S. and other countries in order to determine the details of the pharmaceutical waste stream to help devise a solution (Table 1.8). Kuspis & Krenzelok (1996) examined the disposal methods of 100 community-based pharmacies around Pennsylvania. They found that pharmacies sent all non-distributed, unopened, expired medications back to pharmaceutical companies for a return credit. Of the remainder medications, 15% of pharmacists prefer on-site incineration, 17% preferred disposal by a biohazard waste company, and 68% dispose of medications by placing them in the garbage or flushing them down the sink or toilet.

A pharmacy-based survey by Braybrook et al. (1999) in the United Kingdom collected information from 529 people at 18 pharmacies over eight weeks in order to characterize the reasons for medication return. Items were most commonly returned because the medication was stopped or switched (42%), followed by an excess supply or just cleaning out the closet (20%), patient death (16%), and medication stopped by the patient (14%). If this survey were representative of the entire Health Authority in Britain, as much as £800,000 a year (or 1.5% of the annual prescribing budget) would be wasted (Braybrook et al., 1999).

A survey completed in King County, WA (which includes Seattle) gathered data on the quantity and nature of pharmaceutical waste streams and drug waste management practices from a variety of businesses including doctor's offices, specialty and outpatient facilities, veterinary offices, ambulatory/surgical centers, hospitals, pharmacies, and nursing/boarding homes (Oliver and Chapman, 2003). The study found that a reverse distributor is the most common disposal route (6,500 pills), while flushing pills down the drain is the second most common (6,188 pills). Twenty-seven national reverse distributors were contacted via telephone to identify types of services offered, acceptance policies, and other general information about the industry (Chapman, 2003). Of the 23 reverse distributors that provide services to King County, most only provide mail-in service and all accept controlled substances and prescription drugs. Household drugs were typically accepted only under certain conditions, such as the drug was returned through the pharmacy that dispensed it; the drug was not a controlled substance; patient health information subject to privacy laws was protected; and the reverse distributor held the contract as a "returns department" for the manufacturer of the returned drug.

Boivin (1997) conducted an 85-bed nursing home study in Ontario to calculate the costs of medication waste for the period of October 17 to November 20, 1996. Nursing homes are required to keep appropriate records of surplus prescribed drugs, and from this Boivin calculated that 13.14% of dispensed medication was wasted. The study also divided medication waste data into nine different categories. Topical agents were the most expensive class of medications returned (27.17% of total cost of returned medication), followed by respiratory (26.09%), and then neurologic and endocrinologic (17.65%) medications.

Table 1.8: Summary of institutional surveys

Survey	Type of Survey	Location	Information Collected
Kuspis and Krenzelok, 1996	100 community and hospital pharmacies	Tri-state region (PA, NY, NJ)	Disposal methods.
Boivin, 1997	85-bed nursing home	Ontario, Canada	Cost of wasted medications.
Braybrook et al., 1999	Returns by 529 people at 18 pharmacies	United Kingdom	Reasons for return.
Chapman, 2003	27 reverse distributors	National	Identify services offered, acceptance policies, and other general information about the industry.
Oliver and Chapman, 2003	60 businesses	King County, WA	Quantity and nature of pharmaceutical waste streams and drug waste management practices.
Crisostomo et al., 2006	Community-based pharmacy study of 572 patients	Portugal	Quantity and cost of wasted medications.
Hauser, 2006	51 patients at one hospice	Chicago, IL	Type, quantity, and cost of wasted medications.

Hauser (2006) examined the type, quantity, and value of wasted medications at one hospice where 51 patients died during the study period, and found that a total of 4,762 milliliters (mL), 2,495.5 tablets, and 67 patches were disposed of, averaging to 9.7 drugs per patient. The estimated cost of these medications was $5,558.75 if purchased as generics and $10,535.85 if purchased as brand names, or an average of $109.00 or $206.59 per patient, respectively. These studies have shown that large quantities of money are wasted along with the actual unused or expired drugs.

Consumer Surveys

Only a limited number of end-user surveys on pharmaceutical disposal have been completed, but collected information includes patient disposal practices and reasons for disposal, beliefs, waste quantities and costs, number of medicine containers, and storage times (Table 1.9). Kuspis and Krenzelok (1996), Morgan (2001), Seehusen and Edwards (2006), and the BAPPG (2006) are surveys taken at several convenient locations, such as a poison control center, retirement community, army medical center, and pharmaceutical collection event, respectively. Bound and Voulvoulis (2005) and the Washington Citizens for Resource Conservation (WCRC) (2006) are the most rigorous surveys to date; both are randomly sampled phone surveys.

Four surveys show that trash is the most common disposal practice, and two surveys found that the sink or toilet is the most common practice. It must be noted that the method of data collection was not uniform in these studies (different disposal categories; some allow more than one answer for disposal method while others do not; etc.). The WCRC survey also compared the disposal practices for various demographic groups. Interestingly, younger residents (aged 18 to 54 years) are more likely to dispose of unused or expired medicines in

the trash, while residents aged 55 or older are more likely to use the sink or toilet. The WCRC survey (2006) is the only random sample survey that was conducted in the United States, and it is unclear whether its results apply uniformly to the rest of the nation.

Table 1.9: Summary of routes of disposal as determined by surveys in percent

Disposal Method	Kuspis and Krenzelok, 1996	Boivin, 1997	Bound and Voulvoulis, 2005	BAPPG, 2006	Seehusen and Edwards, 2006	WCRC, 2006
Trash	54	31	63.2	45.2	--	52
Sink or toilet	35.4	46 (toilet)	11.5	28	35.2 (sink); 53.8 (toilet)	20
Pharmacy	1.4	17	21.8	--	22.9	2
Doctor	--	--	--	--	--	1
Return to health care provider	--	--	--	--	14	--
Physician	--	2	--	--	--	--
Store at home	--	--	--	--	54.2	--
HHW event	--	--	--	16.1	--	--
Gave away	--	--	--	2.1	11	--
Other	--	4	3.5	16.1	--	1
Does not apply	9.2	--	--	--	--	23

Note: all units are in %; "- -" means question was not investigated in survey**.**

Another method of disposal is not really what it implies. Drug recycling, or drug donation is a new concept that is currently being tested in two states (California and Oklahoma). Drug recycling entails collecting unused pharmaceuticals from nursing homes, wholesalers, and manufacturers in order to redistribute them to medically indigent patients. This type of program has the potential to minimize the amount of wasted drugs and also help those that cannot afford their medications and would go without treatment due to the cost. Drug recycling is not intended to take advantage of medically indigent patients in any way.

One issue related to drug recycling is expiration dates. Questions arise over whether or not a drug that is past its expiration date can still be recycled. Expiration dates on

pharmaceuticals were required by law in 1979 to determine a date where the manufacturer guarantees the full potency and safety of the drug (Altschuler, 2002). An expiration date does not mean how long or how safe the drugs actually are. In fact, many drugs still have the same potency 10 to 15 years from their expiration date, and many medical authorities state that it is generally safe to take them no matter how expired they are (Altschuler, 2002). In some respects, an expiration date is purely a marketing ploy; if a bottle of aspirin lasted 10 years, a consumer would be less likely to buy more. A study commissioned by the U.S. Army about 20 years ago confirms these results. The FDA analyzed over 100 prescription and over-the-counter drugs and determined that 90% of the drugs had shelf lives of up to 15 years past the expiration date (Altschuler, 2002). Joel Davis, a former FDA expiration-date compliance chief noted that there are a few exceptions to this rule, most notably: nitroglycerin, insulin, and some liquid antibiotics. Storing drugs and antibiotics in a refrigerator can drastically increase their shelf lives as well. Therefore, consuming recycled, expired drugs will usually not lead to harmful health effects and may be more beneficial than not taking needed medications at all.

Senator Joe Simitian of California sponsored Senate Bill 798 that allows for drug recycling. Under the Bill's stipulations, the medication cannot be a controlled substance and cannot have been in the possession of a patient or any individual member of the public. Patient confidentiality must be maintained, the drugs must be unexpired, unopened, and in tamper-proof packaging; persons and entities accepting, disposing, and dispensing of pharmaceuticals must also be protected against liability.

Not surprisingly, there are a number of concerns surrounding drug recycling that need to be addressed before it is implemented on a full-scale basis, but it is a viable option to reduce the waste stream reaching surface waters, and to also help those in need.

There is no clear solution for the development of regulations or the proper disposal of pharmaceuticals by end-users. Disposal guidelines, such as those recently released by the White House Office of National Drug Control Policy (ONDCP) should be more widely advertised within local communities in order to reduce the presence of pharmaceuticals in surface waters. Policy options to address pharmaceutical disposal include permanent collection at household hazardous waste facilities or other locations such as pharmacies; a mail-back program; special collection events; or drug recycling. Controlled substance regulations present a barrier to implementing any of these programs as only law enforcement officials may accept controlled substances from end-users.

1.10 Research Objectives

The presence of antibiotics, other pharmaceuticals, and endocrine disrupting compounds are increasingly detected in surface waters due to incomplete removal by wastewater treatment plants and nonpoint sources. The scope of this research project is to:

- Determine the aqueous concentration and persistence of antibiotics in wastewater and receiving waters in a watershed that includes a drinking water source and recreational area.

  Estimate the concentration of one antibiotics in the watershed at locations where samples were not collected using the BMEGUI geostatistical software,

and to also determine any spatial and/or temporal variations during the study period.

- To complete an environmental hazard assessment of detected antibiotics.
- To investigate possible policy implications in order to reduce the presence of pharmaceuticals in surface waters.

2. MATERIALS AND METHODS

2.1 Materials

Antibiotic standards purchased from Sigma-Aldrich (St. Louis, MO) include: sulfathiazole, sulfamerazine, sulfamethazine, sulfamethizole, sulfachlorpyridazine, sulfamethoxazole, sulfadimethoxine, erythromycin, tylosin, roxithromycin, trimethoprim, lincomycin, norfloxacin, sarafloxain, flumequine, levofloxacin, pipemidic acid, oxolinic acid, minocycline, oxytetracycline, tetracycline, demeclocycline, chlortetracycline, doxycycline; all were 99% pure. Isotopically labeled standards ($^{13}C_6$-sulfamethoxazole, $^{13}C_3$-ciprofloxacin, and $^{13}C_2$-erythromycin) were purchased from Cambridge Isotope Laboratories (Andover, MA). The internal standard, simatone, was purchased from Accustandards (New Haven, CT). Erythromycin-H_2O was prepared at the University of North Carolina laboratory with the erythromycin standard and the method described by McArdell et al. (2003) and Ye, 2005). Ciprofloxacin was purchased from ICN Biochemicals (Irvine, CA) and Enrofloxacin was purchased from Fluka (Bachs, Switzerland).

HPLC-grade methanol, acetonitrile, and formic acid were purchased from Fisher Scientific (Pittsburgh, PA). Disodium ethylenediamine tetraacetic acid (Na_2EDTA) and sodium azide were purchased from Aldrich (Milwaukee, WI). Laboratory grade water (LGW) was prepared in the University of North Carolina laboratory using a water purification system (Pure Water Solutions, Hillsborough, NC). The system filters chloraminated tap water to 1 µm, removes residual disinfectants, reduces total organic carbon

to less than 0.2 ppm with an activated carbon resin, and reduces conductivity to 18 Mohm with mixed bed ion-exchange resins.

2.2 Cleaning Procedures

All non-volumetric glassware was detergent washed in a dishwasher, and then soaked in a 10% nitric acid bath. The glassware was removed and rinsed three times with LGW and then placed in a 110°C oven overnight or until thoroughly dry (about 4 hours). Volumetric glassware (pipettes, volumetric flasks, and graduated cylinders) were manually detergent washed with tap water, rinsed three times with LGW, then MeOH, and allowed to dry on a clean Kimwipe placed in a clean plastic bucket that was placed in a safety hood. Caps and septa were soaked in a clean beaker filled with a mixture of LGW and detergent for an hour or more, rinsed with LGW, MeOH, and then dried on a clean Kimwipe.

Tubing used for solid phase extraction (SPE) was rinsed with LGW followed by MeOH promptly after use. This was done by connecting the tubing to plastic cartridges (containing no sorbent) to a vacuum manifold. With the vacuum on, LGW was aspirated through each of the tubes for 30-60 seconds, followed by MeOH. The ends of the tubing were then rinsed with LGW. The tubes were then allowed to vacuum dry before being placed back in storage for later use.

2.3 Stock Solution Preparation

Stock solutions were prepared at 1 g/L concentrations following details listed in Appendix A. Antibiotic, surrogate, and internal standard solutions were stored in a freezer at approximately -15ºC and were typically held for no longer than three months (tetracyclines, quinolones, macrolides, and lincomycin) or one year (sulfonamides and trimethoprim).

Working mixtures of the 26 antibiotics were freshly prepared within two days of each sampling event. Fresh working solutions were prepared prior to a sampling event.

2.4 Sample Collection

Grab samples were collected from wastewater treatment plant effluent at the point where the effluent discharges from the WWTP and mixes with stream water, and at various downstream locations during each sampling event. Local utilities were contacted for site access permission when needed and to coordinate sampling events. It was agreed that local utilities would remain anonymous for publication and communication outside of involved researchers and collaborators. One upstream sampling point, the point of entry of wastewater effluent, and two downstream sampling locations were identified on each stream. Various locations throughout the receiving reservoir were sampled during the final sampling event, while only one location in the reservoir was sampled more frequently. This sampling point is located at an influent pipe for a drinking water treatment plant. The distance from the influent pipe varied from 9 – 11 miles from the second downstream sampling point in each of the streams. Sampling in the reservoir was conducted by a local utility due to the location of the pipe. Criteria for identifying sampling locations were mainly based on the practicality and accessibility of the location as all three streams flowed through a forested, intermittently swampy, area downstream from the WWTP point of discharge. Upstream locations ranged from 400 feet to 2 miles from the WWTPs; distance between the downstream sampling points varied (Figure 2.1). Samples from each stream were collected on the same day, but it was not possible to collect samples for all three streams in one day. However, samples from all three streams and the reservoir were collected within 1-3 days of each other in the direction of flow.

Normal or worst-case (drought) conditions were desired for sampling events. Weather conditions such as previous rainfall were considered when planning a sampling event. Normal stream flow was determined by investigating stream flow and stream gage data for each stream using the U.S. Geological Survey (USGS) real-time stream data. This was possible because a USGS monitoring station was located at or near a downstream location on each stream of the three streams involved in the study. The study was conducted during times of extreme drought so dilution via rainfall was a negligible issue.

Between 2 and 4 liters were collected at each sampling location depending on the type of analyses performed. Two liters were needed for the antibiotic analysis and one liter for the yeast estrogen screen (YES) bioassay. These volumes ensured a sufficient amount of sample in order to perform the analyses and determine analyte concentrations using the method of standard addition. All water samples were either collected in pre-cleaned 1-L amber bottles (Laboratory Supply Distributors, Mt. Laurel, NJ) and capped with screw caps containing removable Teflon-lined septa, or pre-cleaned 4-L amber bottles that previously contained HPLC MeOH (Fisher Scientific, Pittsburgh, PA). Sodium azide, a preservative used to inhibit bacterial growth, was placed in the amber bottles at 100 µg/L. The sodium azide solution was prepared by measuring out the appropriate mass and dissolving it in LGW. The solution was stored in a refrigerator at 4°C for up to 6 months. After samples were collected, they were placed inside a cooler stocked with frozen ice packs to keep them stable during transport. Upon arrival at the University of North Carolina laboratory, samples were immediately placed in a dark 4°C refrigerator until analysis. Aqueous samples were processed within 24-36 hours.

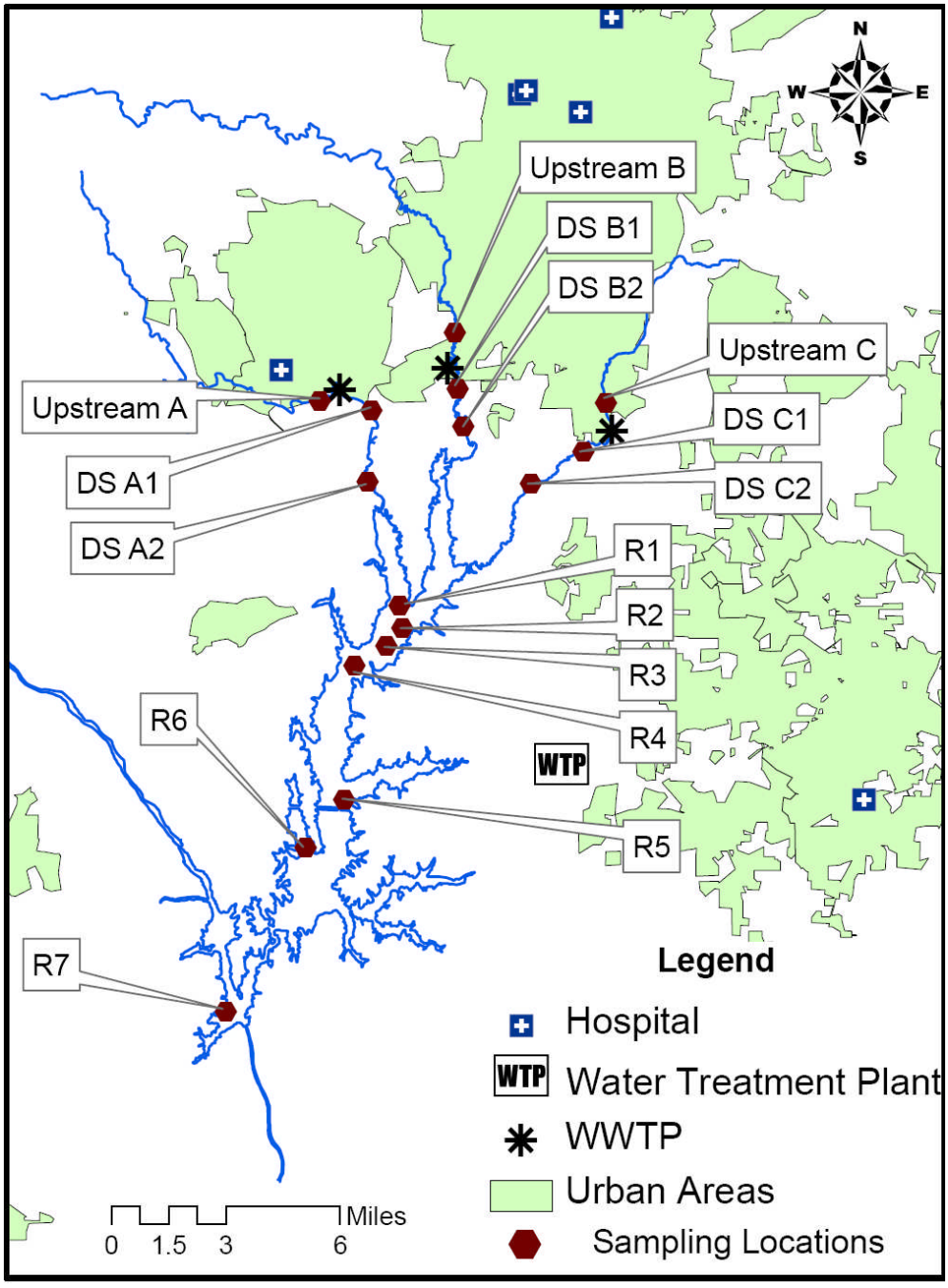


Figure 2.1: Sampling locations within watershed

2.5 Sample Preparation

2.5.1 Sample Preparation for Antibiotic Analysis

Sample preparation for the antibiotic analysis was adopted from previous work in our laboratory (Richards, 2007; Ye, 2005). Samples were filtered first with 0.7 µm glass microfiber filters (Fisher Scientific, Hampton, NH), and then followed by 44 mm, 0.45 µm nylon filters (GE Water & Process Technologies, Trevose, PA) into 1000 mL Buchner flasks. The filtrate was then divided into six 250 mL amber bottles using a 250 mL volumetric flask for standard addition. The remaining sample was then stored in a dark 4°C refrigerator for backup analysis if necessary. The 250 mL filtrates were spiked with 25 µL of a mixture containing each of the surrogate standards at a concentration of 0.5 mg/L to obtain an individual surrogate concentration of 50 ng/L. The filtrates were then capped and the bottle inverted three times to mix the contents. Four of the six 250 mL bottles were spiked with an increasing concentration of the working mixture of the 26 antibiotics typically at 20, 100, 500, and 1000 ng/L which captures the range of expected concentrations in the unspiked samples. The antibiotic and surrogate standard mixtures were spiked into the filtrate using micropipettes and disposable glass capillaries that ranged from 10 to 250 µL. Two of the 250 mL bottles were left unspiked. All six 250 mL amber bottles were then spiked with a solution of 25 µL of 1.25 mg/L Na_2EDTA in LGW to prevent complexation of target analytes with any metals in the sample. The filtrate was then pH adjusted to between 5.8 and 6 using 2% formic acid in LGW that is prepared weekly.

The samples were then extracted using 200 mg Strata X polymeric reversed phase extraction cartridges (Phenomenex, Torrance, CA). First, the cartridges were conditioned

using 6 mL HPLC-grade MeOH, 3 mL of 0.1% formic acid in MeOH (volume/volume), and two aliquots each of 6 mL LGW. The samples were then extracted through the cartridges at a maximum flow rate of 5 mL/min using a 12- or 24-fold vacuum manifold (Fisher Scientific, Pittsburgh, PA). After extraction but before the cartridges dried out, the cartridges were rinsed with 5 mL of LGW, and then dried under a vacuum pressure of 15-18" Hg for approximately 10-15 minutes.

At this point the analytes of interest are retained on the solid phase of the cartridges. They are eluted into 10 or 13 mL conical vials using 2 x 4 mL of 2% formic acid in MeOH. The extract was either stored in a freezer for later analysis or blown-down immediately to 50 µL with a gentle stream of nitrogen gas at 99.999% purity. The 50 µL volume was measured by visually comparing the volume with that to another vial injected with 50 µL of LGW. The vials were covered with aluminum foil to prevent photodegradation while in the blow-down apparatus. After concentrating the eluent to 50 µL, it was reconstituted to 250 µL with 9:1 LGW:MeOH (volume/volume) using a 500 µL syringe. 10 µL of a 1.25 mg/L concentration of simatone was spiked under the surface of the reconstituted extract using a 10 µL syringe. The conical vials are then capped with Teflon-lined screw-on caps, briefly vortexed (Thermolyine Maxi-Mix, Dubuque, IA), and then allowed to settle for 15 minutes while covered with aluminum foil.

After settling, the extract is removed from each vial with a 500 µL syringe, the volume is measured and recorded to later account for any uncertainties about the data, and is filtered into labeled 2 mL autosampler vials containing 250 µL glass inserts (Laboratory Supply Distributors, Mt. Laurel, NJ) with 4 mm, 0.45 µL PTFE syringe filters (Laboratory Supply Distributors, Mt. Laurel, NJ). The autosampler vials were then capped with

polypropylene screw thread closures containing polytetrafluoroethylene septa (Laboratory Supply Distributors, Mt. Laurel, NJ). The extracts were stored in a -20°C freezer until analysis on a Varian 1200L liquid chromatography tandem mass spectrometer (Varian, Walnut Creek, CA) with a maximum holding time of 7 days.

2.5.2 Sample Preparation for Yeast Estrogen Screen Bioassay

The recombinant yeast estrogen screen (YES) assay is one of many used to measure the potential estrogenicity of pollutants in surface and wastewaters. The YES assay was developed by incorporating the human estrogen receptor (hER) gene into the main chromosome of a yeast strain (Routledge and Sumpter, 1996). The hER gene provides estrogen receptors that, when activated, cause production of the enzyme β-galactosidase. The β-galactosidase can then react with chlorophenol-red-β-D-galactopyranoside (CPRG), a chromogenic substrate added to the growth medium. This causes a color change that is measured through optical absorbance to determine the amount of enzyme activity and consequently, the amount of estrogenic activity in wastewater effluent and downstream surface waters (Routledge and Sumpter, 1996).

500 mL of each sample was filtered through 1.5 μm glass filters and placed in a 500 mL amber bottle (Laboratory Supply Distributors, Mt. Laurel, NJ). Strata-X 200 mg RP cartridges were conditioned with 3 mL MtBE, 3 mL MeOH, and 6 mL LGW. Samples were run through the cartridges at a flow rate not exceeding 8 mL/min. The cartridges were washed with 3 mL 40% MeOH in LGW, 3 mL LGW, and 3 mL 10% MeOH and 2% NH_4OH. The cartridges were dried for 1 hour under maximum vacuum pressure (18-20" Hg), and then eluted with 5 mL MtBE:MeOH (9:1) into 10 mL conical vials (Laboratory

Supply Distributors). Extracts were blown down to dryness with nitrogen gas and reconstituted to 200 μL with EtOH. Samples were then taken through the YES assay.

The YES assay protocol was modified from Routledge and Sumpter (1996). All solutions used for the YES assay were prepared in sterilized bottles, stored at 4°C, and filter-sterilized before use with a Uniflow-25 disposable 0.25 μm syringe filter. Solutions were discarded in the event of visible turbidity. The solutions prepared were as follows:

- 20% w/v solution of D-(1)-glucose
- 4 mg/mL solution of L-aspartic acid
- 24 mg/mL L-threonine
- 20 mM copper (II) sulfate solution
- 10 mg/mL stock solution of chlorophenol red-ß-D-galactopyranoside (CPRG)
- 0.8 mg/mL solution of $Fe_2(SO_4)_3$
- 0.02 mg/mL solution of biotin
- 25 mg/mL solution of chloramphenicol

The minimal medium was created by adding the following components into 1 L of LGW. The medium was autoclaved at 121˚C for at least 15 minutes and stored at room temperature.

- 13.61 g KH_2PO_4
- 1.98 g $(NH_4)_2SO_4$
- 4.2 g KOH
- 0.2 g $MgSO_4$
- 1 mL $Fe_2(SO_4)_3$ solution (1 g/L)
- 50 mg L-leucine
- 50 mg L-histidine
- 50 mg adenine
- 20 mg L-arginine-HCl

- 20 mg L-methionine
- 30 mg L-tyrosine
- 30 mg L-isoleucine
- 30 mg L-lysine-HCl
- 25 mg L-phenylalanine
- 100 mg L-glutamic acid
- 150 mg L-valine
- 375 mg L-serine

The vitamin solution was created by adding the following components into 180 mL of filter-sterilized LGW. The solution was stored at 4˚C.

- 8 mg thiamine
- 8 mg pyridoxine
- 8 mg pantothenic acid
- 40 mg insitol
- 20 mL of biotin solution

Each time the yeast was inoculated, a new yeast plate was streaked on an agar plate made in the laboratory. The following components were added to 1 L of LGW, autoclaved at 121˚C for at least 15 minutes, poured into sterile plates in 15 mL aliquots, and stored in Ziploc bags at 4˚C.

- 60 g Sabourad's Dextrose Agar (Difco)
- 3 mL chloramphenicol solution (available as 25 mg/mL)

2.5.3 YES Assay Protocol

Minimal medium (45 mL aliquots) was added to a sidearm flask and several culture flasks and capped. The number of culture flasks depends upon the number of samples, but

generally one culture flask can be used for about 3 96-well plates, and one culture flask was used as a control. Both aliquots were autoclaved at 121˚C for at least 15 minutes. A growth medium was also added to the minimal medium on the day of inoculation. The following components were added to an autoclaved 50-mL beaker and filter-sterilized using a 60-mL sterile disposable syringe and then added to the autoclaved minimal medium in the sidearm and culture flasks:

- 5 mL glucose solution
- 1.25 mL L-aspartic acid solution
- 0.5 mL vitamin solution
- 0.4 mL L-threonine solution
- 125 µl copper (II) sulfate solution

The plates were inoculated using a culture of the yeast strain by selecting a single colony from the agar plate and transferring into the growth medium using a sterilized metal microbial loop. The inoculated culture was grown in a 30˚C temperature-controlled room on a shaker table at 100 rpm until it reached an absorbance of 1.0, read at 640 nanometers in a Molecular Devices E*max* Precision Microplate Reader, Model 383109. Assay plates were prepared by adding 20-µL aliquots of the extracted samples, a 38 µg/L estradiol standard, and a negative control (growth medium only) in the first well and then serially diluted in a 96 well plate. 20 µL of ethanol was added to each well in the well plate except for the last column of wells. 20 µL of sample was transferred from each column and mixed with the next column to create 50% dilutions along the columns. The solutions in the wells were then allowed to completely evaporate in a biosafety hood.

1 mL of 10 mg/mL sterile CPRG solution was added to the sidearm flask. A small volume (250 µL) of this solution was added to each well in the 96-well plate that was

previously plated with standards and samples. The plate was then covered with Parafilm® and placed in the 30˚C room. The plate was read after 72 hours of inoculation using a Molecular Devices E*max* Precision Microplate Reader, Model 383109. The standard operating procedure for the YES assay analysis can be found in Appendix C.

2.6 Liquid Chromatography Tandem Mass Spectrometry Methods (LC/MS-MS)

The samples prepared for antibiotic analysis were analyzed with a liquid chromatography triple quad tandem mass spectrometer (LC/MS-MS) (Varian 1200L, Varian Inc., Walnut Creek, CA) with a dual off-axis electrospray ionization (ESI) interface that was connected to a solvent delivery module (ProStar 210, Varian Inc., Walnut Creek, CA) and autosampler (ProStar 430, Varian Inc., Walnut Creek, CA).

Three methods were designed for the chromatographic separation for a subset of the 26 antibiotics in each class (Ye, 2005). 20 µL of the extract was injected three times when analyzing for all 26 antibiotics. A Varian Pursuit C-18 guard column (3 cm x 2 mm, 3 µm) attached to a Varian Pursuit C-18 analytical column (15 cm x 2 mm, 3 µm) were used in the separation of the antibiotics. The mobile phase was A: 0.1% formic acid in LGW and B: HPLC-grade acetonitrile. The mobile phases were freshly prepared before analysis and filtered through 0.2 µm nylon fiber filters (Millipore Billerica, MA). The flow rate remained constant and 0.2 µL/min while the gradient varied throughout the chromatographic run. The methods were labeled as “SAMA”, “QL”, and “TC”. The “SAMA” method was used to separate the sulfonamide, macrolides, lincomycin, trimethoprim, surrogate standards $^{13}C_6$-sulfamethoxazole and $^{13}C_2$-erythromycin, and the internal standard simatone. The “QL”

method separated the quinolones, the surrogate standard $^{13}C_3$-ciprofloxacin, and simatone, and the "TC" method separated the tetracyclines, the surrogate standard meclocycline, and simatone (Table 2.1). The gradient for each method is presented in Table 2.2.

Table 2.1: Antibiotics within each LC-MS/MS method

Antibiotic Class	Analyte	Group Acronym
Sulfonamide	Sulfachlorpyridazine	"SAMA"
	Sulfadimethoxine	
	Sulfamerazine	
	Sulfamethazine	
	Sulfamethizole	
	Sulfamethoxazole	
	Sulfathiazole	
Macrolide	Erythromycin-H_2O	
	Roxithromycin	
	Tylosin	
Lincosamide	Lincomycin	
Diaminopyrimidine	Trimethoprim	
Fluoroquinolone	Ciprofloxacin	"QL"
	Enrofloxacin	
	Flumequine	
	Levofloxacin	
	Norfloxacin	
	Sarafloxacin	
Quinolone	Oxolinic Acid	
	Pipemedic Acid	
Tetracycline	Chlortetracycline	"TC"
	Demeclocycline	
	Doxycycline	
	Minocycline	
	Oxytetracycline	
	Tetracycline	

Positive ionization was used to identify ions during mass spectrometry. Electrospray parameters had been previously optimized and remained constant during antibiotic analyses

(Ye, 2005). Optimized parameters are shown in Table 2.3. The drying and nebulizer gases were nitrogen and the collision gas was argon.

Table 2.2: Gradients for "SAMA", "QL", and "TC" methods

"TC" Method

Time	% A	% B
0.00	82	18
5.00	50	50
17.00	50	50
19.00	0	100
21.00	0	100

"QL" Method

Time	% A	% B
0.00	90	10
20.00	0	100
22.00	0	100
37.00	0	100

"SAMA" Method

Time	% A	% B
0.00	85	15
15.00	50	50
19.00	50	50
21.00	0	100
34.00	0	100

Table 2.3: Optimized parameters for antibiotic analyses on LC-MS/MS

Nebulizer needle voltage (V)	5000
Detector voltage (V)	1900
Shield voltage (V)	600
Ion-transfer voltage (V)	60
Drying gas flow (L/min)	4
Drying gas temperature (°C)	300
Nebulizer gas flow (L/min)	1
ESI chamber temperature (°C)	50
Collision gas pressure (mTorr)	2.6

Precursor and product ions were determined during direct infusion of known standards at a concentration of 10 µg/L in MeOH at a flow rate of 20 µL/min (Ye, 2005). Two product ions detected at the highest signal strength were identified for each antibiotic and the internal standard while only one product ion was identified for the surrogate standards. Precursor and product ions and optimal collision voltages are listed in Table 2.4. Underlined product ions indicate that they were rarely detected above noise in actual samples. The analytes listed at the bottom of each section are the surrogate standards.

Table 2.4: Analyte precursor, product ions, and optimal collision voltages

Analyte	Precursor Ion	Product Ions (Collision Voltage, eV)	
		Major	Minor
Sulfachlorpyridazine	256.0	156.0 (13)	108.0 (20)
Sulfadimethoxine	311.0	156.0 (15)	108.0 (15)
Sulfamerazine	265.0	156.0 (16)	108.0 (18)
Sulfamethazine	279.0	186.0 (16)	92.0 (18)
Sulfamethizole	271.0	156.0 (12)	108.0 (19)
Sulfamethoxazole	254.0	156.0 (14)	108.0 (20)
Sulfathiazole	256.0	156.0 (13)	108.0 (20)
Erythromycing-H_2O	716.4	158.0 (22)	558.0 (22)
Roxithromycin	837.5	158.0 (26)	<u>159.0 (26)</u>
Tylosin	916.6	174.0 (32)	<u>101.0 (38)</u>
Trimethoprim	291.0	230.0 (23)	123.0 (21)
Lincomycin	407.0	126.0 (23)	359.0 (18)
$^{13}C_2$-Erythromycin	718.4	160.0 (22)	--
$^{13}C_6$-Sulfamethoxazole	260.0	162.0 (14)	--
Ciprofloxacin	332.0	288.0 (17)	245.0 (23)
Enrofloxacin	360.0	316.0 (18)	245.0 (25)
Flumequine	262.0	244.0 (15)	202.0 (30)
Levofloxacin	362.0	318.0 (16)	344.0 (20)
Norfloxacin	320.0	276.0 (17)	233.0 (23)
Oxolinic acid	262.0	244.0 (15)	216.0 (27)
Pipemedic acid	304.0	286.0 (15)	215.0 (29)
Sarafloxacin	386.0	342.0 (17)	299.0 (26)
$^{13}C_3$-Ciprofloxacin	336.0	291.0 (17)	--
Chlortetracycline	479.0	444.0 (17)	154.0 (24)
Demeclocycline	465.0	448.0 (17)	430.0 (26)
Doxycycline	445.0	428.0 (17)	154.0 (24)
Minocycline	458.0	441.0 (18)	283.0 (41)
Oxytetracycline	461.0	426.0 (17)	443.0 (11)
Tetracycline	445.0	410.0 (18)	427.0 (12)
Meclocycline	477.0	460.0 (18)	--
Simatone (internal standard)	198.0	128.0 (18)	100.0 (22)

Note: Analytes shown in *italic* font are surrogate standards, values <u>underlined</u> indicates that they are not consistently seen above a signal to noise ration greater than 10; -- indicates that no minor ions were present for these analytes.

3. Results

3.1 Antibiotic Analysis by LC-MS/MS

Analyte detection in a sample used three criteria for positive analyte identification: 1) relative retention time to the internal standard simatone was within 2% of the value obtained in standard solutions; 2) the target ion for that analyte was found at that retention time; and 3) at least one product ion (Table 2.4) with a signal-to-noise (S:N) ratio greater than three was obtained under the predefined breakdown conditions for the parent ion. Chromatographic peak integration was completed manually or automatically using integration methods programmed into the Varian Workstation V.6.8 software. Typical retention times for each analyte are shown in Table 3.1. While retention times varied slightly depending on the matrix, instrument usage and conditions, a match in relative retention time between a known analyte and an unknown analyte means that the retention times of a particular peak for an unspiked and spiked sample varied no more than 2% for a single chromatographic run. The analytes' presence is also confirmed when at least one product ion is identified at a matching retention time.

The internal standard simatone was targeted during each chromatographic run although it eluted at different times due to differences in the gradient program. Relative area, which uses the peak area of the internal standard during the same chromatographic run

designed for the class of the targeted antibiotics, was used to quantify analytes and will be discussed in Section 3.1.2.

Table 3.1: Typical analyte chromatographic relative retention time

Method	Analyte	Relative Retention Time (min)
"SAMA"	Sulfathiazole	0.625
	Sulfamerazine	0.797
	Sulfamethazine	0.895
	Sulfamethizole	0.968
	Sulfachlorpyridazine	1.19
	Sulfamethoxazole	1.32
	Sulfadimethoxine	1.59
	Tylosin	1.72
	Erythromycin-H2O	1.89
	Trimethoprim	0.723
	Lincomycin	0.650
"QL"	Pipemidic acid	0.863
	Norfloxacin	0.984
	Ciprofloxacin	1.01
	Levofloxacin	1.01
	Enrofloxacin	1.02
	Sarafloxacin	1.11
	Flumequine	1.74
"TC"	Minocycline	0.761
	Oxytetracycline	0.910
	Tetracycline	1.075
	Demeclocycline	1.209
	Chlortetracycline	1.299
	Doxycycline	1.373
	Meclocycline	1.522

Note: Retention times determined from extract of Stream A wastewater effluent September 22, 2007; relative retention times were determined using column dimensions and gradients listed in Section 2.6

Example chromatograms showing chromatographic peaks for each analyte in each of the three methods (SAMA, QL, and TC) are shown in Figures 3.1, 3.2, and 3.3. An extracted ion chromatogram is created by plotting the intensity of a chosen mass to charge ratio (m/z)

as a function of retention time. Therefore, any peak shown on these chromatograms was specifically targeted from both of its known ion breakdowns. Surrogate standards $^{13}C_6$-sulfamethoxazole, $^{13}C_3$-ciprofloxacin, and $^{13}C_2$-erythromycin were not included in any of these chromatograms because their retention times match those of their analyte pair and the chromatographic peaks would not be distinguishable (i.e. $^{13}C_6$-sulfamethoxazole and $^{12}C_6$-sulfamethoxazole have the same retention time). Targeting specific ion breakdowns allows differentiation between these analytes to be possible. The internal standard simatone was also not pointed out in these chromatograms as the retention time differed in each method, but it was targeted in every run.

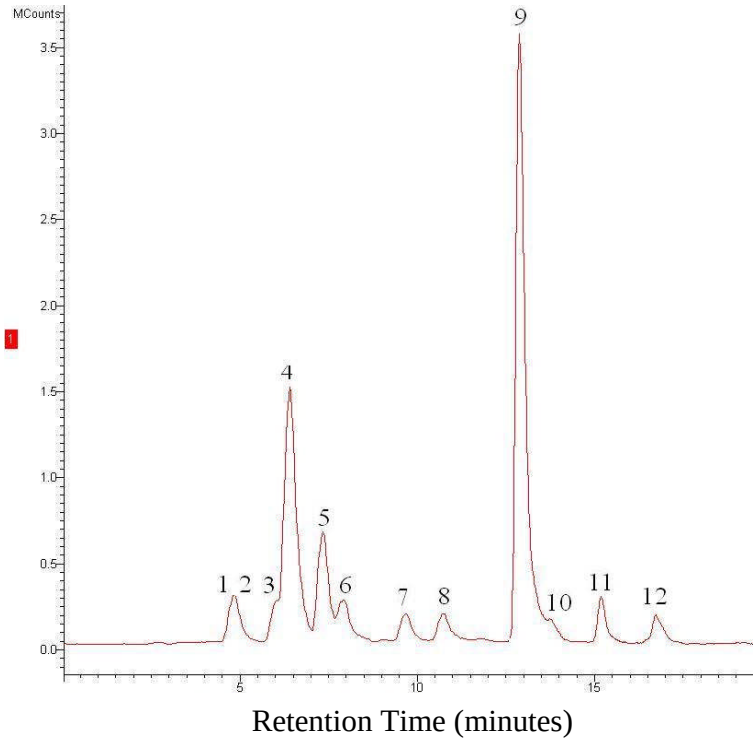


Figure 3.1: Extracted 12 ion chromatogram for SAMA antibiotics spiked in surface water at 500 ng/L (Stream A 9/22/07)

(1) Sulfathiazole; (2) Lincomycin; (3) Trimethoprim; (4) Sulfamerazine; (5) Sulfamethazine; (6) Sulfamethizole; (7) Sulfachlorpyridazine; (8) Sulfamethoxazole; (9) Sulfadimethoxine; (10) Tylosin; (11) Erythromycin-H_2O; (12) Roxithromycin

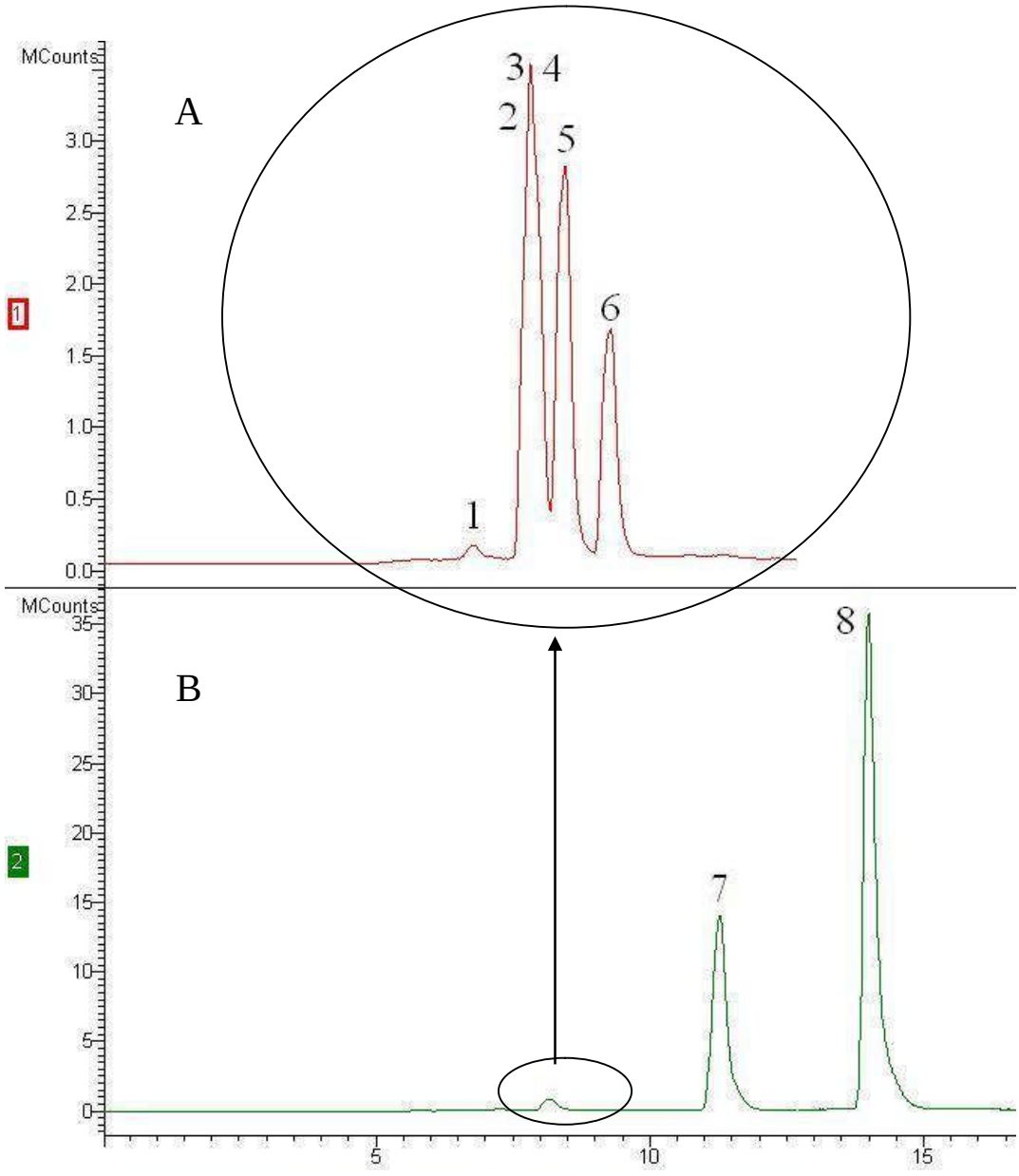


Figure 3.2: Extracted 8 ion chromatogram for QL antibiotics spiked into surface water at 500 ng/L (Stream A 9/22/07)

(1) Pipemidic Acid; (2) Norfloxacin; (3) Levofloxacin; (4) Ciprofloxacin; (5) Enrofloxacin; (6) Sarafloxacin; (7) Oxolinic Acid; (8) Flumequine

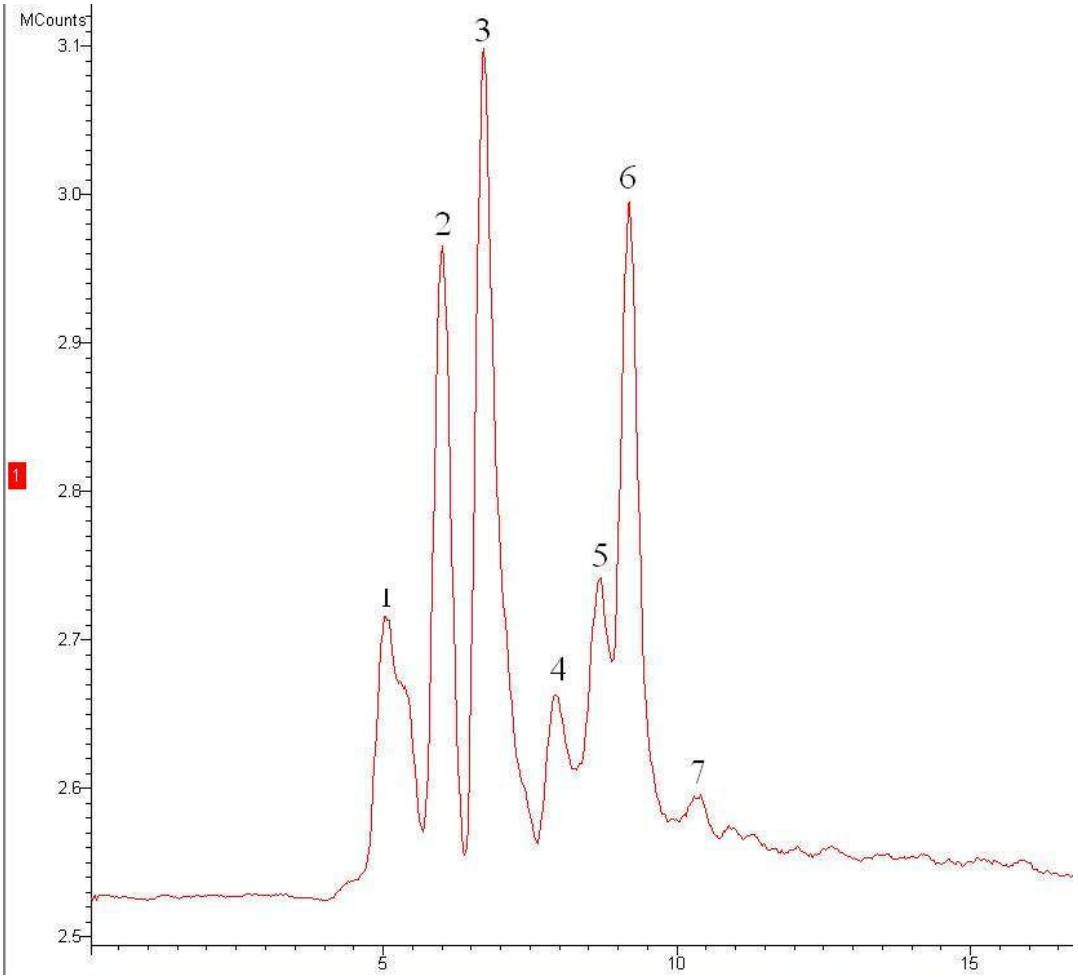


Figure 3.3. Extracted 7 ion chromatogram for TC antibiotics spiked into surface water at 500 ng/L (Stream A 9/22/07)

(1) Minocycline; (2) Oxytetracycline; (3) Tetracycline; (4) Demeclocycline;
(5) Chlortetracycline; (6) Doxycycline; (7) Meclocycline

Once analytes were identified, each extracted ion chromatographic peak was integrated, either manually or automatically, to determine the area under the peak, or instrument response, which is later used to quantify the analytes. Using the Varian Workstation 6.8 software, each peak is individually targeted from the overall chromatogram

by identifying the specific ion breakdown for the analyte of interest. Peaks for both the major and minor product ion for each analyte were targeted, but only one product ion is required for quantitation.

Figure 3.4 is an example set of ion breakdown chromatograms for extracts from a spiked and unspiked surface water sample that illustrates the basic concepts in interpreting LC-MS/MS data. The chromatograms are for extracts of the same surface water; chromatograms A and B are for extracts of the unspiked water of unknown concentration, while chromatograms C and D are for extracts of the same water spiked with the 26 antibiotic mixture listed in Table 3.1 at 1000 ng/L as part of a standard addition calibration curve. The major ion breakdowns for trimethoprim were targeted: 291 > 230 at -23 eV in Figure 3.4A and Figure 3.4C; and 291 > 123 at -21 eV in Figure 3.4B and Figure 3.4D. All of these ions are detected at the same retention time (± 0.1 minutes), indicating a specific match between trimethoprim in the unspiked and spiked sample. By use of a standard addition calibration curve, the integrated areas can then be used to quantify the concentration of trimethoprim in the unspiked sample.

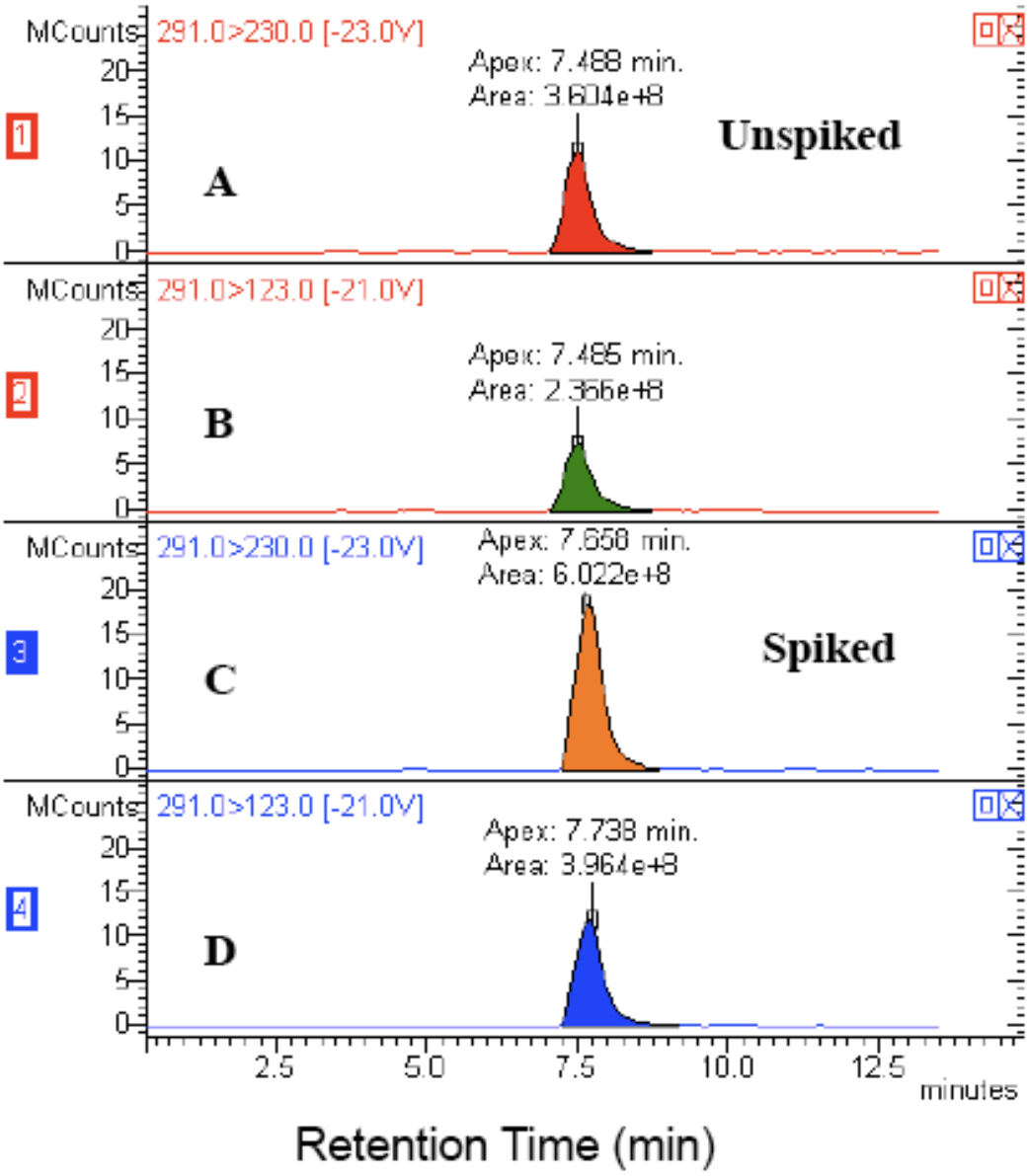


Figure 3.4: Ion chromatographic peak integration example

(A) Trimethoprim major product ion targeted in extract of unspiked surface water sample; (B) Trimethoprim minor product ion targeted in the same sample; (C) Trimethoprim major product ion in extract from same sample spiked at 1000 ng/L; (D) Trimethoprim minor product ion in extract from (C)

3.1.2 Data Quantitation by Standard Addition for Antibiotic Analyses

In standard addition, a calibration curve is created which allows the original concentration of a given analyte in an unspiked sample to be determined by extrapolation. Calibration curves must be prepared for each analyte in each sample matrix. Environmental samples from each individual matrix are divided into several equal portions, with unspiked samples in duplicate and the remaining samples spiked at increasing concentrations with the analytes. Typical spike concentrations for surface and wastewater are 20, 50, 100, 500, and 1000 ng/L and are selected to cover the range of expected analyte concentrations in the sample. A calibration curve is then created by plotting the relative area against the spike concentrations. The relative area is defined in equation 3.1, where the "quantifiying ion" for each analyte is the major product ion listed in Table 2.4. The response of the internal standard used in this calculation is that of simatone for the breakdown (m/z = 198 to m/z = 128) that is present in the particular chromatogram of interest.

$$Relative\ Area = \frac{Quantifying\ \ ion\ \ response}{Internal\ \ standard\ \ ion\ \ response} \qquad \text{(eq. 3.1)}$$

A visual representation of this concept is provided in Figure 3.5 for a surface water sample from stream B (10/5/07).

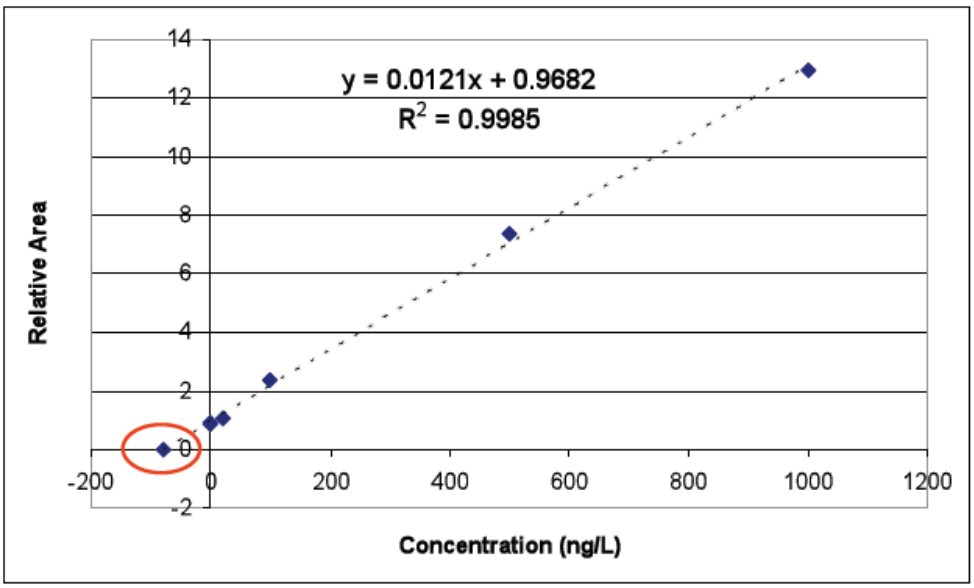


Figure 3.5: Illustration of extrapolation of standard addition calibration curve to calculate analyte concentration in surface water (Stream B 10/5/07)

A linear regression is conducted in order to find the linear relationship for the calibration. Typical regression correlation coefficient (R^2) values for the calibration curves were greater than 95%, which was used in this study as acceptable precision across the concentration range. When R^2 values were lower than 95%, outlier data points were removed from the calibration curve. From the calibration curve, the concentration in the unspiked sample can be determined by regressing the line back to the x-axis, which is represented by the circle drawn on Figure 3.5. The concentration can be determined using equation 3.2, viz:

$$\text{Concentration}_{\text{Unspiked}} = \frac{y - Intercept}{Slope} = \frac{0.9682}{0.0121} = 80 \text{ ng/L} \qquad \text{(eq. 3.2)}$$

Analyte identity is confirmed by comparing the ratio of the response intensities of the major and minor product ions (see Table 2.4) in each unspiked sample with that of the spiked samples.

Isotopic dilution is another technique used to quantify analytes and is based on using isotopically labeled compounds that behave physically and chemically like its unlabeled counterpart as the two compounds only differ by a few mass units. A surrogate standard will also have the same physical and chemical properties to a compound in the same chemical class as its unlabeled compound. Twenty-six antibiotics were targeted in this study, but only four surrogates were available ($^{13}C_6$-sulfamethoxazole, $^{13}C_3$-ciprofloxacin, $^{13}C_2$-erythromycin, and meclocycline). It is not possible for these four surrogates to accurately represent all twenty-six of the antibiotics, which is why standard addition was solely used for quantitative analyses.

Standard addition is a time-consuming process since calibrations need to be made for each matrix for each sampling event. However, for accurate quantitation, standard addition is necessary to compensate for matrix effects and recovery loss. The degree to which matrix effects alter signal response is difficult to predict because organic carbon content and composition vary largely in surface and wastewaters. Compensation for this variation with calibration curves in each matrix is a valuable contribution of the method of standard addition.

3.1.3 Linearity, Detection, and Quantitation Limits

The range of linearity of the LC-MS/MS instrument responses were evaluated by injecting 20 μL of standard solutions of the target analytes in 9:1 (volume:volume) LGW:MeOH (HPLC grade) in the range of 0.5 to 100 μg/L representing the range in which the analytes are expected to be detected in the final concentrated extracts (Ye, 2005). R^2 values were typically 0.999. Instrumental detection limits (IDL), previously determined by

Ye (2005), were calculated as three times the standard deviation of instrument response from ten consecutive injections of standard solutions in the range of 5 to 100 μg/L, depending on the instrumental sensitivity for a particular analyte.

Ye (2005) previously determined the limits of detection (LOD) and limits of quantitation (LOQ) in our laboratory using the same instrument and analytical methods in order to evaluate the lower range of linearity for analytes taken through the whole analytical procedure. LOD and LOQ are respectively defined as the analyte concentration in a sample that would give a minimum S:N for the analyte of 3 and 10. Values for IDL, LOD, and LOQ are all shown in Table 3.2.

The practical quantitation limit (PQL) is defined as the lowest spike level used in the method of standard addition. This value is relevant to this study because an analyte may still be detected outside of the range of calibration if the detected value is less than the lowest spike level. The value can be semi-quantified, but it must be reported as detected below the PQL. In this study, 20 ng/L was the PQL for surface waters and wastewater effluent because the targeted breakdown ions could not consistently be distinguished from the noise at spike levels less than 20 ng/L.

Table 3.2: Detection and quantitation limits of target analytes in LGW (Ye, 2005)

Analyte	IDL (pg)	LOD (ng/L)	LOQ (ng/L)
Sulfachlorpyridazine	12	1.0	6.7
Sulfadimethoxine	10	1.0	3.5
Sulfamerazine	18	1.0	5.6
Sulfamethazine	14	1.0	2.5
Sulfamethizole	16	1.0	4.7
Sulfamethoxazole	8.0	1.0	5.4
Sulfathiazole	10	1.0	8.2
Erythromycin-H_2O	100	0.5	1.4
Roxithromycin	100	0.5	1.0
Tylosin	80	0.5	1.0
Lincomycin	5.0	NA	NA
Trimethoprim	8.0	0.5	1.3
Ciprofloxacin	40	1.0	2.7
Enrofloxacin	20	1.0	5.0
Flumequine	2.0	0.5	1.0
Levofloxacin	NA	NA	NA
Norfloxacin	60	2.0	5.6
Sarafloxacin	40	0.5	2.1
Oxolinic acid	2.0	1.0	3.0
Pipemidic acid	80	3.0	10
Chlortetracycline	16	2.0	6.5
Demeclocycline	20	3.0	12
Doxycycline	10	1.0	3.5
Minocycline	40	6.0	32
Oxytetracycline	10	1.0	4.4
Tetracycline	12	0.5	3.2

Note: IDL = Instrument Detection Limit; LOD = Limit of Detection; LOQ = Limit of Quantitation; NA = Not Applicable. Lincomycin is not extracted in quenched, chlorine samples, and levofloxacin was not a target analyte at the time of IDL, LOD, and LOQ evaluation. Volume injected to determine IDL was 20 μL.

3.1.3 Quality Assurance and Quality Control

The objectives of quality assurance and quality control measures are to achieve precision, accuracy, and comparability. Components of quality control incorporated into this study include solvent blanks, method blanks, replicate samples, matrix spikes, and calibration curves. Solvent blanks were injected along with every sample set analyzed on the LC-

MS/MS prior to, within, and at the end of each sample run to help ensure column equilibrium and to prevent any carryover of contamination between methods. The internal standard simatone, which was targeted during every chromatographic run and used to calculate relative area for quantitation using the method of standard addition, was also used as an instrument performance check solution to confirm proper instrument performance. The retention time and peak area were used, and if these values differed significantly from a previous instrument performance test or sample run, the instrument was deemed to be not performing within specifications and measures were taken to fix the problem.

Duplicate samples help to estimate precision, and so one set of duplicates for the unspiked sample was analyzed with each sample batch in this study. None of the spiked samples were processed in duplicate due to the more critical need to complete the extraction of a complete batch of samples in a 24-36 hour period. In order for the analysis of duplicate samples to be considered acceptable, the relative percent difference (RPD) should be less than or equal to 20%. In this study, the RPD of the analysis of duplicates of the unspiked samples was monitored as a measure of quality control. Table 3.3 presents the percentage of samples that were not considered acceptable. These RPDs were calculated by comparing the concentrations obtained from the major and minor targeted breakdown ions for all detected analytes in all of the samples during the entire study period. The data presented in the second and third columns are the percentage of instances when RPD of the measured concentrations of the duplicates using the major and minor ions were greater than 20% in either a wastewater effluent, stream, or reservoir sample.

Table 3.3: Duplicate quality control and acceptance criteria from all samples sets in all three streams, WWTP effluent, and reservoir samples (n = 70)

Sampling Location	20% RPD
A	6.0
B	5.7
C	9.1
Reservoir	0.0

Note: RPD for locations A, B, and C include both treated wastewater effluent and surface water samples; 0.0 values for the reservoir sites indicate that no set of unspiked duplicate samples resulted in a relative percent difference greater than 20%.

The data in this table suggest that duplicate analysis when using the 20% RPD criteria yields poor precision between 5.7% and 9.1% of the time for all samples, suggesting that the method or technique occasionally yields poor precision. The values between streams may be different from each other due to different percentages of natural organic matter or carbon content that may be affecting extraction processes to varying extents. The RPD for the streams include treated wastewater and surface water samples whereas the reservoir samples are just surface water. Fewer samples were also collected in the reservoir (n=10) compared to the streams (n=60) and the reservoir water was generally clearer than the stream water, which would explain the differences in RPD between the stream and reservoir samples. One antibiotic class was not found to generate more precise results than another.

The samples are put through several processing steps such as extraction, blow-down, reconstitution, etc. where human error may have an impact. Time is a major factor when processing these samples, and human error could be due to the necessity of processing up to twelve samples within a few days to ensure integrity in the analysis of samples collected. Eliminating this error is difficult with manual processing. Matrix effects may also be contributing to different calculated concentrations between the unspiked duplicates as well.

Ye (2005) states that co-extracted matrix components in water can compete for charges during electrospray ionization, resulting in signal suppression or enhancement. Signal suppression increases with the amount of organic matter in the sample, and this may have been a factor in the different instrument responses of extracts from surface and wastewaters. However, quantitation of analytes should not be greatly affected by matrix interferences because standard addition calibration curves are prepared for every analyte in each sample batch to help compensate for recovery loss and matrix effects.

3.1.4 Antibiotic Occurrence in the Targeted Watershed

The watershed is located in a rapidly expanding region in the Southeastern United States that is mainly fed by three smalls streams to the north and a large river to the south that are all impacted by wastewater inputs. The watershed tributaries flow eastward through a major basin in the state and eventually into the Atlantic Ocean. This study focuses on the sections of the three northern streams that are impacted by WWTPs and at two points downstream of these plants as the streams flow into a drinking water reservoir (Figure 2.1 is reprinted below for reference). The most frequently sampled point in the reservoir was located near the intake pipe to a drinking water treatment plant (R5 site). A large portion of the watershed in which streams A, B, and C are located is urban and home to almost 1.1 million people. The reservoir supplies a source of drinking water to almost 1 million of those people. The WWTPs that discharge to the streams investigated in this study all use similar treatment processes (Figure 3.6).

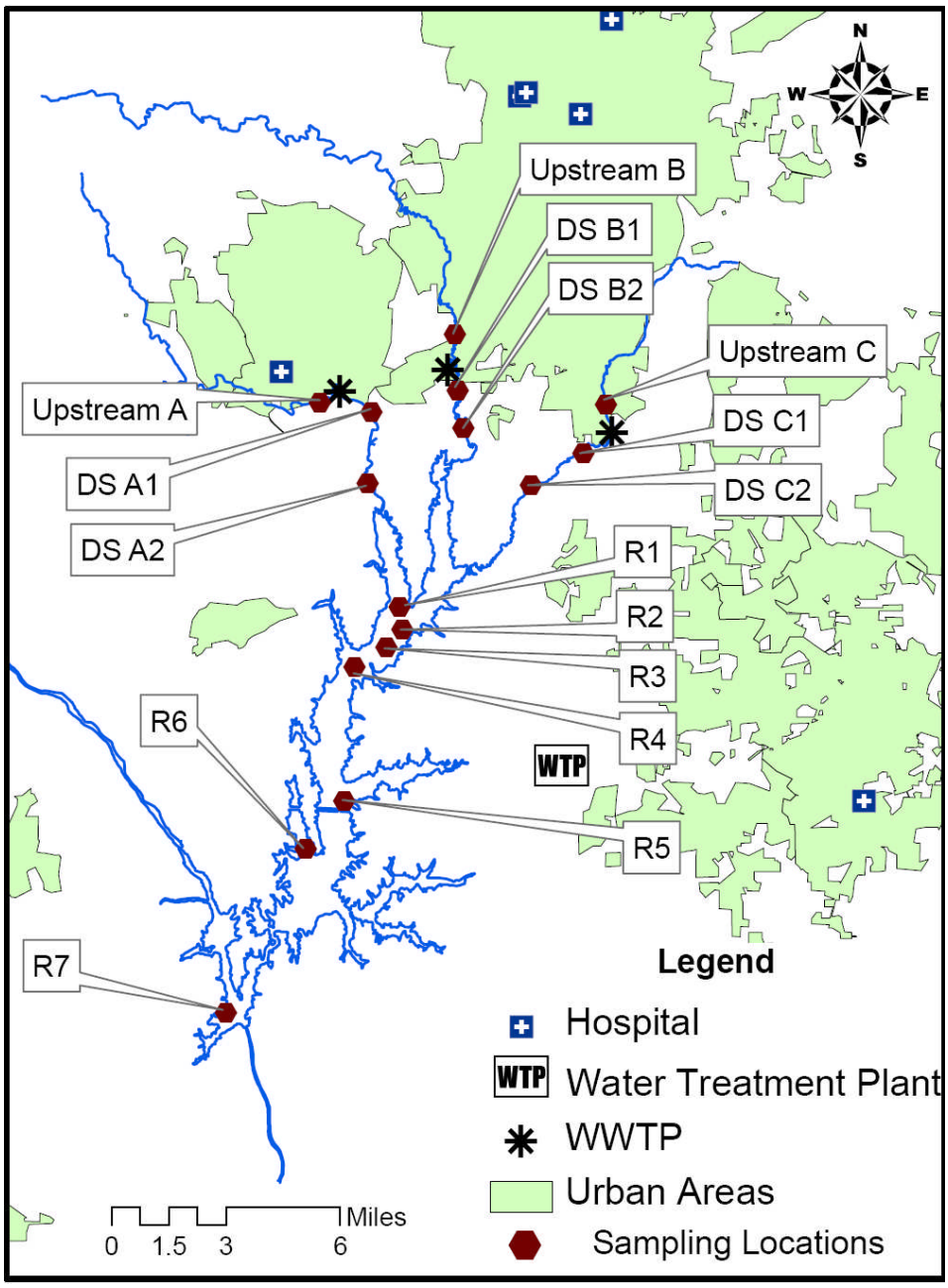


Figure 2.1 (reprinted): Sampling locations within watershed

Ultraviolet light (UV) irradiation is the main method of disinfection prior to discharging the effluent to surface waters from all three WWTPs. In the final phase of the

treatment process, water flows through a channel with several banks of UV bulbs that emit light in the ultraviolet range, effectively killing pathogenic organisms. UV light is safer for the receiving stream since unlike free chlorine, it leaves no chemical residue in the water.

Figure 3.6: Wastewater treatment plant diagram (City of Durham, 2006)

WWTPs A and B each collect waste from similarly sized populations of about 50,000 people, including residential and commercial zones, hospitals, research universities, and several nursing homes. WWTP C differs in that it only collects waste from a commercial area that employs about 38,000 people. WWTP A and C are permitted to discharge up to 12 MGD, although WWTP A averages about 10 MGD and WWTP C averages about 4 MGD. WWTP B is permitted to discharge 20 MGD, and the average daily flow is 16 MGD.

Effluent flows, as reported by the plant managers at the time of sampling, and stream flows measured by United States Geological Survey (USGS) monitoring stations located at either of the downstream sampling points are presented in Table 3.4. In general, peak flows occurred from 9 AM until about 4 PM and it is during this time that antibiotic concentrations in the wastewater are likely to be greatest and, therefore, to give rise to the highest environmental impact.

Table 3.4: Sampling dates, effluent flow rates, USGS stream flow rates, and percent of stream flow attributed to wastewater effluent

Date	Stream	Time	Effluent Input Flow (ft^3/s)	Stream Flow (ft^3/s)	% Attributed to Effluent
8/9/07	A	8:15 AM	3.3	7.8	43
8/10/07	B	9:25 AM	14	15	96
8/10/07	C	11:15 AM	6.7	7.5	89
8/24/07	A	10:15 AM	14	17	85
8/29/07	B	11:00 AM	17	17	98
8/29/07	C	12:30 PM	7.2	9.2	78
9/18/07	A	3:00 AM	13	13	99
9/20/07	B	10:15 AM	13	15	87
9/20/07	C	12:20 PM	7.1	9.8	72
10/3/07	A	11:00 AM	14	15	96
10/3/07	B	12:20 PM	7.6	11	69
10/5/07	C	1:50 AM	7.6	9.2	82
12/11/07	A	2:18 AM	15	15	97
12/11/07	B	12:25 PM	16	19	83
12/11/07	C	11:05 AM	6.7	8.8	76

The downstream sampling sites are heretofore referred to as DS the letter for the appropriate stream and then either a 1 or a 2 to signify how far downstream from the WWTP it is (for example DS A2 refers to the second downstream sampling location on stream A). All three receiving streams flow through urban areas before effluents discharge into them,

while the surrounding areas downstream of the WWTPs are mainly forested and intermittently swampy with little to no residential impact. A creek that flows through a small farm flows into stream A in-between the DS 1 and DS 2 sites, but it was not sampled during this study due to inaccessibility. No other potential nonpoint sources for the targeted compounds have been identified on any of the streams. Streams B and C both flow through constructed waterfowl impoundments (DS B2 and DS C2) that are basically dammed up portions of the streams to serve as a habitat for various waterfowl and aquatic life. It must be noted that there is a large fish population in these impoundments and that the local residents do fish for food at these locations.

Streams A, B, and C contribute the majority of the water to the northern half of the drinking water reservoir. The USGS found that the hydraulic retention time of a plug of water in the upper reaches is about 418 days, whereas the hydraulic retention time in the southern half of the reservoir is about 4 days. This suggests that there is sufficient exposure time for the antibiotics to adsorb to sediments and undergo photodegradation in the northern half of the reservoir where this study was undertaken.

The study period was from August through December 2007 and a total of 70 samples were collected within the watershed (see Appendix D for measured data). Real-time stream data from the USGS can be obtained by going to the USGS Real-time water data website: http://waterdata.usgs.gov/nwis/rt. From here, a U.S. state can be selected, or a stream name can be typed into the "select sites by name or number" box. In the "predefined displays" drop down box, select "Daily Stage and Streamflow". Depending on the number of USGS monitoring sites on the specified stream or river, multiple locations may come up. Select the desired site, and the desired time on the next page. Figure 3.7 displays an example of a graph

of the daily stream flow of Morgan Creek at the DS A1 site during the period of April 1, 2008 through April 9, 2008. The average flow on April 5, 2008 was approximately 12 cubic feet per second (cfs). Alternatively, one may choose to view the data in a tabular format, which is easier to determine stream flow and stream gage. The tabular format is not presented here due to the length of the table. A USGS monitoring station was located at the DS A1, DS B2, and DS C2 sites.

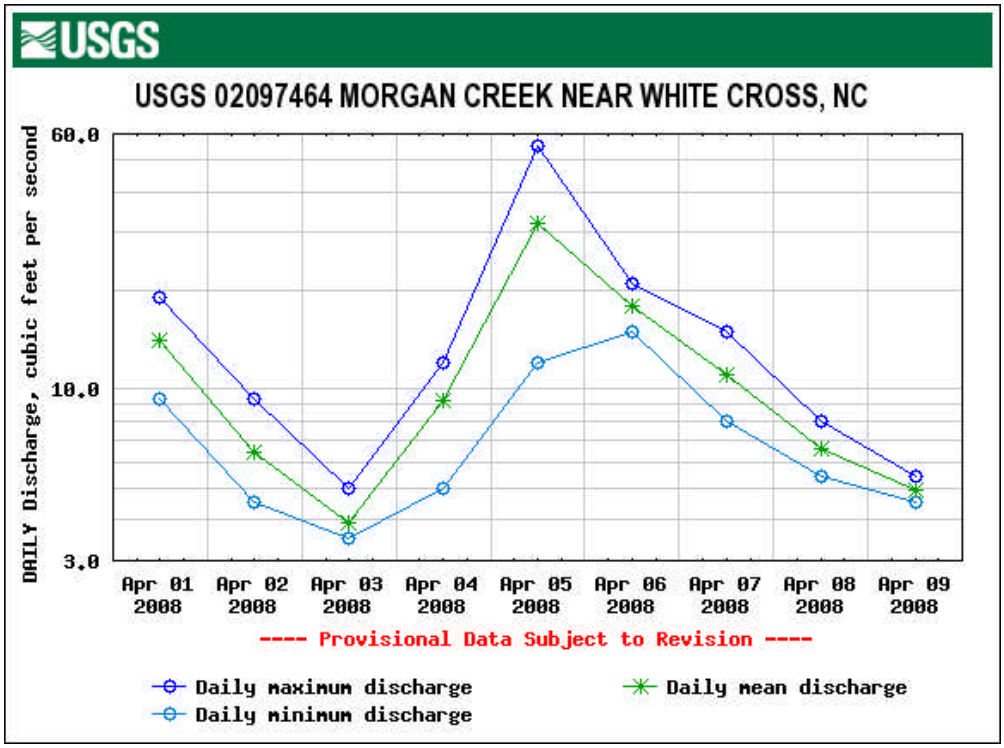


Figure 3.7: Example of a USGS Real-time daily stream flow graph

Distances from sampling locations and to the reservoir are presented in Table 3.5 below. The WWTP effluent was sampled at the point where the effluent was being discharged into each of the streams. The distance listed for the second downstream points to the reservoir is the distance to the point where each stream flows into the reservoir, and is at a point where sampling did not occur. The distances from the second downstream sampling

points to the influent pipe to the drinking water treatment plant (R5) varied between 9 to 12 miles.

Table 3.5: Distances between sampling locations

Location	Distance (miles)
WWTP A - DS A1	0.5
DS A1 - DS A2	2
DS A2 - Reservoir	0.9
Reservoir - R5	9
WWTP B - DS B1	0.1
DS B1 - DS B2	1.4
DS B2 - Reservoir	2
Reservoir - R5	12
WWTP C - DS C1	1.2
DS C1 - DS C2	1.9
DS C2 - Reservoir	2.4
Reservoir - R5	11

Samples were collected when the stream was at base flow and rainfall had not occurred at least three to seven days beforehand. Heavy rainfall was not an issue as the region was in an extreme drought during the sampling period. However, rainfall did occur between November and December 2007 and stream flow increased as can be seen in Table 3.4. Effluent flow at the outfall into the stream was obtained from plant charts during the time of sampling, and varied with each plant depending upon the time of day. During most of the study, the major component of stream flow was treated wastewater effluent. Stream flow was less than 50% effluent on only one occasion for Stream A on 8/9/07 (Table 3.4).

General summary tables presenting the maximum and minimum concentrations of the antibiotics that were detected above the PQL of 20 ng/L are given for each stream and the reservoir over five sampling events (Tables 3.10 through 3.13). Individual sampling event

data for stream A are presented in Tables 3.5 through 3.9, but the complete data set for streams B and C can be found in Appendix D.

In some cases, the sample matrix generated elevated noise in the ion chromatogram, while in other instances, extended instrument usage and contamination on the electrospray needle led to an increase in noise. A typical sample run batch usually lasted about 15 hours, and cleanliness of the electrospray chamber and shield became an issue. This is illustrated in Table 3.9 where sulfathiazole is detected at 160 ng/L in the wastewater effluent, but is also detected at 144 ng/L with only one breakdown ion in the DS A2 site. Occasionally concentrations were found to increase downstream of a particular location (see Table 3.6 for sulfamethoxazole and trimethoprim). There was a lack of nonpoint sources on each of the streams so this increase may be attributed to the fact that the antibiotics could have accumulated from the previous day/night or did not undergo attenuation processes overnight due to a lack of sunlight at certain locations.

Table 3.6: Antibiotic occurrence data in ng/L for stream A 8/9/07

Antibiotic	Upstream	Effluent	DS A1	DS A2
Sulfamethoxazole	< 20	26	124	814
Sulfathiazole	< 20	54	97	< 20 (11)
Sulfadimethoxine	< 20	359	37	< 20 (7)
Erythromycin	< 20	527	316	40
Trimethoprim	< 20	< 20 (14)	170	66
Lincomycin	< 20	< 20 (4)	< 20	< 20
Ciprofloxacin	< 20	154	104	43
Levofloxacin	< 20	269	148	< 20
Sarafloxacin	< 20	< 20	< 20	< 20
Tetracycline	< 20	< 20	< 20	< 20 (0.5)

Note: Concentrations are in ng/L; < 20 indicates that analytes were not detected above a S:N ratio above 3 in chromatograms; < 20 (--) indicates that the analyte was detected above a S:N ratio above 3 in chromatograms, but concentration was below PQL of 20 ng/L. Underlined values indicate that only one MS/MS product ion was used to confirm the analyte.

Table 3.7: Antibiotic occurrence data in ng/L for stream A 8/25/07

Antibiotic	Upstream	Effluent	DS A1	DS A2
Sulfamethoxazole	< 20	206	188	94
Sulfathiazole	< 20	66	21	81
Sulfadimethoxine	< 20	448	26	< 20 (13)
Erythromycin	< 20	693	32	209
Trimethoprim	< 20	< 20 (19)	36	109
Lincomycin	< 20	296	51	< 20
Ciprofloxacin	< 20	161	21	< 20 (12)
Levofloxacin	< 20	148	122	< 20
Tetracycline	< 20	< 20 (3)	121	< 20

See Table 3.6 for legend

Table 3.8: Antibiotic occurrence data in ng/L for stream A 9/22/07

Antibiotic	Upstream	Effluent	DS A1	DS A2
Sulfamethoxazole	< 20	2828	2860	1480
Sulfathiazole	< 20	217	287	< 20
Trimethoprim	< 20	168	195	77
Sulfadimethoxine	< 20	35	< 20 (19)	40
Erythromycin	< 20	218	181	98
Lincomycin	< 20	24	< 20 (1)	< 20 (17)
Ciprofloxacin	< 20	55	< 20 (14)	< 20 (3)
Levofloxacin	< 20	338	162	27
Tetracycline	< 20	26	< 20 (10)	< 20

See Table 3.6 for legend

Table 3.9: Antibiotic occurrence data in ng/L for stream A 10/5/07

Antibiotic	Upstream	Effluent	DS A1	DS A2
Sulfamethoxazole	< 20	1777	1749	1245
Sulfathiazole	< 20	160	130	144
Trimethoprim	< 20	87	147	60
Sulfadimethoxine	< 20	< 20 (16)	53	30
Erythromycin	< 20	140	103	85
Lincomycin	< 20	< 20 (8)	< 20 (15)	< 20 (4)
Ciprofloxacin	< 20	< 20 (19)	35	< 20
Levofloxacin	< 20	253	171	27
Enrofloxacin	< 20	< 20 (0.3)	< 20 (13)	< 20 (1)
Tetracycline	< 20	< 20 (13)	< 20	< 20

See Table 3.6 for legend

Table 3.10: Antibiotic occurrence data in ng/L for stream A 12/11/07

Antibiotic	Upstream	Effluent	DS A1	DS A2
Sulfamethoxazole	< 20	3255	2531	1584
Trimethoprim	< 20	106	238	59
Sulfdimethoxine	< 20	27	20	459
Sulfathiazole	< 20	713	436	335
Erythromycin	< 20	224	153	122
Lincomycin	< 20	< 20 (6)	< 20 (1)	< 20 (5)
Ciprofloxacin	< 20	55	< 20 (18)	< 20
Levofloxacin	< 20	338	157	33
Enrofloxacin	< 20	16	10	49
Tetracycline	< 20	20	214	< 20

See Table 3.6 for legend

Of the 26 antibiotics targeted in stream A, 10 were detected during a 5-month sampling period over five sampling events (Table 3.11). Twelve and eleven of the 26 antibiotics were detected in streams B and C, respectively (Tables 3.12 and 3.123. The

reservoir 5 site was sampled on four occasions (8/25, 9/22 10/3, and 12/6), whereas the other six reservoir points were only sampled once (12/6) (Table 3.14).

Table 3.11: Maximum and minimum antibiotic occurrence in Stream A in ng/L (n=20)

Antibiotic	Effluent		DS A1		DS A2	
	Min	Max	Min	Max	Min	Max
Sulfamethoxazole	26	3255	124	2860	94	1584
Trimethoprim	< 20 (14)	168	36	238	59	109
Sulfadimethoxine	< 20 (16)	448	< 20 (19)	37	< 20 (7)	40
Sulfathiazole	54	713	21	436	< 20 (11)	335
Erythromycin	140	693	32	316	40	209
Lincomycin	< 20 (4)	296	< 20 (1)	51	< 20 (4)	< 20 (17)
Ciprofloxacin	< 20 (19)	161	< 20 (14)	104	< 20 (3)	43
Levofloxacin	148	338	122	171	< 20	33
Enrofloxacin	< 20 (0.3)	< 20 (16)	< 20 (13)	10	< 20 (1)	49
Tetracycline	< 20 (3)	26	< 20 (10)	214	< 20	< 20 (0.5)

See Table 3.6 for legend; Data for period 8/9/07 to 12/7/07

Table 3.12: Maximum and minimum antibiotic occurrence in Stream B in ng/L (n= 20)

Antibiotic	Effluent		DS B1		DS B2	
	Min	Max	Min	Max	Min	Max
Sulfamethoxazole	1419	3006	237	1337	155	1936
Trimethoprim	42	1396	190	651	98	240
Sulfadimethoxine	< 20 (14)	500	< 20	198	< 20 (10)	91
Sulfathiazole	< 20	238	< 20 (7)	195	< 20	127
Erythromycin	75	173	58	155	37	97
Lincomycin	< 20 (3)	28	< 20 (3)	< 20 (14)	< 20 (5)	25
Ciprofloxacin	35	86	< 20 (2)	84	< 20	< 20 (18)
Levofloxacin	< 20 (19)	261	< 20 (9)	251	< 20 (1)	168
Sarafloxacin	< 20	198	36	88	< 20 (13)	131
Norfloxacin	< 20	51	< 20	< 20 (12)	< 20	< 20
Enrofloxacin	< 20	42	< 20	302	< 20	< 20 (10)
Tetracycline	< 20	42	< 20	< 20 (10)	< 20	< 20 (16)

See Table 3.6 for legend; Data for period 8/9/07 to 12/7/07

Table 3.13: Maximum and minimum antibiotic occurrence in Stream C in ng/L (n = 20)

Antibiotic	Effluent C		DS C1		DS C2	
	Min	Max	Min	Max	Min	Max
Sulfamethoxazole	556	1304	198	1379	302	1199
Trimethoprim	< 20 (11)	14	< 20 (2)	55	< 20 (2)	28
Sulfathiazole	< 20	337	< 20 (0.04)	< 20	< 20	< 20
Erythromycin	53	65	36	155	< 20 (5)	87
Lincomycin	< 20 (0.5)	61	< 20 (2)	161	< 20 (2)	24
Sulfadimethoxine	165	25	75	296	< 20 (12)	111
Ciprofloxacin	< 20 (1)	< 20 (13)	161	< 20 (10)	< 20	< 20 (17)
Levofloxacin	47	62	< 20	< 20 (17)	< 20 (2)	< 20
Sarafloxacin	< 20	127	< 20	< 20 (12)	< 20	< 20 (10)
Enrofloxacin	< 20 (10)	65	< 20	< 20 (4)	< 20	242
Tetracycline	< 20	34	< 20	< 20	< 20	< 20

See Table 3.6 for legend; Data for period 8/9/07 to 12/7/07

Table 3.14: Maximum and minimum antibiotic occurrence in the reservoir (ng/L) (n=10)

Antibiotic	Reservoir 1	Reservoir 2	Reservoir 3	Reservoir 4	Reservoir 5		Reservoir 6	Reservoir 7
					Min	Max		
Sulfamethoxazole	< 20 (14)	64	60	144	< 20 (13)	64	223	238
Erythromycin	< 20 (15)	< 20 (0.02)	< 20 (17)	21	< 20 (6)	44	< 20 (12)	75
Trimethoprim	< 20	37	< 20 (12)	30	< 20	37	< 20 (18)	46
Lincomycin	< 20 (15)	< 20 (1)	21	23	< 20	44	< 20 (7)	59
Sulfadimethoxine	< 20	< 20	< 20	< 20	< 20	< 20 (4)	< 20	< 20
Ciprofloxacin	< 20 (5)	< 20	< 20 (1)	< 20 (5)	< 20 (1)	< 20 (4)	< 20 (0.3)	35
Levofloxacin	< 20 (6)	< 20	< 20 (3)	< 20 (2)	< 20 (1)	< 20 (10)	< 20 (4)	45
Norfloxacin	< 20 (6)	< 20	64	< 20	< 20	< 20 (16)	< 20 (1)	51
Enrofloxacin	< 20(14)	< 20	62	51	< 20	< 20 (2)	< 20 (5)	38
Tetracycline	30	< 20	< 20 (4)	< 20 (3)	< 20	< 20	< 20 (9)	< 20

3.2 Estrogenic Activity within Watershed: YES Assay Results

Estrogenic activity in the aqueous samples collected in the watershed was determined using the YES assay. QA/QC measures included the use of LGW blanks during YES assay analyses, positive and negative spike controls, and duplicate samples. Stanford (2007) previously determined the PQLs for the YES assay by analyzing a series of estradiol (E2) dilutions in LGW. A 38,000 ng/L working solution of E2 was diluted volumetrically eight times so that the final concentrations ranged from 38,000 ng/L to 148 ng/L. Each dilution was then plated in duplicate as described in Appendix B, and analyzed as described in Appendices B and C. Using this method, the PQL used for this study is 15.2 ng/L, assuming 100% recovery (Stanford, 2007).

In order to determine the estrogenicity of the samples, the effective concentration halfway between the baseline and the maximum of the yeast response (EC_{50}) from the samples is compared to the EC_{50} of the yeast response for the estrogen standard (E2). This is done by creating sigmoidal dose-response plots using the program R (R Foundation for Statistical Computing, Vienna, Austria) (instructions in Appendix C). An illustrative plot of an E2 control is presented in Figure 3.8. The maximum color absorbance is 4 because this is the maximum reading obtained by the *Emax* plate reader.

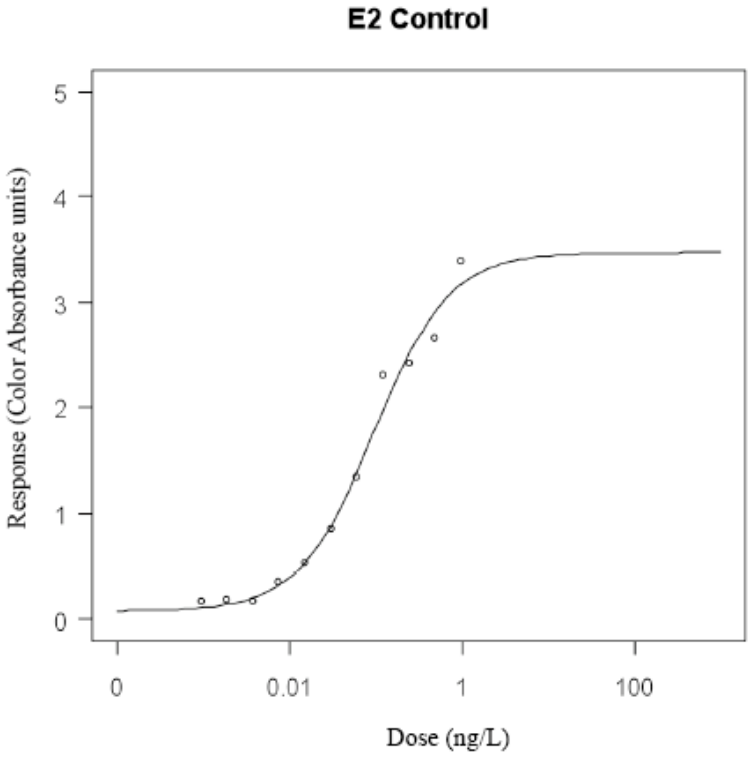


Figure 3.8: Dose-response curve for estradiol (E2) control

Using the EC_{50} values determined from the dose-response curves, the estradiol equivalence (EEQ) and estrogenicity for each sample is determined. The EEQ of the E2 control is equivalent to 1 since it is the control. EEQ is calculated using equation 3.3.

$$\text{EEQ} = \frac{EC_{50}\ of\ E2\ Control}{EC_{50}\ of\ Sample} \qquad \text{(eq. 3.3)}$$

The working solution of the E2 control is 38,000 ng/L, and 20 μL of that is plated, evaporated, and then reconstituted to 250 μL. Therefore, the in-well concentration is actually 3040 ng/L for the first well and is serially diluted by half for each subsequent well. The EEQ for each sample is then regressed back to the original concentration plated into the wells. The original concentration is obtained by dividing the initial E2 concentration (38,000 ng/L)

by a concentration factor of 2500 (the original 500 mL aqueous sample is concentrated to 200 μL) to equal 15.2 ng/L. The EEQ values are multiplied by this value of 15.2 ng/L to obtain a value of estrogenic activity in the aqueous samples. Table 3.15 presents average results from YES assay analyses for all of the samples collected at each location. A total of four YES assays were performed throughout the study period for the dates (8/25/07, 9/22/07, 10/5/07, and 12/7/07). The standard error is a measure of error associated with the regression curve and is indicative of how well the curve fits the data points. It is automatically calculated when processing the data with the program R.

Table 3.15: Average results from YES assay analyses

Sample	EC_{50} (ng/L)	EEQ	Estrogenicity (ng/L) (= EEQ * 15.2 ng/L)	Standard Error (ng/L)
Upstream A	12.6	0.012	0.186	5.2
Effluent A	3.15	0.065	0.986	0.5
DS A1	5.47	0.137	2.08	0.8
DS A2	6.56	0.038	0.574	1.0
Upstream B	4.37	0.081	1.23	0.3
Effluent B	12.6	0.016	0.241	3.1
DS B1	5.21	0.045	0.681	0.6
DS B2	6.92	0.031	0.476	1.5
Upstream C	5.50	0.044	0.668	2.9
Effluent C	5.51	0.053	0.808	0.6
DS C1	5.56	0.067	1.01	1.0
DS C2	6.44	0.114	1.74	0.6
Reservoir 5	5.15	0.067	1.03	1.1

Note: EEQ = Estradiol equivalence

Average EC_{50} values for each sample are within the same order of magnitude, and all are below 13 ng/L, which is orders of magnitude lower than EC_{50} values observed where adverse effects are seen in various trophic levels (see Table 4.1). The calculated estrogenicity in the upstream surface water samples is lower than that of the wastewater

effluent as expected, except for stream B where the upstream estrogenic activity is higher than downstream concentrations. The upstream site on stream B was in a waterfowl impoundment and the fish and bird waste may have led to an increase in estrogenicity. Also, the water at this site was murky and more or less stagnant and this may have impacted the YES assay in some way. All samples were collected using the same method and were treated similarly after collection.

Estrogenicity decreased downstream of the point of wastewater entry in streams A and B, but increased slightly between the first and second downstream points in stream C. The difference in estrogenicity is not consistent between the two downstream points in streams A and B however. Estrogenicity was measured to be about 3.6 times less between the two points in stream A, approximately 1.4 times less in stream B, and 0.58 times greater in stream C. There was still estrogenicity measured in the R5 site (0.689 ng/L), and it is similar to what is observed in the wastewater and stream water samples so it appears that compounds exhibiting estrogenic activity were un-attenuated over this distance.

4. INTERPRETATION

4.1 Antibiotic Occurrence

All of the 26 targeted antibiotics were below detection limits upstream of the wastewater plants, so that detection of these chemicals downstream of wastewater discharge suggests this point source as a significant contribution of antibiotics in this watershed. The antibiotics that were detected most frequently in each of the three streams are:

- Sulfamethoxazole
- Trimethoprim
- Erythromycin
- Lincomycin
- Sulfadimethoxine
- Sulfathiazole
- Ciprofloxacin
- Levofloxacin
- Norfloxacin
- Enrofloxacin

Although there generally were not any temporal trends, the highest concentrations of antibiotics occurred on December 11, 2007. This time period coincides with winter and a higher likelihood for people to succumb to illness and take these antibiotics.

In general, antibiotic concentrations were seen to decrease downstream of the input from the WWTP, as would be expected without additional inputs of non-point source pollution. However, during the 8/9/07 sampling event for streams A and B, the WWTP effluent concentrations for sulfamethoxazole, trimethoprim, lincomycin, sulfathiazole, and sulfadimethoxine were less than the downstream concentrations (see Tables D.1 and D.2 in Appendix D). This can possibly be explained by the fact that the effluent samples were collected in the early morning hours (when effluent flow and possibly concentration of these antibiotics were at their lowest), and the plug of effluent from the previous night hours that had since moved downstream may have had a higher concentration of antibiotics in the water. Wastewater effluent is constantly being introduced into the water column and if there is little to no flow from upstream, as was the case during a large portion of the summer of 2007, the antibiotics could accumulate in a downstream area because the half-lives of these compounds can be several days (Schmitt-Kopplin et al., 1999; Andreozzi et al., 2003; Verma et al., 2007). Desorption from sediments could also be another mechanism for increasing concentrations downstream of the WWTP discharge.

Many of the fluoroquinolones and tetracyclines were either not detected or not found above detection limits (as defined in QA/QC criterion – see Section 3.1.3) in the effluent and in surface water samples. This is expected due to their high K_{oc} values (Table 1.5), which indicate an ability to sorb to solids during wastewater treatment and/or to stream sediments. Because the sulfonamides have lower sorption coefficients, these antibiotics would be expected at higher concentrations in the effluent and surface waters. Stream sediments were not investigated in this study, but sorption coefficients can be useful as a relative predictor of antibiotics present in sediment as compared to those in the aqueous phase.

Antibiotics detected in the drinking water reservoir include sulfamethoxazole, erythromycin, trimethoprim, lincomycin, and enrofloxacin (see Tables D.16 and D.17). The reservoir site sampled most frequently was Reservoir 5 in which 3 of the 26 targeted antibiotics were detected above 20 ng/L in all instances except the 8/25/07 sampling event, which was the first time this site was sampled. As the drought continued, it can be hypothesized that the antibiotics that did not adsorb to the sediment persisted in the stream over a distance of 9-12 miles from the second downstream points in each stream to the Reservoir 5 site, where the influent pipe to the drinking water treatment plant is located. The treated drinking water at this plant was also analyzed in late May 2007, but none of the targeted antibiotics were detected above the PQL.

4.3 Estrogenicity in the Targeted Watershed

Estrogenic activity was detected and ranged from 0.19 to 2.1 ng/L in the surface waters and from 0.24 to 0.99 ng/L in the treated effluent (Table 3.15). Estrogenic activity in upstream samples was detected, indicating that there were compounds in this water that induced an estrogenic response. The average activity of the upstream B samples was much higher than in the downstream samples (1.23 ng/L versus 0.241, 0.681, and 0.476 ng/L in the effluent and sequential downstream samples). Upstream nonpoint source pollution that may contain estrogenic compounds, which could degrade before reaching the WWTP, may be impacting stream B. The upstream A and C samples had a response much lower than downstream samples indicating that the effluent did contribute estrogenic activity. The raw wastewater was not sampled in this study, but if estrogenic compounds had been present it appears that the wastewater treatment processes are effective in removing estrogenic compounds from the aqueous phase. Peer-reviewed studies have quantified estrogenic

activity in domestic wastewater treatment effluent in the range of 1.7 to 49 ng/L, and between 0.04 and 1.5 ng/L in waters downstream from wastewater treatment plants (Hashimoto et al., 2007; Leusch et al., 2006; Baronti et al., 2000). Results in this current study are at the lower end of this range.

Although the biosolids at each of these plants were not sampled, there is a concern that they contain estrogenic activity and that runoff from biosolids may have an impact on surface waters during rain events (Parks, 2006). It must be noted that the biosolids from WWTP A are applied to local farmland and have the ability to runoff into surface waters during rain events, and possibly contaminate the drinking water reservoir. The farmland is located next to a creek (that was not involved in this study), which flows into a river that passes through the southern end of the reservoir. The creek and farmland are more than 25 miles upstream from the reservoir 7 sampling point, which is located in the area where this river entered the watershed. About 32-113 kg/acre/year are applied to 1,044 acres of farmland adjacent to the creek (Parks, 2006).

4.4 Measured Environmental Hazard

A range of EC_{50} values taken from published literature (Table 4.1) have been used to calculate the measured environmental hazards for the surface and wastewaters (Tables 4.2 through 4.6) using equations 1, 2, and 3 as described in Section 1.7.1. Toxicological studies do not use the same species; hence, there is a range of EC_{50} values and species presented in Table 4.1. The European Medicines Agency (EMEA) (2006) states that blue-green algae are the species of choice to conduct toxicological studies related to antibiotics. It was not possible to find a set of toxicological studies in the literature that involve the targeted antibiotics using the same test species let alone studies focused exclusively on blue-green

algae, but the species presented in Table 4.1 are neither the least, nor the most sensitive, and can be considered a set of representative species. Cyanobacteria are found to be one of the most sensitive species to ciprofloxacin. Trimethoprim and sulfathiazole were found to be the least toxic to different species of green algae. Different species will have different EC_{50} values for every chemical investigated, so calculated hazard values will change depending on what EC_{50} value is used, but the assessment factor or 1000 is supposed to account for these intra- and interspecies differences. It is important to note that these hazard values are just estimates and are not meant to be absolute values of a hazard or risk.

Table 4.1: EC_{50} values used for hazard quotient calculations

Antibiotic	EC_{50} (µg/L)	Species	Reference
Sulfamethoxazole	81	*L. gibba* (aquatic plant)	a
Trimethoprim	16 mg/L	*R. salina* (green algae)	b
Sulfamethoxazole/ Trimethoprim	275	*S. capricornutum (green algae)*	c
Norfloxacin	22	*V. fischeri* (luminescent marine bacteria)	d
Ciprofloxacin	5	*M.aeruginosa* (cyanobacteria)	e
Erythromycin	36.6	*S. capricornutum* (green algae)	c
Sulfadimethoxine	230	*S. capricornutum* (green algae)	c
Lincomycin	23.8	*D. magna* (crustacean cladocera)	f
Levofloxacin	81	*L. gibba* (aquatic plant)	a
Enrofloxacin	840	*V. fischeri* (luminescent marine bacteria)	g
Sarafloxacin	150	*S. capricornutum* (green algae)	b
Sulfathaizole	17.2 mg/L	*C. vulgaris* (green algae)	h
Tetracycline	400	*L. gibba* (aquatic plant)	a

a. Brain et al. (2004); b. Holten Lützhøft et al. (1999); c. Eguchi et al. (2004); d. Backhaus et al. (2000); e. Halling-Sørensen (2000); f. Isodori et al. (2005); g. Hernando et al. (2006); h. Baran et al. (2006).

Tables 4.2 through 4.6 present calculated hazard values using the maximum measured concentrations for each stream and reservoir sampling point during the entire study period. There are no specific units for the hazard value, and as explained in Section 1.7.1, a "low hazard" ranges from 0.5 to 2, a "medium hazard" from 2 to 10, and a "high hazard" from 10 +. Values shown in red represent a "high hazard", those in orange represent a "medium hazard" and those in blue represent a "low hazard". Only those antibiotics that were detected are presented in each of the tables, and the environmental hazard for the combined effect of sulfamethoxazole and trimethoprim has also been calculated. Eguchi et al. (2004) found an enhanced growth inhibitory effect when *S. capricornutum* (green algae) were exposed to a mixture of these two antibiotics (EC_{50} = 0.275 mg/L) when compared to the adverse effect of sulfamethoxazole and trimethoprim alone (1.53 mg/L and 80.3 mg/L, respectively). Synergistic effects of antibiotics and other PhACs are largely unknown, but the possibility is there.

Sulfamethoxazole poses the largest calculated environmental hazard in all three streams and both downstream locations because it was detected at the highest concentrations. Calculated hazards are found to decrease downstream, but erythromycin, ciprofloxacin, and the combined effects of sulfamethoxazole and trimethoprim in stream A have a calculated "medium hazard" in DS A2, just 0.9 miles from the reservoir. All other compounds are found to have a "low hazard" in the DS A2 site (Table 4.2).

Table 4.2: Maximum calculated hazard quotients for Stream A

Antibiotic	EC_{50} (µg/L)	DS A1		DS A2	
		MEC (µg/L)	Hazard	MEC (µg/L)	Hazard
Sulfamethoxazole	81	2.86	35	1.6	20
Trimethoprim	16000	0.238	0.01	0.11	0.007
SMX + TMP	275	3.10	11	1.7	6.2
Sulfadimethoxine	230	0.037	0.16	0.04	0.18
Sulfathiazole	172000	0.436	0.003	0.33	0.002
Erythromycin	36.6	0.316	8.6	0.21	5.7
Lincomycin	23.8	0.051	2.1	0.017	0.71
Ciprofloxacin	5	0.104	21	0.043	8.6
Levofloxacin	81	0.171	2.1	0.033	0.41
Enrofloxacin	840	0.010	0.01	0.049	0.06
Tetracycline	400	0.214	0.54	0.001	0.001

Note: SMX + TMP = sulfamethoxazole + trimethoprim; MEC = measure environmental concentration; red = high hazard (10+), orange = medium hazard (2-10), and blue = low hazard (0.5-2); na = not applicable.

In stream B, ciprofloxacin and sulfamethoxazole pose the greatest hazard at the DS B1 site, with ciprofloxacin having a much larger calculated hazard value (Table 4.3). Interestingly, the maximum detected concentration for sulfamethoxazole was found to be higher at the DS B2 site than at the DS B1 site. Because the concentration is higher, the risk is correspondingly higher; the same occurs for erythromycin. The combined effect of SMX + TMP is found to have a "medium hazard" in the DS B2 site; a greater hazard than at the DS B1 site due to the increase in concentration of sulfamethoxazole. Lincomycin and levofloxacin also pose a "medium hazard" at the DS B1 site, but the hazard decreases downstream to a "low hazard" for lincomycin. Despite the decrease in concentration of ciprofloxacin (84 ng/L to 18 ng/L), it still poses a "medium hazard" to the environment at the DS B2 site. The DS B2 site is a waterfowl impoundment that supports a large fish population that is caught by some of the local population. Although very few toxicological

studies have investigated plants and animals at higher trophic levels, adverse effects seen in algae, bacteria and plants may be seen in fish as well. DS B2 is about 2 miles from the entrance to the reservoir, so there is still a considerable distance during which the compounds could adsorb to sediments or undergo photodegradation, therefore decreasing the concentration and consequently, the hazard.

Table 4.3: Maximum calculated hazard quotients for Stream B

Antibiotic	EC_{50} (µg/L)	DS B1		DS B2	
		MEC (µg/L)	Hazard	MEC (µg/L)	Hazard
Sulfamethoxazole	81	1.34	17	1.9	24
Trimethoprim	16000	0.65	0.04	0.2	0.015
SMX + TMP	275	1.99	1	2.2	7.9
Sulfadimethoxine	230	0.20	0.85	0.09	0.40
Sulfathiazole	172000	0.20	0.001	0.13	0.001
Erythromycin	36.6	0.15	0.4	0.10	2.7
Lincomycin	23.8	0.01	3.5	0.02	1.04
Ciprofloxacin	5	0.08	50	0.02	3.6
Levofloxacin	81	0.25	3.7	0.17	2.07
Sarafloxacin	150	0.09	0.1	0.13	0.88
Enrofloxacin	840	0.30	0.36	0.01	0.01
Tetracycline	400	0.01	0.03	0.02	0.040

Note: SMX + TMP = sulfamethoxazole + trimethoprim; MEC = measure environmental concentration; red = high hazard (10+), orange = medium hazard (2-10), and blue = low hazard (0.5-2); na = not applicable.

The hazard for sulfamethoxazole in stream C (Table 4.4) is lower than in the downstream sites of stream A and B, but there is still a “high hazard” at a much lower effluent flow rate (~4 MGD vs. ~10 MGD and ~16 MGD for streams A and B, respectively). Erythromycin, lincomycin and ciprofloxacin pose a “medium hazard” at DS C1, and the hazard remains the same for all three antibiotics except for lincomycin at the DS C2 site. The DS C2 site is also a waterfowl impoundment that many locals fish at, and is approximately 2.4 miles from the reservoir.

Table 4.4: Maximum calculated hazard quotients for Stream C

Antibiotic	EC_{50} (µg/L)	DS C1		DS C2	
		MEC (µg/L)	Hazard	MEC (µg/L)	Hazard
Sulfamethoxazole	81	1.38	17	1.2	15
Trimethoprim	16000	0.05	0.003	0.0	0.002
SMX + TMP	275	1.43	5.2	1.2	4.5
Sulfadimethoxine	230	0.30	1.3	0.11	0.48
Sulfathiazole	172000	< 20	NA	< 20	NA
Erythromycin	36.6	0.15	4.2	0.09	2.4
Lincomycin	23.8	0.16	6.8	0.02	1.02
Ciprofloxacin	5	0.01	2.0	0.02	3.4
Levofloxacin	81	0.02	0.21	< 20	NA
Sarafloxacin	150	0.01	0.08	0.01	0.07
Enrofloxacin	840	0.004	0.005	0.24	0.29
Tetracycline	400	< 20	NA	< 20	NA

Note: SMX + TMP = sulfamethoxazole + trimethoprim; MEC = measure environmental concentration; NA = not applicable; red = high hazard (10+), orange = medium hazard (2-10), and blue = low hazard (0.5-2).

Calculated hazard values for the antibiotics present above quantitation limits at the reservoir 5 site (Table 4.5) are low. The same is true for the rest of the sites sampled in the reservoir except for those at locations reservoir 6 and 7 (Table 4.6), where sulfamethoxazole presents a "medium hazard". Erythromycin, lincomycin, and ciprofloxacin all pose a "medium hazard" at the reservoir 7 site. Reservoir 6 is just downstream from the drinking water treatment plant influent pipe and reservoir 7 is at the very southern end of the reservoir where a major river flows in. About three WWTPs discharge treated effluent into this river and several antibiotics are still present at high levels as the river flows into the reservoir. Because the hydraulic retention time at this area of the reservoir is about 4 days, the pollutants are most likely carried through the reservoir and downstream.

Table 4.5: Maximum calculated hazard quotients for Reservoir 5

Antibiotic	EC_{50} (µg/L)	8/25/07		9/22/07		10/5/07		12/6/07	
		MEC (µg/L)	Hazard	MEC (µg/L)	Hazard	MEC (µg/L)	Hazard	MEC (µg/L)	Hazard
Sulfamethoxazole	81	0.013	0.16	51	0.63	64	0.79	38	0.47
Trimethoprim	16000	0.37	0.02	16	0.001	< 20	NA	7	0.00
SMX + TMP	275	0.383	1.4	67	0.244	64	0.23	45	0.16
Sulfadimethoxine	230	< 20	NA	< 20	NA	< 20	NA	4	0.02
Erythromycin	36.6	0.015	0.41	37	1.01	44	1.2	6	0.16
Lincomycin	23.8	< 20	NA	24	1.01	44	1.8	21	0.88
Ciprofloxacin	5	< 20	NA	11	2.20	4	0.80	3	0.60
Levofloxacin	81	0.010	0.12	1	0.012	7	0.09	3	0.04
Enrofloxacin	840	< 20	NA	0.3	0.0004	2	0.002	1	0.001

Table 4.6: Maximum calculated hazard quotients for reservoir sites on 12/7/07

Antibiotic	EC_{50} (µg/L)	Reservoir 1		Reservoir 2		Reservoir 3		Reservoir 4	
		MEC (µg/L)	Hazard	MEC (µg/L)	Hazard	MEC (µg/L)	Hazard	MEC (µg/L)	Hazard
Sulfamethoxazole	81	0.014	0.17	0.064	0.79	0.06	0.74	0.144	1.8
Trimethoprim	16000	< 20	NA	0.037	0.002	0.012	0.0008	0.03	0.002
SMX + TMP	275	0.014	0.05	0.101	0.37	0.072	0.26	0.174	0.63
Sulfadimethoxine	230	< 20	NA	< 20	NA	< 20	NA	< 20	NA
Erythromycin	36.6	0.015	0.41	0.0002	0.005	0.013	0.36	0.021	0.57
Lincomycin	23.8	0.015	0.63	0.001	0.04	0.021	0.88	0.023	0.97
Ciprofloxacin	5	0.005	1.0	< 20	NA	0.001	0.20	0.005	1.0
Levofloxacin	81	0.006	0.07	< 20	NA	0.003	0.04	0.002	0.02
Enrofloxacin	840	0.014	0.02	< 20	NA	0.062	0.07	0.051	0.06
Norfloxacin	22	0.006	0.27	< 20	NA	0.064	2.9	< 20	NA
Tetracycline	400	0.03	0.08	< 20	NA	0.004	0.01	0.003	0.008

Antibiotic	EC_{50} (µg/L)	Reservoir 5		Reservoir 6		Reservoir 7	
		MEC (µg/L)	Hazard	MEC (µg/L)	Hazard	MEC (µg/L)	Hazard
Sulfamethoxazole	81	0.038	0.47	0.223	2.8	0.238	2.9
Trimethoprim	16000	0.007	0.0004	0.018	0.001	0.046	0.003
SMX + TMP	275	0.045	0.16	0.241	0.88	0.284	1.0
Sulfadimethoxine	230	0.004	0.02	< 20	NA	< 20	NA
Erythromycin	36.6	0.006	0.16	0.012	0.33	0.075	2.0
Lincomycin	23.8	0.021	0.88	0.007	0.29	0.059	2.5
Ciprofloxacin	5	0.003	0.60	0.0003	0.06	0.035	7.0
Levofloxacin	81	0.003	0.04	0.004	0.05	0.045	0.56
Enrofloxacin	840	0.001	0.001	0.005	0.01	0.038	0.05
Norfloxacin	22	< 20	NA	0.001	0.05	0.051	2.3
Tetracycline	400	< 20	NA	< 20	NA	< 20	NA

Antibiotics have been detected in the aqueous phase in this watershed at the ng/L level but not at the acute level (mg/L) likely to induce lethal toxicity to aquatic organisms or to have a significant impact on the growth of plants and bacteria. Antibiotic residues in the ng/L range may contribute to the widespread resistance of bacterial pathogens and post-therapeutic effects, especially from exposure to antibiotics in stream sediments. The toxicity studies cited in this section involve single high-dose exposures over a short time period, and it is difficult to extrapolate the effects resulting from continuous, sub-therapeutic exposure, which is the case in this watershed. More toxicological studies are needed to investigate synergistic and antagonistic effects as well as continuous low-level exposure of PhACs and the vast array of other chemicals in treated wastewater effluent before risk can be thoroughly categorized.

4.5 Rates of Antibiotic Removal from Watershed

Antibiotics were found to decrease as a plug of effluent moved downstream during most sampling events. Removal rates using a first order decay rate are calculated here for sulfamethoxazole and erythromycin, the two most frequently detected antibiotics, using measured concentrations in each stream and for each sampling event to determine approximately how long either of these pollutants remains in the aqueous phase. These removal rates were only calculated from their point of entry to the second downstream point on each stream because there were no additional inflows of water in these areas.

A mass balance is a mathematical accounting of the sources (wastewater effluent) and sinks (adsorption to sediments, photodegradation) of a substance within a system, such as a water body. A mass balance model for a water body is useful to help understand the relationship between the loadings of a pollutant and the levels in the water and sediments.

The law of conservation of matter states that matter is conserved, and if the amount of material that enters a chain of processes is known and the amounts that are added or subtracted are also known, then the decay rate can be estimated. The mass balance can be written as:

$$\frac{\Delta Mass}{\Delta t} = \text{(flux in - flux out of SMX) - first order reaction rate} \qquad \text{(eq 4.1)}$$

Often the reaction rate is due to adsorption of an antibiotic to sediments, biological or photo-degradation, and is also known as a decay rate. The term "decay rate" is commonly used in this situation, but for clarification, the antibiotics are not truly decaying, but are instead being removed from the aqueous phase by attenuation processes such as adsorption to stream sediment, photodegradation, and/or biodegradation. Hence, the term "decay" is being replaced with "removal".

The removal rate is often modeled as a first order reaction, which means that the amount removed is proportional to the amount present at any time (equation 4.2).

$$C = C_0 e^{-kx} \qquad \text{(eq. 4.2)}$$

where the first order reaction rate, *k*, describes the rate of antibiotic removal in surface waters, and is what will eventually be solved for.

Equating the mass balance equation 4.1 for a certain volume V gives:

$$\frac{\Delta VC}{\Delta t} = QC - Q'C' - kVC = -\Delta QC - kVC \qquad \text{(eq. 4.3)}$$

where V is the volume of a stream at a specific sampling point, Q is the effluent flow, C is the concentration of SMX entering the stream, and Q' and C' are the flow and concentration of SMX downstream of the WWTP. Dividing equation 4.3 by the volume of the stream at

the sampling point (V=A*dx, where A is the wet cross sectional area of the stream reach and dx is its length), and taking the limit for Dt→0 and Dx→0, equation 4.4 is obtained. The purpose of this equation is to identify $\frac{\partial C}{\partial x}$.

$$\frac{\partial C}{\partial t} = -\frac{1}{A}\frac{\partial QC}{\partial x} - kC = -\frac{Q}{A}\frac{\partial C}{\partial x} - \frac{C}{A}\frac{\partial Q}{\partial x} - kC \qquad \text{(eq. 4.4)}$$

It was assumed during this study that the stream flows were at a steady state, or $\frac{\partial C}{\partial t} = 0$, due to a lack of additional flow from rainfall. When substituting $\frac{\partial C}{\partial t} = 0$ into equation 4.4 and solving for $\frac{\partial C}{\partial x}$, equation 4.5 is obtained.

$$\frac{\partial C}{\partial x} = -\left(\frac{kCA}{Q} + \frac{C\partial C}{Q\partial x}\right) = -\left(\frac{k}{u} + \frac{1}{Q}\frac{\partial Q}{\partial x}\right)C \qquad \text{(eq. 4.5)}$$

where $u = \frac{Q}{A}$ is the velocity of water passing by USGS monitoring stations, which were located at a downstream location on each stream where samples were collected for antibiotic analysis. The term $\frac{1}{Q}\frac{\partial Q}{\partial x}$ accounts for the dilution of the upstream flow where the antibiotics were below the PQL. On several sampling dates, the upstream flow was near 0 cubic feet per second (cfs or ft^3/s). When this occurred, the upstream flow was assumed to be equivalent to the downstream flow in order to fit the model.

Since the distances between the sampling points were relatively short and there was no rainfall during the study period, it is reasonable to assume that $\frac{1}{Q}\frac{\partial Q}{\partial x}$ is constant.

Therefore, integrating equation 4.5 and solving for C leads to equation 4.6, which is equivalent to equation 4.2 presented earlier.

$$C = C_o \exp -\left(\frac{k}{u} + \frac{1}{Q}\frac{\partial Q}{\partial x}\right)x \quad = C_o \exp(-Kx) \qquad \text{(eq. 4.6)}$$

where $K = \left(\frac{k}{u} + \frac{1}{Q}\frac{\partial Q}{\partial x}\right)$. Prior to solving for *K*, the concentration of the antibiotic in the effluent, C_o, must be converted into a stream concentration that takes into account the dilution effect from the stream that the effluent is flowing into. The stream flow must be greater than the effluent flow (i.e. $Q_0 > Q_{outlet}$). This is done using equation 4.7.

$$C_o = \frac{C_{outlet} * Q_{outlet}}{Q_0} \qquad \text{(eq. 4.7)}$$

In order to determine *K* in equation 4.6, the natural logarithm of the concentration of the antibiotic at the sampling locations (x_0 or the point of effluent discharge, x_1, and x_2) is divided by the initial concentration of the antibiotic in the effluent, C_0, and plotted against the distance (x) from the WWTP to each of the downstream sampling locations is plotted (Figure 4.1). The slope is equivalent to *K* in units of ft^{-1}.

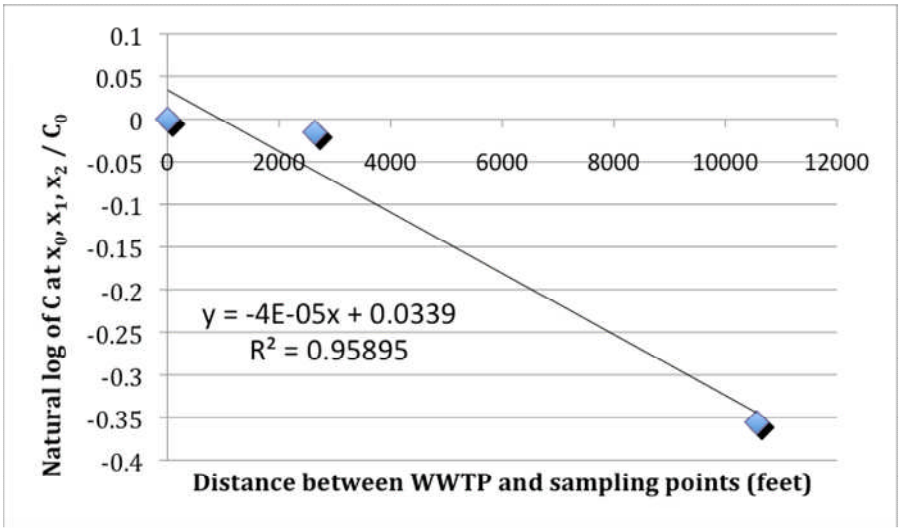


Figure 4.1: Illustrative plot to determine K for Stream A on 10/5/07

Crude cross-sectional areas of the streams at each of their respective sampling points were determined using a measuring tape and yardstick. The dilution flow contribution per unit distance along the creek $\frac{\partial Q}{\partial x}$ and the average velocity (u) of the water for each stream and day of sampling was then calculated. This value is then plugged into equation 4.8, where k is the first order removal rate. This equation is obtained by solving for k in equation 4.6.

$$k = \left(K - \frac{1}{Q}\frac{\partial Q}{\partial x} \right) u \qquad \text{(eq. 4.8)}$$

The half-life of the antibiotics is then determined using equation 4.9.

$$t_{1/2} = \frac{\ln(2)}{k} \qquad \text{(eq. 4.9)}$$

Removal rates and half-lives ($t_{1/2}$) have been calculated for sulfamethoxazole and erythromycin in all three streams for each sampling date because these were the only two antibiotics detected at every sampling point on every sampling day. As mentioned earlier,

there were some instances where concentrations increased downstream of the WWTP. Decay rates and half-lives were not presented on days where this effect occurred. Table 4.7 presents sulfamethoxazole decay rates (*k*) in units of s^{-1}, and Table 4.8 presents the corresponding $t_{1/2}$ values.

Sulfamethoxazole is observed to undergo removal from the aqueous phase at a slower rate in stream C than in streams A and B (an average of 3.9×10^{-6} s^{-1} versus 1.7×10^{-5} s^{-1} and 1.1×10^{-4} s^{-1}). Stream characteristics play a large part in the fate of pollutants. Stream B flows through a swampy area where there is a greater chance for adsorption to stream sediments. Stream C is deeper and wider than the other two streams, and pollutants would be expected to undergo less photodegradation and adsorption to stream sediments. The size and shape of stream A falls in-between the other two.

Table 4.7: Removal rates (k) for sulfamethoxazole (SMX)

Date	Stream A SMX k (s^{-1})	Stream B SMX k (s^{-1})	Stream C SMX k (s^{-1})
8/9/07	na	7.15E-05	3.85E-06
8/25/07	3.38E-05	5.49E-05	2.13E-06
9/22/07	1.41E-05	1.16E-04	1.65E-06
10/3/07	8.78E-06	1.95E-04	5.65E-06
12/11/07	1.23E-05	1.29E-04	6.15E-06
Mean	1.72E-05	1.13E-04	3.88E-06
STDEV	1.12E-05	5.51E-05	2.02E-06
Variance	1.26E-10	3.03E-09	4.08E-12
95% CI	1.10E-05	5.40E-05	1.98E-06

Note: na = not applicable; STDEV = standard deviation; CI = confidence interval

Data is not presented for the stream A 8/9/07 sampling event because SMX was not found to decrease downstream and, therefore, a removal rate was not calculated. The half-

lives increase slightly in stream A as the study progresses (0.24 days on 8/25 vs. 0.65 days on 12/11). The half-lives do not vary greatly between streams, but stream C was shown to have the greatest half-lives. It seems as if seasonal differences may have an influence on the half-lives of SMX because they are not consistent throughout the period of the study.

Rate of removal and half-lives for erythromycin are presented in Tables 4.9 and 4.10. Erythromycin was found to increase downstream of the WWTP on 10/3/07 but the removal rate was not calculated because it was not found to decrease downstream. The decay rates of erythromycin are within the same order of magnitude as those for sulfamethoxazole and the same pattern is seen with respect to the order of removal by stream (i.e k for stream C < A < B).

Table 4.8: Half-life ($t_{½}$) values for sulfamethoxazole in days

Date	Stream A $t_{1/2}$ (day)	Stream B $t_{1/2}$ (day)	Stream C $t_{1/2}$ (day)
8/9/07	na	0.11	2.1
8/25/07	0.24	0.15	3.8
9/22/07	0.57	0.07	4.9
10/3/07	0.91	0.04	1.4
12/11/07	0.65	0.06	1.3
Mean	0.59	0.09	2.7
STDEV	0.28	0.04	1.6
Variance	0.08	0.002	2.4
95% CI	0.27	0.041	1.5

Note: na = not applicable; STDEV = standard deviation; CI = confidence interval

Table 4.9: Removal rates (k) for erythromycin

Date	Stream A k (s^{-1})	Stream B k (s^{-1})	Stream C k (s^{-1})
8/9/07	5.13E-05	1.82E-05	9.64E-07
8/25/07	2.33E-05	3.26E-05	2.98E-06
9/22/07	1.62E-05	9.59E-05	6.23E-06
10/3/07	1.02E-05	1.63E-04	na
12/11/07	6.21E-06	2.68E-04	4.45E-06
Mean	1.40E-05	1.16E-04	3.66E-06
STDEV	7.44E-06	1.03E-04	2.23E-06
Variance	5.54E-11	1.05E-08	4.99E-12
95% CI	7.29E-06	1.01E-04	2.19E-06

Note: na = not applicable; STDEV = standard deviation; CI = confidence interval

The erythromycin half-life values (Table 4.10) calculated for stream A are found to increase with time, while those for stream B decrease slightly. The half-life calculated using the stream C 8/25/07 data shows a much larger value than for the other sampling events, and this skews the average half-life so that it is much higher than those obtained from the three other sampling events, and also contributes to the very large variance for this data set. This is most likely due to the large decrease in concentration from DS C1 to DS C2 (316 ng/L to 40 ng/L).

Table 4.10: Half-live ($t_{½}$) values for erythromycin

Date	Stream A $t_{1/2}$ (day)	Stream B $t_{1/2}$ (day)	Stream C $t_{1/2}$ (day)
8/9/07	0.16	0.44	8
8/25/07	0.34	0.25	2.7
9/22/07	0.50	0.08	1.3
10/3/07	0.78	0.05	na
12/11/07	1.3	0.03	1.8
Mean	0.61	0.17	3.5
STDEV	0.44	0.17	3.2
Variance	0.20	0.03	11
95% CI	0.39	0.15	2.8

Note: na = not applicable; STDEV = standard deviation; CI = confidence interval

The half-lives for both sulfamethoxazole and erythromycin seem to be the most consistent from month to month in stream B, whereas those for streams A and C vary quite a bit. Published half-life values for sulfamethoxazole and erythromycin in a natural, aqueous setting are scarce. In a lab-controlled study, Andreozzi et al. (2003) calculated the half-life of SMX in salt and organic-free bi-distilled water (buffered to 5.5 with NaH_2PO_4 and KH_2PO_4 salts) caused by photodegradation to be 2.4 days in the presence of nitrates, and 3.3 days in the presence of humic acids. Humic acids are thought to act as a photosensitizer towards sulfamethoxazole, and there may be a higher proportion of humic acids in stream C that may be causing the increased persistence of both antibiotics (Andreozzi, et al. 2003). This same study found other pharmaceutically active compounds to have degradation rates of 5.0, 10.6, and 16.8 days for diclofenac, ofloxacin, and propranolol, indicating that sulfamethoxazole more readily degraded in the presence of UV light. In relation to this current study, these literature findings suggest that even though sulfamethoxazole was detected at the highest concentrations among all antibiotics studied, it undergoes photo-degradation relatively quickly and will therefore be less likely to adsorb to stream sediments. Compounds that are less likely to undergo photo and biodegradation and that also have high sorption coefficients are expected to be found in the stream sediment. Published studies investigating removal rates and half-life values for erythromycin in terms of a natural setting were not found and so it is difficult to compare the values calculated here.

4.6 BMEGUI Maps

As described in Section 1.8, the BME framework is a validated model that can be used to estimate concentrations of environmental pollutants in different matrices (Christakos and Serre, 2000a and b; Serre et al., 2003; Serre et al., 2004). In this current study, the

space/time random field (S/TRF) of sulfmethoxazole, SMX(p), is used to represent the uncertainties and natural variability associated with sulfamethoxazole at any space/time point p = (s, t) of interest, where s is the geographical coordinate, and t is the time. The BME space/time mapping analysis provides a rigorous mathematical framework used to process the physical data for SMX in this watershed.

The mean function $m_x(p) = \overline{X(p)}$ of the S/TRF characterizes trends and systematic structures in space and time, while the covariance function in equation 4.1 expresses relevant correlations;

$$c_x(p, p') = \overline{[X(p) - \overline{X}(p)][X(p') - \overline{X}(p')]} \qquad \text{(eq. 4.1)}$$

where: c_x denotes the covariance
p and p' are spatial points
$X(p)$ denotes random fields
a subscripted x represents random variables

The BME space/time analysis framework can be used to predict concentrations of SMX at unmeasured points, $p_{K,}$ given the total knowledge base available. The total knowledge base available, K, is broken down into two subsequent knowledge bases: the general knowledge base, G, and the site-specific knowledge base, S, so that $K = G \cup S$. Knowledge base G is general in that it is based on watershed characteristics such as its space/time moments, relevant flow and transport laws, and any other assumptions that may apply. The site-specific knowledge base, S, represents measured data obtained at certain locations within the watershed. S can be further broken down into hard and soft data, but only hard data were included in the model used here (see concentrations for SMX in Appendix D). Hard data are

accurate measurements, whereas soft data are probabilistic descriptions of the possible values for SMX at certain points.

The BME model consists of three main approaches, and more detailed information on the theory and equations behind the BME model is provided in Christakos et al. (2002).

1) Structural stage: *G*, which refers to values of the pollutant in the watershed at all mapping points is considered, where $p_{map} = (p_{hard}, p_{soft}, p_k)$.

2) Specificatory stage: The *S* knowledge base is then expressed in terms of exact measurements χ_{hard} and a variety of soft data χ_{soft}. Then, $\chi_{map} = (\chi_{hard}, \chi_{soft}, \chi_k)$.

3) Integration stage: The total knowledge base, $K = G \cup S$, is assimilated by means of an operation Bayesian conditionalization rule that produces a posterior density function (PDF), f_K, providing a full stochastic description of SMX at any estimation point p_k of interest within the watershed.

The dataset used in this model includes all measured SMX data (n = 54) in treated wastewater effluent and surface water samples during the entire study period from August 9, 2007 through December 11, 2007 at a total of 16 monitoring locations (see Figure 2.1 for sampling locations within the watershed). Data measured upstream of the WWTPs were not included in the model because SMX was below quantitation at these locations, and including these points would have temporally skewed the dataset.

An exploratory data analysis was conducted to determine how the data are distributed in the raw and log form (Figure 4.2). Relevant statistics for the dataset in both raw and log form can be found in Table 4.11. The natural log (ln) of the data is a more normal shape

when compared to the raw data, and was chosen for further analysis within the model; all concentrations are herein reported as ln-ng/L.

Table 4.11: Statistics for SMX raw and natural log distribution

Statistics	Raw (ng/L)	Natural Log
Mean	1001	6.29
Std. Deviation	892	1.36
Skewness	0.861	-0.739
Kurtosis	-0.092	-0.395

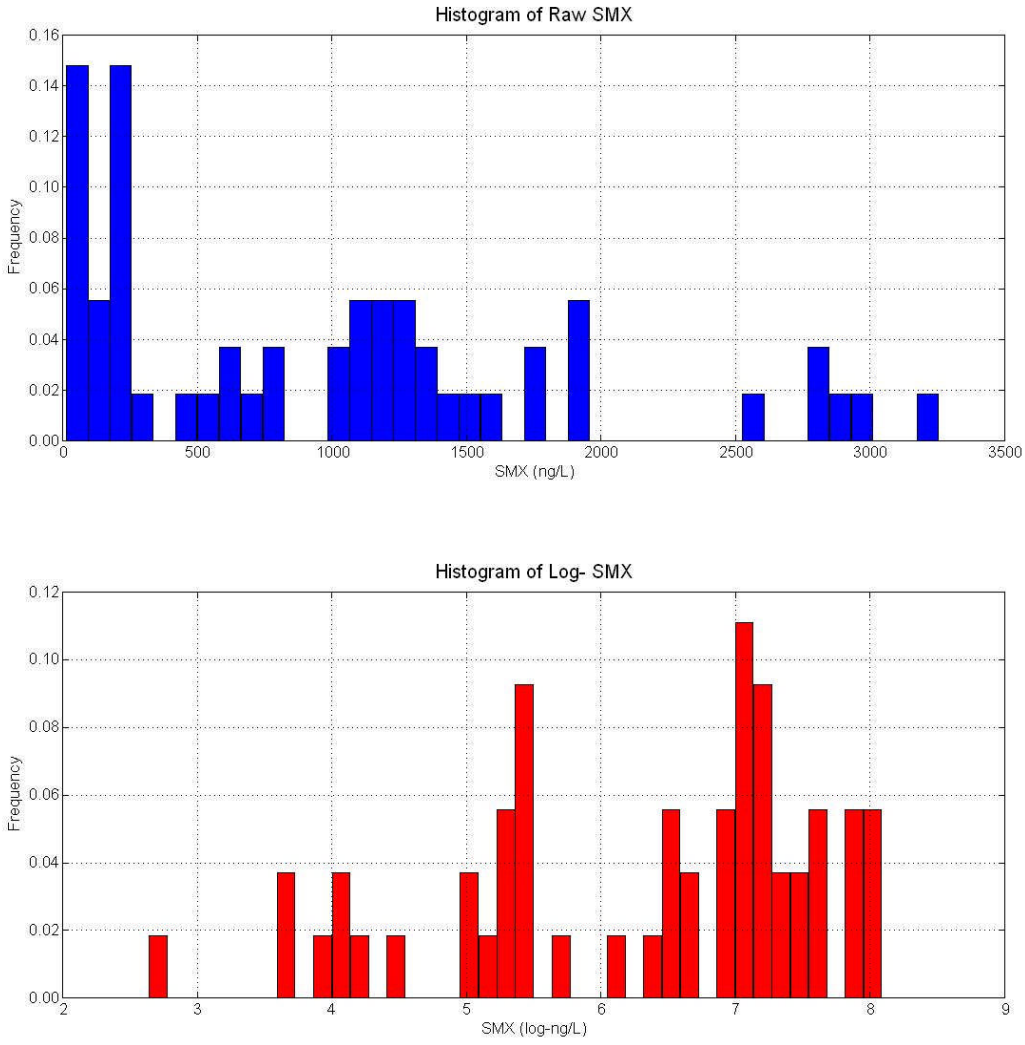


Figure 4.2: Histogram of SMX (ng/L) measurements in raw and natural log form

Temporal trends for SMX in the final effluent of each of the three WWTPs during the study period are presented in Figures 4.3. The points on the graph correspond to actual sampling events. Day 1 is equivalent to August 1, 2007. Sampling began on August 9, 2007 and ended December 11, 2007. The concentration of SMX in the effluent mostly increases over time during the study period at all three WWTPs, but there is a significant drop in concentration at WWTP B and C during late August and September.

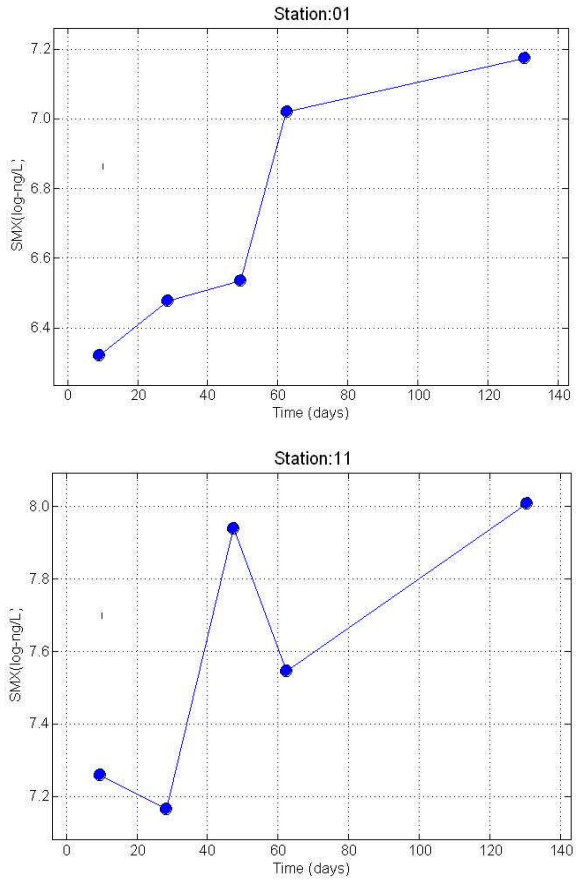


Figure 4.3: Temporal trend of SMX in ln-ng/L in effluent for WWTP A (Station 01), WWTP B (Station 11) and WWTP C (Station 13)

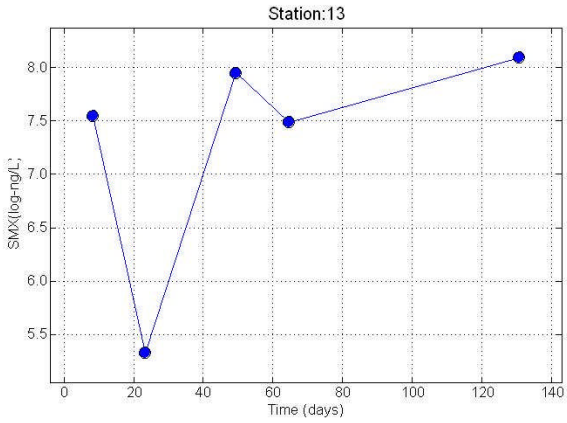


Table 4.3 (cont.): Temporal trend of SMX in ln-ng/L in effluent for WWTP A (Station 01), WWTP B (Station 11) and WWTP C (Station 13)

The temporal trend for all of the stream and reservoir measurements remains steady during August to October, but then decreases by half the concentration just before the final sampling event in early December when the concentration spikes up again (Figure 4.4). The mean trend is shown as the dashed line, and it essentially smoothes the spatiotemporal fluctuations of the data. Overall, the concentration of sulfamethoxazole fluctuates, but there do not appear to be any definite trends during the study period.

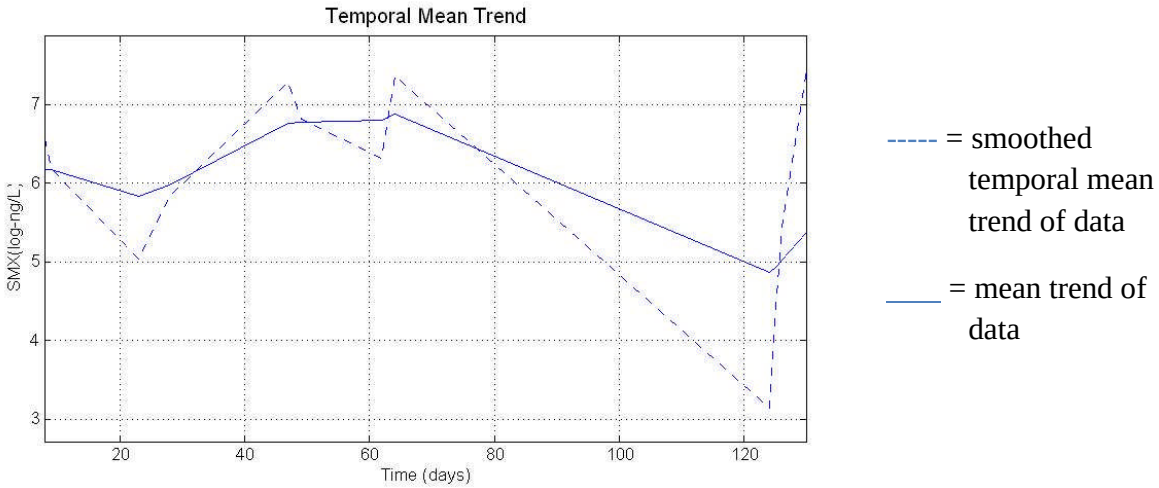


Figure 4.4: Temporal mean trend at all locations for SMX in ln-ng/L

A space/time covariance analysis has been used to calculate the spatial and temporal components of the covariance of the residual of the data since its mean trend was removed. The residual data are assumed to be homogeneous and stationary, which implies that the covariance between two space/time points $\boldsymbol{p}=(\boldsymbol{s},t)$ and $\boldsymbol{p}'=(\boldsymbol{s}',t')$ is only a function of the spatial lag (i.e. the spatial distance) $r=\|\boldsymbol{s}-\boldsymbol{s}'\|$ and time lag (i.e. the time difference) $\tau=|t-t'|$ between these two space/time points. The covariance $c(\boldsymbol{p},\boldsymbol{p}')$ between points $\boldsymbol{p}$ and $\boldsymbol{p}'$ can be written as:

$$c(\boldsymbol{p},\boldsymbol{p}') = c(r=\|\boldsymbol{s}-\boldsymbol{s}'\|,\ \tau=|t-t'|),$$

where r is the spatial lag and τ is the temporal lag

There are two steps in modeling the covariance. First the covariance values for different spatial and temporal lags were estimated and are referred to as experimental covariance values. Then a covariance model was fitted to the experimental covariance values using three space/time separable components as shown below:

$$\begin{aligned} c(r,\tau) &= \sum_{i=1}^{N} c_{0i}\, c_{ri}(r) c_{ti}(\tau) \\ &= 0.65\exp(-\frac{3r}{0.12})\exp(-\frac{3r}{13}) + 0.24\exp(-\frac{3r}{5.0})\exp(-\frac{\pi\tau}{25}) + 0.135\exp(-\frac{3r}{0.99})\exp(-\frac{3r}{60}) \end{aligned}$$

More detailed information on what each of these terms represents can be found in Christakos et al. (2002).

The spatial and temporal components of the data are presented in Figure 4.5. The y-axis in both plots represents the covariance of the data set. The x-axis in the first plot represents the spatial lag in degrees, and represents the temporal lag in days in the second plot. The spatial lag is more or less the distance that the data correspond to each other and

the temporal lag is equivalent to how well the data correspond to each other over time. For reference, 1 degree is approximately 111 km, so 0.01 degrees is approximately 1.1 km.

The data correlate spatially very well within about 0.03 degrees, or about 3 km. This indicates that the model can estimate concentrations accurately within this distance around actual sampled points assuming that the measured data is similar to days that were not sampled during the study period, but estimated concentrations outside this range may not be as reliable. There appears to be a slight temporal trend as concentrations are seen to fluctuate up and down about every ten days.

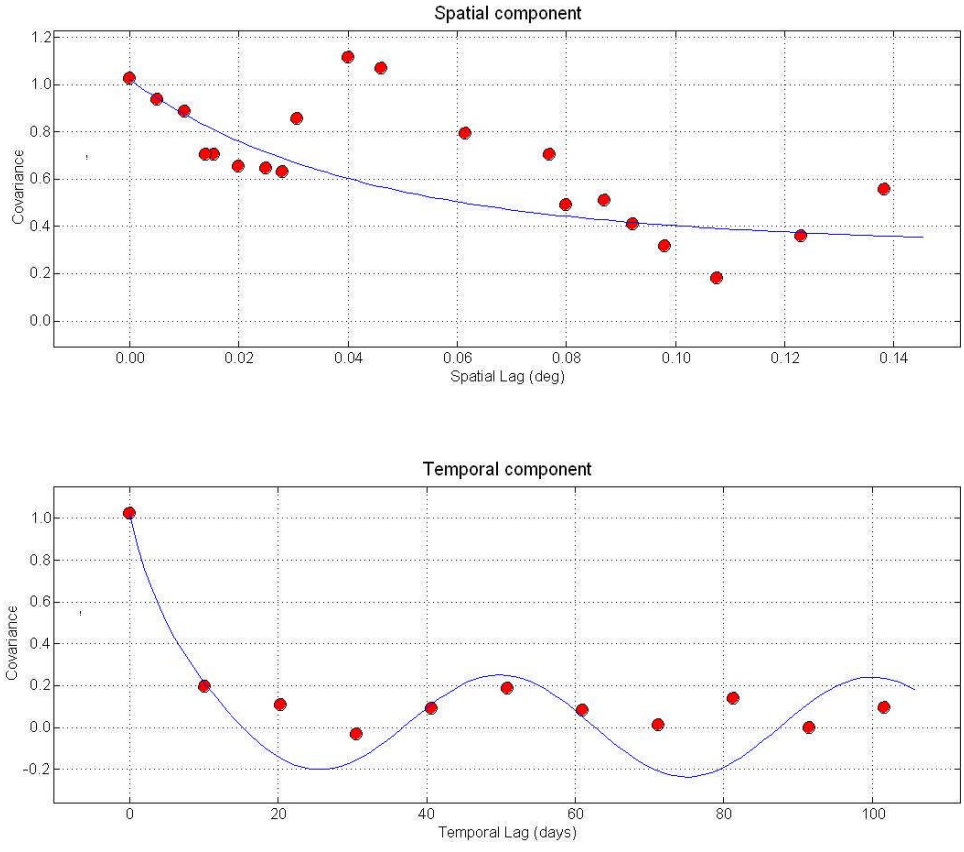


Figure 4.5: Spatial and temporal covariance of the mean trend removed ln-transformed SMX data

Using the BME method, the general knowledge base consisting of the mean trend and covariance model is combined with the site-specific knowledge base consisting of all of the SMX data to obtain a BME posterior PDF which estimates the concentration of SMX at any point within the whole watershed at various days throughout the study period. Maps have been produced for various days during the study showing the targeted area and estimated concentrations in the form of color shading, where a dark red is equivalent to the highest concentrations and a dark green is equivalent to the lowest concentrations (Figure 4.6). The upper and lower bounds of the color shades are provided in the map legend in units of ln-ng/L.

The area of greatest concentration is at the point of origin, the WWTP. From there the concentration of SMX is estimated to decrease downstream until the reservoir 6 point, and then increase again in the southern portion of the reservoir due to the additional input coming from the river flowing through the reservoir (R7). This trend is generally observed over the duration of the study as can be seen in all four maps for days 9, 23, 49, and 64, which correspond to August 9, August 23, September 18, and October 3, 2007.

It is interesting to note how differently the model predicts concentrations in the stream locations upstream of the WWTPs. On day 9, the model predicts upstream areas in streams A and B to be significantly higher than they should be considering SMX was below detection limits at this point. This is because upstream data was not included in the model, but it does not explain why estimated concentrations for stream C are so low. As time progresses, the estimated concentrations in the stream portions generally increase.

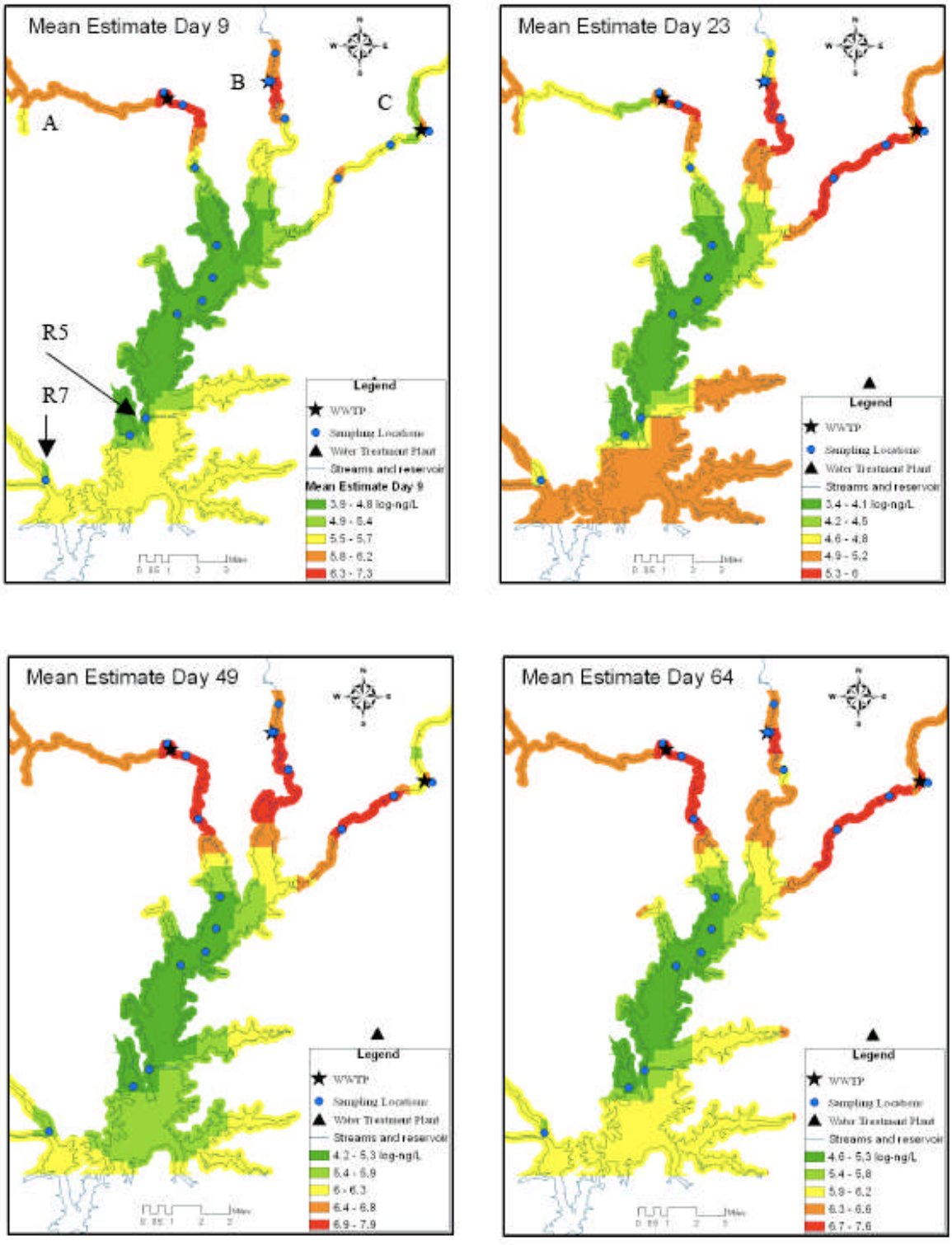


Figure 4.6: BMEGUI mean estimation maps for SMX in ln-ng/L on various days after August 1, 2007

The majority of the drinking water reservoir is estimated to have low concentrations of SMX over the study period, which agrees with the measured data. From a public health perspective, this is good because the green areas are where most of the boating and fishing occurs, and this is also the area where the drinking water treatment plant influent pipe is (R5 site).

Estimated concentrations fluctuate greatly over the study period. This point was sampled in a large river that is impacted by three upstream WWTPs and was found to have the highest concentration of SMX in the reservoir during the 12/11/07 sampling event. But, estimated concentrations are only high around August 23 (Mean estimate day 23 map). In order for the model to more accurately represent the observed concentrations, upstream data needs to be included in the model, as well as "soft" data points that estimate concentrations based on removal rates calculated for the specific sampling events.

Overall, the BMEGUI framework allows one to rigorously process the space/time variability of a pollutant in a watershed, the uncertainty in the site-specific data, and the relevant flow and transport governing laws. The model estimates pollutant concentrations at any point within the targeted watershed where a low number of samples were collected due to site accessibility and budgetary constraints, and can be more accurate than those obtained by classical modeling methods lacking the flexibility of BME. These estimation maps can visually portray spatial and temporal trends of a pollutant as well as areas of concern within the watershed, which may help to assess the uncertainty and risks associated with water quality in the targeted watershed, and can also be used to improve future sampling plans.

5. Conclusions

The original purpose of this research project was to investigate the occurrence and persistence of twenty-six antibiotics in a watershed that supplies a drinking water and recreational reservoir in a rapidly expanding area in the Southeastern United States. The research objectives are restated to frame this discussion within the context of the original purpose of this study:

- Determine the aqueous concentration and persistence of antibiotics in wastewater and receiving waters in a watershed that includes a drinking water source and recreational area.
- To complete an environmental hazard assessment of detected antibiotics and in the watershed.
- Estimate the concentration of a frequently detected antibiotic in surface waters at locations where samples were not taken using the BMEGUI geostatistical software, and to also determine any spatial and/or temporal trends during the study period.
- To investigate possible policy implications in order to reduce the presence of pharmaceuticals in surface waters.

The results of this study will be used to discuss how these objectives have been met.

5.1 Antibiotic Occurrence in the Targeted Watershed

Throughout this study, anywhere from 10 to 12 of the targeted antibiotics were detected in at least one aqueous sample collected from at least one of the three streams. At least one and up to eight antibiotics were detected in the reservoir samples during a specific sampling event. Detection criteria for an analyte's presence assessed with LC-MS/MS analysis were three-fold: 1) matching chromatographic retention time (within 2%) of unspiked and spiked calibration samples; 2) confirmation by at least one of the two targeted product ions from the analyte's parent ion (as listed in Table 2.4); and 3) matching relative ion abundance between standards and sample extracts. Quality assurance measures included the use of solvent blanks, method blanks, method blanks with standards, replicate samples, matrix spikes, and standard addition calibration curves for quantitation with typical linear R^2 values of 95% and above. Precision was relatively good, with only 5.2% of the total number of samples generating a RPD greater than 20% (Section 3.1.3).

Watershed and wastewater treatment plant information along with occurrence summary tables presenting the maximum and minimum concentrations of antibiotics detected within each stream during the entire study period are presented in Section 3.1.4. The sulfonamides were the most frequently detected class of targeted antibiotics with concentrations ranging from 1 ng/L to 3.3 μg/L, followed by the fluoroquinolones with concentrations from 1 to 338 ng/L. The tetracyclines were rarely detected in the treated effluent or surface water samples, most likely due to their high sorption coefficients when compared to those of sulfonamides and fluoroquinolones (Table 1.5). Sulfamethoxazole, trimethoprim, and erythromycin were present in each stream and were detected at the highest concentrations in every sampling event. The detected antibiotics and their concentrations are

similar to those found in other studies (Vanderford et al., 2003; Kolpin et al., 2002; Lissemore et al., 2006; Nakata et al., 2005) as discussed in Section 1.5. Sulfamethoxazole was detected at relatively high concentrations in this watershed, but this may be due to the fact that the area was under strict water conservation measures and a "normal" usage of antibiotics may have been concentrated by measures such as not flushing the toilet as often. This would concentrate any PhACs passing through the body in the toilet water before being flushed. Dilution as a primary attenuation process should affect all analytes in the same manner regardless of antibiotic class or chemical properties, and since the whole area was practicing strict conservation measures and there was minimal rainfall during the study, the concentration of sulfamethoxazole was similar in each of the three streams.

Table 5.1 presents a summary of detected antibiotics during the study period, where an x represents detection during at least one of the sampling events and a red star indicates that the antibiotic was detected during every sampling event. Areas where neither an x nor a red star is present indicate that the antibiotic was not detected at all. In general, the variety of antibiotics present and their concentration in the three streams were similar despite the fact that the daily volume of effluent discharged into each of the streams differed. Wastewater treatment processes are comparable, as are the population sizes and absence of nonpoint sources of pollution.

There were two antibiotics that were not consistently measured in each of the three streams, sarafloxacin and norfloxacin. These two antibiotics were only occasionally detected in the wastewater effluents at all three treatment plants, and only found in the waterways after the 9/22/07 sampling event. These results seem to indicate a seasonal usage pattern, but since norfloxacin is commonly prescribed to treat gonorrhea, prostrate, and urinary tract

infections and sarafloxacin is both a human and veterinary antibacterial drug, this does not seem to be the case. Sarafloxacin is commonly used as an antibacterial for aquarium fish, and this is the most likely source for this antibiotic in the treated wastewater effluent.

Table 5.1: Summary of overall antibiotic occurrence

	Stream A	Stream B	Stream C	Reservoir
Sulfachlorpyridazine				
Sulfadimethoxine	★	✗	★	✗
Sulfamerazine				
Sulfamethazine				
Sulfamethizole				
Sulfamethoxazole	★	★	★	★
Sulfathiazole	★	✗	✗	✗
Erythromycin-H_2O	★	✗	★	✗
Roxithromycin				
Tylosin				
Lincomycin	★	✗	★	✗
Trimethoprim	★	★	★	✗
Ciprofloxacin	★	✗	✗	✗
Enrofloxacin	★	✗	✗	✗
Flumequine				
Levofloxacin	✗	★	✗	✗
Norfloxacin		✗		✗
Sarafloxacin		✗	✗	
Oxolinic acid				
Pipemidic acid				
Chlortetracycline				
Demeclocycline				
Doxycycline				
Meclocycline				
Minocycline				
Oxytetracycline				
Tetracycline	✗	✗		✗

Note: x = analyte was detected during at least 1 sampling event; red star = analyte was detected during every sampling event.

Figure 5.1 presents a graphical summary, in a logarithmic scale, of the average sum of detected antibiotics by stream over the entire study period. This figure is intended purely for comparison purposes to illustrate that there was not a specific class of antibiotics that was detected in one stream compared to another.

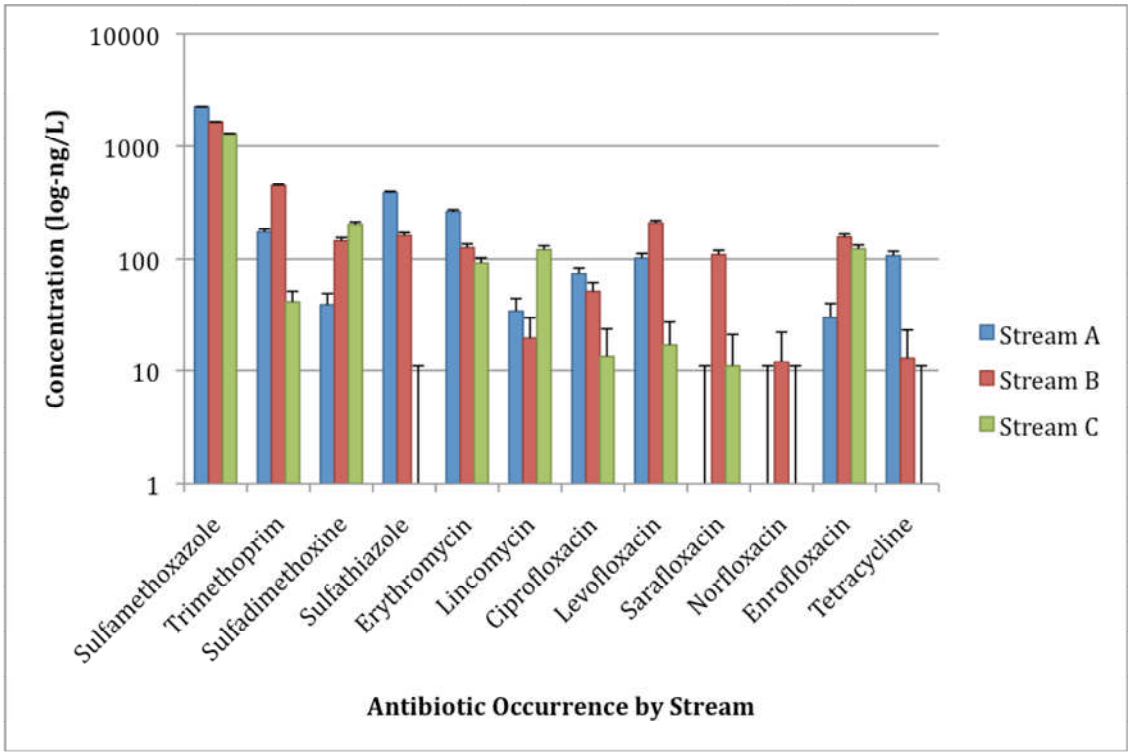


Figure 5.1: Average antibiotic occurrence by stream for comparison purposes (log-ng/L)

Each stream has slightly different characteristics; stream A is slow-moving and its size falls in-between the other two streams, stream B branches off and flows through a very swampy area, and stream C is wider and deeper than both streams A and B. Implications of stream characteristics are discussed in further detail in Section 5.4.

Several antibiotics were detected in the reservoir 1 through 6 locations (see Table 3.13) at much lower concentrations than found in the streams, and the overall decrease in antibiotic concentrations is thought to be caused by photodegradation and adsorption to stream sediment. Several more antibiotics were found in the aqueous phase at the reservoir 7 location, which is where a major river impacted by three wastewater treatment plants enters the reservoir (Table 3.13).

The BMEGUI framework with modern spatiotemporal geostatistics was used to process information about the space/time variability of sulfamethoxazole in this watershed and governing flow and transport laws in order to obtain statistically estimated concentrations of sulfamethoxazole at locations within the watershed that were not sampled. Estimation maps created by the model generally agree with the measured data that the areas of greatest concern coincide with streams A and B (see Figure 4.6). The BMEGUI maps illustrate how the concentration of SMX is greatest at its point of entry into the watershed (i.e. the wastewater treatment plant discharge), and then decreases as a plug of water from each of the three streams flows into the reservoir. The model estimates that the area of the reservoir in the vicinity of the drinking water treatment plant influent pipe has a low predicted aqueous concentration of SMX. SMX is found to increase in the southern portion of the reservoir due to the impact of another river flowing into it at that point only around the end of August. There are some discrepancies for estimated concentrations in the southern portion of the watershed, but this may be remedied by including upstream hard data and soft data points for the reservoir locations.

The model is useful in that it can predict analyte concentrations in areas and on days when samples were not taken, which is beneficial to this study due to a prevalence of site

inaccessibility and budgetary constraints. However, some weaknesses should be noted. First, the model predicts concentrations over a specified area and it does not distinguish between land, air, or water so the reader must consider this when interpreting the data. Second, there can be a large amount of variance associated with the expected concentrations due to a lack of sampling points in the study area or a lack of sampling events, etc. Third, weather events are not taken into account when predicting concentrations, so if sampling data were all collected during a period of heavy rainfall, the estimations would probably differ from estimations of a dry spell, etc. Fourth, the estimations are subject to how the modeler manipulated the model to generate the estimations, and may differ from person to person.

Suggestions to overcome these limitations are generating more data by sampling more frequently over a larger temporal and spatial period so that the model can more accurately predict concentrations throughout the watershed; "soft" data can also be generated by estimating concentrations using fate and transport laws at specific locations. Despite these limitations, it is a useful tool that shows general trends over a whole study area during a specific time and can be valuable in watershed management practices.

5.2 Estrogenic Activity in the Targeted Watershed

The aquatic environment is the ultimate sink for most chemicals, whether natural or man-made. However chemicals and products are disposed of, whether in landfill sites or via drains to WWTPs, they, or their degradation products, will enter the aquatic environment and some will cause estrogenic responses in aquatic life. Estrogenic activity was detected in the surface and treated wastewater samples at concentrations up to 2 ng/L, which is equivalent to an EEQ to estradiol of 0.14 (Table 3.15). Samples taken upstream of the WWTPs on streams A and C were found to have estrogenicity, but the input of treated wastewater effluent

increased the estrogenic activity at the point where the effluent and stream water mix in streams A and C. The opposite occurred in stream B, where the estrogenic activity at the upstream sampling point was higher than the effluent itself (1.23 ng/L vs. 0.241 ng/L). Other pollutants that were in the upstream surface water that are estrogenically active may have caused this increased response at the upstream location. These pollutants may also have been removed from the aqueous phase between the upstream and WWTP sites. The estrogenicity then decreased downstream, most likely due to attenuation processes of the estrogenic compounds, and was measured at an average 0.7 ng/L at the reservoir 5 site (location of drinking water influent pipe).

The YES assay is easy to use, but there are major reservations due to a lack of standardization, strain differences between yeast, and the unique physiology of yeast as compared to mammalian cells. Another major limitation of the YES assay is that it cannot determine the exact pollutant(s) which is/are causing an estrogenic response, and cannot distinguish between agonists and antagonists. Wastewater effluent is composed of a wide variety of chemicals and this is a major limitation to using the YES assay for this matrix. Major advantages of the YES assay include ease of use (because cells do not have to be continuously transformed), short-term duration, the ability to quantify results without using toxic materials, and the ability to use results as a comparative tool in a watershed setting.

5.3 Calculated Environmental Hazard in Targeted Watershed

The environmental hazard was calculated using measured concentrations and various EC_{50} values taken from the published literature for a wide variety of species. An assessment factor of 1000 was applied to the EC_{50} value in order to account for inter- and intra-species variability.

Sulfamethoxazole, ciprofloxacin, and erythromycin were consistently shown to have the greatest environmental hazard in all three streams and reservoir locations. Calculated environmental hazard values were found to decrease with increasing distance from the point of entry due to lower concentrations of the antibiotics, but there is still a hazard associated with the antibiotics in the drinking water reservoir.

It is difficult to assess what this means in terms of the aquatic life in the watershed. Present knowledge indicates that pharmaceutical residues in the aquatic environment are unlikely to pose a hazard for acute toxicity within the environment and the public. Environmental concentrations of the antibiotics detected in this watershed are at least 48 times less than known EC_{50} values (smallest difference between measured antibiotics and listed EC_{50} values in Table 4.1), but environmental concentrations are continuous whereas most toxicological studies are single dose exposures. When looking at all investigated antibiotic classes, the sulfonamides were the most frequently detected and therefore may present the greatest environmental threat.

There is a general lack of chronic toxicity data on PhACs, particularly for higher trophic levels, such as fish. More research about potential long-term ecotoxicological effects, particularly with respect to endocrine disruption, immunological status, or gene activation and silencing during long-term exposure is in order as well as the potential for additive and antagonistic effects within chemical mixtures. Due to the low concentrations of antibiotics seen in treated drinking water and source waters, public health effect are thought to be minimal, but subtle changes and disturbances may have negative consequences for aquatic life, and until more studies are completed it is difficult to assess what occurrence studies mean in terms of environmental hazard.

5.4 Impact of Watershed Characteristics on Occurrence and Persistence of Antibiotics

Watershed characteristics greatly affect the occurrence, fate, and transport of PhACs in the environment. Watersheds, receiving wastewater discharge and non-point sources of pollution from agricultural run-off are more susceptible to PhAC intrusion, and those areas closer to the source are expected to have higher concentrations relative to those areas farther away from the source due to attenuation processes such as sorption, dilution, photodegradation, and biodegradation. Land use surrounding the watershed is also closely related to the type of PhACs that can enter the watershed (i.e. urban, rural, agricultural, etc.). For example, an area that is home to several CAFOs and rainfall may have higher antibiotic concentrations in the surrounding watershed due to non-point source pollution than a semi-arid, urban area.

The size and average daily stream or river flow can also affect PhAC attenuation. PhACs found in a large, shallow, slow-moving stream would be more likely to adsorb to stream sediment and undergo photo- or biodegradation. Sediment transport is more likely in a high flow stream as high flow will agitate and move sediment to a greater extent than a low flow stream. Also, PhACs found in rivers that are narrow and deep would be less likely to adsorb to stream sediment or undergo photodegradation because there would be less contact with stream sediment, and sunlight may not be able to penetrate at certain depths.

As much information as possible was obtained about the watershed, such as potential point and non-point sources, land use practices, flow and stream characteristics, and wastewater treatment processes in order to assist with data interpretation. The streams investigated in this study are located in the same watershed and have relatively similar

characteristics. All three flow through a semi-urban area, are impacted by wastewater discharge, and are not impacted by non-point sources of pollution downstream of the WWTP. After wastewater effluent is discharged into these streams, they flow through a heavily forested/intermittently swampy area before entering a recreational reservoir that also serves as a drinking water source.

There are, however, differences in stream size. Stream A is shallower and narrower than streams B and C. Stream B branches off and then flows through a very swampy area before entering the reservoir, and stream C is deeper and wider in most locations when compared to streams A and B. These differences may have a significant impact on attenuation processes. For example, because stream C is wider in comparison to the other two streams, PhACs in the aqueous phase may be less prone to undergo photodegradation or adsorb to stream sediment, whereas the characteristics of stream B would most likely result in a greater percentage of PhACs in the aqueous phase more readily adsorbing to the stream sediment. This is, in fact, observed when comparing the rate of removal (k) values calculated using measured data for sulfamethoxazole and erythromycin (see Tables 4.7 and 4.9). Stream C has k values that are lower than streams A and C, which indicates that both of these antibiotics are found to undergo slower rates of removal from the aqueous phase. This is also clear when looking at the half-lives ($T_{½}$) of both sulfamethoxazole and erythromycin (Tables 4.8 and 4.10). During the study period, sulfamethoxazole was found to have average $T_{½}$ values of 1.58, 0.09, and 2.5 days in streams A, B, and C, respectively. Erythromycin was found to have average $T_{½}$ of 0.61, 0.17, and 3.5 days in streams A, B, and C, respectively. These values indicate that aqueous concentrations of antibiotics in stream B

undergo the fastest rate of removal from the aqueous phase, followed by stream A, and finally stream C.

5.5 Reduction of Antibiotics Through Wastewater Treatment Technologies

WWTPs are a major potential PhAC point source to the aquatic environment, while also providing a major opportunity for centralized removal processes. Conventional WWTPs effectively remove solids, nutrients, and biodegradable organic matter but are not typically designed to remove PhACs during normal operation.

Individual PhACs have distinct chemical and physical properties that suggest potentially different mechanisms and locations in the WWTP treatment train for removal or reduction. Biological transformations, the effects of mixtures, hydraulic and temperature variations, analytical limitations, and the combination of treatment processes in a WWTP can contribute to uncertainties in PhAC removal. Advanced treatment such as ozonation, activated carbon, and membrane filtration are receiving considerable attention, while research into a better understanding of removal in conventional treatment (primary, secondary and tertiary) remains active. It is difficult to justify upgrading conventional wastewater treatment systems when the effects of PhACs in the environment are largely unknown. If, and when adverse toxicological effects evidence is sufficient to warrant additional treatment, it is practicable that abatement design will not be tailored to an individual compound but based rather on overall capacity to remove trace contaminants. Until then, research in this area remains of fundamental importance.

5.6 Public Health Significance

Antibiotics can be considered an indicator of anthropogenic contamination in surface and drinking water. In this case study, several antibiotics, most notably sulfamethoxazole, ciprofloxacin, and erythromycin, have been found to have an environmental hazard as described in Section 4.4. The environmental and human risk associated with PhACs in watersheds and drinking water reservoirs are largely unspecified. Little is known about long-term exposure to sub-therapeutic doses of antibiotics and other compounds, either individually or as a mixture through drinking water. Drug resistant pathogens are an increasing threat to the health care industry, and the presence of PhACs in surface waters may be a cause of this phenomenon. Little is also known about transformation products of PhACs that may also pose a public health threat. Exposure through recreation in the reservoir, or consuming fish from the waterfowl impoundments along these streams is also of concern. If antibiotics are present in the waterways, it can be assumed that other pollutants are present as well, and the results from this study indicate that environmental concentrations of antibiotics in the aqueous phase validate public health and antibiotic resistance concerns.

5.7 Policy Implications

Policy implications were discussed in Section 1.10. There are several regulatory agencies involved in the development and dispersion of pharmaceuticals, as well as their fate. It is important to coordinate research between government, pharmaceutical companies, academia, and the public in order to formulate a plan to decrease the presence of anthropogenic compounds in the environment.

Controlling pollution at its source is a better option than spending large amounts to

upgrade current wastewater and water treatment plant infrastructures. Effective source control reduces the consumed quantities as well as the ecological exposure of selected compounds by educating the public and medical professionals on the consequences of PhACs in the environment. It is of paramount concern to decrease the prevalence of these compounds in the environment because of the unknown effects of long-term, sub-therapeutic exposure of these compounds.

Several suggestions to help decrease this waste in the environment are:

- modify current regulations that impose stricter testing by pharmaceutical companies and encourage the development of more readily biodegradable drugs,
- develop plain language educational programs to inform medical professionals and the public of the fate and potential environmental/health hazards of these compounds,
- promote alternative or "natural" medicines, as well as discourage over-prescription and overuse to decrease reliance on these anthropogenic sources of pollution,
- educate pharmacies, medical professionals, and the public on how to properly dispose of prescription and non-prescription drugs,
- better coordination between the FDA and EPA when conducting environmental assessments in order to establish maximum contaminant levels,
- create worst-case scenario model ecosystems or watersheds to determine maximum contaminant levels, and to create predictive models to investigate fate and transport of PhACs, and

- create a comprehensive monitoring program that includes compounds that are indicators of each major source of PhACs to the environment (for example, antibiotics or caffeine as a tracer for WWTP effluents) and frequently used compounds in high use.

Sharing information and opening up the discussion to the public without pushing false assumptions and scare tactics will result in consumer and corporate responsibility and hopefully a decrease in the environmental presence of these pollutants.

5.8 Recommendations for Future Study

This study has shown that antibiotic occurrence in the aqueous phase is prevalent in an impacted watershed that supplies a drinking water reservoir. Investigating sediment samples in impacted watersheds is also helpful to understand the sorption mechanisms for PhACs, to further understand their fate and transport and provide a more complete mass balance that in turn will assist in predictions of environmental hazard. Investigating WWTPs that utilize different treatment processes would help to determine which processes are more effective in PhAC removal at the full-scale plant level. The impact of PhACs in the environment is still largely unknown, and more in-depth toxicological studies are warranted in order to understand the effects of long-term, continuous exposure of the aquatic environment to a range of compounds. Improvements in analytical methods, as well as developing standard methods in all matrices should be considered to ensure more comparable research results.

5.9 Parting Thoughts

Knowledge of the presence of antibiotics and other pharmaceutically active compounds in the environment has increased due to improved analytical methods for their detection, and an increase in peer-reviewed publications on the subject matter. This study was conducted to investigate the occurrence of 26 antibiotics using validated analytical methods and LC-MS/MS identification, and corresponding estrogenic activity within a drought-ridden watershed impacted by wastewater effluent in the Southeastern United States. It is unique in that the study period investigated three streams that flowed over a short distance directly into a drinking water reservoir and coincided with an extreme drought, which resulted in treated wastewater effluent feeding the northern half of a drinking water reservoir. The study is not entirely representative with respect to the long-term temporal evolution of this watershed. No other case study known to the author has previously investigated the occurrence of antibiotics in a watershed during a period of extreme drought. This also allowed the removal rates of two of the most prevalently detected antibiotics, sulfamethoxazole and erythromycin, to be determined because there was no additional stream flow other than the wastewater effluent during the study period. The use of the BMEGUI framework also allowed the author to reliably estimate concentrations of sulfamethoxazole within the whole watershed despite a limited number of sampling sites. Other studies of this nature have only relied on measured data to create a picture of what was occurring in investigated areas. BMEGUI concentration estimations can be used in future watershed management issues concerning emerging contaminants and possibly other water quality criteria.

Case studies such as this are beneficial as they can give us insight into the occurrence and fate of anthropogenic pollution originating from a point source in a “worst-case” scenario. They also provide a basis for toxicological and health impact studies on environmental levels of PhACs that are critical to the development of a regulatory framework.

Appendix A: Standard Operating Procedure for Antibiotic Analysis using LC/MS-MS

Purpose: This standard operating procedure (SOP) describes the analysis of 26 antibiotics including 7 tetracyclines, 7 sulfonamides, 7 (fluoro)quinolones, 3 macrolides, trimethoprim, and lincomycin in wastewater effluent and surface water samples at ng/L levels using solid phase extraction and liquid chromatography electrospray tandem mass spectrometry.

1. Preparation of solutions

Supplies:

- balance
- 10 and 25 mL volumetric flasks
- 100 mL beakers
- glass funnel
- 10 and 40 mL amber vials and Teflon-lined caps
- spatula
- weighing dishes – whatever size available
- 5 ¾ inch pasteur pipettes
- 250 mL and 1 L amber bottles with Teflon-lined caps
- 10 mL conical vials with Teflon-lined caps

Parts from Laboratory Supply Distributors (Mt. Laurel, NJ):

- amber glass autosampler vials, graduated spot (LSD Part #20211AS-1232)
- autosampler vial 10mm/425 black closure with hole (LSD Part #41360-10)
- 0.45 μm syringe filters (LSD Part #NN8012-100)
- 250 μL Glass LVI with prospering inserts (LSD Part #21100-11)

Reagents:
Antibiotic standards purchased from Sigma-Aldrich (St. Louis, MO) include: sulfathiazole, sulfamerazine, sulfamethazine, sulfamethizole, sulfachlorpyridazine, sulfamethoxazole, sulfadimethoxine, erythromycin, tylosin, roxithromycin, trimethoprim, lincomycin, norfloxacin, sarafloxain, flumequine, levofloxacin, pipemidic acid, oxolinic acid, minocycline, oxytetracycline, tetracycline, demeclocycline, chlortetracycline, doxycycline; all are 99% pure.

Isotopically labeled standards ($^{13}C_6$-sulfamethoxazole, $^{13}C_3$-ciprofloxacin, and $^{13}C_2$-erythromycin) were purchased from Cambridge Isotope Laboratories (Andover, MA).

The internal standard, simatone, was purchased from Accustandards (New Haven, CT).

Erythromycin-H_2O was prepared at the University of North Carolina laboratory with the erythromycin standard and the method described by McArdell et al. (2003) and Ye, 2005).

Na_2EDTA and sodium azide were purchased from Aldrich (Milwaukee, WI).

Location of Reagents:
The antibiotic, internal standard simatone, and surrogate standard stock and working solutions are stored in Freezer D. Neat standards of minocycline, meclocycline, ciprofloxacin, pipemidic acid, flumequine, erythromycin, and levofloxacin standards are stored in Fridge A. Neat standards of sulfamethazine, sulfamethoxazole, and EDTA are stored in the Organics cabinet by hoods. All other neat standards are stored in Freezer D. Formic acid is also stored in Fridge A.

Solvents:
- HPLC-grade methanol (Fisher Scientific)
- LGW/methanol (1:1, 0.2% HCl, v/v)
- HPLC-grade acetonitrile (Fisher Scientific)
- HPLC-grade formic acid (Fisher Scientific)

1.1 Preparation of antibiotic stock solutions (see Table 1)

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

- Tare the scale on the weighing balance (Fisher Scientific Accu-124D Dual Range, Serial #15806759) after placing the weighing dish
- Close the glass door of the balance before weighing
- Use the spatula to transfer a predetermined amount (Table 1) of antibiotic neat standard to the weighing dish (record exact weight to 4 decimal places)
- Use 5 ¾" pasteur pipette 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
- Transfer the solution into an amber vial of separate volume and cap with Teflon-lined cap
- Label the vial with antibiotic name, concentration, initials, and date of preparation and store in Freezer D
- Clean the spatula thoroughly (with LGW and then methanol) and dry thoroughly with Kimwipes
- Brush off scale gently between use

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

- Tare the balance and then weigh the volumetric flask without stopper; tare the balance
- Add antibiotic neat standard directly into the volumetric flask through a filter funnel using a spatula
- Add appropriate solvent directly into the flask with a 5 ¾" Pasteur pipette
- Cap the flask and invert 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 (10 mL, or use a 40 mL vial for 25 mL of stock solution) and cap with Teflon-lined cap
- Calculate the actual concentration
- Label the vial with antibiotic name, concentration, initials, and date and store in Freezer D

Note:

- 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.

- Store all solutions in Freezer D at about 10°F, except for aqueous solutions, which should be kept in Fridge A at about 45°F. The stock solutions are stable for at least 3 months (tetracyclines, quinolones, macrolides, lincomycin, and surrogate standards) and 1 year (sulfonamides, trimethoprim, simatone) when stored in freezer.

- Usually takes ~ 1-2 days to prepare stock solutions for all 26 antibiotics

- Antibiotic standards are stored in the little blue box in Freezer D

Table 1. Preparation of Stock Solutions of Antibiotics

Analyte	Molecular Weight	HCl or salt form	Stock Conc. (mg/L)	Amount of Neat Std. (g)	Solvent	Volume (mL)
Minocycline	457.5	494	1000	0.0108	MeOH	10
Oxytetracycline	460.4	496.9	1000	0.0108	MeOH	10
Tetracycline	444.4	480.9	1000	0.0108	MeOH	10
Demeclocycline	464.9	501.4	1000	0.0108	MeOH	10
Chlortetracycline*	478.9	515.4	400	0.0135	MeOH	25
Doxycycline	444.4	480.9	1000	0.0108	MeOH	10
Meclocycline	476.9	695	1000	0.0146	MeOH	10
Sulfamerazine*	264.3	287.3	400	0.0109	MeOH	25
Sulfathiazole	255.3	278.3	1000	0.0109	MeOH	10
Sulfamethazine*	278.3	-	400	0.01	MeOH	25
Sulfamethizole	270.3	-	1000	0.01	MeOH	10
Sulfachlorpyridazine	284.7	-	1000	0.01	MeOH	10
Sulfamethoxazole	253.3	-	1000	0.01	MeOH	10
Sulfadimethoxine	310.3	-	1000	0.01	MeOH	10
Trimethoprim*	290.3	-	400	0.01	MeOH	25
Ciprofloxacin	331.3	367.8	1000	0.0111	LGW	10
Norfloxacin*	319.3	-	400	0.01	LGW/MeOH 1:1, 0.2% HCl, v/v	25
Enrofloxacin*	359.4	395.9	400	0.011	LGW/MeOH 1:1, 0.2% HCl, v/v	25
Sarafloxacin*	385.4	-	80	0.008	90% LGW : 10% MeOH	100
Pipemidic acid*	303.3	-	400	0.01	LGW/MeOH 1:1, 0.2% HCl, v/v	25
Oxolinic acid*	261.2	-	400	0.01	ACN (2% NH_4OH, v/v)	25
Flumequine	261.2	-	1000	0.01	ACN (2% NH_4OH, v/v)	10
Tylosin	916.1	1066	1000	0.0116	MeOH	10
Erythromycin	733.9	-	1000	0.01	MeOH	10
Roxithromycin	837.1	-	1000	0.01	MeOH	10
Lincomycin	406.5	443.5	1000	0.0109	MeOH	10

Note: * = not easily soluble.

1.2 Mixture of each antibiotic group (usually prepared at 20 mg/L)

Group 1: six tetracyclines (TCs) — minocycline, meclocycline, 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

Procedure

1. Calculate the volume of antibiotic stock solution to be added to 10 mL of methanol.
   $C_{stock} * V_{stock} = V_{mixture} * C_{mixture}$

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

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

4. Pipette the stock solution (V_{stock}) and inject below liquid in the volumetric flask (begin with ones that need the largest V_{stock})
   - Adjust the micropipette volume setting to the desired value
   - Clean the micropipette tip with MeOH and dry with Kimwipe
   - Insert capillary tube, tighten, and check
   - Withdraw antibiotic solution (V_{stock})
   - Wipe off solvent outside of capillary with Kimwipe
   - Inject *under* liquid level and shake the pipette gently
   - Remove glass capillary
   - Rinse tip with wash MeOH and dry with Kimwipe
   - Repeat process for each injection

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

6. Repeat for all antibiotic groups

7. Store the solutions in labeled amber glass vials capped with Teflon-lined caps in Freezer D

1.3 Mixture of 26 antibiotics at 0.5 mg/L in MeOH

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

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

$C_{mixture}$: 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 mg/L * V_{taken} = 0.5 mg/L * 10 mL

V_{taken} = 0.25 mL

2. Prepare the mixture of 25 antibiotics using the same technique as described in Section 1.1.2

1.4 Preparation of Surrogate and Internal Standard Solutions

1.4.1 Mixture of surrogate standards at 0.5 mg/L

1. Calculate the volumes of stock solutions of surrogate standards ($^{13}C_6$-sulfamethoxazole, $^{13}C_3$-ciprofloxacin, $^{13}C_2$-erythromycin, and meclocycline) to be taken to prepare a mixture of the 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 mg/L * 5 mL

2. Prepare the mixture in HPLC-grade MeOH using the same technique as described Section 1.1.2.

1.4.2. Internal standard (I.S.) solution

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

- Make dilution of the simatone stock solution to a desired I.S. concentration of 1.25 mg/L by adding 125 μL of stock solution to 10 mL MeOH

- Pour working solution into an amber vial, cap with Teflon-lined cap, label the vial, and store in Freezer D

1.5 Preparation of other solutions

1.5.1 0.1% Formic acid in methanol (also referred to as acidified methanol; used to condition and, later, to elute compounds from SPE cartridges)

Measure 200 mL HPLC-grade MeOH into a 250 mL amber bottle and add 200 µL formic acid with micropipette. Cap the bottle with a Teflon-lined cap and invert 3 times.

*** Prepare fresh solution within one week of analysis***

1.5.2 0.25 g/L Na_2EDTA stock solution in LGW (added to samples to prevent metal complexation before SPE)

Weigh 0.025g Na_2EDTA, transfer to a 100 mL volumetric flask, and dissolve in LGW. (Na_2EDTA is stored in Organics cabinet; use most recently opened bottle).

1.5.3 Solvent mixture of LGW and methanol (10% MeOH: 90% LGW) (for reconstitution of samples after blow-down)

Measure 180 mL LGW and 20 mL methanol, respectively, and mix in a 250 mL amber bottle. Cap with Teflon-lined cap.

1.5.4 2% Formic acid in LGW (for pH adjustment of water samples)

Measure 200 mL LGW and pour into 250 mL amber bottle. Inject 4 mL formic acid with 5 mL glass pipette and bulb below the surface. Cap bottle with Teflon-lined cap and invert 3 times.

2. Sample processing

2.1 Sample Collection

1. Samples should be collected in 1 L amber bottles

2. Rinse bottle with sample 3 times before collecting the appropriate volume

3. When collecting actual sample, wear latex gloves, put bottle below surface and fill to the top, cap with Teflon-lined cap

4. Bottle should be labeled with sample name, collector name, date, and time

2.2 Sample 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, usually a 1 L Buchner flask

3. The surface/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, only use nylon filter (0.45 μm)

4. Connect the vacuum and place the filter into the system using forceps

5. Wet the filter with a small amount of LGW

6. Switch on vacuum

7. Pour about 100 mL of water sample into the sample reservoir and let it filter through under vacuum. Stop vacuum before the filter dries, swirl the 100 mL around in flask, then dump out (this serves to condition the glassware and further remove any residue). Pour water sample into the reservoir and filter the rest of the sample; 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)

2.3 Solid phase extraction (SPE)

2.3.1 Sample Preparation

After filtration, each 1 L sample is divided into six aliquots of 250 mL each: two for the unspiked samples and four for spiked samples. (Note: at least 2 spike levels are needed for standard addition).

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. To prevent contamination of sample from glassware, this volume is sometimes estimated. To be more precise, use a 250 mL volumetric flask to measure volume and then pour into bottles.

3. Addition of surrogate standards

The surrogate standard mixture is a mixture of 4 surrogates including $^{13}C_6$-sulfamethoxazole, $^{13}C_3$-ciprofloxacin, and $^{13}C_2$-erythromycin, and meclocycline at a concentration 0.5 mg/L.

Spike the surrogate standard mixture prior to antibiotic mixture to avoid contamination and because surrogates monitor the entire sample processing procedure.

a) Calculate the volume of surrogate mixture to add to 250 mL. The desired concentration is 50 ng/L so, for a 250 mL sample, the volume of the mixture required is calculated as follows:

$$\frac{C_{surrogate} \cdot V_{sample}}{C_{Surrogate\ Stock}} = \frac{0.05\frac{\mu g}{L} \cdot 250mL}{500\frac{\mu g}{L}} = 0.025mL = 25\mu L$$

b) Use a micropipette with an appropriate volume range (ie 10 – 50 μL) and adjust to the desired volume

c) Inject surrogate mixture (from nonspiked to spiked samples with increasing concentrations) using the same technique as described in section 1.1.2. The same volume of the surrogate standard mixture is added to each bottle.

4. Spike of antibiotics

a) Prepare the 26-analyte mixture (see section 1.1.3) on the day of extraction

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

c) The spike levels for raw water are usually 20, 50, 100, and 500 ng/L

d) The spike levels for finished drinking water are usually 2, 5, and 10 or 20 ng/L

e) The spike levels for treated wastewater effluent are usually 20, 100, 500, and 1000 ng/L

f) Calculate the amount of antibiotic mixture to spike for each increasing spike level. For example, if a spike concentration in a 250 mL sample of 50 ng/L is desired, then the volume of a 0.5 mg/L mixture required would be:

$$\frac{C_{analyte} \cdot V_{sample}}{C_{mixture\,of\,25}} = \frac{50\frac{ng}{L} \cdot 250mL}{0.5\frac{mg}{L}} = 0.025mL = 25\mu L$$

g) Inject the antibiotic mixture below the surface using the same techniques as discussed earlier.

5. Addition of Na_2EDTA at 1 mg/L

Na_2EDTA is added to prevent complexation between the analytes and the metals in water. 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).

a) Prepare 0.25 g/L Na_2EDTA stock solution (section 1.3.2)

b) Calculate the amount of Na_2EDTA stock solution to add. For a 250 mL sample, the volume of the 0.25 g/L working solution required is calculated as follows:

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

c) Addition of Na_2EDTA to samples:
- Fill a 10 mL graduated pipette with the EDTA solution and dispense 1 mL of this solution to each sample in its 250 mL amber bottle on *top* of the liquid level of the sample

- Cap and invert the amber bottle once
- Repeat for every sample

6. pH adjustment

Notes:
- The pH meter (Fisher Scientific) is located on the first bench
- When not in use, store the electrode in buffer storage solution and put 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 electrode with LGW and dry again with Kimwipe

j. Place electrode in pH 4 buffer, switch from "standby" to "pH" and adjust the "slope" knob only to pH 4.00 (get as close as possible)

k. Switch back to "standby", remove electrode from pH 4 buffer, rinse and replace in pH 7 buffer. Place in "pH" mode and read. If measurement is within 0.1 pH units, the meter is ready to use. If not, the buffer solution should be replaced, and if same problem occurs, then the electrode needs to be replaced.

l. Switch back to "standby" mode and insert the electrode in pH 4 buffer

2) Measure pH of samples starting with the unspiked samples

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 with surface and wastewater samples)
- 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 2% formic 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 from solid phase extraction is the same for each sample

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 unspiked sample goes below 5.8, mix it with the other portion of the unspiked samples and readjust pH
d. RECORD 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, rinse the electrode and store in the pH 4 buffer solution

4) Repeats steps 2) and 3) above for each of the samples in order from lowest to highest spiked sample in each sample set.

2.3.2 Solid phase extraction

Preparation

1. Equipment Needed:
    a. vacuum manifold (Supelco Visiprep 24)
b. solid phase cartridges cartridges (Strata-X 6 mL or equivalent 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

<u>1. Preconditioning of SPE columns</u>

Notes:
- Start preconditioning before sample pH adjustment because it takes ~10 minutes
- Precondition using MeOH, which cleans and activates HLB or Strata-X sorbent, and acidified MeOH, which is later used as the eluting solvent
- LGW is used to wash the MeOH out of the cartridge

1) Add ~ 6mL MeOH 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 MeOH has almost completely passed through the cartridge, add ~ 1 mL of acidified MeOH (0.1% formic acid in MeOH) (one squeeze of 5 ¾" pasteur pipette; or use 1 mL or larger pipette)

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

5) Apply 6 mL of LGW to each cartridge twice and try not to introduce any air bubbles (air bubbles can fill pores of stationary phase and reduce effectiveness). When about 3 mL of LGW is remaining in cartridge, proceed to extraction.

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 sample amber bottle, insert one small, precleaned tubing into each bottle (try to get tubings that have equal length if possible), and connect the tubing fittings to the SPE cartridge with a piece of tape (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 a 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 by ~50 mL) and the level should be the same in each bottle

7) Complete checks
a. make sure tubing is at very bottom of amber bottles
b. no leak around cartridge and t-adaptor
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 passed 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 mm Hg, or push 3 syringe volumes of air through each cartridge.

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) Clean SPE tubing: use tubes designated for cleaning tubing (in a labeled plastic bag in drawer). Put them on each of the valves used, connect tubing to the top. Get two beakers, one for LGW and another for wash MeOH. Apply a vacuum and suck LGW through tubing, followed by methanol. Keep the vacuum on until tubing is dry. Place tubing back in SPE drawer.

14) Rinse MeOH through each orange valve on the manifold that was used (where each cartridge was connected).

15) Wipe off water on the underside of the manifold cover.

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

17) Pour extraction waste into sink.

3. Elution
(~ 15 minutes)
Note:
- Use acidified MeOH (0.1% formic acid in MeOH) prepared no more than 1 week prior to extraction because formic acid may evaporate over time

1) Locate ~10 mL conical vials (1 per sample) and one Teflon-lined cap for each tube
******* Make sure that filter syringe can fit into the test tubes first ********

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

3) Place white plastic rack inside the manifold

4) Place cartridges on the manifold without T-adaptors (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 MeOH to each cartridge using a 10 mL pipette, allow to pass 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 sealed bag in Freezer D. Do NOT throw away cartridges until all analyses are complete.

2.4 Solvent reduction

Notes:
- This process takes ~ 2 hours
- Can begin to set up blow down during elution

2.4.1 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 blow-down tubes from the drawer under the blow-down apparatus.

3. Check the nitrogen tank in advance (to make sure there is enough nitrogen head to use for blow down) by opening valve on top of the tank and checking to see if the pressure is > 500 psi. Stop using the nitrogen tank after its pressure drops 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. Fill holes at least half-way with LGW.

5. Wait ~ 5 minutes for the block to heat up.

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

7. Place conical vials in the heating block. Place

8. Lower and secure the top.

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

10. Open the nitrogen tank and adjust the flow rate so that the nitrogen blows gently with minimum disturbance to the eluent.

- More samples necessitate higher flow rates 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 the moveable snorkel above the blow-down apparatus down as low as possible.

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

13. Check the liquid level in the conical vials approximately every 15 minutes, and lower the top if necessary as the liquid evaporates.

14. Add 50 µL of LGW/ methanol (9:1) mixture to an empty conical test tube using the designated syringe located in the drawer to the left of the LC-MS/MS. Cap with Teflon-lined cap. This vial will is labeled as the solvent blank + internal standard (internal standard is added later).

15. Blow down SPE eluents to ~ 50 µL in the conical vial. Visually compare to the solvent blank prepared in previous step. **NEVER blow down to dryness!**

16. After the desired volume is achieved, turn off heating block and close the nitrogen tank.

17. Clean tubing with wash MeOH and place on a Kimwipe to dry. After they dry, place them back in the designated drawer.

2.4.2 Reconstitution

1. Estimate the residual volume of SPE eluent by visually comparing to solvent blank + I.S. conical vial:

a) If the estimated sample volume is > 50 µL, make another solvent blank in a separate conical vial with 70 µL of LGW/methanol solvent mixture, and try to estimate the level of the sample (it might be ~ 60 µL).

b) Record estimated volume on the label of each conical vial.
(Note: the sample extract after blow down often looks slightly yellow due to the presence of natural organic matter in water).

2. Rinse the 500 µL syringe that is located in the drawer to the left of the LC-MS/MS with HPLC-grade MeOH three times.

3. Fill a 10 mL beaker with LGW/MeOH solvent mixture (9:1).

4. Reconstitute each sample to a final volume of 250 µL with the LGW/MeOH solvent mixture (9:1) using the 500 µL syringe starting with the unspiked samples and then moving on to increasing spike levels. Add reconstituting solvent above the liquid level of the sample and avoiding touching the sides of the conical vial. If syringe does not touch sides of conical vials, then it does not have to be rinsed in-between reconstituting each sample.

5. Rinse syringe with wash MeOH after reconstitution of all samples.

2.4.3 Addition of internal standard simatone

1. Clean the designated 10 µL syringe (located in the drawer to the left of the LC/MS-MS) with HPLC-grade MeOH three times, and then pull up 10 µL of internal standard (I.S.) solution at 1.25 mg/L (see Section 1.2.2) and discard to waste at least three times.

2. Add I.S. solution to each sample and into solvent + internal standard blank conical vials.

1) Add 10 µL of 1.25 mg/L I.S. solution to each conical vial (if the volume in each conical vial is not 250 µL, then the volume to add needs to be recalculated).

Technique:
a. Draw I.S. solution slowly into the 10 µL syringe making sure that no air bubbles are drawn up (if air bubbles are present, put the solution in the syringe to waste and try again).
b. Inject I.S. *under* the eluent surface in each conical vial. Add I.S. solution from unspiked sample to spiked samples with increasing concentration.
c. Re-cap vials after I.S. injection and place back in test tube rack.
d. Rinse syringe needle with wash MeOH and dry with a Kimwipe between every injection.

3. Vortex each sample using the vortexor (Thermolyne Maxi Mix Plus, model #M63215).

4. After vortexing each sample, slowly turn conical vial around to cover the surface area of the conical vial with the extract liquid that was not reached by vortexing (sometimes there are solids stuck to the vial).

5. Let each sample sit for 10-15 minutes covered with aluminum foil in a vial rack to allow liquid settle to the bottom of the vial.

2.4.4 Syringe filtration of sample extracts

1 . Preparation:

a) Gather a vial rack and autosampler rack, 250 µL glass inserts, 2 mL amber autosampler vials, and Teflon-lined plastic screw caps

b) Label each autosampler vial with sample ID, name, and date.

c) Place a glass insert into each vial.

2. Syringe filtration of extracts:

a) Locate 0.45 µm pore size syringe filters from the LC-MS drawer

b) Use the 500 µL syringe to slowly draw sample from conical vial starting with samples of increasing concentration.

c) Measure the total volume to verify that it is ~ 250 µL and record in lab book.

d) With sample still in syringe, unscrew the needle and attach a syringe filter.

e) Slowly push sample through the syringe filter directly into the glass insert in the labeled autosampler vial.

****Be careful when pushing sample through syringe filter. If pressure increases too much, the filter may pop and the sample extract may be lost.****

The syringe filter can be changed for a new one by pulling up air with syringe to pull out any liquid in the actual filter. Then replace the old filter with a new one and continue to push the sample through.

f) After sample is filtered, take filter off the syringe, draw in air, re-attach the filter, and push the remaining sample into the vial.

g) Close the vial with appropriate cap.

h) Tap the vial to get rid of bubbles in the insert.

i) Rinse syringe twice in-between each sample using the LGW/MeOH solvent.

j) Repeat steps 1 – 10 for each sample.

Cleaning: rinse syringe 3 times with LGW/MeOH solvent, then rinse syringe with HPLC-grade MeOH. Remove the plunger and unscrew the syringe and rinse each with wash MeOH and place on a KimWipe to dry.

Autosampler vials with extracts can be stored in Freezer D until LC-MS/MS analysis.

2.5 Overall clean-up:

1. Collect all dishes/parts/tubes/etc. in a plastic bucket and fill with tap water and soap mixture (by the sink) if dishes are not to be immediately washed.

2. Pipettes:
   a) Rinse with tap water and then with LGW, inside and outside
   b) Rinse tips and the whole pipette with wash MeOH over a waste bucket
   c) Place a large KimWipe or paper towel inside a clean, plastic bucket and then place the pipettes in the bucket to dry overnight.
   d) Place a label on the bucket with your name and date.

4. T-adaptors: rinse with LGW and then wash MeOH and put back in beaker in the SPE drawer

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

a) Wash bottles in dishwasher in MHRC Room 1206 on setting 3. (If not trained in use of the dishwashers, then seek out someone who knows).

b) After the dishwashing cycle is complete, submerge amber bottles in 10% nitric acid bath in the teaching lab (MHRC Room 1204) for about 6 hours. (Must know how to use acid bath before using).

c) After soaking in the acid bath, carefully remove the bottles following the proper protocol and place in a clean plastic bucket. Take the bucket back to MHRC Room 1210 and rinse each bottle 3 times with LGW.

d) Place amber bottles in oven overnight to dry. After completely dry, cover openings with aluminum foil and put back in proper storage cabinet.

6. Caps: soak in 500 mL beaker with a mixture of LGW and soap for a few hours. Rinse with LGW three times, and then rinse with wash MeOH, then place on a KimWipe to dry.

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

3.1 Equilibration of LC system

1. Check mobile phase levels (make sure there is at least 3 inches of liquid in 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 0.2 µm nylon filter)
-mobile phase B: Acetonitrile (filter with 0.2 µm nylon filter)

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

1) Connect a 10 mL plastic syringe to the mobile phase A fitting under the degasser (tubing is labeled as A or B).
2) Switch T valve so it is facing to the left to open it.
3) Slowly pull out mobile phase to get all of the bubbles out. Close the T-valve before removing syringe, and then discard any mobile phase solution in syringe to a waste bucket.
5) Repeat for mobile phase B.

<u>Priming:</u>
This needs to be done when the pumps have not been used within ~ 24 hours.

1) Open black B valve at the bottom of the pump station to divert the mobile phase to waste

2) Prime pump A first.

3) Push “Stop” on the pump screen

4) Push “Prime”, and then set the flow rate (do not exceed a flow of 5 mL/min; 1 mL/min is a good flow rate). Pressure should be around 330 – 400 psi. If pressure is much higher, stop priming and check that black B valve is open. If pressure continues to increase, there may be a blockage in the tubing.

5) Prime for ~ 2 minutes

6) Push “Stop” again

7) Repeat process for mobile phase B (pressure should be around 220 psi)

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

3. If the instrument has been used within 24 hours: before starting the pumps 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. 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) Unscrew tubing from the column and cap column ends with end caps found in LC-MS drawer

2) Connect red tubes directly using a union (found in LC-MS drawer)

3) On the main window, open file → activate method → go to methods directory under Varian WS → methods folder → select “flushing” method → open

4) Maximize the Prostar/Dynamax window to monitor the flow rate and mobile phase composition (leave in inject position to prevent corrosion to interface)

5) Flush for about 5 - 10 minutes

6) Maximize Prostar/Dynamax window again

7) Click “Stop Pumps” to stop flushing

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, 3 μm)

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 a plastic bag in the drawer

7. Check to see if shield inside electrospray chamber is clean, if not:
1) Moisten Kimwipe with LGW and wipe shield, ensuring that no water enters the MS
2) Repeat with methanol
3) Clean needle tip with methanol

3.2 LC-MS/MS analysis

Method Activation

-The 26 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 (QL) → tetracyclines (TC)

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

2. Make configuration adjustments to the method (should not be necessary if nothing has changed).
- 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 (ie 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
    - 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 a sequence

- File → new sample list → Varian WS → data → Your Name → create new folder and label using whichever labeling method is best for you
    - 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 solvent blank to equilibrate the LC column and to check 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.

Every few months, make a 10 µg/L test solution of the antibiotic mixture and analyze using the three methods to determine if the retention times have shifted.

6. Set up instrument parameters and turn on the ESI-MS (*before* you start the sequence)
    - Open N2 dewar all the way
- Select API auto on main screen
    - drying gas temp at 300ºC
    - API house temp at 50ºC
    - Click OK
    - Click icon with green arrow in the upper left to turn on electrospray
- Wait until drying gas temperature gets to 160ºC, 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 come to room temperature after being removed from the freezer

- Check that the clear glass solvent vial (called transport vial) behind the rack is full of the reconstituting solvent (90% LGW 10% MeOH)

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 (use only if the run stops at night or when no one is around to turn instrument off)

4. Data Analysis

4.1 Integration of analyte peaks

4.1.1 Manual integration

Software: Varian WS Work station 6.8.

Notes: targeted analyte ions must be integrated separately

1. Click on the Review/Process MS Data button

- Find data file

- Open chromatogram on the screen; an ion list will show up on left and the chromatogram for each ion breakdown will appear by clicking on it

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)

3. Adjust chromatograph
- Right click on chromatogram → select local chromatograph plot preferences → chromatogram plot → under filtrating → smooth data (points 5 or 7) AND remove spikes → OK

4. Zoom in to enlarge peak
    - Adjust x axis by dragging mouse along axis
    - Adjust on y axis by just clicking

5. Integrate peak
    - Select "set click and drag action" → integrate area
- Draw a straight line along the base of the peak
    - Enter area and retention time into Excel

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 (eg. 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. Select close

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

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 methods folder, select a representative 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 major targeted breakdown ion of I.S. (m/z = 198 > 128) (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 relative percent of difference (RPD) using the formula below. Use two significant figures.

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

Appendix B: Yeast Estrogen Screen (YES) Assay Protocols

This protocol is modified from Routledge and Sumpter 1996 Environmental Toxicology and Chemistry 15 (3): 241-248. Modifications include the use of plate-grown cultures to inoculate liquid media, less dilution of the growth culture into the assay medium and increased CPRG concentration. They yeast strain used for this YES-assay are the human estrogen receptor (hER) transfected Saccharomyces cerevisiae provided under agreement with Prof. J.P. Sumpter at Brunel University, UK. The modified SOP was written by Chad Roper and Ben Stanford.

Materials

Spectrophotometer (Visible Range)
96-well Plate Reader (Molecular Devices, EMax)
Shaker table

125 mL sidearm flask with metal cap
125 mL culture flask with metal cap
100 mL beaker
1 L or more screw top glass bottle
4 x 250 mL or more screw top bottles
Disposable sterile syringes with luer lock tip (60 mL)
Sterile syringe filters (0.2 μm for filter sterilization), disposable
Disposable filter sterilization flasks
Disposable sterile petri plates
Disposable sterile 96 well plates
Disposable sterile 96 well plate covers
Parafilm
10 mL sterile pipets (with pipet bulb)
V shaped wells for multichannel pipetting (autoclavable) with cover
8 Channel Pipettor (250 μL) and tips
Pipettor (20 μL) and tips
Aluminum foil
Autoclave tape
Ethanol (EtOH), high purity (denatured is ok)

Solutions:

- 500 mL solution of D-(1)-glucose 20% w/v solution
- 100 mL solution of L-aspartic acid 4 mg/mL solution
- 100 mL of L-threonine 24 mg/mL
- 25 mL 20 mM copper (II) sulfate solution
- 10 mg/mL stock solution of chlorophenol red-ß-D-galactopyranoside (CPRG) in LGW
- 100 mL solution 0.8 mg/mL solution of $Fe_2(SO_4)_3$
- 100 mL solution of 0.02 mg/mL solution of biotin
- 25 mL solution of chloramphenicol at 25 mg/mL (dissolve in ~2-3 mL EtOH first,

then add sterile water to 25 mL)

- 10 mg/L estradiol (E2) in ACN (for positive control row in assay)
    - 38 μL of 10 mg/L E2 working solution diluted to 10 mL EtOH

Minimal Media

Amounts are per liter LGW. The solution should be autoclaved at 121°C for a minimum of 15 minutes, and then stored at room temperature. In the event of precipitation during autoclaving, filter sterilize the solution into 500 mL sterile flasks.

- 13.61 g KH_2PO_4
- 1.98 g $(NH_4)_2SO_4$
- 4.2 g KOH
- 0.2 g $MgSO_4$
- 1 mL $Fe_2(SO_4)_3$ solution (1 g/L)
- 50 mg L-leucine
- 50 mg L-histidine
- 50 mg adenine
- 20 mg L-arginine-HCl
- 20 mg L-methionine
- 30 mg L-tyrosine
- 30 mg L-isoleucine
- 30 mg L-lysine-HCl
- 25 mg L-phenylalanine
- 100 mg L-glutamic acid
- 150 mg L-valine
- 375 mg L-serine

Vitamin Solution

Amounts below should be added to 180 mL LGW, then the solution should be filter sterilized into a sterilized screw top bottle and stored at 4°C.

- 8 mg thiamine
- 8 mg pyridoxine
- 8 mg pantothenic acid
- 40 mg insitol
- 20 mL of biotin solution

Sabauroud's Dextrose Agar with Chloramphenicol

Amounts given are per liter LGW. The solution should be autoclaved at 121°C for a minimum of 15 minutes, allowed to cool to 60°C, and then be poured into sterile plates in aliquots of 15 to 20 mL. Once the agar has set and cooled completely, the plates should be stored at 4°C in plastic Ziploc bags for up to one year.

- 60 g Sabourad's Dextrose Agar (Difco)
- 3 mL chloramphenicol solution (available as 25 mg/mL)

Propagation and Storage of Yeast Strain

Every two weeks (4 weeks maximum), a single colony should be selected from the current agar plate with the YES-human estrogen receptor (hER) culture and that colony should be streaked out on a new agar plate using proper technique. Agar plates should be wrapped in Parafilm after the colony has been streaked.

The new agar plate should be grown for 48 to 72 hours at 30°C and then stored in a refrigerator at 4°C. This then becomes the current agar plate for use.

In the event that the assay ceases to work due to the selection of a revertant colony of yeast incapable of responding to estrogenic compounds, the previous plate can be used for an inoculum and new streak plate. Dispose of old plates after 3 generations of colonies have been streaked onto new plates.

Assay Protocol (Day 1)

Preparation:

- Light Bunsen burner and work within a 1 foot radius to ensure sterility of minimal medium.
- Add 45 mL minimal medium to sidearm culture flask using a graduated cylinder, and put cap on. Label with autoclave tape. This will be the flask that grows the stock colony culture. Create one of these for every 8 flasks that will be inoculated (see chart below).
- Add 45 mL minimal medium to culture flask and put cap on. Label with autoclave tape (use the chart below to determine the number of these flasks that will be needed).

# of 96-well plates needed	# of culture flasks needed	# of stock colony side-arm culture flasks needed
2	1	1
4	2	1
6	3	1
8	4	1
10	4	2
12	5	2
14	6	2
16	7	2
18	8	3
20	8	3
22	9	3
24	10	3

- Wrap 100 mL beaker in aluminum foil.
- If pipette tips are not sterile, place pipette tips in box/rack, and label with autoclave tape.

- Wrap V-shaped well for multichannel pipetting and cover in foil (if not already sterile)
- Autoclave all of the above items at 121°C for 15 minutes on the liquids cycle.
- While hot, move all autoclaved items into biosafety cabinet and allow to cool.
- Light Bunsen burner and work within 1 foot radius to ensure sterility of solutions. Mix the following in the sterile 50 mL beaker using sterile pipettes. If sterile pipettes are not available, briefly wave the pipette through the flame of Bunsen burner to sterilize. *Note: amounts are per 45 mL minimal medium to be inoculated!
    - 5 mL glucose solution
    - 1.25 mL L-aspartic acid solution
    - 0.5 mL vitamin solution
    - 0.4 mL L-threonine solution
    - 125 µl copper (II) sulfate solution

- Draw this mixture into the 60 mL sterile disposable syringe and attach the syringe filter. Note the volume. Dispense the appropriate portion of the solution into one of the sterile minimal medium sidearm flasks aseptically (flame both the tip of the filter and lip of the flask). Add the remaining volume to each of the remaining flasks. **The volume will be approximately 7 mL added per flask**. Remember to flame lips of glassware and lids before putting caps back on.

Inoculation of Growth Medium

- Remove current streak plate of hER culture from refrigerator.
- In biosafety cabinet, remove Parafilm from plate.
- Flame loop the full length that will extend into the culture flask. If no loop is available, melt the end of a glass Pasteur pipette (when end closes, it's sterile).
- Select a single colony from the plate.
- Remove the lid.
- Touch loop to blank agar surface to cool it. Remove single colony from plate with loop. Immediately replace the plate lid.

- Open sidearm flask, flame the lip of the flask, and insert the loop with the colony into the media. Try not to touch the sides of the flask. Stir media with loop to remove colony. Remove loop from flask, flame the lip of the flask and cap the flask. Flame loop again.
- Put inoculated culture sidearm flask and other culture flasks into 30°C warm room on shaker table (approximately 100-130 rpm on continuous mode) until its optical density reaches ~1.0 (approximately 18-24 hours). The OD_{640} is often at about 0.6 to 0.8 after 24 hours, depending on the size of the colony used to inoculate the stock culture. The lower O.D. solutions still work just fine in the assay with no impact on sensitivity. Just add a bit more of this culture in the step below (e.g. 8 mL is fine for 0.6 O.D.). If, however, an OD_{640} of 0.6 has not been reached within 24 hours, discard the solution and start over.
    - The UV spectrophotometer is in Dr. Aitken's laboratory in MHRC. Turn on UV spec, open UV Spec Software. Select 'Spectrophotometer' on upper tabs, then 'Set Wavelength', select 650 nm. Hold cap onto sidearm flask and carefully insert arm into cuvette holder; be very careful not to spill any of the growth medium.

Preparation of Assay Plates (Day 2)

- Prepare fresh E2 working solution in EtOH in (38 μL in 10 mL EtOH from 10 mg/L E2 solution in ACN) volumetric flask from stock solution stored in freezer. This will be used for the positive control, row 1 on 96-well plates.
- In biosafety cabinet, cut open 96 well-plate and cover with sterile lid, label both plates and lids with a number and place lids on plates and set aside.
- **For EtOH-based samples**:
    - Pour some HPLC-grade EtOH into a small beaker. Draw up 3 volumes of EtOH with syringe and discard to waste.
    - Add 20 μL of E2 standard with syringe to the first two wells of row 1.
    - Clean syringe with HPLC-grade EtOH.
    - Add 20 μL of EtOH to first two wells in row B.

- Samples will be plated in duplicate (i.e. two rows for 1 sample). Add 20 μL of sample 1 to first two wells starting with row C. Add 20 μL of sample 1 to first two wells to row D.
- Repeat for subsequent samples, plating each sample in duplicate and cleaning syringe with EtOH in-between samples.

- Each row can be sequentially diluted with EtOH across the plate such that the effective volume of starting solution is decreased by half in each well. Pour some EtOH into a V-shaped well, set micropipettor to 20 μL, and put on sterile pipette tips. Pull up 20 μL of EtOH. Start at column 10 and inject 20 μL of EtOH into the left corners of each well in each previous column, stopping at column 2. The volume in the wells in column 2 will now be 40 μL.
- Pick up 20 μL in column 2 and mix 3-4 times by pulling the volume up and blowing it out. After mixing, pick up 20 μL and inject it into column 3. Mix up column 3, pick up 20 μL, and blow out into column 4. Repeat the process until 20 μL is in the wells of column 11. Leave column 12 empty!
- One row of negative controls (EtOH) and blanks should be included on each plate. Leave the last column (column 12) on the plate completely empty as a "blank" calibration for the plate reader. Allow solvents to evaporate completely by placing the plate (uncovered) in the laminar flow cabinet for 30 to 60 minutes. Once dry, cover with lid until ready to inoculate with yeast.
- **For aqueous samples:**
  - The same dilution scheme as the EtOH-based samples should be used without the added evaporation step. In the steps below where the assay medium is added, adjust volume to 230 μL instead of 250 μL used with the evaporated EtOH samples.

Dilution for Assay (after EtOH is evaporated)

- Into each flask to be used for the assay, add 0.5 mL of the 10 mg/mL sterile CPRG solution (10 mg of CPRG into 10 mL LGW). Solution in the culture flasks will turn yellow.
- Using a disposable, sterile 10 mL pipette, collect 5 mL (or the appropriate volume given the actual OD – a greater volume if OD is < 1.0) of the OD 1.0 culture in the **sidearm flask** and aseptically (flame the exterior of the pipette containing the culture briefly and flame the lip of the culture flask containing the assay medium) transfer that volume to a **culture flask**.
    - If using more than 1 culture flask, pour all of the solutions in the culture flasks into one culture flask to create a homogeneous mixture.
- Flame the lip of the culture flask again and cap the flask.
- Mix the culture in the flask gently for approximately 1 minute.
- Open the foil on the V-shaped well for multichannel pipetting.
- Open the cap on the culture flask and flame the lip.
- Pour the dilute culture into the V-shaped well.

Assay

- Change volume on micropipettor to 250 μL. Dispense inoculated assay solution 250 μL per well in the 96 well plate from V-shaped reservoir using the 8-channel pipettor and sterile pipette tips. (Use only 230 μL if working with aqueous samples!).
- Completely cover plate with Parafilm (about 2.5 lengths). If plate is not completely covered, the liquid in wells will evaporate and the evaporated wells cannot be used in the dose-response curve.
- Cover plate with lid and press down. 96 well plate wells should be sealed by Parafilm.
- Tap side of plate to mix well contents.
- Put plate(s) in 30°C warm room on counter.

Reading the Assay

72 hours after inoculation, the plate(s) can be read (though an additional 24 hours may be needed for full color development). The plate reader is in Dr. Nylander-French's laboratory on the 3rd floor of MHRC. Turn on plate reader, the password to the computer is leena123$!, open Shortcut to SPF. Select 'Experiment', then 'New Experiment'. Open the template and select which wells contain the standard, which contain samples, and which are blanks.

The lid and Parafilm should be removed and the plate inserted into the plate reader. Select 'Setup' and 'Set Wavelength' to measure absorbances at 562 nm (have to select 570 nm though) for chlorophenol red and 650 nm for turbidity should be recorded. Blank wells must be included in each plate as the software automatically corrects the remaining wells based on the absorbance of the empty ones. Data should be saved by date and plate number and then exported as a text file using the software export tool (File > Export . . .). **Remember to bring a usb key to take data off computer.** Remember to close software and turn off instrument when finished.

Data imported in Microsoft Excel should be manipulated as follows:

To obtain the plate reading that corrects for turbidity caused by the assay, subtract A_{650} from A_{570}. If multiple experiments are compared and the data need to be normalized, the relative absorbance (R) could also be converted into logistic form: $R = [(A_{562} - A_{650})\text{-}\min(A_{562}\text{-}A_{650}) / [\text{Max}(A_{562} - A_{650}) - \min(A_{562}\text{-}A_{650})]$, where "Max" is the maximum response for a given row of samples and "min" is the minimum response for a given row of samples. Simple sigmoidal dose-response plots can be created by placing the concentration on the x-axis with the absorbance (or relative abasorbance) on the y-axis.

In order to compare the results, the concentration which elicits 50% of the maximum response (EC50, also known as ED50) needs to be estimated for each compound and/or sample tested. This can be done by regression of the data according to the dose-response equation:

$$\textbf{!Syntax Error, +}\ y = \min + [(\max - \min) / (1 + 10^{(\log EC_{50} - x)\text{Hillslope}}]$$

where min = minimum corrected response plateau

max = maximum corrected response plateau for E2

y = response

x = log concentration

hill slope = slope of the sigmoidal dose-response curve at its midpoint

When the percent relative response is used, note that min = 0% and max = 100% for 17-β estradiol (E2). E2 is used as the standard reference compound for this assay and the maximum absorbance value is taken from the E2 control samples (Huber, Ternes et al., 2004). Each plate should theoretically have its own E2 "calibration curve" for proper reference.

The EC_{50} is used to compare relative estrogenicity for each compound/sample tested. For samples with unknown concentrations of estrogens, concentration is replaced with the concentration factor (relative to extraction from original sample). The hill slope taken from the E2 curve is used as a constant hill slope for the unknowns. E2 equivalents (EEQs) are expressed as EC_{50} for E2 / EC_{50} for the unknown.

For information on data manipulation using R-software, see Appendix C.

A note about data analysis: The original Routledge and Sumpter (1996) paper as well as several current papers use a simple 50% of maximum response estimation (e.g. draw a line at the 50% response and find where this intersects on the graph, then determine the corresponding x-axis concentration). Though this method has been accepted in peer-reviewed literature, it does not allow for calculation of statistical variation and confidence limits within data sets. By using the dose response equation, 90% (or higher) confidence intervals can be calculated for the EC_{50} and subsequent EEQs.

Clean Up

- All plates that have been inoculated (or that came in contact with yeast) should be autoclaved prior to disposal.

- All culture media that has been inoculated should be autoclaved prior to putting them down the drain.
- All glassware should be soap and water washed. Any glassware that has been acid washed should be rinsed with lab grade water and dried prior to use in this protocol.
- All disposable items that have had contact with media containing inoculated culture media should be autoclaved prior to diposal.

Routledge, E.J. and J.P. Sumpter (1996). Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environmental Toxicology and Chemistry 15 (3) pp. 241 – 248.

Appendix C: Instructions for Working with YES Data in R 2.4.0

In order to minimize the difficulty of working with a programming language like R 2.4.0 the following set of instructions have been prepared for the specific analysis of data generated by the yeast estrogen screen (YES) assay. Sections of these instructions were generated by Prof. Nina Cedergreen of Aarhus Unviersity, Denmark and were presented in the workshop "Statistical Assessment of Dose-Response Curves with Free Software" presented at the SETAC Montreal Conference, November 2006. The original instructions were for general dose-response curve and have since been modified in this research project to be specific to dealing with the YES assay. The original instructions were provided by Dr. Cedergreen with permission to use and modify as needed. As such, they are being included as an appendix to this dissertation with acknowledgement to Dr. Cedergreen and her colleague, Christian Ritz, who co-designed and led the workshop.

1. Software Installation

Assuming that the necessary software has been provided, double-click on the file R-2.4.0-win32.exe and install on the local computer. Follow all screen prompts.

Once installed, two packages must be installed to add functionality to the program for working with dose-response curves. On the upper tool bar click Packages > Install package(s) from local zip file… From there navigate to the folder from where R was installed and select plotrix_2.1-1.zip and drc_1.0-5.zip. Install each of those packages and follow screen prompts.

If the R software is not available it can be downloaded from http://www.r-project.org/ . Once it has been installed, the "drc" and "plotrix" packages can be downloaded under the *Packages* menu. Once downloaded, the installation instructions above can be followed.

2. Preparing Data in Excel for Export to R

There are two ways to import data into R from Excel: copy and paste from the clipboard or directly import from a comma separated format (.csv) file. In general, data in Excel need to be stored in .csv format, one worksheet only. Data also need to be in column format: Column 1 heading = "concentration"; 2 = "curve"; 3 = "Response"; 4 = "Type". "Type" is another label for "curve". The "curve" column should always have an associated number with a given group of data within the sample set (e.g. 1, 2, 3, etc.) while the "Type" column will be given names for each group of data within the sample set (e.g. E2 Control, Sample Extract 1, Negative Ctrl, etc.). Note that everything in R is

case sensitive. The column headings may be changed, just make sure to note the changes and place the correct text into the program.

The easiest way to prepare data is to have an Excel document where the first tab or "worksheet" is set up with the proper column headings. Once the data are in place and the summary worksheet is active, select *save as…* from within Excel, choose the ".csv" format, and give the file a new name (e.g. Data for Export to R.csv). The file is now ready for manipulation in R.

Another way to move small bits of data into R is to use the clipboard. Here, the user does not have to change anything to .csv format, but it is important to capture the column headings. Without the column headings, R uses the default headings and will not recognize the dose-response commands. Simply highlight the data with column headings, and press *ctrl-C* to copy. The data is now on the clipboard for importing into R.

3. R Start-up Commands and Data Import

From this point forward, all text in ***italicised, bold, Courier font*** can be directly copied and pasted into R as a command line. The ***black text*** must be used as is. Any ***text written in red*** can be changed to suit the name and/or functionality required by the user. The red text could be broadly called "***Dataname***". Use a period instead of a space to separate words in the dataname.

The first step to using R for the analysis of dose-response curves is to load the DRC package. This must be done every time the program is started. To load the DRC package type:

> ***library(drc)***

Once the DRC library is active, then data can be imported from Excel. For import of an entire .csv file, type:

> ***YeastE2*<- *read.csv(file.choose(), dec=".", sep=",", header=T)***

In the case of importing data from the clipboard, type:

YeastE2.Clipboard*<- *read.delim(file="clipboard", row.names=NULL)

The exact table or selection can be shown on screen by typing the dataname alone:

>***YeastE2***

The ***row.names=NULL*** gets around the error stating that each row must have unique name. The

command basically puts in a unique value for each row. For more information type,

>help(read.delim)

or type

>help(read.table)

To see a list data files associated with the workspace type:

>ls()

To clear the screen, press ctrl-L

4. Data Analysis and Generation of Results

There are many different models included in the DRC curve which can be used to describe a dose-response curve. The user may access these through any of the help commands or by referencing the "Statistical assessment of dose-response curves with free software: Collection of examples" packet created by Christian Ritz and Jens C. Streibig. The main model used for the YES assay is a four-parameter logistic model described by:

$$f(x,(b,c,d,e)) = c + \frac{d-c}{1+\exp\{b(\log(x)-\log(e))\}}$$

where b = hill slope

c = lower limit

d = upper limit

e = EC_{50}

In R, the four-parameter logistic function is named ***l4()***. In the case where the user's data contains only one dose response curve, the program will fit a best-fit curve by typing

>model <- multdrc(Response ~ concentration, curve, fct=l4(), data = YeastE2)

The above command will work for multiple curves, but the error associated with the curves is much higher than if they are treated singly. In the case where the data file contains multiple curves, simply break the analysis into subsets for each curve:

>model.curve1 <- multdrc(Response ~ concentration, fct=l4(), data = subset(YeastE2, curve == 1))

To view the numeric output of the results type

>summary(model)

To view the results graphically type

>plot(model)

The "plot" command alone does not generally provide enough detail to adequately interpret the data. For example, in the default "plot" settings data points below 0.01 are shown as x=0 and subsequently are masked or do not show up on the graph. The line of text input shown below will provide parameters for the most common changes necessary for each plot. Note that the order of text within the parentheses does not matter. As such, items may be moved out or added in to the parentheses as needed for each plot.

>plot(model, ylim=c(0,5), xlim=c(0,1000), conLevel=(0.0001), xlab="Dose", ylab ="Response", main= "Main Title")

Explanation:

> ***ylim*** sets the min to 0 and the max to 5 (or whatever values are entered); same for ***xlim***.
>
> ***conLevel*** forces all values above the number entered will be shown as their true value, not zero as in the default settings.
>
> ***xlab, ylab***, and ***main*** provide labels/titles for the plot

Other functions which can be added to the ***plot()*** command include:

> ***Type = "points"*** shows all points instead of the average of points as in the default
>
> ***legendPos=c(100,1))*** fixes the legend at the desired x, y coordinates
>
> ***add=TRUE*** allows the user to add multiple plots to the currently displayed plot
>
> ***col=c(1,3,5,9)*** or ***col=TRUE*** adds colors to the plots. To get help on the numerical values for color names, type ***help(plot)*** or ***help(col)*** or ***help(color).***
>
> ***ldw=c(1)*** changes the line width. Larger numbers make bolder lines; the number defines the number of pixels used to create the line width.
>
> ***lty=0*** makes the model line disappear while still plotting the points (lty = line type). The ***lty*** command can also be used to modify dash/dot and color settings.

pch=19 can be used to modify the point characteristics (e.g. change dots to open triangles, filled triangles, squares, etc.). A full string of values can be accessed in help(plot)

There are numerous lines of text towards the end of this document that describe different things the user can do with the program and how to examine and manipulate the data. At this point the instructions will focus on what to type in order to process a YES data set with multiple curves and describe how to interpret the data.

The section of the model fit command line that is ***fct=l4()*** invokes the program to solve for each parameter b, c, d, and e. In many cases it is desirable to force upper limits, lower limits, and/or slope values based upon the behavior of the estradiol positive control standard or samples of similar matrix and concentration. The command line needed to change the ***fct=l4()*** model from the default settings to the user settings looks like this:

model.modified <- multdrc(Response ~ concentration, Type, fct=l4(fixed=c(NA, 0.1, 3.6, NA)), data = YeastE2)

OR,

model.modified.curve1 <- multdrc(Response ~ concentration, fct=l4(fixed=c(NA, 0.1, 3.6, NA)), data = subset(YeastE2, curve == 1))

The ***l4(fixed=c(NA, 0.1, 3.6, NA))*** portion of the command line can be set to ***l4(fixed=c(NA, NA, NA, NA))*** in order to return to all default settings. "NA" specifies default settings while numerical values specify user input. Each of the input parameters within the parentheses refer to b, c, d, and e in order. Of course, "e" should never be specified (since "e" corresponds with the EC_{50} value for which the program is trying to solve). The lower limit (c) will rarely be zero since the lowest corrected response is typically around 0.11 absorbance units. Similarly, the upper response (d) cannot exceed 4 (the maximum absorbance possible on from the plate reader) and is typically between 3.1 and 3.8. The hillslope (b) should be modified to reduce the standard error associated with each line. Visually (using the plot() command) this corresponds to the fitted line evenly splitting the data and passing through as many points as possible. Numerically this corresponds with a decrease in standard error shown in the ***summary(model)*** command. Both numerical and graphical data should be visually inspected to ensure a proper line fit. The user may compare two model results using an ANOVA test to determine statistical significance as follows:

>anova(model, model.modified)

Please note that during the data analysis phase, if the user does not change/modify the model name each time then the data from the previous model will be overwritten. This is important when wanting to compare models and compare samples.

As data are processed, plots should be copied and pasted into a Microsoft Word document. R does not have the option of uniquely naming each plot and then recalling the plot later. Therefore, the plots should be saved as soon as they are generated. Plots may be copied and pasted using the *copy* and *paste* commands in the edit menu. The user may specify whether to export/copy as a windows metafile or as a bitmap image.

Similarly, numerical data should be copied and pasted into Microsoft Word. The simplest way to do this is at the end of processing all data, invoke the program to produce summaries of all the models obtained. e.g.:

>summary(model.1)

>summary(model.2)

>summary(model.3)

>summary(model.4)

…etc.

Copy the whole lot into Word and/or into a blank Microsoft Excel spreadsheet. In Microsoft Excel, the data can be force into column format by selecting from the toolbar *Data>Text to Columns…* after selecting all of the data in the first column.

5. Convergence Errors

In the case of hormesis where yeast die off in high concentration extracts, the program will give a convergence error like the following:

Error in mdrcOpt(opfct, startVec, optMethod, derFlag, constrained, warnVal, :

Convergence failed

This is due to the programs inability to determine where in the data set the maximum value and minimum values can be found. The user has two options: The easiest option is to go back into the data set (in Excel) and delete the upper hormesis/die-off data points. From here, copy the data back into R and then use the data available to fit the slope and expected upper limit for the assay. The other option involves use of the five-parameter logistic equation (***fct=l5()***) to account for hormesis. The former approach has been validated in Chapter 3 of this dissertation while the latter approach has not been tested here.

6. Programming Information from Original Workshop

The commands listed below are those originally provided by Nina Cedergreen at the "Satistical Assessment of Dose-Response Curves with Free Software" workshop. Note that the # sign is used to signify a new line. This was done to allow the user to copy and paste entire sections of programming and instruction into R. Lines with the # sign at the beginning are not recognized as commands by R, so they are simply ignored.

One curve only in the data set

#For fitting one dose-response curve to a model (model name is given in the manual) you write:

model<-multdrc(Response~dose, fct=l3(), data = Dataname)

To see a graph of your model and data averages write:

plot (model)

To get the parameters of your function, write:

summary(model)

#The t-value tests whether the parameter is significantly different from zero. If c is not (p > 0.05), a # model without a lower limit is recommended.

To check for homogeneity of variance in your data, you can inspect a plot of the residuals versus # the model predicted values:

plot(fitted(model), residuals(model))

If the variance is not homogeneous, data should be transformed. We use a box-cox

transformation. This is done by adding the following term (boxcox=TRUE). If you have missing

value in your dataset, you can use (na.action=na.omit):

tmodel<-multdrc(response~dose, fct=l4(), data = Dataname, boxcox = TRUE, na.action=na.omit)

Remember. If your data-set contains zero or negative values, they cannot be transformed using

boxcox.

If you want to know for example the ED_{10} or the ED_{90} you write:

ED(tmodel, c(10,90))

Multiple curves

To get a view over your multiple curves, write:

plotraw(response~dose, curve, data = Dataname, trellis=TRUE)

To fit a model to the data, write:

mult<-multdrc(response~dose, curve, fct=l4(), data = Dataname, boxcox = T)

#To fit multiple data with one common parameter (here the upper limit. Parameter names are #arranged alphabetically)
multa<-multdrc(response~dose, curve, fct=ml3a(), data = Dataname, collapse=data.frame(curve, 1,curve, curve))

#To do F-test between models, do this:

anova(mult, multa)

#If no significant difference between these two models, then use the simpler of the two

summary, plots, residuals, ED-values ect. are as for one curve.

Binomial endpoint

Binominal data is distributed differently from gradual data, and therefore need to be described

with a different model. Note that also the data has to be set up differently with a column for both

the response (for example “immobile”) and the total number of individuals. An l2 model fits a

curve which is defined with an upper limit of one and a lower limit of zero. Apart from l2, the

same models can be used for binominal data as for gradual data.

bin* <- *multdrc(no/total~dose, weights =total, fct = l2(), type = "binomial", data = Dataname)

To fit more than one curve, to receive a summary, plots, residuals, ED-values ect. The same

commands are used as for gradual data.

Fixing a parameter at a certain value

A parameter can be fixed using the five parameter logistic model, where the parameters are listed #alphabetically: *b, c, d, e, f*, with *f* being a parameter describing hormesis. Setting *f* to one reduces #the model to a four parameter logistic model (l4) model. The example fixes *c* at 0.5 while #estimating *b, d* and *e*:

model*<-*multdrc(response~dose, fct=logistic(fixed=c(NA, NA, 4, NA, 1), data = Dataname))

Graphs

There are several commands to use, to modify your graphs. Some of the most used follow here:

The value you want your controls to be situated at (conLevel= 0.1), the name you will put instead

of the control value (conName= "control"), the labels of your axes (xlab = dose, ylab= response),

the limits of your axes (ylim= c(0,10)), if you want to see all your data (type= "points"), or if you # want to hide the data (obs = "none"). Colours can be added on the graph (col = TRUE). The

command are just added to the plot-command line as in the example:

plot(model, conlevel = 0.1, xlab = "Concentration (mM)", ylab = "Freshweight (g)")

If you want to know more about the use of R as a programme to solve statistical problems you could consult the http://www.r-project.org/ and browse through the manuals. A good way to start is to go to the contributed documentation.

7. DRC Help File for “Plot” Copied and Pasted From R.2.4.0

DRC Plot Instructions

plot.drc(drc)

Plotting fitted curves for a drc object

Description

'plot' displays fitted curves and observations in the same plot window, distinguishing between curves by different plot symbols and line types.

Usage

```
## S3 method for class 'drc':
plot(x, ..., level = NULL, broken = FALSE, col = FALSE,
conLevel, conName, grid = 100, legend, legendText, legendPos,
type = "average", obs, lty, log = "x", pch, xlab, ylab, xlim, ylim,
bcontrol = NULL, xt = NULL, xtlab = NULL, yt = NULL, ytlab = NULL,
add = FALSE)
```

Arguments

`x`	an object of class 'drc'. For instance, 'lwd=2' or 'lwd=3' increase the width of plot symbols.
`...`	additional arguments.
`level`	vector of character strings. To plot only the curves specified by their names.
`broken`	logical. If TRUE the x axis is broken provided this axis is logarithmic (requires the CRAN package 'plotrix').
`col`	either logical or a vector of colours. If TRUE default colours are used. If FALSE (default) no colours are used.
`conLevel`	numeric. Dose level below which the dose is zero (the amount of stretching on the x-axis above zero). Default is 1e-2.
`conName`	character string. Name on x axis for dose zero. Default is '"0"'.
`grid`	numeric. Number of points in the grid used for plotting the fitted curves.
`legend`	logical. If TRUE a legend is displayed.

`legendText` a character string or vector of character strings specifying the legend text.

`legendPos` numeric vector of length 2 giving the position of the legend.

`type` a character string specifying how the originals observations should be plotted. There are 4 options: "average" (default), "none" (only the fitted curve(s)), "obs" (only the data points), "all" or "points" (all data points).

`obs` Outdated argument. Use `type`.

`lty` a numeric vector specifying the line types.

`log` a character string which contains "'x'" if the x axis is to be logarithmic, "'y'" if the y axis is to be logarithmic and "'xy'" or "'yx'" if both axes are to be logarithmic. The default is "x". The empty string "" yields the original axes.

`pch` a vector of plotting characters or symbols (see `points`).

`xlab` an optional label for the x axis.

`ylab` an optional label for the y axis.

`xlim` a numeric vector of length two, containing the lower and upper limit for the x axis.

`ylim` a numeric vector of length two, containing the lower and upper limit for the y axis.

`bcontrol` a list. Controlling the appearance of the break (if 'broken' is TRUE).

`xt` a numeric vector containing the positions of the tick marks on the x axis.

`xtlab` a vector containing the tick marks on the x axis.

`yt` a numeric vector, containing the positions of the tick marks on the y axis.

`ytlab` a vector containing the tick marks on the y axis.

`add` logical. If TRUE then add to already existing plot.

Details

Suitable labels are automatically provided.

The use of `xlim` allows changing the x-axis, extrapolating the fitted dose-response curves.

See `colors` for the available colours.

Value

An invisible data frame with the values used for plotting the fitted curves. The first column contains the dose values, and the following columns (one for each curve) contain the fitted response values.

Author(s)

Christian Ritz and Jens C. Streibig Contributions from: Xiaoyan Wang

See Also

plotraw plots the observations only.

Examples

```
## Fitting models to be plotted below
model1 <- multdrc(MEANLR~MM, data=FA)
model2 <- multdrc(MEANLR~MM, data=FA, fct=l3())  # lower limit fixed at 0

## Plotting observations and fitted curve for 'model1'
plot(model1)

## Adding fitted curve for 'model2'
plot(model2, add = TRUE, type = "none",
col = 2, lty = 2)

## Fitting model to be plotted below
model3 <- multdrc(SLOPE~DOSE, CURVE, data=PestSci)

## Plot with no colours
plot(model3, main="Different line types (default)")

## Plot with default colours
plot(model3, col=TRUE, main="Default colours")

## Plot with specified colours
plot(model3, col=c(2,6,3,23,56), main="User-specified colours")

## Plot of curves 1 and 2 only
plot(model3, level=c(1,2), main="User-specified curves")

## Fitting another model to be plotted below
model4 <- multdrc(weight~conc, data=hormesis)
```

```
## Using the argument 'conLevel'. Compare the plots!
par(mfrow=c(2, 2))
plot(model4, main="conLevel=1e-2 (default)")  # using the default
plot(model4, conLevel=1e-4, main="conLevel=1e-4")
plot(model4, conLevel=1e-6, main="conLevel=1e-6")
plot(model4, conLevel=1e-8, main="conLevel=1e-8")
par(mfrow=c(1,1))

## Using the argument 'broken'
plot(model1, conLevel = 0.1, broken = TRUE)
plot(model1, conLevel = 0.1, broken = TRUE,
bcontrol=list(style="zigzag"))

## Removing models from work space
rm(model1, model2, model3, model4)
```

Appendix D: Antibiotic Occurrence in Streams and Reservoir

Concentrations are in ng/L; < 20 indicates that analytes were not detected above a S:N ratio above 3 in chromatograms; < 20 (--) indicates that the analyte was detected above a S:N ratio above 3 in chromatograms, but concentration was below PQL of 20 ng/L. Underlined values indicate that only one MS/MS product ion was used to confirm the analyte.

Table D.1: Stream A 8/9/07 Sampling Event

Antibiotic	Upstream	Effluent	DS A1	DS A2
Sulfamethoxazole	< 20	26	124	814
Sulfathiazole	< 20	54	97	< 20 (11)
Sulfadimethoxine	< 20	359	37	< 20 (7)
Erythromycin	< 20	527	316	40
Trimethoprim	< 20	< 20 (14)	170	66
Lincomycin	< 20	< 20 (4)	< 20	< 20
Ciprofloxacin	< 20	154	104	43
Levofloxacin	< 20	269	148	< 20
Sarafloxacin	< 20	< 20	< 20	< 20
Tetracycline	< 20	< 20	< 20	< 20 (0.5)

Table D.2: Stream B 8/9/07 Sampling Event

Antibiotic	Upstream	Effluent	DS B1	DS B2
Sulfamethoxazole	< 20	1419	1153	240
Sulfathiazole	< 20	< 20 (17)	195	97
Sulfadimethoxine	< 20	< 20 (14)	56	< 20 (12)
Trimethoprim	< 20	42	651	98
Sulfamerazine	< 20	189	43	80
Erythromycin	< 20	86	155	73
Lincomycin	< 20	< 20 (6)	< 20 (5)	25
Ciprofloxacin	< 20	77	no data	< 20 (12)
Levofloxacin	< 20	261	no data	< 20 (7)
Sarafloxacin	< 20	67	no data	82
Norfloxacin	< 20	< 20 (19)	< 20	< 20
Tetracycline	< 20	< 20 (11)	< 20	< 20 (16)

Note : nd = no data, measurements were not collected at this site on this day

Table D.3: Stream C 8/9/07 Sampling Event

Antibiotic	Upstream	Effluent	DS C1	DS C2
Sulfamethoxazole	< 20	556	198	302
Sulfadimethoxine	< 20	165	75	< 20 (12)
Trimethoprim	< 20	< 20 (19)	55	28
Erythromycin	< 20	58	36	55
Lincomycin	< 20	< 20 (0.5)	< 20 (13)	24
Ciprofloxacin	< 20	35	< 20	< 20
Levofloxacin	< 20	47	< 20	92
Sarafloxacin	< 20	127	< 20	< 20
Enrofloxacin	< 20	< 20 (18)	< 20	< 20
Tetracycline	< 20	34	< 20	< 20

Table D.4: Stream A 8/25/07 Sampling Event

Antibiotic	Upstream	Effluent	DS A1	DS A2
Sulfamethoxazole	< 20	206	188	94
Sulfathiazole	< 20	66	21	81
Sulfadimethoxine	< 20	448	26	< 20 (13)
Erythromycin	< 20	693	32	209
Trimethoprim	< 20	< 20 (19)	36	109
Lincomycin	< 20	296	51	< 20
Ciprofloxacin	< 20	161	21	< 20 (12)
Levofloxacin	< 20	148	122	< 20
Tetracycline	< 20	< 20 (3)	121	< 20

Table D.5: Stream B 8/25/07 Sampling Event

Antibiotic	Upstream	Effluent	DS B1	DS B2
Sulfamethoxazole	< 20	1292	237	237
Sulfathiazole	< 20	238	< 20 (7)	127
Trimethoprim	< 20	437	190	208
Sulfadimethoxine	< 20	< 20 (16)	< 20	< 20 (10)
Sulfamerazine	< 20	134	108	< 20 (11)
Erythromycin	< 20	75	58	41
Lincomycin	< 20	< 20 (3)	< 20 (14)	< 20 (8)
Ciprofloxacin	< 20	35	< 20 (2)	< 20
Levofloxacin	< 20	< 20 (19)	< 20 (9)	< 20 (12)
Sarafloxacin	< 20	121	36	< 20 (13)
Norfloxacin	< 20	< 20 (5)	< 20	< 20
Enrofloxacin	< 20	< 20	< 20	< 20
Tetracycline	< 20	< 20 (9)	< 20	< 20

Table D.6: Stream C 8/25/07 Sampling Event

Antibiotic	Upstream	Effluent	DS C1	DS C2
Sulfamethoxazole	< 20	651	443	651
Sulfathiazole	< 20	113	< 20	< 20
Sulfadimethoxine	< 20	208	128	111
Trimethoprim	< 20	< 20 (11)	< 20 (2)	< 20 (5)
Erythromycin	< 20	53	45	46
Lincomycin	< 20	< 20 (1)	161	< 20 (2)
Ciprofloxacin	< 20	62	< 20 (6)	< 20 (9)
Levofloxacin	< 20	241	< 20 (12)	< 20 (10)
Sarafloxacin	< 20	65	< 20 (4)	< 20 (9)
Enrofloxacin	< 20	274	21	235
Tetracycline	< 20	< 20	< 20	< 20

Table D.7: Stream A 9/22/07 Sampling Event

Antibiotic	Upstream	Effluent	DS A1	DS A2
Sulfamethoxazole	< 20	2828	2860	1480
Sulfathiazole	< 20	217	287	< 20
Trimethoprim	< 20	168	195	77
Sulfadimethoxine	< 20	35	< 20 (19)	40
Erythromycin	< 20	218	181	98
Lincomycin	< 20	24	< 20 (1)	< 20 (17)
Ciprofloxacin	< 20	55	< 20 (14)	< 20 (3)
Levofloxacin	< 20	338	162	27
Tetracycline	< 20	26	< 20 (10)	< 20

Table D.8: Stream B 9/22/07 Sampling Event

Antibiotic	Upstream	Effluent	DS B1	DS B2
Sulfamethoxazole	< 20	1809	1337	806
Sulfadimethoxine	< 20	184	198	91
Trimethoprim	< 20	696	672	240
Sulfathiazole	< 20	173	27	26
Erythromycin	< 20	173	152	97
Lincomycin	< 20	28	< 20 (3)	24
Ciprofloxacin	< 20	35	60	< 20 (11)
Levofloxacin	< 20	40	251	23
Sarafloxacin	< 20	130	56	131
Norfloxacin	< 20	< 20 (17)	< 20 (12)	< 20
Enrofloxacin	< 20	< 20 (18)	302	< 20 (10)
Tetracycline	< 20	< 20	< 20	< 20

Table D.9: Stream C 9/22/07 Sampling Event

Antibiotic	Upstream	Effluent	DS C1	DS C2
Sulfamethoxazole	< 20	689	1106	1019
Sulfathiazole	< 20	185	110	< 20
Trimethoprim	< 20	< 20 (13)	< 20 (5)	< 20 (4)
Erythromycin	< 20	65	56	38
Lincomycin	< 20	< 20 (1)	< 20 (2)	< 20 (3)
Ciprofloxacin	< 20	67	< 20 (0.13)	< 20 (13)
Levofloxacin	< 20	282	< 20 (14)	< 20 (5)
Sarafloxacin	< 20	80	< 20 (12)	< 20 (18)
Enrofloxacin	< 20	311	29	215
Tetracycline	< 20	< 20	< 20	< 20

Table D.10: Stream A 10/5/07 Sampling Event

Antibiotic	Upstream	Effluent	DS A1	DS A2
Sulfamethoxazole	< 20	1777	1749	1245
Sulfathiazole	< 20	160	130	144
Trimethoprim	< 20	87	147	60
Sulfadimethoxine	< 20	< 20 (16)	53	30
Erythromycin	< 20	140	103	85
Lincomycin	< 20	< 20 (8)	< 20 (15)	< 20 (4)
Ciprofloxacin	< 20	< 20 (19)	35	< 20
Levofloxacin	< 20	253	171	27
Enrofloxacin	< 20	< 20 (0.3)	< 20 (13)	< 20 (1)
Tetracycline	< 20	< 20 (13)	< 20	< 20

Table D.11: Stream B 10/5/07 Sampling Event

Antibiotic	Upstream	Effluent	DS B1	DS B2
Sulfamethoxazole	< 20	1894	nd	155
Trimethoprim	< 20	1396	nd	98
Sulfadimethoxine	< 20	500	nd	49
Sulfathiazole	< 20	43	nd	< 20
Erythromycin	< 20	153	nd	37
Lincomycin	20	20	nd	< 20 (18)
Ciprofloxacin	< 20	86	nd	< 20 (18)
Levofloxacin	< 20 (10)	114	nd	168
Sarafloxacin	< 20	198	nd	58
Norfloxacin	< 20	51	nd	< 20
Enrofloxacin	< 20	42	nd	< 20
Tetracycline	< 20	42	nd	< 20 (16)

Note : nd = no data, measurements were not collected at this site on this day

Table D.12: Stream C 10/5/07 Sampling Event

Antibiotic	Upstream	Effluent	DS C1	DS C2
Sulfamethoxazole	< 20	1118	1379	1051
Trimethoprim	< 20	14	43	< 20 (3)
Sulfathiazole	< 20	242	< 20 (0.04)	< 20 (17)
Erythromycin	< 20	60	155	81
Lincomycin	< 20	55	60	< 20 (9)
Sulfadimethoxine	< 20	214	296	91
Ciprofloxacin	< 20	18	<20 (1)	< 20 (17)
Levofloxacin	< 20	109	< 20 (8)	< 20 (2)
Sarafloxacin	< 20	< 20	< 20	< 20 (7)
Enrofloxacin	< 20	< 20 (10)	< 20 (1)	< 20 (1)
Tetracycline	< 20	< 20	< 20	< 20

Table D.13: Stream A 12/11/07 Sampling Event

Antibiotic	Upstream	Effluent	DS A1	DS A2
Sulfamethoxazole	< 20	3255	2531	1584
Trimethoprim	< 20	106	238	59
Sulfdimethoxine	< 20	27	20	459
Sulfathiazole	< 20	713	436	335
Erythromycin	< 20	224	153	122
Lincomycin	< 20	< 20 (6)	< 20 (1)	< 20 (5)
Ciprofloxacin	< 20	55	< 20 (18)	< 20
Levofloxacin	< 20	338	157	33
Enrofloxacin	< 20	16	10	49
Tetracycline	< 20	20	214	< 20

Table D.14: Stream B 12/11/07 Sampling Event

Antibiotic	Upstream	Effluent	DS B1	DS B2
Sulfamethoxazole	< 20	3006	1210	1936
Trimethoprim	< 20	1270	432	181
Sulfadimethoxine	< 20	293	155	< 20 (17)
Sulfathiazole	< 20	< 20	41	< 20
Erythromycin	< 20	139	108	50
Lincomycin	< 20	25	< 20 (4)	< 20 (5)
Ciprofloxacin	< 20	59	84	< 20 (6)
Levofloxacin	< 20	76	23	< 20 (1)
Sarafloxacin	< 20	< 20	88	97
Tetracycline	< 20	34	< 20 (10)	< 20

Table D.15: Stream C 12/11/07 Sampling Event

Antibiotic	Upstream	Effluent	DS C1	DS C2
Sulfamethoxazole	< 20	1304	791	1199
Trimethoprim	< 20	< 20 (12)	< 20 (6)	< 20 (2)
Sulfathiazole	< 20	337	63	< 20
Erythromycin	< 20	59	57	87
Lincomycin	< 20	61	< 20 (13)	< 20 (2)
Sulfadimethoxine	< 20	25	< 20 (4)	< 20 (11)
Ciprofloxacin	< 20	< 20 (13)	< 20 (10)	< 20 (2)
Levofloxacin	< 20	101	< 20 (17)	< 20
Sarafloxacin	< 20	< 20	< 20 (5)	< 20
Enrofloxacin	< 20	< 20 (13)	< 20 (6)	242
Tetracycline	< 20	< 20	< 20	< 20

Table D.16: Reservoir 5 Sampling Event

Antibiotic	8/25/07 R5	9/22/07 R5	10/5/07 R5	12/6/07 R5
Sulfamethoxazole	< 20 (13)	51	64	38
Erythromycin	< 20 (15)	37	44	< 20 (6)
Trimethoprim	37	< 20 (16)	< 20	< 20 (7)
Lincomycin	< 20	24	44	21
Sulfadimethoxine	< 20	< 20	< 20	< 20 (4)
Ciprofloxacin	< 20	< 20 (1)	< 20 (4)	< 20 (3)
Levofloxacin	< 20 (10)	< 20 (1)	< 20 (7)	< 20 (3)
Norfloxacin	< 20	< 20 (11)	< 20 (16)	< 20 (4)
Enrofloxacin	< 20	< 20 (0.3)	< 20 (2)	< 20 (1)
Tetracycline	< 20	< 20	< 20	< 20

Table D.17: Antibiotic Occurrence in Drinking Water Reservoir 12/11/07 Sampling Event

Antibiotic	R1	R2	R3	R4	R5	R6	R7
Sulfamethoxazole	< 20 (14)	64	60	144	38	223	238
Erythromycin	< 20 (15)	< 20 (0.02)	< 20 (17)	21	< 20 (6)	< 20 (12)	75
Trimethoprim	< 20	37	< 20 (12)	30	< 20 (7)	< 20 (18)	46
Lincomycin	< 20 (15)	< 20 (1)	21	23	21	< 20 (7)	59
Sulfadimethoxine	< 20	< 20	< 20	< 20	< 20 (4)	< 20	< 20
Ciprofloxacin	< 20 (5)	< 20	< 20 (1)	< 20 (5)	< 20 (3)	< 20 (0.3)	35
Levofloxacin	< 20 (6)	< 20	< 20 (3)	< 20 (2)	< 20 (3)	< 20 (4)	45
Norfloxacin	< 20 (6)	< 20	64	< 20	< 20 (4)	< 20 (1)	51
Enrofloxacin	< 20(14)	< 20	62	51	< 20 (1)	< 20 (5)	38
Tetracycline	30	< 20	< 20 (4)	< 20 (3)	< 20	< 20 (9)	< 20
Oxytetracycline	< 20	< 20	< 20 (6)	< 20 (7)	< 20	< 20	< 20
Chlortetracycline	< 20	< 20	< 20 (6)	< 20 (14)	< 20	< 20	< 20

References

Adams, C.; Wang, Y.; Loftin, K.; Meyer, M. (2002). Removal of antibiotics from surface and distilled water in conventional water treatment processes. Journal of Environmental Engineering 128: 253-260.

Ahel, M. and Giger, W. (1993). Partitioning of alkylphenols and alkylphenol polyethoxylates between water and organic solvents. Chemosphere 26: 1471-1478.

Alley,W. (2004). *Regional Groundwater Quality*. Van Nostrand Reinhold, New York, New York.

Altschuler, R. (2002). "Do medications really expire?" Medscape Psychopharmacology Today, September 9, 2002.

Andreozzi, R.; Raffaele, M.; Nicklas, P. (2003). Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environments. Chemosphere 50 (10): 1319-1330.

Associated Press. "Cities rarely release water test results". USA Today, March 10, 2008.

Aymes, S. (2001). *Magic Bullets, Lost Horizons: The Rise and Fall of Antibiotics*. London, New York: Taylor & Francis.

Backhaus, T.; Scholze, M.; Gimme, L.H. (2000). The single substance and mixture toxicity of quinolones to the bioluminescent bacterium *Vibrio fischeri*. Aquatic Toxicology 49: 49-61.

BAPPG. Bay Area Pollution Prevention Group. (2006). Report on the San Francisco Bay Area's Safe Medicine Disposal Days. Available at: http://www.baywise.info/disposaldays/SFBAY_SafeMeds_Report_August2006.pdf.

Baran, W.; Sochackaa, J.; Wardas, W. (2006). Toxicity and biodegradability of sulfonamides and products of their photocatalytic degradation in aqueous solutions. Chemosphere 65 (8): 1295-1299.

Barber, L.B.; Murphy, S.F.; Verplanck, P.L.; Sandstrom, M.W.; Taylor, H.E.; Furlong, E.T. (2006). Chemical loading into surface water along a hydrological, biogeochemical, and land use gradient: A holistic watershed approach. Environmental Science & Technology 40: 475-486.

Barbosa, J.; Barron, D.; Jimenez-Lozano, E.; Sanz-Nebot, V. (2001). Comparison between capillary electrophoresis, liquid chromatrography, potentiometric and

spectrophotometric techniques for evaluation of pKa values of zwitterionic drugs in acetonitrile-water mixtures. Analytica Chimica Acta 437: 309-321.

Baronti, C.; R. Curini; D'Ascenzo, G.; DiCorcia, A.; Gentili, A.; Samperi, R. (2000). Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. Environmental Science & Technology 34 (24): 5059-5066.

Batt, A.; Snow, D.; Aga, D.S. (2006). Occurrence of sulfonamide antimicrobials in private water wells in Washington County, Idaho, USA. Chemosphere 64 (11): 1963-1971.

Boivin, M. (1997). The cost of medication waste. Canadian Pharmaceutical Journal 130 (4): 32-39.

Boxall, A. B. A. (2008). Chapter 5: Fate and transport of veterinary medicines in the soil and environment. *Fate of Pharmaceuticals in the Environment and in Water Treatment Systems*. Diana S. Aga (editor), CRC Press, Boca Raton, FL.

Bound, J. P. and Voilvoilis, N. (2004). Pharmaceuticals in the aquatic environment – a comparison of risk assessment strategies. Chemosphere 56: 1143-1155.

Brain, R.; Johnson, D.; Richards, S.; Sanderson, H.; Sibley, P.; Solomon, K. (2004). Effects of 25 pharmaceutical compounds to *Lemna Gibba* using a seven-day static renewal test. Environmental Toxicological Chemistry 23: 371-382.

Braybrook, S.; John, D. N.; Leong, K. (1999). A survey of why medicines are returned to pharmacies. The Pharmaceutical Journal, 263: 7063.

Brion, F.; Tyler, C.R.; Palazzi, X.; Laillet, B.; Porcher, J.M.; Garric, J.; Flammarion, P. (2004). Impacts of 17[Beta]-Estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-live stages in zebrafish (Danio Rerio). Aquatic Toxicology 68: 193-217.

Brooks, B.W.; Turnera, P.K.; Stanleya, J.K.; Weston, J.J.; Glidewella, E.A.; Forand, Slattery, C.M.; Thomas W. La Pointa, Huggett, D.B. (2003). Waterborne and sediment toxicity of fluoxetine to select organisms. Chemosphere 52 (1): 135-142.

Brown, K. D.; Kulis, J.; Thomson, B.; Chapman, T. H.; Mawhinney, D. B. (2006). Occurrence of antibiotics in hospital, residencial, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. Science of the Total Environment 366 (2-3): 188-192.

Campbell, C.G.; Borglin, S.E.; Green, F.B.; Grayson, A.; Wozei, E.; Stringfellow, W.T. (2006). Biologically directed environmental monitoring, fate, and transport of

estrogenic endocrine disrupting compounds in water: a review. Chemosphere 65: 1265-1280.

CDM, Camp Dresser & McKee Inc. (2004). Guidelines for water reuse. U.S. Environmental Protection Agency. (Vol. 2007) Available at: http://www.epa.gov/ORD/NRMRL/pubs/625r04108/625r04108.pdf. Accessed: September 15, 2007.

Center for Disease Control and Prevention (2007). Press Release October 17, 2007. http://www.cdc.gov/od/oc/Media/pressrel/2007/r071016.htm

CFR. Code of Federal Regulations. (2006). *Code of Federal Regulations*. Office of the Federal Register National Archives and Records Administration. Available: http://www.gpoaccess.gov/cfr/index.htmlCFR, Section 25: http://www.access.gpo.gov/nara/cfr/waisidx_06/21cfr25_06.html, Section 25.22

Chapman, A. I. (2003). Pharmaceutical Reverse Distributor Survey, Final Report. Seattle, WA: Local Hazardous Waste Management Program in King County. Available: www.govlink.org/hazwaste/publications/ReverseDistributorSurvey.pdf.

Choi, S.M.Y.; Sun D.L.; Byung M. (2004). Toxicological characteristics of endocrine-disrupting chemicals: developmental toxicity, carcinogenicity, and mutagenicity. Journal of Toxicology & Environmental Health: Part B (7): 1-32.

Christakos, G.; Bogaert, P.; Serre, M.L. (2000a). Spatiotemporal study of environmental exposure-health effect associations. Journal of Exposure Analysis and Environmental Epidemiology 10 (2): 168-187.

Christakos, G.; Bogaert, P.; Serre, M.L. (2000b). BME analysis of spatiotemporal particulate matter distributions in North Carolina. Atmospheric Environment 34: 3393-3406.

Christakos, G.; Bogaert, P.; Serre, M.L. (2002). *Temporal GIS: Advanced Functions for Field-Based Applications*, Springer-Verlag, New York, N.Y.

City of Durham. (2006). Wastewater Treatment Plant Schematic. City of Durham Sanitary Sewer Report FY 2005-2006, www.durhamnc.gov/departments/wm/pdf/sewer_report_06.pdf)

Conerly, O. (2005). Overview presentation from the U.S. EPA's Office of Water, The U.S. Environmental Protection Agency meeting on pharmaceuticals in the environment (pp. 3-4). Las Vegas, NV.

Cooper, R. L. and Kavlock, R. J. (1997). Endocrine disruptors and reproductive development: a weight-of-evidence overview. Journal of Endocrinology 152: 159-166.

Crisostomo, S., Mendes, Z., Marques, F. B., Martins, A. P., Rodrigues, V., & Ribeiro, C. F. (2006). Quantifying the amount and cost of wasted medicines in Portugal. Pharmacoepidemiology and Drug Safety, 15: S1-S316.

D'Ascenzo, G.; Di Corcia, A.; Gentili, A.; Mancini, R.; Mastropasqua, R. (2003). Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities. Science of The Total Environment 302 (1-3): 199-209.

Daughton, C. G. (2000). PPCPs as environmental pollutants: Frequently asked questions from the public, media, and scientific communities. Available at: http://www.epa.gov/nerlesd1/chemistry/pharma/faq.htm.

Delmas, J.D; Chapel, A.M.; Gaudin, V.; Sanders, P. (1997). Pharmacokinetics of flumequine in sheep after intravenous and intramuscular administration: bioavailability and tissue residue studies. Journal of Veterinary Pharmacology and Therapeutics 20 (4): 249-257.

Delepee R.; Pouliquen H.; Le Bris H. (2004). The bryophyte *Fontinalis antipyretica* Hedw. bioaccumulates oxytetracycline, flumequine and oxolinic acid in the freshwater environment. Science of the Total Environment 322: 243-253.

DHHS. Department of Health and Human Services (1998). Guidance for industry: environmental assessment of human drug and biologics applications (Vol. July 1998 CMC 6 Revision 1): U.S. Department of Health and Human Services, Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER).

Diaz-Cruz, M.; Silvia; López de Alda, J.; Barceló, D. (2006). Determination of antimicrobials in sludge from infiltration basins at two artificial recharge plants by pressurized liquid-extraction liquid chromatography-tandem mass spectrometry. Journal of Chromatography A 1130 (1): 72-82.

Donn, J.; Mendoza, M.; Pritchard, J. (2008). "AP Probe Finds Drugs in Drinking Water", Associated Press, March 9.

DTSC. California Department of Toxic Substances Control (2003). Regulatory Issues for waste pharmaceutical and personal care products management. Available at: http://www.dtsc.ca.gov/PollutionPrevention/Pharmaceutical_Regulatory.cfm.

Eguchi, K.; Nagaseb, H.; Ozawaa, M.; Endoha, Y.S.; Gotoa, K.; Hiratab, K.; Miyamotob, K.; Yoshimura, H. (2004). Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae. Chemosphere 57 (11): 1733-1738.

Ellsworth, A.J. (editor); Witt, D.M.; Dugdale, D.C.; Oliver, L.M. (2004). Mosby's Medical Drug Reference. Mosby-Year Book; St. Louis, MO.

Emmanuel, E.; Perrodin, Y.; Keck, G.; Blanchard, J.-M.; Vermande, P. (2005). Ecotoxicological risk assessment of hospital wastewater: a proposed framework for raw effluents discharging into urban sewer network. Journal of Hazardous Materials A117: 1-11.

Enick, O.V. and Moore, M.M. (2007). Assessing the assessments: Pharmaceuticals in the environment. Environmental Impact Assessment Review 27 (8): 707-729.

Erikson, C.; Webber, K.; Fitzpatrick, S. (2005). Environmental assessment for pharmaceuticals, workshop on pharmaceuticals in the environment. Las Vegas, NV.

European Medicines Evaluation Agency (EMEA) (2006). Guideline on the environmental risk assessment of medicinal products for human use. Doc. Ref. No. EMEA/CHMP/SWP/4447/00. London, June 1, 2006.

Farkas, M. H.; Berry, J. O.; Aga, D. S. (2008). Chapter 9: Antibiotic transformation in plants via glutathione conjugation. *Fate of Pharmaceuticals in the Environment and Water Treatment Systems*. Diana S. Aga (editor). CRC Press, Boca Raton, FL.

Ferrari, B.; Paxéusb, N.; Giudicec, R. L.; Pollioc, A.; Garric, J. (2003). Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. Ecotoxicology and Environmental Safety 55 (3): 359-370.

Finlay-Moore, O.; Hartel; P. G.; Cabrera, M. L. (2000). 17β-estradiol and testosterone in soil and runoff from grasslands amended with broiler litter. Journal of Environmental Quality 29 (5): 1604-1611.

Food and Drug Administration (1998). FDA's guidance on *Environmental Assessment of Human Drug and Biologics Applications* (July 1998): available at http://www.fda.gov/cder/ guidance/index.htm

Food and Drug Administration (2001). FDA's guidance on *Environmental Impact Assessments (EIAs) for Veterinary Medicinal Products (VMPs)*; VICH GL6 (March 7, 2001) Available at http://www.fda.gov/cvm/guidance/guide89.doc.

Food and Drug Administration (2002). Title 21 - Food and Drugs Chapter 13, Drug abuse prevention and control; Subchapter I, Control and enforcement; Part B, Authority To Control; Standards and Schedules, Sec. 812: Schedules of controlled substances. Available at: http://www.dea.gov/pubs/csa/812.htm#b.

Gao, H. and Zepp, R.G. (1998). Factors influencing photoreactions of dissolved organic matter in a coastal river of the Southeastern United States. Environmental Science & Technology 32: 2940-2946.

Gibs, J.; Stackelberg, P.E.; Furlong, E.T.; Meyer, M.; Zaugg, S.D.; Lippincott, R.L. (2007). Persistence of pharmaceuticals and other organic compounds in chlorinated drinking water as a function of time. Science of the Total Environment 373 (1): 240-249.

Gobel, A.; McArdell, C. S.; Suter, M. J.-F.; Giger, W. (2004). Trace determination of macrolide and sulfonamide antimicrobials, a human sulfonamide metabolite and trimethoprim in wastewater using liquid chromatography coupled to electrospray tandem mass spectrometry. Analytical Chemistry 76 (16): 4756-4764.

Golet, E.; Alder, A.; Giger, W. (2003). Environmental exposure and risk assessment of fluoroquinolone antibacterial agents in wastewater and river water of the Glatt Valley watershed. Environmental Science & Technology 36 (17): 3645-3651.

Grist, E.P.M.; Wells, N.C.; Whitehouse, P.; Brighty, G.; Crane, M. (2003). Estimating the effects of 17α-Ethinylestradiol on populations of the fathead minnow *Pimephales promelas*: are conventional toxicological endpoints adequate? Environmental Science & Technology 37: 1609-1616.

Halling-Sorensen, B.; Nielson, S.N.; Lanzky, P.F.; Ingerslev, F.; Lutzh_ft, H.C.H.; Jorgensen, S.E. (1998). Occurrence, fate, and effects of pharmaceutical substances in the environment – a review. Chemosphere 2: 357-393.

Halling-Sorensen, B. (2000). Algal toxicity of antibacterial agents used in intensive farming. Chemosphere 40: 731-739.

Hashimoto, T.; Onda, K.; Nakamurac, Y.; Tadab, K.; Miyac, A.; Murakami, T. (2007). Comparison of natural estrogen removal efficiency in the conventional activated sludge process and the oxidation ditch process. Water Research 41 (10): 2117-2126.

Hauser, J. M. (2006). Down the drain: The cost of medications wasted in hospice [Letter to the editor]. Journal of Pain and Symptom Management, 31 (5): 379-380.

Heberer, T. (2002). Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicology Letters 131 (1-2): 5-17.

Hermens, J.; Canton, H.; Jansen, P.; De Jong, R. (1984). Quantitative structure-activity relationships and toxicity studies of mixtures of chemicals with anaesthetic potency: acute lethal and sublethal toxicity to *Daphnia magna*. Aquatic Toxicology 5: 143-154.

Hernando, M.D.; Mezcua, M.; Fernandez-Alba, A.R.; Barcelo, D. (2006). Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. Talanta 69: 334-342.

Hirano, T.; Yasudaa, S.; Osakaa, Y.; Kobayashia, M.; Itagakia, S.; Iseki, K. (2006). Mechanism of the inhibitory effect of zwitterionic drugs (levofloxacin and grepafloxacin) on carnitine transporter (OCTN2) in Caco-2 cells. Biochimica et Biophysica Acta (BBA) – Biomembranes 1758 (11): 1743-1750.

Hirsch, R.; Ternes, T.A.; Haberer, K.; Kratz, K-L., (1999). Occurrence of antibiotics in the aquatic environment. Science of the Total Environment 225 (1,2): 109-118.

Ho, Pak-Leung; Que, Tak-Lun; Chiu, Susan S.; Yung, Raymond W.H.; Ng, Tak-Keung; Tsang, Dominic N.C.; Seto, Wing-Hong, Lau, Y-L. (2004). Fluoroquinolone and other antimicrobial resistance in invasive *Pneumococci*. Hong Kong, 1995-2001. Emerging Infectious Disease 10 (7).

Holten Lützhøft, H.-C.; Halling-Sørensen, B.; Jørgensen, S.E. (1999). Algal toxicity of antibacterial agents applied in Danish fish farming. Environmental Contamination Toxicology 36 (1): 1-6.

Huang, Y.-W.; Twidwell, D.L.; Elrod, J.C. (2003). Occurrence and effects of endrocrine disrupting chemicals in the environment. Practice Periodical of Hazardous, Toxic & Radioactive Waste Management 7: 241-252.

Huber, M.M.; Canonica, S.; Park, G.Y.; von Gunten, U. (2003). Oxidation of pharmaceuticals during ozonation and advanced oxidation processes. Environmental Science & Technology 37 (5): 1016-1024.

Isidori, M.; Lavorgna, M.; Nardelli, A.; Pascarella, L.; Parrella, A. (2005). Toxic and genotoxic evaluation of six antibiotics on non-target organisms. Science of the Total Environment 346: 87-98.

Karthikeyan, K. G. and Meyer, M. T. (2006). Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. Science of the Total Environment 361 (1-3): 196-207.

Kay, P.; Blackwell, P.A.; Boxall, A.B.A. (2005). Transport of veterinary antibiotics in overland flow following the application of slurry to arable land. Chemosphere 59 (7): 951-959.

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

Kim, Y.; Cho, J.; Kim, I.; Vanderford, B.; Snyder, S. (2007b). Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking and wastewaters. Water Research. 41 (5): 1013-1021.

Kolpin, D.; Furlong, E.; Meyer, M.; Thurman, M.; Zaugg, S.; Barber, L.; Buxton, H. (2002). Pharmaceuticals, hormones, & other wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. Environmental Science & Technology 32: 1202-1211.

Kolpin, D. W.; Skopec, M.; Meyer, M. T.; Furlong, E. T.; Zaugg, S. D. (2004). Urban contribution of pharmaceuticals and other organic wastewater contaminants to streams during differing low flow conditions. Science of the Total Environment 328: 119-130.

Konemann, H. (1981). Fish toxicity tests with mixtures of more than two chemicals: A proposal for a quantitative approach and experimental results. Toxicology 19: 229-238.

Kostich, M.S. and Lazorchak, J.M. (2007). Risks to aquatic organisms posed by human pharmaceutical use. Science of the Total Environment 389 (2-3): 329-339.

Kuspis, D. A. and Krenzelok, E. P. (1996). What happens to expired medications? A survey of community medication disposal. Veterinary and Human Toxicology, 38 (1): 48-49.

Lalumera, G.M.; Calamari, D.; Galli, P.; Castiglioni, S.; Crosa, G.; Fanelli, R. (2004). Preliminary investigation on the environmental occurrence and effects of antibiotics in aquaculture in Italy. Chemosphere 54 (5): 661-668.

LaVille, N.; Aït-Aïssa, S.; Gomez, E.; Casellas, C.; Porcher, J.M. (2004). Effects of human pharmaceuticals on cytotoxicity, EROD activity and ROS production in fish hepocytes. Toxicology 196 (1-2): 41-55.

Leusch, F.D.L.; H.F. Chapman; van den Heuvel M.R.; Tan B.L.L.; Gooneratne S.R.; Tremblay L.A. (2006). Bioassay-derived androgenic and estrogenic activity in municipal sewage in Australia and New Zealand. Ecotoxicology and Environmental Safety 65 (3): 403-411.

Lindberg, R.H.; Bjorklund, K.; Rendahl, P.; Johansson, M.I.; Tysklind, M.; Anderson, B.A.V. (2007). Environmental risk assessment of antibiotics in the Swedish environment with emphasis on sewage treatment plants. Water Research 41: 613-619.

Lindsey, M. E.; Meyer, M.; Thurman, E. M. (2001). Analysis of trace levels of sulfonamide and tetracycline antimicrobials in groundwater and suface water using solid-phase extraction and liquid chromatography/mass spectrometry. Analytical Chemistry 73 (19): 4640-4646.

Lissemore, L.; Hao, C.; Yang, P.; Sibley, P. K.; Marbury, S.; Soloman, K. R. (2006). An exposure assessment for selected pharmaceuticals within a watershed in Southern Ontario. Chemosphere 64 (5): 717-729.

Mann, J. (1999). *The Elusive Magic Bullet: The Search for the Perfect Drug*. Oxford, New York Oxford University Press (UK).

Matsui, Y.; Knappe D.R.U.; Takagi, R. (2002). Pesticide adsorption by granulated activated carbon adsorbers. 1. Effect of natural organic matter preloading on removal rates and model simplification. Environmental Science & Technology 36 (15): 3426-3431.

McArdell, C.S.; Molnar, E.; Suter, M.J-F.; Giger, W. (2003). Occurrence and fate of macrolide antibiotics in wastewater treatment plants and in the Glatt Valley watershed, Switzerland. Environmental Science & Technology 37 (24): 5479-5486.

McEvoy, G. K. (2004). American Hospital Formulary Service (AHFS) Drug Information, the American Society of Health-System Pharmacists, Inc., Bethesda, MD; pp. 62, 85, 225, 282, 379, 402, 420, 448.

Merck Manuals (2003). Online Medical Library. Antibiotics. Available at: http://www.merck.com/mmhe/sec17/ch192/ch192a.html. February 2003.

Merck Index, 12th Ed. (1996). Division of Merck Research Laboratories, Merck & Co., Inc. 1996.

Morgan, T. M. (2001). The economic impact of wasted prescription medication in an outpatient population of older adults. The Journal of Family Practice 50 (9): 779-781.

Nakata, H.; Kannan, K.; Jones, P. D.; Giesy, J. P. (2005). Determination of fluoroquinolone antibiotics in wastewater effluents by liquid chromatography-mass spectrometry and fluorescence detection. Chemosphere 58 (6): 759-766.

Nowara, A.; Burhenne, J.; Spiteller, M. (1997). Binding of fluoroquinolone carboxylic acid derivatives to clay minerals. Journal of Agricultural Food Chemistry 45: 1459-1463.

ODC, Office of Diversion Control (2007a). Questions and Answers. U.S. Department of Justice, Drug Enforcement Agency. Available at: http://www.deadiversion.usdoj.gov/faq/general.htm#pre_med.

ODC, Office of Diversion Control. (2007b). Registration Application. U.S. Department of Justice, Drug Enforcement Agency. Available at: http://www.deadiversion.usdoj.gov/drugreg/reg_apps/onlineforms.htm.

OECD (1992). Environment monograph No. 59 Paris 1992. Report of the OECD workshop on the extrapolation of laboratory aquatic toxicity data to the real environment. OCDE/GD (92) 169.

Oliver, D. and Chapman, A. I. (2003). Pharmaceutical waste survey, final report. Seattle, WA: Local hazardous waste management program in King County.

ONDCP, Office of National Drug Control Policy. (2006). Proper disposal of prescription drugs. Available at: http://www.whitehousedrugpolicy.gov/drugfact/factsht/proper_disposal.html. Accessed September 12, 2007.

Parks, A. (2006). Indicators of non-point source pollution in surface waters originating from biosolids. University of North Carolina at Chapel Hill.

Pereira, V. (2005). Analytical methods validation and bench scale UV treatment of pharmaceutical compounds and contrast media in water. University of North Carolina at Chapel Hill.

Ph:ARM Team, Washington State (2007). Pharmaceuticals from Households: A return mechanism Executive Summary. December, 2007. www.govlink.org/hazwaste/publications/Pharmaceuticals%20from%20HouseholdsExec12-19-07.pdf

Physicians' Desk Reference (2005). Thomson PDR: Montvale, NJ. Pp. 828, 2103.

Plumb, D.C. (2005). *Plumb's Veterinary Drug Handbook (5th ed.)*, Blackwell Publishing, Ames, IA (2005): 1311.

Rajapakse, N.; Silva, E.; Scholze, M.; Kortenkamp, A. (2004). Deviation from additivity with estrogenic mixtures containing 4-Nonylphenol and 4-Tert-Octylphenol detected in the E-Screen assay. Environmental Science and & Technology 38: 6343-6352.

Reddy, K.N. and Locke, M.A. (1994). Molecular properties as descriptors of octanol-water partition coefficients of herbicides. Water, Air, & Soil Pollution 86 (1-4): 389-405.

Reverse Distributors Working Group (RDWG) (2003). Reverse distributors working group meeting minutes. Available at: http://www.deadiversion.usdoj.gov/mtgs/reverse/mtg07092003.htm.

Richards, L. (2007). Antibiotic Fate and Transport in Aqueous and Sediment Watershed Environments. University of North Carolina at Chapel Hill.

Rodriguez-Mozaz, S.; Lopez de Alda, M.J.; Barcelo D. (2004). Monitoring of estrogens, pesticides and bisphenol A in natural waters and drinking water treatment plants by solid-phase extraction-liquid chromatography-mass spectrometry. Journal of Chromatography A 1045 (1-2): 85-92.

Routledge, E.J. and Sumpter, J.P. (1996). Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environmental Toxicology & Chemistry 15 (3): 241-248.

Sacher, F.; Lange, F. T.; Brauch, H.-J.; Blankenhorn, I. (2001). Pharmaceuticals in groundwaters: Analytical methods and results of a monitoring program in Baden-Württemberg, Germany. Journal of Chromatography A 938 (1-2): 199-210.

Santos, J.L.; Aparicio, I.; Alonso, E. (2007). Occurrence and risk assessment of pharmaceutically active compounds in wastewater treatment plants: Seville City (Spain). Enviroment International 33 (4): 596-601.

Saptoka, A.; Curriero, F.; Gibson, K.; Schwab, K. (2007). Antibiotic-resistant *enterococci* and fecal indicators in surface water and groundwater impacted by a concentrated swine feeding operation. Environmental Health Perspectives 115 (7): 1040-1045.

SciFinder Scholar, American Chemical Society (2006). Chemical information for chlortetracycline, sulfamethoxazole, oxolinic acid, sulfathiazole, sulfamerazine. Accessed October 2006, www.cas.org/SCIFINDER/SCHOLAR/index.html.

Schmidtt-Kopplin, P.; Burhenne, J.; Freitag, D.; Spiteller, M.; Kettrup, A. (1999). Development of capillary electrophoresis methods for the analysis of fluoroquinolones and application to the study of influence of humic substances on their photodegradation in aqueous phase. Journal of Chromatography A 837: 253-265.

Schwaiger, J.; Ferling, H.; Mallow, U.; Wintermayr, H.; Negele, R.D. (2004). Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part I: histopathological alterations and bioaccumulation in rainbow trout. Aquatic Toxicology 68 (2): 141-150.

Seehusen, D. A. and Edwards, J. (2006). Patient practices and beliefs concerning disposal of medications. Journal of the American Board of Family Medicine, 19 (6): 542-547.

Serre, M.L.; Kolovos, A.; Christakos, G.; Modis, K. (2003). An application of the holistochastic human exposure methodology to naturally occurring Arsenic in Bangladesh drinking water. Risk Analysis 23 (3): 515-528.

Serre, M.L.; Carter, G.; Money, E. (2004). Geostatistical space/time estimation of water quality along the Raritan river basin in New Jersey. Computational Methods in Water Resources 2004 International Conference, Elsevier, vol. II: 1839-1852.

Servos, M. R.; Bennie, D.T.; Burnisonb, B.K.; Jurkovicb, A.; McInnisb, R.; Nehelib, T.; Schnellc, A.; Setob, P.; Smythb, S.A.; Ternes, T.A. (2005). Distribution of

estrogens, 17β-estradiol and estrone, in Canadian municipal wastewater treatment plants. Science of The Total Environment 336 (1-3): 155-170.

Smith, C. A. (2002). Managing pharmaceutical waste - What pharmacists should know. Journal of the Pharmacy Society of Wisconsin, Nov/Dec 2002: 17-22.

Smith, C. A. (2005). Presentation abstract: Environmental stewardship of waste pharmaceuticals from a hospital perspective, EPA National Center for Environmental Research Meeting, Las Vegas, NV, August 23-25.

Snyder, S. A.; Westerhoff, P.; Yoon, Y.; Sedlak, D. L. (2003). Pharmaceuticals, personal care products, and endocrine disruptors in water: implications for the water industry Environmental Engineering Science 20(5): 449-469.

Stanford, B.D. (2007). The fate and transport of steroid estrogens and nonylphenols through soils. The University of North Carolina at Chapel Hill.

Syracuse Research Corporation Databases (2007). North Syracuse, NY: Syracuse Research Corporation. Retrieved November 15, 2007. Available at: http://www.syrres.com/esc/physdemo.htm.

Taggart, M.A.; Senacha, K.R.; Green, R.E.; Jhala, Y.V.; Raghaven, B.; Rahmani, A.R.; Cuthbert, R.; Pain, D.J.; Meharg, A.A. (2007). Diclofenac residues in carcasses of domestic ungulates available to vultures in India. Environment International 33 (6): 759-765.

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

Ternes, T.A.; Meisenheimer, M.; Mcdowell, D.; Brauch, H.J.; Brigitte, H.G.; Preuss, G.; William, U.; Zulei-Seibert, N. (2002). Removal of pharmaceuticals during drinking water treatment. Environ. Sci. Tech. 36: 3855–3863.

Ternes, T.A.; Stüber, J.; Herrmann, N.; McDowell, D.; Ried, A.; Kampmann, N.; Teiser, B. (2003). Ozonation: a tool for removal of pharmaceuticals, contrast media and musk fragrances from wastewater? Water Research 37 (8): 1976-1982.

Teuschler, L.K. (2007). Deciding which chemical mixtures risk assessment methods work best for what mixtures. Toxicology and Applied Pharmacology 223 (2): 139-147.

Thacker, P. D. (2005). Pharmaceutical data eludes environmental researchers. Environmental Science & Technologh 39 (9): 193-194.

Thiele-Bruhn, S. (2003). Pharmaceutical antibiotic compounds in soils – a review. Journal of Plant Nutrition & Soil Science 166: 145-167.

Tolls, J. (2001). Sorption of veterinary pharmaceuticals in soils: a review. Environmental Science & Technology 35 (17): 3397-3346.

U.S. Congress (1996). 1996 Amendments to the Safe Drinking Water Act-Public Law 104-182 104th Congress. Washington, DC. Available at: http://www.epa.gov/safewater/sdwa/text.html.

U.S. Environmental Protection Agency (1998). Guidelines for ecological risk assessment, Risk Assessment Forum. Washington, D.C., p. 114.

U.S. Environmental Protection Agency (1999). Method 1668, Revision A: Chlorinated Biphenyl Congeners in Water, Soil, Sediment, and Tissue by HRGC/HRMS.

U.S. Environmental Protection Agency (2000). Supplementary guidance for conducting health risk assessment of chemical mixtures. EPA/630/R-00/002. ORD/NCEA. Cincinnati, Ohio.

U.S. Environmental Protection Agency (2005). Nonpoint source pollution: The nation's largest water quality problem. Washington, D.C.

U.S. Environmental Protection Agency (2007a). Drinking water contaminant candidate list and regulatory determinations: basic information.

U.S. Environmental Protection Agency (2007b). The National Environmental Policy Act (NEPA) of 1969. http://www.epa.gov/compliance/basics/nepa.html. Accessed December 12, 2007.

Vanderford, B. J.; Pearson, R. A.; Rexing, D. J.; Snyder, S. A. (2003). Analysis of endocrine disruptors, pharmaceuticals, and personal care products in water using liquid chromatography/tandem mass spectrometry. Analytical Chemistry 75 (22): 6265-6274.

Verma, B.; Headley, J.V.; Robarts, R.D. (2007). Behaviour and fate of tetracycline in river and wetland waters on the Canadian Northern Great Plains. Journal of Environmental Health Science Part A 42: 109-117.

Vincent, P. G. (1993). Environmental assessment: U.S. requirements in new drug applications. Journal of Hazardous Materials 35 (2): 211-216.

Vogelsang, C.; Grung, M.; Jantscha, T.G.; Tollefsena, K.E.; Liltved, H. (2006). Occurrence and removal of selected organic micropollutants at mechanical, chemical and advanced wastewater treatment plants in Norway. Water Research 40 (19): 3559-3570.

WCRC. Washington Citizens for Resource Conservation. (2006). SoundStats Report. Available at: http://wastenotwashington.org/Pharmsurvey.pdf.

Wintgens, T. and Gallenkemper, M.T. (2003). Removal of endocrine disrupting compounds with membrane processes in wastewater treatment and reuse. Water Science and Technology 50 (5): 1-8.

Xu, W.; Zhang, G.; Xiangdong, L.; Schichun, Y.; Ping, L.; Zhaohui, H.;Jun, L. (2007). Occurrence and elimination of antibiotics at four sewage treatment plants in Pearl River Delta (PRD), South China. Water Research 14 (19): 4526-4534.

Yang, S. and Carlson, K. (2003). Evolution of antibiotic occurrence in a river through pristine, agricultural, and urban landscapes. Water Research 37 (19): 4645-4656.

Ye, Z. (2005). Occurrence, fate, and transformation of antibiotics during drinking water treatment. University of North Carolina at Chapel Hill.

Zweiner, C. and Frimmel, F.H. (2000). Oxidative treatment of pharmaceuticals in drinking water. Water Research 34 (6): 3530-3537.