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Assessment of the Occurrence and Potential Risks of Antibiotics and their Metabolites in South Florida Waters Using Liquid Chromatography Tandem Mass Spectrometry

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ASSESSMENT OF THE OCCURRENCE AND POTENTIAL RISKS OF

ANTIBIOTICS AND THEIR METABOLITES

IN SOUTH FLORIDA WATERS USING LIQUID CHROMATOGRAPHY TANDEM

MASS SPECTROMETRY

A dissertation submitted in partial fulfillment of

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in

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by

Venkata Reddy Panditi

2013

To: Dean Kenneth G. Furton
       College of Arts and Sciences

This dissertation, written by Venkata Reddy Panditi, and entitled Assessment of the occurrence and potential risks of antibiotics and their metabolites in South Florida waters using liquid chromatography tandem mass spectrometry, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Florida International University, 2013

DEDICATION

This thesis is dedicated to the memory of my beloved mother, Samrajyam. She inspired me through her pain and suffering. I would also like to dedicate this thesis to my wife, Sudha Rani and loving daughter, Hasini, for their understanding, support and unconditional love.

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ABSTRACT OF THE DISSERTATION

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An automated on-line SPE-LC-MS/MS method was developed for the quantitation of multiple classes of antibiotics in environmental waters. High sensitivity in the low ng/L range was accomplished by using large volume injections with 10-mL of sample. Positive confirmation of analytes was achieved using two selected reaction monitoring (SRM) transitions per antibiotic and quantitation was performed using an internal standard approach. Samples were extracted using online solid phase extraction, then using column switching technique; extracted samples were immediately passed through liquid chromatography and analyzed by tandem mass spectrometry. The total run time per each sample was 20 min. The statistically calculated method detection limits for various environmental samples were between 1.2 and 63 ng/L. Furthermore, the method was validated in terms of precision, accuracy and linearity.

The developed analytical methodology was used to measure the occurrence of antibiotics in reclaimed waters (n=56), surface waters (n=53), ground waters (n=8) and drinking

waters (n=54) collected from different parts of South Florida. In reclaimed waters, the most frequently detected antibiotics were nalidixic acid, erythromycin, clarithromycin, azithromycin trimethoprim, sulfamethoxazole and ofloxacin (19.3-604.9 ng/L). Detection of antibiotics in reclaimed waters indicates that they can't be completely removed by conventional wastewater treatment process. Furthermore, the average mass loads of antibiotics released into the local environment through reclaimed water were estimated as 0.248 Kg/day. Among the surface waters samples, Miami River (reaching up to 580 ng/L) and Black Creek canal (up to 124 ng/L) showed highest concentrations of antibiotics. No traces of antibiotics were found in ground waters. On the other hand, erythromycin (monitored as anhydro erythromycin) was detected in 82% of the drinking water samples (n.d-66 ng/L). The developed approach is suitable for both research and monitoring applications.

Major metabolites of antibiotics in reclaimed wates were identified and quantified using high resolution benchtop Q-Exactive orbitrap mass spectrometer. A phase I metabolite of erythromycin was tentatively identified in full scan based on accurate mass measurement. Using extracted ion chromatogram (XIC), high resolution data-dependent MS/MS spectra and metabolic profiling software the metabolite was identified as desmethyl anhydro erythromycin with molecular formula $C_{36}H_{63}NO_{12}$ and m/z 702.4423. The molar concentration of the metabolite to erythromycin was in the order of 13 %. To my knowledge, this is the first known report on this metabolite in reclaimed water. Another compound acetyl-sulfamethoxazole, a phase II metabolite of sulfamethoxazole was also identified in reclaimed water and mole fraction of the metabolite represent 36 %, of the

cumulative sulfamethoxazole concentration. The results were illustrating the importance to include metabolites also in the routine analysis to obtain a mass balance for better understanding of the occurrence, fate and distribution of antibiotics in the environment. Finally, all the antibiotics detected in reclaimed and surface waters were investigated to assess the potential risk to the aquatic organisms. The surface water antibiotic concentrations that represented the real time exposure conditions revealed that the macrolide antibiotics, erythromycin, clarithromycin and tylosin along with quinolone antibiotic, ciprofloxacin were suspected to induce high toxicity to aquatic biota. Preliminary results showing that, among the antibiotic groups tested, macrolides posed the highest ecological threat, and therefore, they may need to be further evaluated with, long-term exposure studies considering bioaccumulation factors and more number of species selected. Overall, the occurrence of antibiotics in aquatic environment is posing an ecological health concern.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABBREVIATION	FULL NAME
AF	assessment factor
ANOVA	analysis of variance
AP	American Press
APCI	atmospheric pressure chemical ionization
BBC	Biscayne Bay Campus
CAS	chemical abstract service
CDDEP	Center for Disease Dynamics, Economics and Policy
CE	collision energy
CEC	contaminants of emerging concern
CH_2Cl_2	dichloromethane
CH_3CN	acetonitrile
CH_3OH	methanol
CID	collision induced dissociation
DWTP	drinking water treatment plant
EC_{50}	median effective concentration
EDTA	Ethylenediaminetetraacetic acid
ESI	electrospray ionization
FDA	Food and Drug Administration
FISh	fragment ion search

FIU	Florida International University
GC	gas chromatography
HESI	heated electrospray ionization
HLB	Hydrophilic-Lipophilic Balance
HRMS	high resolution mass spectrometry
IT	ion trap
K_{ow}	octanol water partition coefficient
LC_{50}	mean lethal concentration
LLE	liquid-liquid extraction
m/z	mass to charge ratio
MDL	method detection limit
MEC	measured environmental concentration
MF	mass frontier
MGD	million gallons per day
MRM	multiple reactions monitoring
MS	mass spectrometry
PCPs	personal care products
PEC	predicted environmental concentration
PEEK	polyether ether ketone
PET	polyethylene terephthalate
PPCPs	pharmaceuticals and personal care products
PNEC	predicted no effect concentration

QqQ	Triple quadrupole
RPLC	Reversed-phase liquid chromatography
RQ	risk quotient
RSD	relative standard deviation
SPE-LC-MS/MS	solid phase liquid chromatography tandem mass spectrometry
SPME	solid-phase microextraction
SRM	selected reaction monitoring
STPs	sewage treatment plants
UPLC	ultra pressure liquid chromatography
WWTP	wastewater treatment plant
XIC	extracted ion chromatograms

CHAPTER 1

Introduction

1.1. Overview

In the recent years, public awareness of the long-term effects of chemical contaminants such as pharmaceutical and personal care products (PPCPs), pesticides, perfluorinated compounds and polychlorinated biphenyls has tremendously increased due to the anticipation of adverse human and ecological health effects. Previously, most of these chemicals were undetected as their environmental occurrence and/or concentrations are usually very low (micrograms down to sub nanogram per liter) and hence were not historically considered as contaminants (Kolpin. 2013). However, the advances in environmental analytical chemistry have resulted in an information explosion regarding these chemicals (Templeton et al., 2009). Moreover, toxicologists and environmental risk assessment experts advise that some contaminants like antibiotics and hormones even at very low levels can show significant and widespread adverse environmental and human health consequences (Purdom et al., 1994; Levy, 1997; Martinez, 2008; Caliman and Gavrilescu, 2009). These chemicals were commonly referred as contaminants of emerging concern (CECs).

Contaminants of emerging concern, such as, pharmaceuticals (e.g. antibiotics, hormones, steroids), personal care products (PCPs) (e.g. cosmetics, moisturizers, antacids, caffeine, fragrances), perfluorinated compounds, and household chemicals (e.g. detergents, deodorizers, degreasers), are continuously released into septic systems all over the world. As a result of constant production and usage, CECs enter the environment, disperse to various compartments, and persist for much longer than originally expected (Kolpin et al., 2002).

Most of the CECs are not regulated in any way and their potential health effects and acute toxicities to the environment are not known (Halling-Sorensen et al., 1998). In the previous years' diluting the contaminated water by releasing it into streams, rivers, or out to sea was considered as a good choice (Osenga, 2013). However, the continued growth in the human population created a huge demand for the Earth's limited supply of fresh water (Kolpin et al., 2002). Thus, protecting the integrity of our water resources is very essential for the present and future needs of human population. With more contaminants being released into fresh water every year, the world has started to think about the long-term effects of this action.

Among several groups of emerging chemical contaminants, pharmaceuticals, and in particular antibiotics, had received lot of attention in the media in the last several years due to the increasing number of diseases becoming resistant to traditional treatments. Previous research showed the widespread occurrence of residual antibiotics in various water ways, such as surface water (river streams, lakes, ponds), sewage effluents, ground water, ocean outfall, and drinking water (Watkinson et al., 2009). Antibiotics were recently classified as a priority risk group due to their high toxicity to algae and bacteria at low concentrations, and their potential to cause resistance among natural bacterial population (Hernando et al., 2006) and therefore, identified for future monitoring studies (Zuccato et al., 2005).

1.2. What are Antibiotics?

Antibiotics are the chemotherapeutic agents that inhibit or abolish the growth of microorganisms, such as bacteria, fungi, or protozoa; they are widely used to treat infectious diseases in human and veterinary medicine, and also in agricultural practices (Kummerer, 2009). The first antibiotics used in human medicine were of natural origin, e.g. penicillin (produced by fungi) and streptomycin (from bacteria). Currently, antibiotics are obtained by chemical modification of compounds of natural origin (e.g. amoxicillin), or chemical synthesis (e.g. sulfamethoxazole).

Antibiotics are a diverse group of chemicals. Based on their chemical structure and mode of action, they can be subdivided into ß-lactams, sulphonamides, quinolones, tetracylines, macrolides, aminoglycosides and others. An overview of important classes of antibiotics is given in Table 1. Antibiotics are often complex molecules with different functionalities within the same molecule, e.g. ciprofloxacin (Kummerer, 2009). Therefore, they can be neutral, cationic or zwitterionic under different pH conditions. Because of different functionalities with in a single molecule, their physico-chemical and biological properties such as log K_{ow} (Cunningham, 2008), photo reactivity, sorption behavior, antibiotic activity and toxicity may change with pH.

Table 1. Target antibiotics and their classification

Antibiotic	CAS number	Classification
Sulfadiazine	68-35-9	Sulfonamide
Sulfathiazole	72-14-0	Sulfonamide
Sulfamerazine	127-79-7	Sulfonamide
Sulfamethazine	57-68-1	Sulfonamide
Sulfamethizole	144-82-1	Sulfonamide
Sulfachlorpyridazine	80-32-0	Sulfonamide
Sulfamethoxazole	723-46-6	Sulfonamide
Sulfadimethoxine	122-11-2	Sulfonamide
Enoxacin	74011-58-8	Fluoroquinolone
Ofloxacin	82419-36-1	Fluoroquinolone
Norfloxacin	70458-96-7	Fluoroquinolone
Ciprofloxacin	85721-33-1	Fluoroquinolone
Danofloxacin	112938-08-0	Fluoroquinolone
Enrofloxacin	93106-60-6	Fluoroquinolone
Sarafloxacin	98105-99-8	Fluoroquinolone
Oxytetracycline	79-57-2	Tetracylcines
Tetracycline	60-54-8	Tetracylcines
Chlortetracycline	57-62-5	Tetracylcines
Doxycycline	564-25-0	Tetracylcines
Meclocycline	2013-58-3	Tetracylcines
Spiramycin	8025-81-8	Macrolides
Clindamycin	18323-44-9	Macrolides
Tylosin	1401-69-0	Macrolides
Erythromycin	114-07-8	Macrolides
Clarithromycin	81103-11-9	Macrolides
Azithromycin	83905-01-5	Macrolides
Roxithromycin	80214-83-1	Macrolides
Lincomycin	154-21-2	Lincosamide
Amoxycillin	26787-78-0	Miscellaneous
Trimethoprim	738-70-5	Quinolone
Nalidixic acid	389-08-2	Quinolone

1.3. Sources of Antibiotics in the environment

1.3.1. Natural sources

Antibiotics are originally of natural origin. Some species of fungi and bacteria naturally existing in soil produce antibiotics for inhibiting the growth of other microorganisms in their territory. For example, a group of Actinomycetes, such as Streptomycetes, existing in soil, produce streptomycin antibiotic; Fungi in the genus pencillium produce pencillins; other examples include aminoglycosides, tetracyclines, novabiocin, etc. (Fleming, 2001; Kawaguchi et al., 2013). Mankind recognized the ability of microbes to produce antibiotics and started using for his own benefits such as treating infectious diseases.

In general, the antibiotic activity varies with bacterial density; for example, in the free water phase, the bacterial density is much lower compared to sewage sludge or soil (Kummerer, 2009). However, the contamination of free water phase with sewage effluents may influence the bacterial proliferation.

1.3.2. Antibiotic production

As the industrial effluents were mostly regulated, the contribution of antibiotic manufacturing plants for the total antibiotic concentrations in sewage influents was assumed to be of minor importance. In contrast, in a recent study by Larsson et al. it was found that, in some developing Asian countries, the antibiotic concentrations in industrial

effluents were up to several mg L^{-1} (Larsson et al., 2007). Considering the fact that trace occurrence of antibiotic residues in pharmaceutical production plants effluents is not uncommon, manufacturing plants in developed countries can also make a significant contribution along with other domestic sources for total antibiotic concentration in sewage treatment plant influents (STPs) (Khetan and Collins, 2007; Lillenberg et al., 2010).

1.3.3. Usage

Antibiotics have been widely used in human and veterinary medicine, as well as in farming and aquaculture for the purpose of prevention or treatment of infectious diseases. Out of several thousand tons of antibiotics consumed every year worldwide. The United States is among the most intensive users according to prescription statistics data (expressed as DDD per day and capita) collected by the Center for Disease Dynamics, Economics and Policy (CDDEP, Washington DC). France is the highest user of antibiotics followed by Greece, Italy, Belgium and the United States. For the first time in 2012, the United States Food and Drug Administration (USFDA) agency released the public document on systemic antibacterial drugs sold in the US, showing that about 3.28 million kilograms of antibiotics sold for human medical use in year 2010, and over 13.06 million kilograms for food-animal use in 2009 (CVM, 2010; Pham, 2012). Moreover, 16,465 kilograms antibiotic active ingredients was used in plant agriculture in the US in 2009 (Stockwell and Duffy, 2012). This means that about 80% of antibiotics sold in the US are for non-human use, and moreover, about 90% of the animal consumption is for

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non-therapeutic   purposes,   such   as   growth   promoters (UCS, Union of Concerned Scientists, 2001). Class wise antibiotics sales data, both in human and food-producing animals, is shown in Table 2 (CVM, 2010; Pham, 2012). ß-lactams (pencillins, cephalosporins and carbapenems) make up the largest share of human use antibiotics, followed by sulphonamides, quinolones and macrolides, whereas in animal husbandry tetracyclines and ionophores were the mostly used.

Table 2. Nationwide antibacterial drug use in humans and food producing animals in the U.S.

Antibiotic class	Annual Totals in Kilograms, Year 2010*	Annual Totals in Kilograms, Year 2009**
Penicillins	1,439,930	610,514
Cephalosporins	502,561	41,328
Sulfa and TMP	479,484	517,873
Quinolones	281,557	---
Macrolides	164,309	861,985
Nitroimidazoles	114,991	---
Tetracyclines	129,183	4,611,892
Lincosamides	69,235	115,837
Carbapenems	13,173	---
Aminoglycosides	6,991	339,678
Oxazolidinones	5,144	---
Monobactams	3,782	---
Ionophores	---	3,740,627
Lipopeptides	1,123	---
Others	67,443	2,227,366
Total	3,278,906	13,067,100

* in humans   ** in food-producing animals

1.3.4. Occurrence

In both humans and animals, consumed antibiotic will be assimilated, and subjected to various metabolic reactions and finally excreted in urine and feces. For antibiotics, elimination through the metabolic processes is not complete, resulting in excretion of unchanged active parent compound along with metabolic products (Hirsch et al., 1999). In humans, the percent excretion of antibiotic as the parent drug is molecule specific and may range from 10 to 60% (Zuccato et al., 2005). On an average, if the volume of all antibiotics used is totaled the metabolic rate is estimated to be 30%, implying that 70% of the used antibiotic is excreted unchanged (Kummerer and Henninger, 2003).

Along with human and animal excretions, the disposed unused and expired medication will pass through the sewage drains, and reach Waste Water Treatment Plants (WWTPs). Sorption (e.g., tetracyclines), hydrolysis (e.g., penicillins), photo and biodegradation play significant role in attenuating antibiotic persistence. However, several investigations have shown that conventional treatment processes employed in WWTPs are not efficient in degrading or removing antibiotics completely (Heberer et al., 2002; Batt et al., 2006; Deblonde et al., 2011, Anquandah et al., 2011). Eventually, they are released in to local aquatic surroundings via WWTPs effluents (Jorgensen and Halling-Sorensen, 2000). Moreover, by the application of antibiotic residue containing animal manure and sludge to agricultural fields, antibiotics leach into surface waters, and/or infiltrating in to ground water (Hirsch et al., 1999). As ground water and surface water are the main sources of drinking water, antibiotics are often detected in drinking waters (Ye et al., 2007;

Watkinson et al., 2009). Figure 1 shows the possible sources and pathways of antibiotics in the aquatic environment (Anderson et al., 2011).
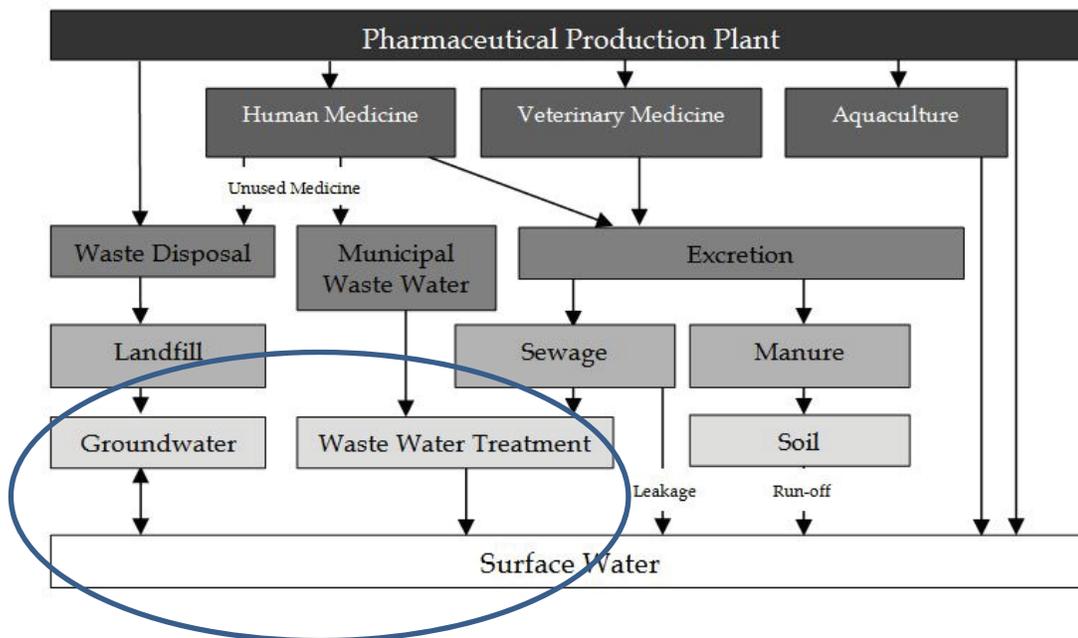
Figure 1. Possible sources and pathways for the environmental occurrence of antibiotics, modified from Anderson et al., 2011.

1.3.5. Significance

Antibiotics have revolutionized medicine in many respects, and countless lives have been saved; their discovery was a turning point in human history. Unfortunately, the use of these wonder drugs has been associated with the rapid appearance of resistant strains (Davies and Davies, 2010). Higher antibiotic consumption and inappropriate use could be the main reasons for rapid spread of antibiotic resistance (Barbosa and Levy, 2000). Many publications reported on occurrence of antibiotics and their resistant strains in

various aquatic environmental compartments (Levy, 1997; Huovinen, 2001; Kummerer and Henninger, 2003; Kummerer, 2004; Martinez, 2008). Chronic exposure to low doses of antibiotics lead to the selective proliferation of resistant bacteria, which could transfer the resistant genes to other unrelated bacteria in a phenomenon called horizontal gene transfer (exchange and fuse of plasmids, chromosome fragments (Bakkali, 2013)). Places where microbial population density is high, such as sewage systems, hospital effluents, and/or animal farms are acting as the reservoirs or sinks for antibiotics and their resistant strains (Holzel et al., 2010). The occurrence of antibiotics in aquatic environments is of ecotoxicological concern too, because of their ability to potentially alter the ecosystem (Kummerer, 2009). Antibiotics can inhibit the growth of microorganisms in sewage treatment systems, and may also have the ability to seriously affect the whole microbial community structure in the local environments, wherever they are present. The effects include but not limited to reduced organic matter degradation (by impacting the biological oxidation processes used in sewage treatment systems), disrupting key bacterial cycles critical to aquatic ecology (nitrification/denitrification), soil fertility (Watkinson et al., 2009).

Antibiotics may adversely affect organisms of different tropic levels such as algae, invertebrates, and to a little extent on fish. Blue-green algae (cyanobacteria) are very sensitive to many antibiotics, such as amoxicillin, spiramycin, sarafloxacin and tetracycline (Boxall et al., 2004). As algae are the basis of the food chain, even slight decreases in the algal population may affect the equilibrium in an aquatic system. In some cases some trophic levels may be completely wiped out, causing the community structure to be remarkably changed (Wollenberger et al., 2000). This effect can make its

way up the food chain and may be the cause of the trends observed towards lower biodiversity. Other ecotoxicological concerns, such as adverse reproductive effects in the early life stages of Daphnia magnam (clarithromycin, trimethoprim, and neomycin), depressed hatching rate of Artemia sp. cysts, and high mortality rate for nauplii, serve as examples of negative effects of antibiotics on aquatic organisms (Macri et al., 1988; Migliore. L, 1993 ; Wollenberger et al., 2000; Kummerer, 2009).

The effects of antibiotics on human health may include: allergic reactions (e.g. ß-lactams), negative interaction of tetracyclines with developing teeth in young children, nephrotoxicity (e.g. gentamicin) and increased sensitivity to light due to quinolones, and because of their antimicrobial activity, a negative interaction within the human gut (Sanchez et al., 2004; Hadjipour, 2011). However, there were no direct evidences to date the effects are caused by the consumption of drinking water containing antibiotic traces. Hence, in addressing the public health concerns over the drinking water quality and identifying the contamination sources, determining their fate in the environment and assessing the potential ecological health risks associated with short and long term exposure to antibiotics in the aquatic environments, efficient analytical methodologies are required for continuous surveillance of antibiotics and their bioactive metabolites at environmentally relevant concentrations.

Consumed antibiotics are converted into metabolites through a biological process called metabolism. Metabolites also reach environment through STPs and other sources along with the parent molecule. Some metabolites can be transformed back to the parent molecule in the WWTPs treatment process E.g., Sulfamethoxazole (Bonvin et al., 2012). Metabolites may have hazardous effects similar to the parent drugs (Bedner and

Maccrehan, 2006). Therefore it is important to study the metabolites along with their parent forms to assess the occurrence, fate and transport of antibiotics in the environment

To address the ecological health concerns, to identify the contamination sources and also to assess environmental fate of antibiotics robust analytical methods are crucial. The main challenges for the detection of antibiotics in environmental compartments include relatively low analyte concentrations (ng/L), complexity of environmental matrices, and diverse physico-chemical properties of the antibiotics. Hence, determination of antibiotics in the environmental matrices requires highly sensitive and selective methods, especially in multiple residue analysis.

1.4. Hypothesis

On the basis of the above mentioned facts, the two main hypotheses for the current study are,

1. It is possible to develop a single, part-per trillion analytical method for the determination of multiple classes of antibiotics.
2. Occurrence of antibiotics or their metabolites in South Florida waters could pose an ecological risk.

1.5. Objectives

1. To develop a robust and high throughput online SPE-LC-MS/MS method that is sensitive enough for the trace level detection of antibiotics in complex environmental water matrices

2. To apply the developed analytical methodology to analyze both treated (drinking waters and reclaimed waters) and untreated environmental water samples (river waters and ground waters)

3. To identify the potential antibiotic metabolites in environmental water samples using a high resolution mass spectrometry

4. To assess the potential ecological risk associated with antibiotic residues in reclaimed and surface waters using available literature on toxicity exposure data

CHAPTER 2

Online solid-phase extraction–liquid chromatography–electrospray–tandem mass spectrometry method for determination of multiple classes of antibiotics

2.1. Introduction

The environmental concentrations of antibiotics are typically low (sub μg -to- ng/L) and generally requires preconcentration for their detection. Though several extraction techniques such as liquid-liquid extraction (LLE) (Koch et al., 2005), solid-phase microextraction (SPME) were previously reported (Balakrishnan et al., 2006; McClure and Wong, 2007), solid phase extraction (SPE) is the most widely used method of choice for the sample preconcentration (Kim and Carlson, 2007).

In the typical SPE preconcentration, sample volumes ranging from 200 mL to 1000 mL will be passed on to SPE sorbents that are preconditioned with organic and aqueous solvents to retain all the analytes of interest. The classical stationary phases (sorbent materials) for solid phase extraction of antibiotics include non-polar phase (e.g., chemically bonded silica with C_8 or C_{18} organic group), ion-exchange phase and polymeric phase. Among them, Oasis MCX mixed mode sorbent to extract polar to medium-polar analytes and the Oasis HLB (Hydrophilic Lipophilic Balanced phase, Waters Corp.) sorbent to extract both polar and nonpolar analytes under the same conditions were the mostly used for the simultaneous extraction of multiple residues with markedly different chemical characteristics (Petrovic et al., 2005; Hernandez et al., 2007; Seifrtova et al., 2009). The retained analytes are eluted with organic solvents of varying polarity. Then the eluents are dried and reconstituted for further analysis (chromatographic separation and determination). The major drawback of this procedure compared to new online SPE is that the sample throughput is very low, takes almost one day to prepare a batch of 12 samples on a typical SPE vacuum manifold.

For the chromatographic separation of antibiotics, in the previous years several methods were reported using gas chromatography (GC) with its high resolving power (Ternes, 2001; Richardson, 2006). However antibiotics are polar, less volatile molecules derivatization is the additional step necessary for GC analysis and thus liquid chromatography is the preferred technique of choice. Reversed-phase liquid chromatography (RPLC) had been commonly used for their separation. Formic acid and ammonium acetate are the widely used additives in mobile phase to enhance the ionization efficiency and to control pH, therefore improved method detection limits in mass spectrometric analysis (Kim and Carlson, 2007).

In both LC─MS and LC─MS/MS analysis of antibiotics, two ionization interfaces, electrospray ionization and atmospheric pressure chemical ionization were commonly used due to their sensitivity and robustness. Between the two ionization methods reported for the analysis of antibiotics, selection of choice is usually based on the polarity of analytes and additives used in mobile phase (Hao et al., 2007). Since antibiotics are polar and moderately non-polar, thermally labile in nature, electrospray ionization (ESI) is the well suited soft ionization technique and by far the most frequently applied ionization technique for the detection of antibiotics, although it is known to be more prone to signal suppression compared to atmospheric pressure chemical ionization (APCI) (Sorensen and Elbaek, 2004; Hernandez et al., 2007). In the literature, use of internal standards, especially isotopic-labeled internal standards, performing matrix-matched calibrations, standard addition methods or simply diluting the samples were described to compensate for matrix effects (Lindsey et al., 2001; Hernandez et al., 2007; Seifrtova et al., 2009).

Among the mass analyzers, both quadrupole (single and triple) and ion trap (IT) were the widely used for MS and MS/MS detection and quantification of antibiotics. In the context of quadrupole mass analyzers, compared to MS, where need to rely on single ion monitoring; MS^2 has the multiple reactions monitoring (MRM) ability, which detects both the precursor ion and fragment ions of analytes. MRM is the sensitive and selective way of measuring analytes (Hao et al., 2007), as it can reduce the matrix interference that leads to false positives especially in environmental matrices where the analyte concentrations are in ng/L levels (Gobel et al., 2004; Vieno et al., 2006; Feitosa-Felizzola et al., 2007). Triple quadrupole (QqQ) is the frequently used MS^2 detector in LC-MS/MS analysis (Hernandez et al., 2007; Trenholm et al., 2008) for the quantitation purposes.

In traditional offline SPE-LC-MS/MS analysis of antibiotics, sample preconcentration by offline solid-phase extraction (SPE) is the time consuming step and hinders the sample throughput. By hyphenating the SPE step with LC-MS/MS, it is possible to lower the analysis time and to increase the sample throughput without significant losses of sensitivity as reported elsewhere (Pozo et al., 2006; Feitosa-Felizzola et al., 2007; Ding et al., 2009; Garcia-Ac et al., 2009; Garcia-Galan et al., 2010, Ramirez et al., 2013). However, most of these studies were either focused on a particular class of antibiotics (Stoob et al., 2005) or very few target antibiotics were selected from each class (Tang et al., 2006). Dinh et. al. described the most comprehensive analytical method for the determination of 23 antibiotics in river waters. Usual sample preparation steps like filtration, pH adjustment and use of EDTA (chelating agent) were required and two runs were performed for each sample to achieve optimal recovery for all classes of antibiotics, thus doubling the analysis time. Moreover, the suitability of the method for other

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complex matrices such as reclaimed water and waste waters was not tested (Dinh et al., 2011). Lopez-Serna et al., reported a comprehensive online SPE method for the analysis of antibiotics, the method included 20 antibiotics in different water matrices with a long run time of 37 min (Lopez-Serna et al., 2010). Thus, there is a need for fast analytical methodology capable of detecting most classes of antibiotics at environmentally relevant concentrations possibly with minimal sample preparation, and suitable for application to different water matrices.

The objective of this study was to develop a comprehensive analytical method for the analysis of multiple classes of antibiotics in various water matrices, aiming to achieve trace levels detection and better recoveries by using an online SPE in combination with LC-MS/MS determination.

2.2. Experimental

2.2.1. Standards, reagents, and solutions

Lincomycin, trimethoprim, amoxycillin, nalidixic acid, tylosin, sulfadiazine, sulfamethoxazole, sulfathiazole, sulfamethazine, sulfamethizole, sulfamerazine, sulfadimethoxine, sulfachlorpyridazine, enoxacin, ciprofloxacin, danofloxacin, norfloxacin, ofloxacin, sarafloxacin, enrofloxacin, roxithromycin, azithromycin, clarithromycin, clindamycin, spiramycin, erythromycin, meclocycline, doxycycline, tetracycline ,oxytetracycline, chlortetracycline were purchased from Sigma–Aldrich

Canada (Oakville, ON). Isotopically labeled antibiotics (Sulfamethoxazole-d4, spiramycin-d3, sulfadiazine-d4, erythromycin-^{13}C-d3, norfloxacin-d5) and demeclocycline were used as surrogates and/or internal standards. Sulfamethoxazole-d4, spiramycin-d3, sulfadiazine- d4, erythromycin-^{13}C-d3 were purchased from Toronto Research Chemicals (Toronoto, Canada) and norfloxacin-d5 from CDN Isotope Laboratories (Quebec, Canada). All isotopically labeled standards had purity higher than 95% (isotopic purity >99%). Optima LC/MS grade formic acid, acetonitrile and water were purchased from Fisher Chemical (Fairlawn, New Jersey, USA). Membrane filters (0.45 μm and 0.2 μm pore size) were purchased from Millipore (Billerica, MA).

Stock solutions of 1 mg/mL were prepared in methanol for all antibiotics except for the fluoroquinolones which were dissolved in 5% NaOH (0.1M)–95% methanol to get uniform solution (Batt and Aga, 2005). Stock solutions of surrogate standards (0.1 mg/mL) were also prepared in methanol. All stock solutions were kept in the dark at −18 ºC and used for no more than one year. Working standard solution (WS1) mixture was prepared by spiking stock solutions of antibiotic in methanol and the spike level was selected based on their instrumental detection. This solution was prepared fresh every 3 months. A dilution factor of 1000 was applied to prepare a second working standard solution in LC/MS grade water on the day of analysis, which was used to prepare calibration solutions and quality control samples for the given analysis batch. The three types of water matrices used in the method development were reclaimed, river and deionized water. Structures of the selected antibiotics are shown in Table 3.

Table 3. Structures of the selected antibiotics

Tetracyclines
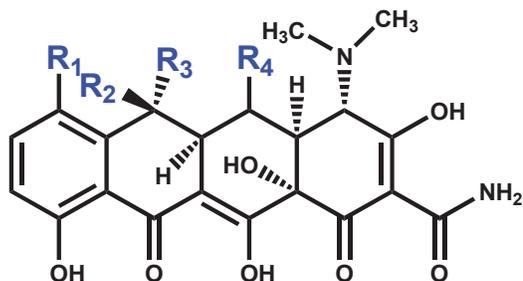
	R$_1$	R$_2$	R$_3$	R$_4$
Oxytetracycline	H	OH	CH$_3$	OH
Tetracycline	H	OH	CH$_3$	H
Chlortetracycline	Cl	OH	CH$_3$	H
Doxycycline	H	H	CH$_3$	OH
Meclocycline	Cl	CH$_2$		OH

Mechanism of action: Protein synthesis inhibitor

Sulfonamides

Sulfadiazine Sulfamerazine Sulfamethazine Sulfadimethoxine

Sulfamethizole Sulfathiazole Sulfamethoxazole Sulfachlorpyridazine
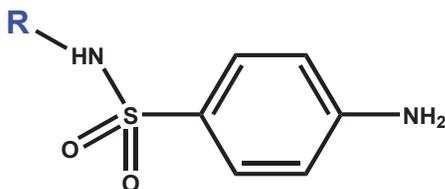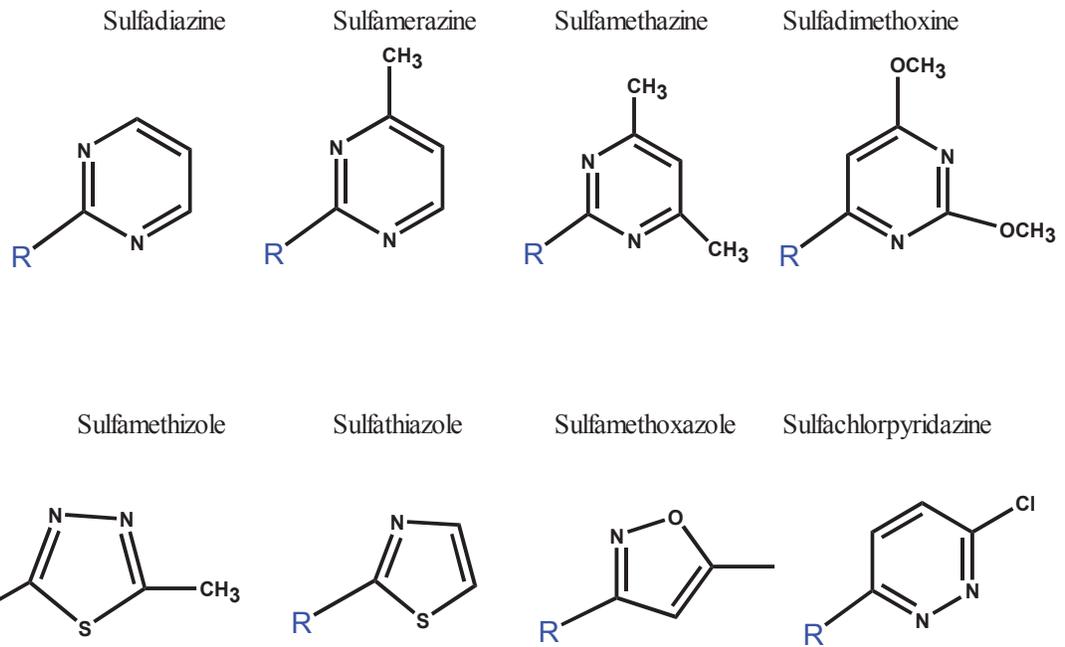
Mechanism of action: competitive inhibitors of the enzyme dihydropteroate synthetase (DHPS), an enzyme involved in folate synthesis

Macrolides

	R_1	R_2	R_3	R_4
Erythromycin	CO	CH_3	H	OH
Roxithromycin	$C-NOCH_2OCH_2CH_2OCH_3$	CH_3	H	OH
Azithromycin	NCH_3		CH_3	OH
Clarithromycin	CO	CH_3	H	OCH_3

	R₁	R₂	R₃	R₄	R₅

	R_1	R_2	R_3	R_4	R_5
Tylosin	CH_2CHO	O	CH_3		
Spiramycin	CH_2CHO		H	H	CH_3
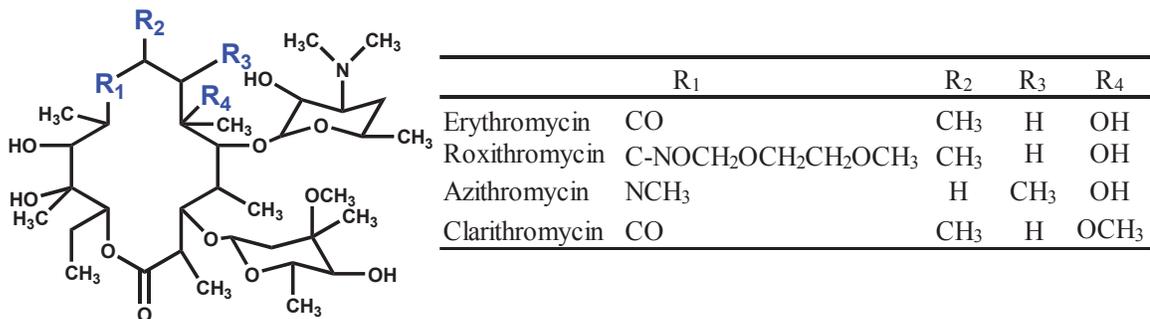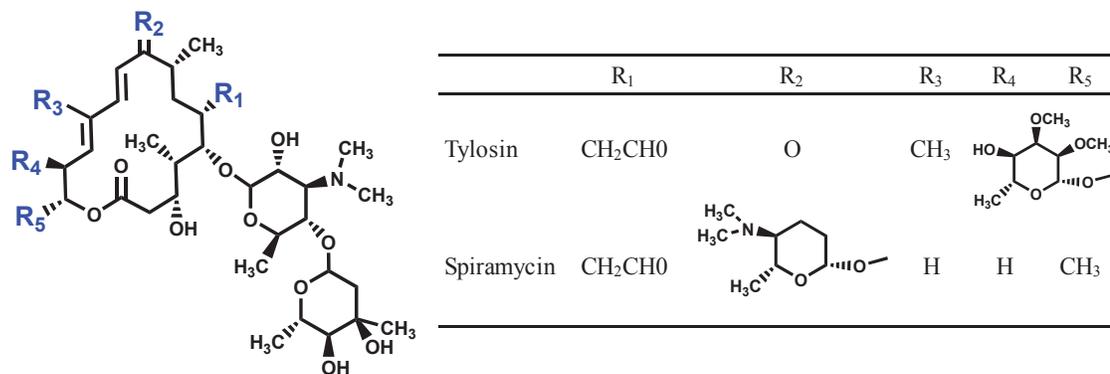
Mechanism of action: protein synthesis inhibitors

Lincosamides

	R
Lincomycin	OH
Clindamycin	Cl
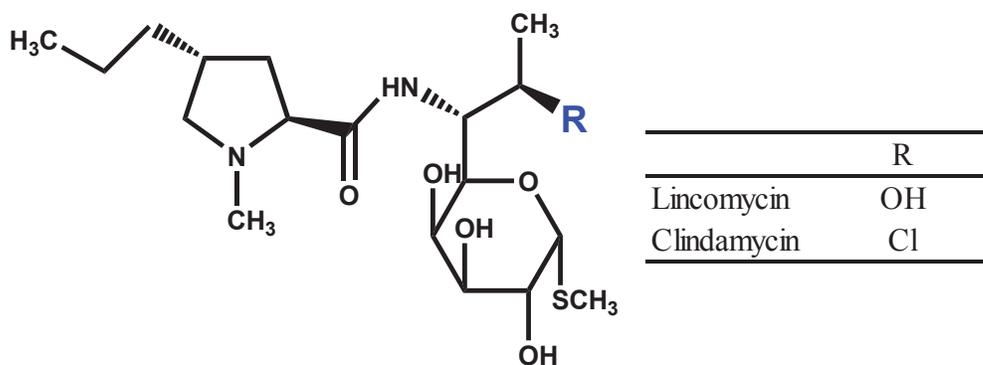
Mechanism of action: protein synthesis inhibitors

Quinolones

	R_1	R_2	R_3	R_4
Ciprofloxacin	C_3H_5	CH		F
Enrofloxacin	C_3H_5	CH	C_2H_5	F
Norfloxacin	C_2H_5	CH		F
Sarafloxacin	(4-fluorophenyl)	CH		F
Danofloxacin	C_3H_5	CH	CH_3	F
Enoxacin	C_2H_5	N		F
Nalidixic acid	C_2H_5	N	CH_3	H
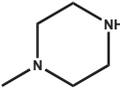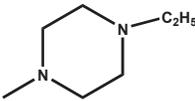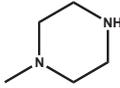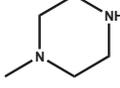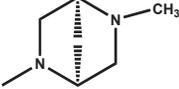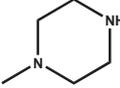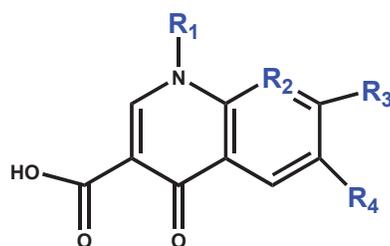
Mechanism of action: inhibit bacterial DNA replication by blocking the enzyme DNA topoisomerase.

2.2.2. Instrumentation

Both sample preconcentration and chromatographic separation were performed using an EQuan system developed by Thermo Fisher Scientific, San Jose, CA, USA. The system allows the injection and preconcentration of up to 20 mL of sample using a high-flow pump (loading pump), and loading column (SPE column); liquid chromatography using a low-flow pump (analytical pump), and analytical column (Figure 2). Sample delivery in the EQuan system was carried out using a HTC-PAL autosampler equipped with a 5 mL injection syringe and a 10 mL (PEEK) loop.

The LC/MS system was equipped with six port dual switching valve that can operate in two different positions. In "load" position, the high-flow pump is connected to sample loop, loading column and to waste in sequential order; and in "inject" position the low flow pump is connected to loading column, analytical column and mass spectrometer in sequential order. At the start up, the divert valve was at the "load" position and the HTC-PAL autosampler was programmed to draw the sample from the vial and inject into the injection loop using the full loop mode. The high-flow Accela UPLC pump was used for sample loading and the low-flow Accela MS pump was used for liquid chromatography. The injected sample from the loop was then transferred to the load column with 100% of solvent A (LC/MS grade water) at a specified flow rate (mL/min). The loading column with all analytes retained was then washed with about 1.5 mL of 100% solvent A (LC/MS grade water) to minimize matrix effects. Then the divert valve was switched to the "inject" position and the column is back flushed into the analytical column using the analytical pump. Column switching between load column and analytical column was performed using a standard 6-port valve. Sample loading and preconcentration was performed on a HyperSep Retain PEP (20mm × 3.0mm I.D) manufactured by Thermo Fisher Scientific, San Jose, CA, USA. Liquid chromatography was carried out using a Hypersil Gold C_{18} column (50mm×2.1mm, 1.9 μm).
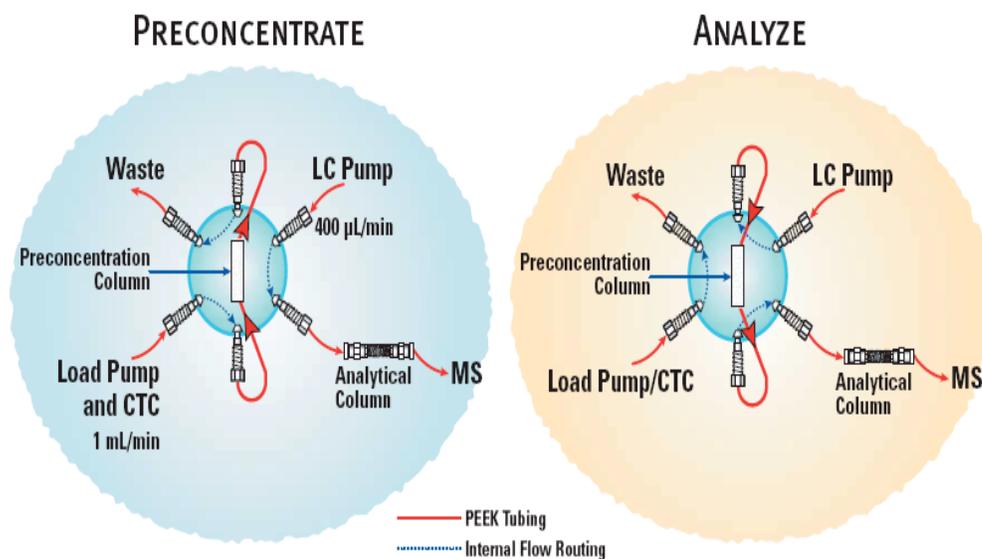
Figure 2. Schematic of online solid phase extraction using Thermo Fisher EQuan system

The LC-MS was equipped with an Ion Max API Heated Electrospray Ionization (H-ESI) Source, operated in positive ionization mode. Where the analytes in solution phase are transformed to gas phase charged droplets with the help of heated auxiliary gas and the ionized. Detection of analytes was performed on a TSQ Quantum Access triple quadrupole Mass Spectrometer (Thermo Scientific, San Jose, CA, USA). For all analytes, optimum ionization conditions and SRM transitions were selected by infusing a 2mg/L individual standard solution through a syringe pump at a flow rate of 50 µL/min. For all analytes, $[M+H]^+$ was selected as the parent ion except for spiramycin and spiramycin-d3 for which the doubly charged ion $[M+H]^{2+}$ was monitored. Subsequent identification of the two most abundant fragment ions and selection of the optimum collision energies (CEs) was carried out in the product ion scan mode. MS/MS optimized parameters for quantitative analysis are shown in Table 4.

Erythromycin is easily converted to its metabolite, anhydro-erythromycin in acidic pH (Kim et al., 2004). In order to verify the efficiency of transformation of erythromycin to its anhydrous form, calibration solutions were prepared by spiking varying levels of stock solutions of erythromycin (or erythromycin -^{13}C-d3) into optima LC/MS water to which 20% optima LC/MS grade formic acid was added. Data was acquired for two SRM transitions 734.2→157.9, 540.14 (erythromycin) and 716.2→157.9, 540.1 (anhydro-erythromycin) and the area ratios were compared. Anhyrdo-erythromycin (m/z 716) accounted for 93.4±2.5% (n=9) in comparison to erythromycin (m/z 734), indicating that more than 90% of erythromycin is converted to anhydro- erythromycin under the source conditions used in this method. Similarly two SRM transitions 738→162.0, 580.4 (labeled standard) and 720.2→161.9, 120.0 (anhydro form of labeled standard) were monitored for erythromycin-^{13}C-d3 indicated a 95.3±0.7% (n=7) conversion of erythromycin -^{13}C-d3. Based on these results, m/z 716 and m/z 720 were used for the quantitation of erythromycin and erythromycin-^{13}C-d3 respectively (Table 4).

Table 4. Optimized parameters for the detection of all analytes and internal standards in MS/MS SRM mode

Antibiotic	Parent ion m/z	Product ions m/z	Collision energy (CE)
Sulfadiazine	250.970	92.118,108.143	32
Sulfathiazole	255.945	92.182,108.128	30
Sulfamerazine	264.994	108.094,171.959	30
Sulfamethazine	279.022	124.102,92.158	28
Sulfamethizole	270.956	92.172,108.110	25
Sulfachlorpyridazine	284.946	92.116,108.111	28
Sulfamethoxazole	253.979	92.181,108.128	32
Sulfadimethoxine	310.986	92.173,108.083	32
Enoxacin	321.033	205.921,234.001	35
Ofloxacin	362.019	260.964,343.992	32
Norfloxacin	361.090	233.003,276.013	32
Ciprofloxacin	332.065	202.972,245.002	55
Danofloxacin	358.059	282.993,340.016	32
Enrofloxacin	360.072	202.984m244.985	40
Sarafloxacin	386.055	298.98,322.000	32
Oxytetracycline	461.045	200.95,426.007	28
Tetracycline	445.066	153.943,410.102	30
Chlortetracycline	479.048	153.949,462.103	25
Doxycycline	445.060	266.895,320.929	25
Meclocycline	476.989	225.998,234.806	30
Spiramycin	422.241	88.130, 174.150	21
Clindamycin	425.073	126.095,377.025	30
Tylosin	916.227	155.92,173.870	28
Erythromycin	716.275	157.953,540.143	25

Clarithromycin	748.266	157.915,558.123	35
Azithromycin	749.500	157.8, 591.3	34
Roxithromycin	837.358	115.992,157.92	34
Lincomycin	407.127	126.114,359.077	30
Amoxycillin	398.034	159.158,348.94	32
Trimethoprim	291.047	260.989,274.99	34
Nalidixic acid	233.013	104.161,186.979	50
Sulfamethoxazole-d4	258.004	96.19,112.100	32
Norfloxacin-d4	325.075	238.042,261.046	35
Spiramycin-d3	423.661	101.097,174.017	21
Sulfadiazine-d4	255.008	96.163,112.145	32
Erythromycin-^{13}C-d3	720.227	120.016,161.943	28
Demeclocycline	465.135	153.980, 288.930	31

The capillary temperature was 375 °C, vaporizer temperature was 350°C and the spray voltage was 4.0 kV. Nitrogen was used as a sheath gas and as an auxiliary gas at a flow rate of 40 and 20 arbitrary units, respectively. Instrument control and data acquisition was performed using Xcalibur 2.1 software (Thermo Scientific, San Jose, CA, USA). Isotopically labeled tetracyclines are not commercially available. Hence, demeclocycline at high concentration (2 µg /mL) was used as an internal standard for the quantification of tetracyclines. LC-MS/MS chromatograms of all target analytes and internal standards, in a fortified and unfortified reclaimed water sample were shown in Figures 3 and 4, respectively.
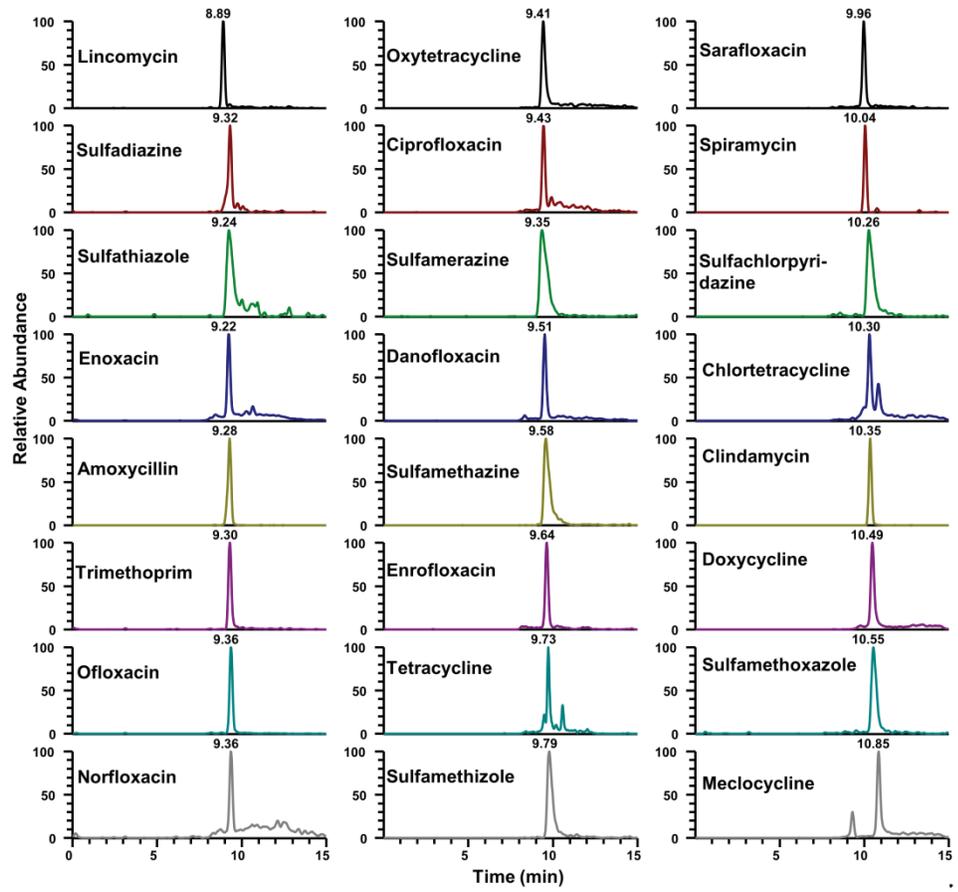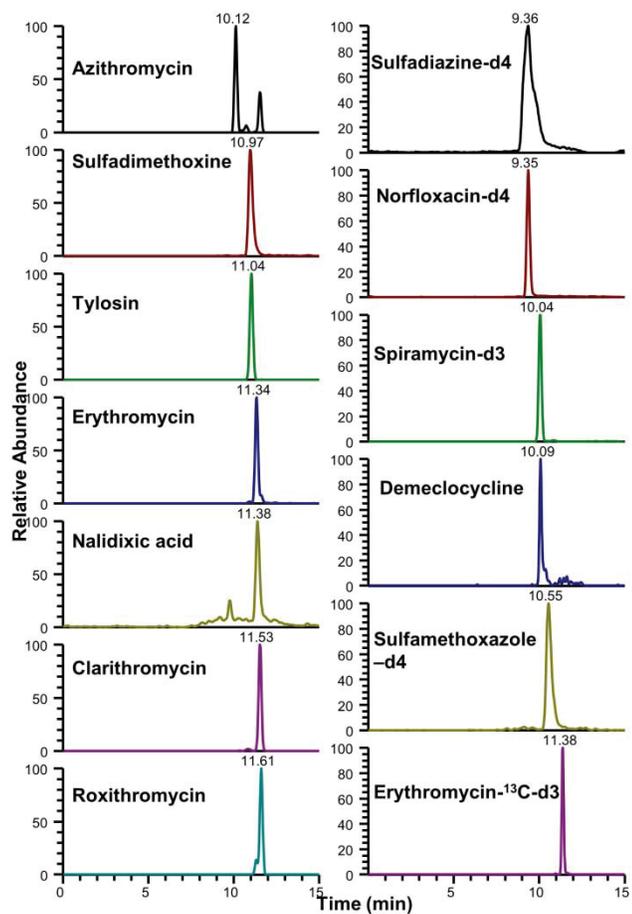
Figure 3. SPE-LC-MS/MS chromatograms of a reclaimed water sample fortified with antibiotics at a concentration equivalent to calibration 5 (CS5), 23-301 ng/L

Figure showing chromatograms of antibiotics with retention times:
Lincomycin 8.08, Oxytetracycline 10.33, Sarafloxacin 10.59; Sulfadiazine 9.35, Ciprofloxacin 9.44, Spiramycin 10.07; Sulfathiazole 11.41, Sulfamerazine 10.26, Sulfachlorpyridazine 8.57; Enoxacin 10.66, Danofloxacin 9.54, Chlortetracycline 10.36; Amoxycillin 11.06, Sulfamethazine 10.24, Clindamycin 9.70; Trimethoprim 9.33, Enrofloxacin 9.59, Doxycycline 10.49; Ofloxacin 9.39, Tetracycline 12.24, Sulfamethoxazole 10.55; Norfloxacin 12.15, Sulfamethizole 9.06, Meclocycline 9.31.

Figure 4. SPE-LC-MS/MS chromatograms of an unfortified reclaimed water sample. Analytes with concentration above MDL include sulfadiazine, trimethoprim, ofloxacin, doxycycline, sulfamethoxazole, meclocycline, azithromycin, tylosin, erythromycin, nalidixic acid and clarithromycin.

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2.2.3. Online SPE-optimization

Three types of loading columns, three sample loading rates (1.0 mL/min, 2.0 mL/min and 5.0 mL/min) and three sample volumes (1.0 mL, 5.0 mL and 10.0 mL) were tested in order to select the best conditions for SPE recoveries and detection limits for both reclaimed and river water matrices. Loading columns included a HyperSep retain PEP (porous polystyrene divinylbenzene, 20 mm x 3.0mm I. D. x 12µm), Hypersil gold aQ (polar endcapped C_{18} phase, 20 mm x 2.1mm I. D. x 12µm) and Hypercarb (porous graphitic carbon, 20 mm x 2.1mm I. D. x 7µm) from Thermo Scientific, San Jose, CA, USA. At least two antibiotics were selected from each class (4 sulfonamides, 3 fluoroquinolones, 2 tetracyclines, 4 macrolides, lincomycin and trimethoprim) to study the matrix effect in reclaimed and river water compared to deionized water (n =3). Among them, the selection was random. In this part of the study sulfamethoxazole-d4 was used as internal standard for quantitation of tetracyclines.

2.2.4. Quantitation of tetrayclines

For all analytes selected under the present study, quantitation was performed using an internal standard approach. Five isotopically labeled internal standards used in the method were norfloxacin d5, sulfamethoxazole d5, sulfadiazine d4, erythromycin ^{13}C d3, spiramycin d3 and demeclocycline. Structural similarity between the analyte of interest and internal standard was chosen as a criterion in selecting the internal standard for the given analyte. Since isotopically labeled tetracycline was not commercially available

during the time of method development, sulfamethoxazole-d5 was chosen as an internal standard based on retention time similarity, for the quantitation of tetracycline compounds.

2.2.5. Matrix effect

The ESI source is highly susceptible to components in the matrix, which may result in signal suppression or enhancement (Mallet et al., 2004; Yang et al., 2005; Kasprzyk-Hordern et al., 2008). In order to understand the degree of ion suppression or enhancement caused by the reclaimed water matrix, its effects were calculated using the equation given below (n=7):

$$\%\text{Matrixeffect} = \left[\frac{(A_S-A_{US})}{A_0}-1\right]*100\% \quad \%\text{Matrixeffect} = \left[\frac{(A_S-A_{US})}{A_0}-1\right]*100\%$$

$$........................ \text{(Equation 1)}$$

where R_s is the peak area ratio of analyte to internal standard (IS) measured in spiked sample matrix, R_{us} is the peak area ratio of analyte to IS measured in unspiked sample matrix and R_0 is the peak area ratio of analyte to IS spiked in deionized water.

2.2.6. Dynamic range and linearity

A 7-point calibration set was freshly prepared by transferring varying levels of working standard solution into a 11-mL glass vial to which 53 μL of 20% optima LC/MS grade formic acid and 50 μL of internal standard mixture in methanol (1 μg/mL of norfloxacin

d5, 0.2 μg/mL of sulfamethoxazole d5, 2 μg/mL of sulfadiazine d4, 1 μg/mL of erythromycin ^{13}C d3, 1μg /mL of spiramycin d3 and 2 μg/mL of demeclocycline) were added and the final volume was made to 10.5 mL with LC/MS grade water. The concentration of all analytes ranged from 2 ng/L to 750 ng/L except for enoxacin, danofloxacin and amoxycillin (20-1500 ng/L). Calibration curves were built with the relative response ratio (area of the analyte standard divided by area of the internal standard) as a function of the analyte concentration. Linear response was observed for all the analytes in the range used (R^2 > 0.99).

The method was applied to reclaimed, river water and drinking waters. In case of reclaimed waters, in order to reduce the effect of the matrix interference, samples were diluted with LC/MS grade water (50:50). Thus, reclaimed water samples were prepared by transferring 10.4 mL of 50:50 diluted sample into a 11-mL vial to which 50μL of 20% optima LC/MS grade formic acid, 50 μL of surrogate mixture in methanol (1 μg/mL of norfloxacin d5, 0.2 μg/mL of sulfamethoxazole d5, 2 μg/mL of sulfadiazine d4, 1 μg/mL of erythromycin ^{13}C d3, 1μg /mL of spiramycin d3 and 2 μg/mL of demeclocycline) were added and the final volume was made to 10.5 mL with LC/MS grade water. Correspondingly, a dilution factor was applied while reporting the results. The river and drinking water samples were injected at their full strength.

The method was further validated in terms of precision which is determined as relative standard deviation (RSD) calculated from repeated injections (n=7) of a 20-100 ng/L spiked matrix (reclaimed water, river water) samples during the same day (repeatability) and on different days (reproducibility).

2.2.7. Method accuracy and detection limits

For the determination of method accuracy in real samples, river and reclaimed water matrices were spiked at two concentration levels representative of typical low and high concentrations (57-755 ng/L) found in those types of water matrices (corresponding MDL is chosen as low level spike and calibration middle point as high level spike, n=7). As real sample matrices may contain target analytes, non-spiked samples were also analyzed and the concentration found was subtracted from the spiked sample concentration. Analyte recovery from different matrices was calculated using the following equation:

$$\% \text{ Method accuracy} = \frac{(C_S - C_{US})}{C_0} * 100 \quad \text{........................ (Equation 2)}$$

Where C_s is the concentration of analyte found in spiked sample matrix, C_{us} is the concentration of analyte measured in unspiked sample matrix and C_0 is the concentration spiked in the sample matrix. In order to assure the quality of the analytical data, a method/procedural blank, a spiked blank, samples duplicates, a matrix spike, and a matrix spike duplicate were analyzed with every sample set (20 samples).

To calculate the method detection limits (MDL), seven replicates of river and reclaimed water samples spiked in the concentration range of 5.7-150 ng/L were analyzed. The MDLs were calculated by multiplying the standard deviation from the seven measurements by the Student t value for six degrees of freedom at the 99% confidence level (t $_{(7-1, 99)}$ = 3.143), according to procedures outlined by the USEPA (USEPA, 2010).

The matrix was spiked at two concentrations levels (n=7) selected based on the sample source.

2.3. Results and discussion

2.3.1. Online SPE

Comparison of the three loading columns tested (HyperSep retain PEP, Hypersil gold aQ and Hypercarb) is shown in the Figure 5. HyperSep Retain PEP column showed the best recoveries for most of the target compounds while both the Hypersil GOLD aQ and the Hypercarb phases showed either high (for ofloxacin) or no retention (for enrofloxacin and norfloxacin), which can be attributed to structural differences between the fluoroquinolones. Thus, HyperSep Retain PEP was selected for further study. This result was expected since the PEP has similar packing material to the Oasis HLB (Hydrophilic lipophilic balance, made of N-vinylpyrrolidone and divinylbenzene) -type cartridges used for enhanced retention of polar analytes in usual offline SPE preconcentration methods (Rao et al., 2008).

For most of the analytes increased breakthrough was observed at high sample loading rates (5.0 mL/min) so the 2.0 mL/min rate was selected to maintain sample throughput. The observed detection limits were lower for both 5.0 mL and 10.0 mL injection volumes compared to 1.0 mL (results were not shown). However, for samples with complex matrix such as reclaimed water, 10 mL showed better detection limits except for enoxacin, ofloxacin, norfloxacin. Therefore, 10.0 mL sample was loaded at a flow rate of

2.0 mL/min and the optimized load pump and analytical pump gradient cycle is shown in Table 5.
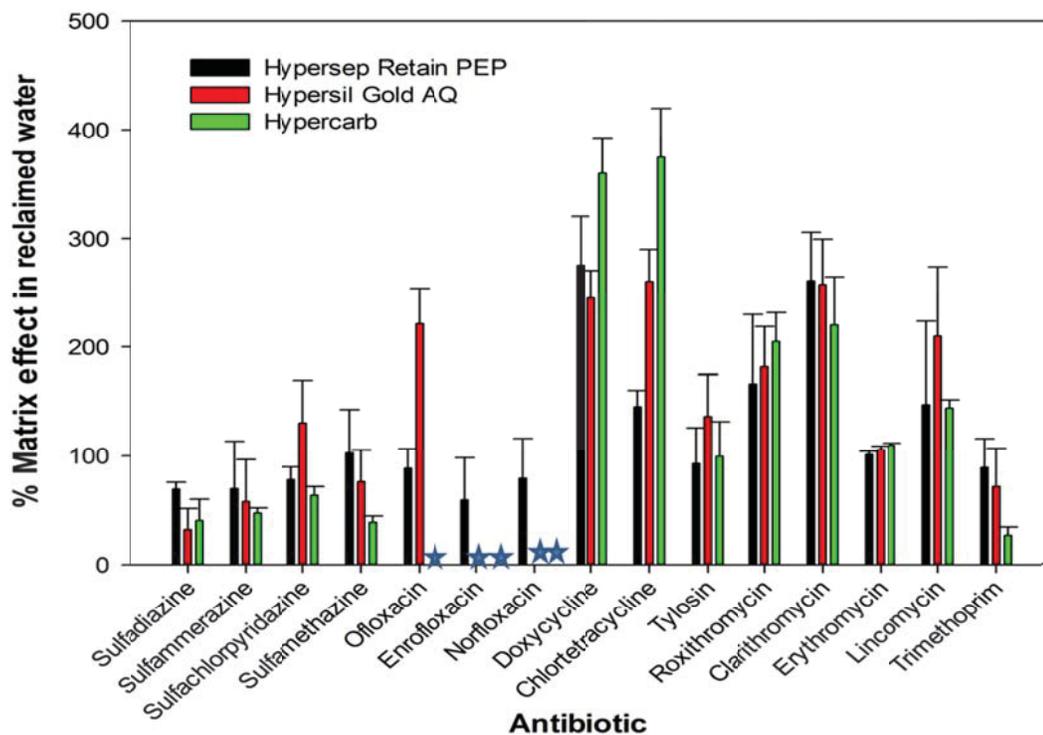
Figure 5. Comparison of loading columns tested for the current study (n=3) Sulfamethoxazole-d4 was used for quantification of tetracyclines instead of demeclocycline (★ = not detected)

Table 5. Mobile phase program for the load pumps (top) and analytical (bottom)

Time (min)	%A (Water)	%B (Methanol)	%C (Acetonitrile)	%D (0.1% Formic acid in water)	Flow (μL min-1)
0.0	100	0	0	0	2000
5.2	100	0	0	0	2000
6.0	0	0	10	90	1000
14.0	0	50	50	0	50
14.2	0	50	50	0	1000
16.0	0	50	50	0	1000
16.2	100	0	0	0	2000
17.0	100	0	0	0	2000

Time (min)	% C (acetonitrile)	% D (0.1% formic acid in water)	Flow (μL min^{-1})
0.0	10	90	220
5.5	10	90	220
9.5	50	50	220
10.6	95	5	220
13.8	25	75	220
17.0	10	90	220

2.3.2. Quantitation of tetrayclines

As seen in the Figure 5, the percent recovery of tetrayclines in reclaimed waters ranged from 150-375% and this result was independent of the type of the loading column chosen. As shown in Table 6, among tetracyclines, % matrix effect (shown as signal suppression or enhancement) varied between -46 to +170%. Positive value indicates signal enhancement and negative value indicate signal suppression. Although, small deviations in the recovery and/or matrix effect are expected among different tetracyclines

due to minor differences in the structure (Table 3), the ranges observed couldn't be explained solely based on the structure. The differences in the affinity of tetracycline and sulfamethoxazole-d5 for the stationary phase, which in turn depend on their structure, could possibly explain the observed discrepancy. This led to the selection of an alternative internal standard, demeclocycline from the same family of antibiotics. To overcome any errors introduced by demeclocycline present in environmental water samples, samples were spiked with demeclocycline at much higher concentration. The updated matrix effects results shown in Table 6 support the use of demeclocycline instead of sulfamethoxazole-d5. Therefore, demeclocycline was used as an internal standard for the quantitation of tetracycline in the later parts of the study.

Table 6. Signal suppression/enhancement values of tetrayclines in reclaimed waters using sulfamethoxazole-d5 as internal standard

Antibiotic	% Signal suppression/enhancement in reclaimed water using Sulfamethoxazole-d5 as internal standard	% Signal suppression/enhancement in reclaimed water using demeclocycline as internal standard
Oxytetracycline	-46.1	0.5
Tetracycline	-23.6	1.5
Chlortetracycline	3.20	4.8
Doxycycline	-10.6	7.8
Meclocycline	170	-12

2.3.3. Matrix effect

Matrix effects were calculated using equation 1. Positive values indicate signal enhancement and negative values indicate signal suppression (Table 7). The results indicated that the effect of matrix was not the same for all classes of antibiotics and it ranged from ± 10 (tetracyclines) to ± 50 (fluoroquinolones), similar to those observed by Lopez-Serna et al. 2010 (Lopez-Serna et al., 2010), i.e., showing higher variation for fluoroquinolones in comparison to other antibiotics. For most of the classes, the signal enhancement or suppression is within ±30%, suggesting that the use of one internal standard per class is sufficient to provide accurate measurements. The selection of the individual internal standard was based on the similarity of structure and/or the similarities of elution times with the target analytes.

Table 7. Signal suppression/enhancement values in reclaimed water sample matrix

Antibiotic	% Signal suppression/enhancement in reclaimed water
Sulfadiazine	1.2
Sulfathiazole	21
Sulfamerazine	3.7
Sulfamethazine	0.8
Sulfamethizole	-14
Sulfachlorpyridazine	24
Sulfamethoxazole	23
Sulfadimethoxine	0.5
Enoxacin	59
Ofloxacin	-21
Norfloxacin	-17
Ciprofloxacin	14
Danofloxacin	-53
Enrofloxacin	-23
Sarafloxacin	8.0
Oxytetracycline	0.5
Tetracycline	1.5
Chlortetracycline	4.8
Doxycycline	7.8
Meclocycline	-12
Spiramycin	7.5
Clindamycin	15
Tylosin	-13
Anhydroerythromycin	27
Clarithromycin	-31
Azithromycin	25
Roxithromycin	5.6
Lincomycin	11
Amoxycillin	11
Trimethoprim	-45
Nalidixic acid	13

The developed method was validated in terms of precision and accuracy. The intra-and inter-day precision of the method was good indicated by relative standard deviations between 4.3-16.4 and 6.8-21.6, respectively (Table 8).

Table 8. Intra- and inter- day precision for all analytes expressed as %RSD

Antibiotic	Intra-day precision %RSD (n=5)	Inter-day precision %RSD (n=3)
Sulfadiazine	8.2	10.2
Sulfathiazole	6.5	6.8
Sulfamerazine	4.3	9.5
Sulfamethazine	9.1	8.6
Sulfamethizole	5.4	10.6
Sulfachlorpyridazine	11.1	10.8
Sulfamethoxazole	7.4	10.6
Sulfadimethoxine	12.4	15.8
Enoxacin	15.4	20.4
Ofloxacin	4.8	10.4
Norfloxacin	11.2	16.6
Ciprofloxacin	4.6	7.8
Danofloxacin	14.8	21.4
Enrofloxacin	7.5	8.4
Sarafloxacin	12.9	10.7
Oxytetracycline	7.2	11.5
Tetracycline	5.8	10.1
Chlortetracycline	10.4	9.8
Doxycycline	8.4	14.9
Meclocycline	12.4	9.7
Spiramycin	15.8	21.2
Clindamycin	10.8	13.3
Tylosin	7.2	9.5
Anhydroerythromycin	4.5	7.1
Clarithromycin	8.5	10.2
Azithromycin	12.5	12.8
Roxithromycin	5.6	7.4
Lincomycin	11.1	15.8
Amoxycillin	16.4	21.6
Trimethoprim	5.8	9.9
Nalidixic acid	10.4	11.2

2.3.4. Method accuracy and detection limits

Both reclaimed water and river water matrices (n=7, except for tetracyclines n=4) were tested to calculate method accuracy using equation 2. Results were compared in Figure 6. In both matrices, the method accuracy ranged from 50-150% for most of the analytes.
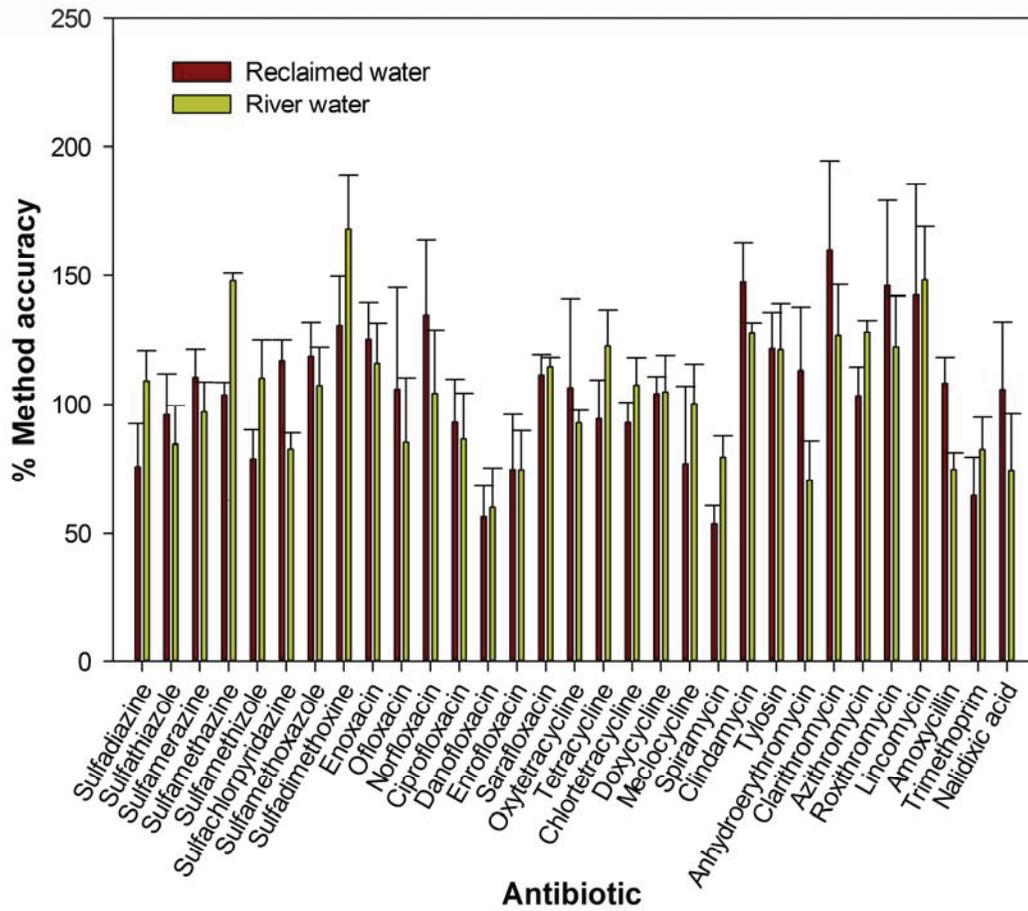
Figure 6. Recovery studies for 31antibiotics in river and reclaimed water matrices (n=7 for all classes of antibiotics except tetracyclines (n=4)

The statistically calculated MDLs are compared in Figure 7 and tabulated in Table 9, implying that matrix components were influencing the method sensitivity and antibiotic detection.
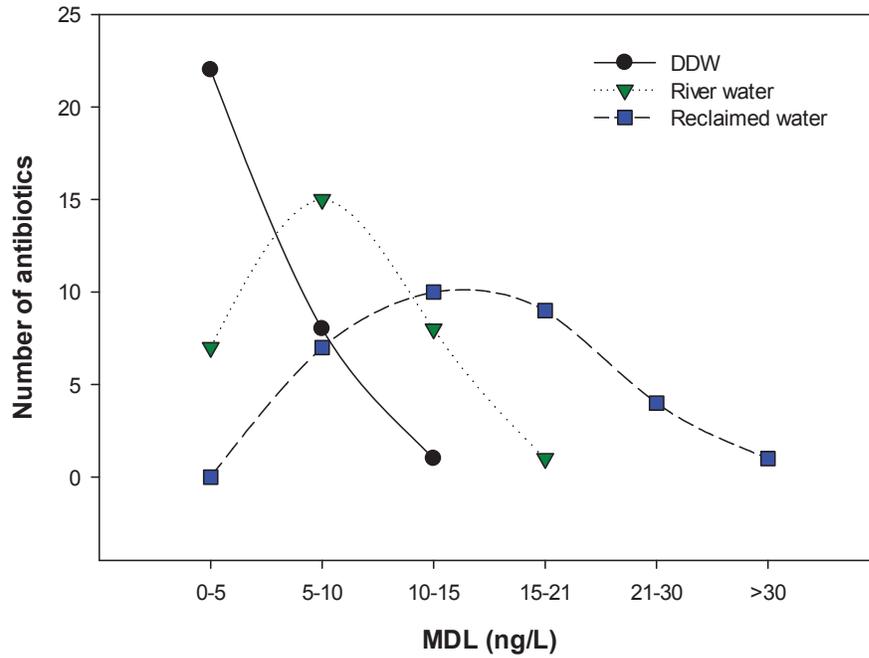
Figure 7. Comparison of MDLs in deionized water, river water and reclaimed water (n=7)

Table 9. Method detection limits of target analytes in deionized, river and reclaimed water matrices

Antibiotic	MDL in Deionized water (ng/L)	MDL in river water (ng/L)	MDL in reclaimed water (ng/L)
Sulfadiazine	7.91	8.52	20.2
Sulfathiazole	6.06	7.18	20.5
Sulfamerazine	1.69	7.23	15.2

Sulfamethazine	1.32	5.81	9.75
Sulfamethizole	3.15	10.2	16.3
Sulfachlorpyridazine	5.40	9.70	10.1
Sulfamethoxazole	4.60	6.40	16.6
Sulfadimethoxine	3.01	10.4	13.6
Enoxacin	6.73	14.5	63.1
Ofloxacin	1.77	9.24	28.4
Norfloxacin	2.35	6.82	21.1
Ciprofloxacin	4.44	5.91	11.9
Danofloxacin	14.4	20.5	28.0
Enrofloxacin	2.02	3.54	7.66
Sarafloxacin	3.96	4.12	5.50
Oxytetracycline	2.86	3.56	7.83
Tetracycline	3.69	5.96	14.1
Chlortetracycline	9.74	11.4	14.2
Doxycycline	1.28	7.86	15.6
Meclocycline	2.08	12.1	25.0
Spiramycin	6.58	11.5	18.5
Clindamycin	4.51	4.61	5.58
Tylosin	4.81	8.40	10.2
Erythromycin	6.50	7.82	8.85
Clarithromycin	1.85	5.47	10.7
Azithromycin	3.81	6.15	10.8
Roxithromycin	1.21	4.53	11.8
Lincomycin	1.81	2.24	7.70
Amoxycillin	3.10	14.9	23.1
Trimethoprim	1.58	3.19	12.0
Nalidixic acid	5.22	11.2	15.2
Average	4.18	8.10	16.1

2.4. Conclusions

An Online Solid phase extraction (SPE) and liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS) method was developed for the simultaneous determination of 31 antibiotics in various water matrices. An Ion Max API Heated Electrospray Ionization (HESI) source operated in the positive ionization mode with two selected reaction monitoring (SRM) transitions was used per antibiotic for positive identity and quantification performed by the internal standard approach, to correct for matrix effects and any losses in the online extraction step. The advantages of developed analytical methodology were, required small sample volume (10 mL), very little sample preparation and high throughput, the total sample run time was 20 minutes. The method had MDLs in the range of 1.2-9.7 (except danofloxacin), 2.2-15, 5.5-63 ng/L in deionized water, surface water and reclaimed waters, respectively and hence has the potential to measure analytes at their environmental concentrations. The method accuracy in matrix spiked samples ranged from 50-150% for the studied antibiotics. Furthermore, the method was validated in terms of precision, accuracy and linearity. And the present method is easy to adopt by analytical labs for regular day to day antibiotic analysis.

CHAPTER 3

Reclaimed water analysis

(Panditi, V., Batchu, S., Gardinali, P., 2013. Analytical and Bioanalytical Chemistry, 405 (18), 5953-5964)

3.1. Introduction

Water is an integral part of life. The amount of water used by human society for different activities on a global scale is shown in Figure 8 (Levine and Asano, 2004). The world population is estimated to be increasing at a rate of about 1.2% per year (UN, 2003) and this steady growth in population demands more water supplies. Earth's limited natural water resources have made humans to think of reuse/reclamation of water to supplement the increasing demand for fresh water. More importantly the sewage effluents containing hazardous chemicals and pathogenic microorganisms that released in to natural aquatic environments were negatively affecting the healthy ecosystems (Boxall, 2004; Kummerer, 2009; Ding and He, 2010; Wang and Gardinali, 2012). Hence, many countries in the world realized the potential effects in both public and environmental health point and taking necessary steps to restrict the contamination of natural water bodies. Reclamation of wastewater is one of the methods of choice widely practiced. Reclaimed water can be defined as the end product of wastewater reclamation that meets water quality requirements for biodegradable materials, suspended matter and pathogens. Different applications of reclaimed water include landscape irrigation, agricultural irrigation in both food and non-food crops, ground water recharge and recreational purposes (Levine and Asano, 2004).
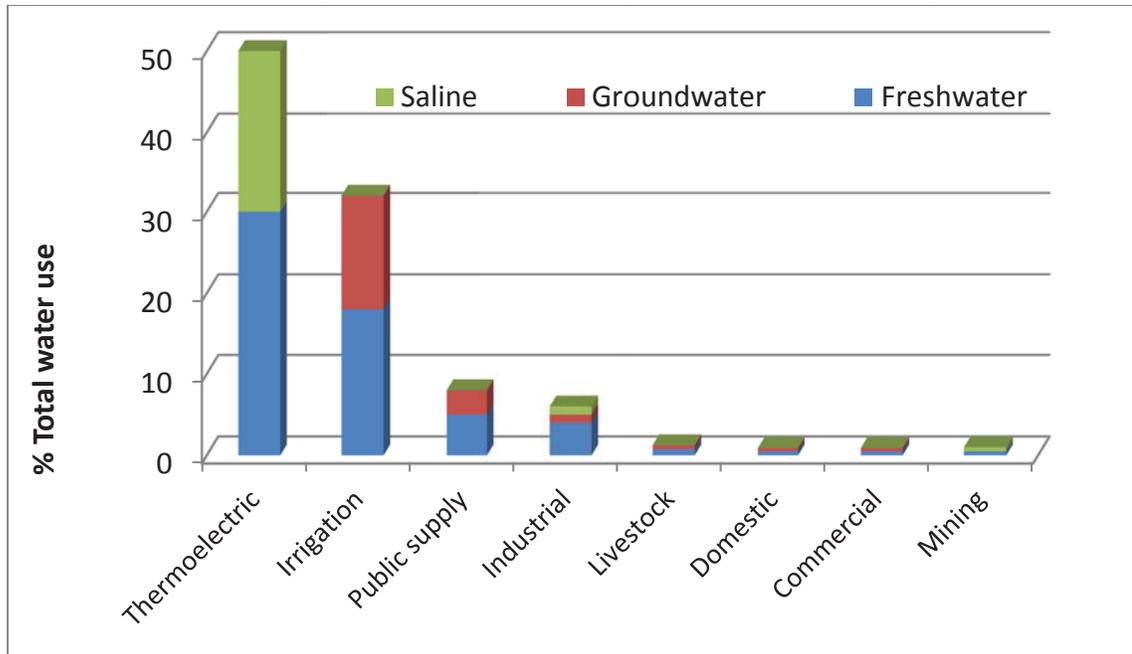
Figure 8. The amount of water used for potable and nonpotable applications on a global scale

In Florida, state law supports water reuse efforts. According to Section 403.064(1), F. S., reuse is a critical component of meeting the state's existing and future water supply needs while sustaining natural systems (CMPD, 2010). In Miami Dade County, the city of Miami supports 20-year irrigation projects to recharge the Biscayne aquifer with highly treated reclaimed water. These projects will be implemented by North and Central District Wastewater Treatment plants (CMPD, 2010). According to Miami Dade Water and Sewer Department's reuse feasibility update report in 2006, 7.9 % of wastewater produced (16.2 MGD) is reused. In addition to the existing capacity, the department is planning to produce an additional 18.75 MGD of reclaimed water by the end of 2013 (MDWASD, 2007b). Various steps involved in producing reclaimed water from the wastewater include microfiltration through a series of membranes (0.1 to 10 μm), reverse

osmosis, treatment with hydrogen peroxide followed by photolysis with UV light (MiamiDade, 2013). To date, FIU Biscayne Bay Campus receives reclaimed water from Miami-Dade North District Waste Water Treatment Facility, which has a capacity to treat 380,000 m^3/day of water (Figure 9). The capacity of the existing reuse system for FIU irrigation is 1.5 million gallons per day (MGD) for irrigating 40 acres of landscape (MDWASD, 2007a). Although reclaimed water is a processed wastewater it may contain residual amounts of biologically active antibiotics which can show adverse effects on sensitive ecosystems. Moreover, as the reclaimed water is used for many different purposes to supplement the fresh water needs, it is necessary to ensure that the reclaimed water is safe for reuse. Hence the quality of reclaimed waters must be monitored.

The main aim of this study is to analyze reclaimed waters for residual antibiotics to determine their concentration profiles, detection frequency and total mass loads estimation using online SPE-LC/MS/MS. To understand significant variation if any, among the different chemical classes the mass loads results were compared with annual antibiotic sales data.
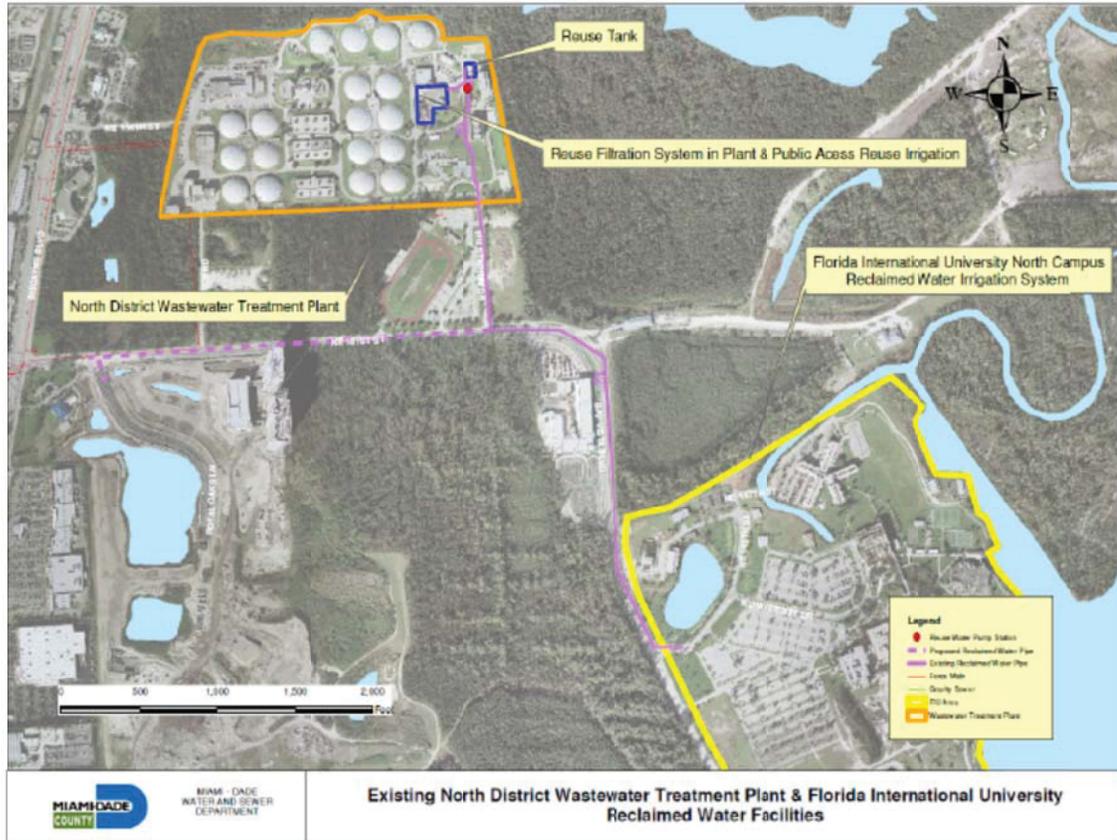
Figure 9. Existing North District Wastewater Treatment plant (circled in orange) and Florida International University reclaimed water facilities (circled in yellow)

3.2. Sample collection and preparation

All reclaimed water samples were collected directly from the sprinkler systems after they were flushed for at least 5 minutes once a week from November 2010 until October 2011 (56 samples). After collection, all samples were immediately transported to the laboratory and sequentially filtered through the 0.45 µm glass fiber filter and finally through 0.2 µm membrane filter to minimize any potential for biodegradation. Filtered samples were stored in the dark at -18ºC until the time of analysis.

3.3. Applicability of the method to reclaimed water samples

The method developed was applied to assess the levels of the target compounds in reclaimed waters and the concentrations below the MDL are considered as not detected for the calculation of mean and frequency of detection.

The results for reclaimed waters were plotted as box plots from the highest to the lowest concentration and frequency of detection is shown in parenthesis (Figure 10). The blue line in each box plot indicates mean annual concentration while top and bottom limits represent the 25th and 75th percentile of the distribution respectively.
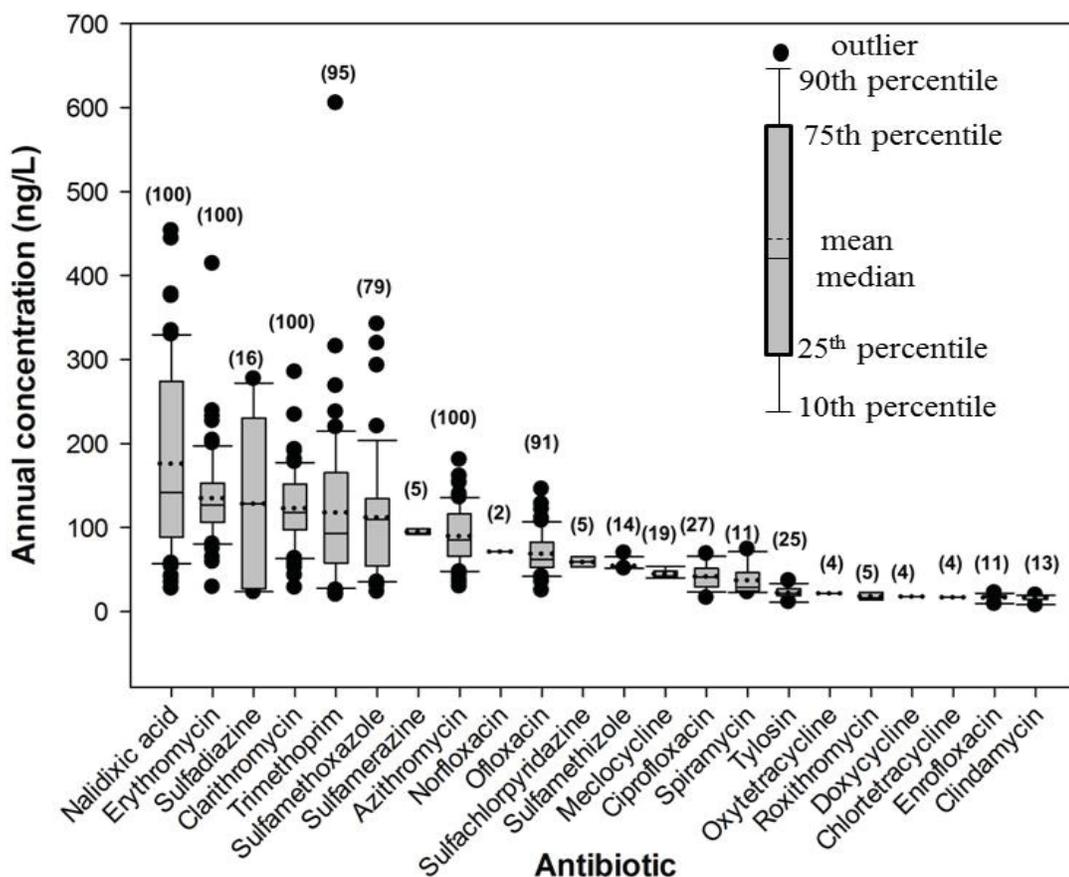
Figure 10. Distribution of antibiotics in reclaimed waters (frequency of detection shown in parenthesis). The boundaries of box plot cover 25th-75th percentile, the center line indicates median of the sample population, error bars (whiskers) above and below the box refer to 90th and 10th percentiles. The dotted line in each box plot indicates mean annual concentration (n=56).

Detailed values of mean, median and the concentration range are shown in (Table 10). For nalidixic acid, trimethoprim and clarithromycin, the mean concentration was moderately higher than the median concentration indicating that majority of the samples

55

have rather high concentrations. The most frequently detected antibiotics were nalidixic acid, erythromycin (monitored as anhydroerythromycin), clarithromycin, azithromycin, trimethoprim, ofloxacin, and sulfamethoxazole. The same antibiotics have been frequently reported in wastewater effluents in many other studies (Nakata et al., 2005; Feitosa-Felizzola et al., 2007; Segura et al., 2007a; Segura et al., 2007b; Gulkowska et al., 2008; Watkinson et al., 2009; Gao et al., 2012).

Table 10. Reclaimed water statistics for the target analytes (n=56).

Antibiotic	Annual mean concentration (ng/L)	% Frequency of detection	Concentration range (ng/L)	Median (ng/L)
Nalidixic acid	176	100	27.1 - 453	142
Erythromycin	135	100	28.5 - 414	127
Sulfadiazine	128	16	<mdl - 276	128
Clarithromycin	123	100	27.9 - 284	118
Trimethoprim	118	95	<mdl - 605	92.9
Sulfamethoxazole	112	79	<mdl - 341	110
Sulfamerazine	94.8	5	<mdl - 100	93.0
Azithromycin	89.7	100	29.3 - 180	85.0
Norfloxacin	71.3	2	71.3	71.3
Ofloxacin	68.8	91	<mdl - 127	61.8
Sulfachlorpyridazine	58.9	5	<mdl - 66.9	58.8
Sulfamethizole	54.4	14	<mdl - 69.2	51.7
Meclocycline	44.5	19	<mdl - 53.8	41.8
Ciprofloxacin	41.6	27	<mdl - 68.0	40.8
Spiramycin	37.3	11	<mdl - 73.7	28.4
Tylosin	22.3	25	<mdl - 36.3	21.7
Oxytetracycline	21.1	4	<mdl - 21.1	21.7
Roxithromycin	17.9	5	<mdl - 25.1	15.8
Doxycycline	17.7	4	<mdl - 17.7	17.7
Chlortetracycline	16.9	4	<mdl - 16.9	16.9
Enrofloxacin	16.7	11	<mdl - 21.9	17.2
Clindamycin	16.3	13	<mdl - 19.1	18.6

Meclocycline, ciprofloxacin, tylosin were moderately detected, with frequency of detection 19-27%. Tylosin was detected less frequently compared to other macrolides, although its usage has been increasing in the last decade (MAF, 2010). This might reflect its higher removal efficiency in sewage treatment plant (Chang et al., 2010) rather than patterns in consumption. Sulfonamides are most commonly used both in humans and veterinary medicine to treat a variety of infections. The sulfonamides more frequently used for this purpose are sulfamethoxazole, sulfadiazine, sulfamerazine and sulfathiazole (Lopes et al., 2011). Trimethoprim is another antibiotic agent often co-administered with sulfamethoxazole to enhance treatment against a variety of bacterial infections (Masters et al., 2003). In the present study, sulfamethoxazole and sulfadiazine were detected frequently and at rather higher concentration. Trimethoprim was also detected in all reclaimed water samples analyzed due to its heavy consumption and/or incomplete removal in the WWTPs. Similar results were also observed by Chang et al in sewage treatment effluents (Chang et al., 2010).

Seasonal variation is observed in Florida with wet season ranging from May to October and dry season from November to April. Daily effluent flow rate (MGD) data was obtained from the Miami-Dade North District Waste Water Treatment Facility and was multiplied by the individual concentration of all antibiotics (ng/L) to obtain the mass load of antibiotics entering the receiving waters using the equation 3. The same was plotted against each month starting from November 2010 to October 2011 in Figure 11. In month of February 2011, sprinklers were not turned on for more than a week due to maintenance and only one reclaimed water sample was collected in the entire month. As seen in the Figure 11, the total antibiotic mass loads released in the reclaimed water were high from

the month of November to March except February and then gradually decreased until September. It seems like daily mass loads were lower in wet season compared to dry season indicating that dilution effects could have played a role in the occurrence of targeted analytes in the reclaimed water. Lei Jiang et al. (Jiang et al., 2011) also observed a similar trend in seasonal variations in overall antibiotic concentrations, i.e., higher concentrations in December (the low water season) and lower concentrations in June (the high water season) for river water samples. However, as the measured concentrations were normalized with daily flow rates the daily mass loads should be nearly constant if there is no significant variation in antibiotic consumption pattern or no changes in reclamation process during the study period. In a recent study by Zhang et al. reported that there is a seasonal variation in antibiotic prescriptions in the United States. According to their study highest prescription rates were from January through March and lowest from July through September (Zhang et al., 2012). It is likely that the data represented here may have been influenced by seasonal variations in antibiotic consumption and possibly contributes to the observed differences in mass loads of antibiotics. The total mass load of antibiotics in reclaimed waters was 472 and 614 g/day in wet and dry seasons respectively.

$$mass\ load\ \left(\frac{g}{day}\right) = concen.\ \left(\frac{ng}{L}\right) x\ flow\ rate\ \left(\frac{L}{day}\right) x\ \left(\frac{1}{10^9} x \frac{g}{ng}\right)$$

…………… (Equation 3)
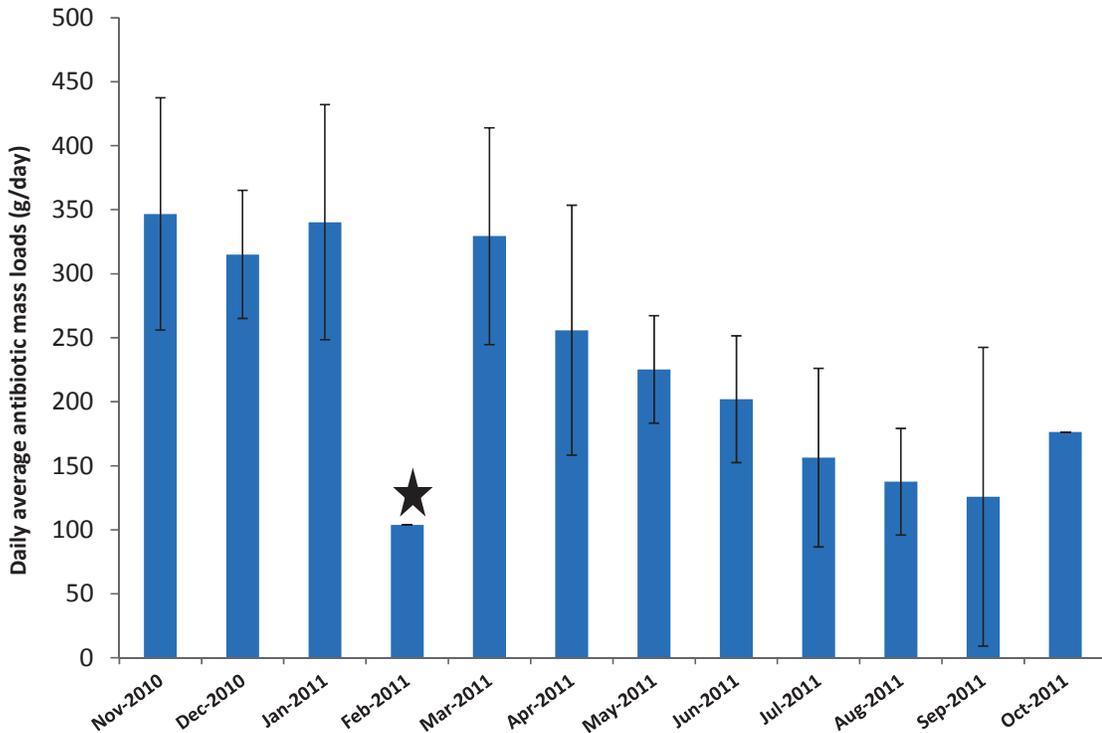
Figure 11. Month wise mass loads (mg/day) distribution of antibiotics in reclaimed waters

★For February 2011, only one reclaimed water sample was collected due to sprinklers maintenance

Daily Mass load per capita, in µg/day/person of antibiotics was calculated based on the size of the served population Miami Dade North district according to United States Census 2011 (USCB, 2012) (Table 11). These results are comparable to levels of antibiotics found in effluent waters from other studies in North America and Europe (Karthikeyan and Meyer, 2006; Lindberg et al., 2006). The presence of antibiotics in reclaimed waters suggests insufficient removal by waste water treatment process

resulting in the potential chronic discharge of antibiotics into the environment. This also

shows a clear need for developing new water treatment technologies.

Table 11. Estimated mass loads (µg/day/person) of targeted analytes in reclaimed waters

Antibiotic	Mass loading in wet season (µg/day/peson)	Mass loading in dry season (µg/day/person)
Sulfadiazine	35	127
Nalidixic acid	77	86
Trimethoprim	37	74
Clarithromycin	47	70
Erythromycin	55	70
Sulfamethoxazole	49	57
Sulfamerazine	45	46
Azithromycin	43	44
Ofloxacin	27	38
Sulfachlorpyridazine	24	30
Sulfamethizole	26	26
Spiramycin	17	22
Ciprofloxacin	19	21
Meclocycline	21	14
Roxithromycin	8	9
Clindamycin	5	9
Doxycycline	--	8
Chlortetracycline	--	8
Tylosin	11	7
Enrofloxacin	8	7
Oxytetracycline	10	--
Norfloxacin	34	--

3.3.1. Mass loads of antibioitcs versus consumption data

According to a recent report by USFDA 3.28 million kilograms of antibiotics were sold for human medical use in 2010 (Pham, 2012). The estimated mass loads of different classes of antibiotics in reclaimed waters was plotted as a function of their sales in 2010 and shown in Figure 12 (n=56). The high correlation coefficient (R^2=0.73) indicates a positive correlation between sales and mass loads detected. The graph shows that inspite of low consumption, macrolide antibiotics were detected at rather high concentration which could be explained based on their high photostability compared to other classes of antibiotics (Vione et al., 2009; Batchu, 2013).
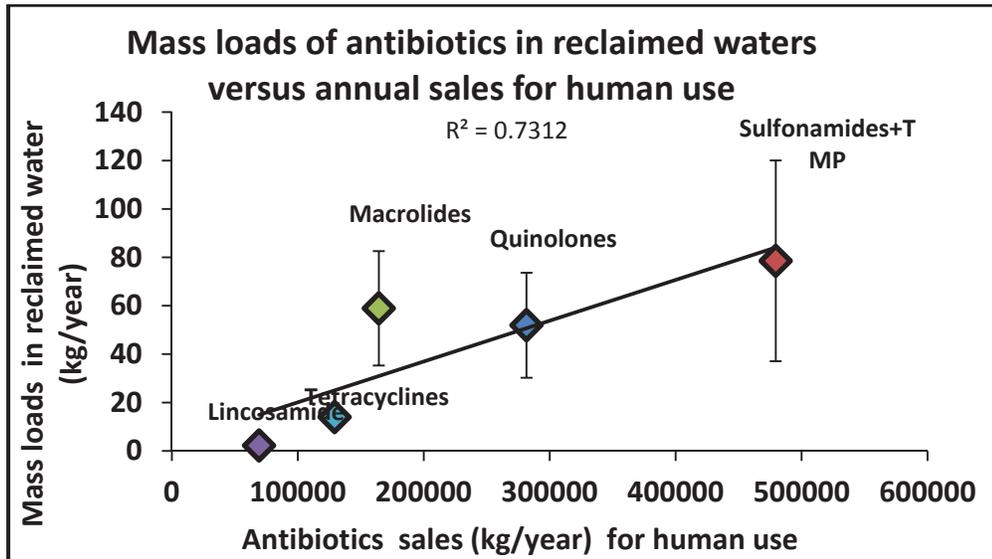
Figure 12. A plot of antibiotics sales in 2010 and their estimated mass loads from the present study

3.4. Conclusions

Detection of antibiotics in reclaimed water indicates that conventional wastewater process used were not sufficient in the complete removal of antibiotics. The developed online SPE-LC-MS/MS method was successfully applied for the quantitation of antibiotics in reclaimed waters collected over a period of one year (n=56). 22 out of 31 selected antibiotics were detected in reclaimed waters reaching up to a maximum concentration of 604 ng/L. Nalidixic acid, erythromycin, clarithromycin and azithromycin were detected in all samples analyzed and at higher concentrations (average concentration: 90-176 ng/L). Other most frequently detected antibiotics were trimethoprim, sulfamethoxazole and ofloxacin. Mass loads of antibiotics released in reclaimed waters plotted against sampling month showed seasonal variation i.e., highest from the month of November through March (except February) and then gradually decreased until September, following general antibiotic prescription statistics. Based on the results, Positive correlation was observed between the mass loads of antibiotics released in reclaimed waters and their annual consumption except for macrolides. The possible reason could be the recalcitrant nature of macrolides such as high photostability unlike other antibiotics. Finally, mass load per capita of each antibiotic in reclaimed water was reported. Detection of antibiotics in reclaimed water indicates that conventional wastewater management practices are not effective in the complete removal of antibiotics.

CHAPTER 4

Surface water analysis

Data from Miami Dade water samples (Panditi, V., Batchu, S., Gardinali, P., 2013. Analytical and Bioanalytical Chemistry, 405 (18), 5953-5964).

4.1. Introduction

Antibiotics are considered as emerging contaminants of concern due to their potential adverse effects on ecosystem and human health (Wollenberger et al., 2000; Kummerer, 2009; Ding and He, 2010). Antibiotics reach sewage treatment plant from various sources such as domestic, industrial wastes and agricultural run offs. Both present and previous studies have showed that the current treatment employed in the sewage treatment plants does not lead to the complete removal of antibiotics; as a result residual antibiotics are continuously released into water ways via wastewater effluents (Feitosa-Felizzola et al., 2007; Gartiser et al., 2007; Panditi et al., 2013) and thus have a large potential to effect the aquatic environment. Various waste disposal options followed by sewage treatment plants include ocean outfalls, deep well injection, soakage pits, drain fields and canals.

 In South Florida, waste disposal alternatives are deep well injection and ocean outfalls and surface water (canal) discharges following secondary wastewater treatment, filtration and nutrient removal (Bloetscher et al., 2005). Broward and Miami-Dade counties dispose approximately 510 MGD of the treated effluents collectively into ocean outfalls and deep well injections (Struhs, 2003).

Previous studies have shown that antibiotics from wastewater discharges were relatively persistent and make their way into the surrounding surface waters as far as 100 meters (Batt et al., 2006). Not only that antibiotics reach surface waters through traditional waste disposal pathway but also through agricultural runoffs (Davis et al., 2006), if the sludge form the sewage treatment plant used for landfill or as fertilizer in agriculture (Holzel et al., 2010), to a little extent poorly maintained leaky sewage pipelines and septic systems

close to surface waters. So far there were no studies reported on antibiotic concentrations in South Florida surface waters. Therefore, it is essential to determine the frequently detected antibiotics and their environmental concentration in South Florida surface waters to understand the potential ecological risk.

In the present study, surface water samples were collected from the major canals in South Florida, under Miami Dade and Broward Counties. Miami River passes through downtown Miami and reaches Biscayne Bay. It has a long history of water quality problems from wastewater intrusions from aging leaky sewage collection and pumping systems and receives large amounts of domestic effluents (Gardinali, 2002; MRC, 2002) and hence included in the present study.

4.2. Sample collection and preparation

500-mL of the surface waters were collected from Miami River and major canals passing through Miami-Dade (n=35) and Broward counties (n=18) in a Polyethylene terephthalate (PETE) bottle. The samples from Broward County were provided by a Broward County Environmental Monitoring lab. The details of the sampling locations are shown in Table 12 and Figure 13. Samples HWO1 and HWO2 were collected from Hollywood waste water treatment plant ocean outfalls.

Table 12. Surface waters sampling site, latitude and longitude details

Sample name	Latitude	Longitude
Florida City canal (1)	25°26'53.85"N	80°27'30.12"W
Florida City canal (2)	25°26'53.94"N	80°24'47.75"W

North Canal drive (1)	25°27'46.37"N	80°24'38.06"W
North Canal drive (2)	25°27'46.18"N	80°22'16.36"W
Mowry Canal (1)	25°28'25.23"N	80°24'38.25"W
Mowry Canal (2)	25°28'16.21"N	80°23'1.74"W
Military Canal	25°29'21.52"N	80°21'44.03"W
Princeton Canal	25°31'10.41"N	80°21'48.12"W
Black Creek Canal (1)	25°32'56.45"N	80°20'50.97"W
Black Creek Canal (2)	25°32'39.54"N	80°19'56.39"W
Cutler Canal	25°36'34.96"N	80°19'2.05"W
C113 Canal	25°30'25.21"N	80°28'44.82"W
Snapper Creek Canal (1)	25°40'4.19"N	80°16'55.14"W
Snapper Creek Canal (2)	25°41'29.85"N	80°18'11.41"W
Coral Gables Canal	25°42'19.09"N	80°15'34.96"W
Miami River (1)	25°46'51.56"N	80°12'38.49"W
Miami River (2)	25°46'11.01"N	80°11'29.40"W
Canal 11 (1)	25°51'11.72"N	80°11'47.23"W
Canal 11 (2)	25°52'16.70"N	80°14'33.28"W
Canal 12	25°52'30.68"N	80°10'59.29"W
Canal 13 (1)	25°55'42.53"N	80° 9'30.50"W
Canal 13 (2)	25°55'46.86"N	80°10'3.80"W
OS1	26° 4'96.36"N	80° 6573.21"W
OS2	26° 6'13.41"N	80° 5'61.59"W
OS3	26° 9'59.68"N	80° 5'28.02"W
Port Everglades PE1	26° 5'61.68"N	80° 6'30.36"W
Pharm 1	26° 5'12.76"N	80° 5'54.73"W
Pharm 2	26° 1'20.83"N	80° 6'12.85"W
Pharm 3	26° 0'21.57"N	80° 6'10.98"W
Pharm 4	25°59'24.15"N	80° 6'8.12"W
HWO1	26° 0'35.79"N	80° 5'4.17"W
HWO2	26° 1'0.56"N	80° 5'5.51"W
QC-37	26° 8'27.30"N	80° 6'24.96"W
QC-10	26° 8'35.55"N	80° 7'3.83"W
QC-15	26° 6'59.81"N	80° 8'10.61"W
QC-38	26° 5'32.55"N	80° 6'46.58"W
QC-39	26° 4'9.97"N	80° 7'0.16"W
QC-24	26° 3'27.43"N	80° 8'55.55"W
QC40	26° 2'9.90"N	80° 7'1.75"W
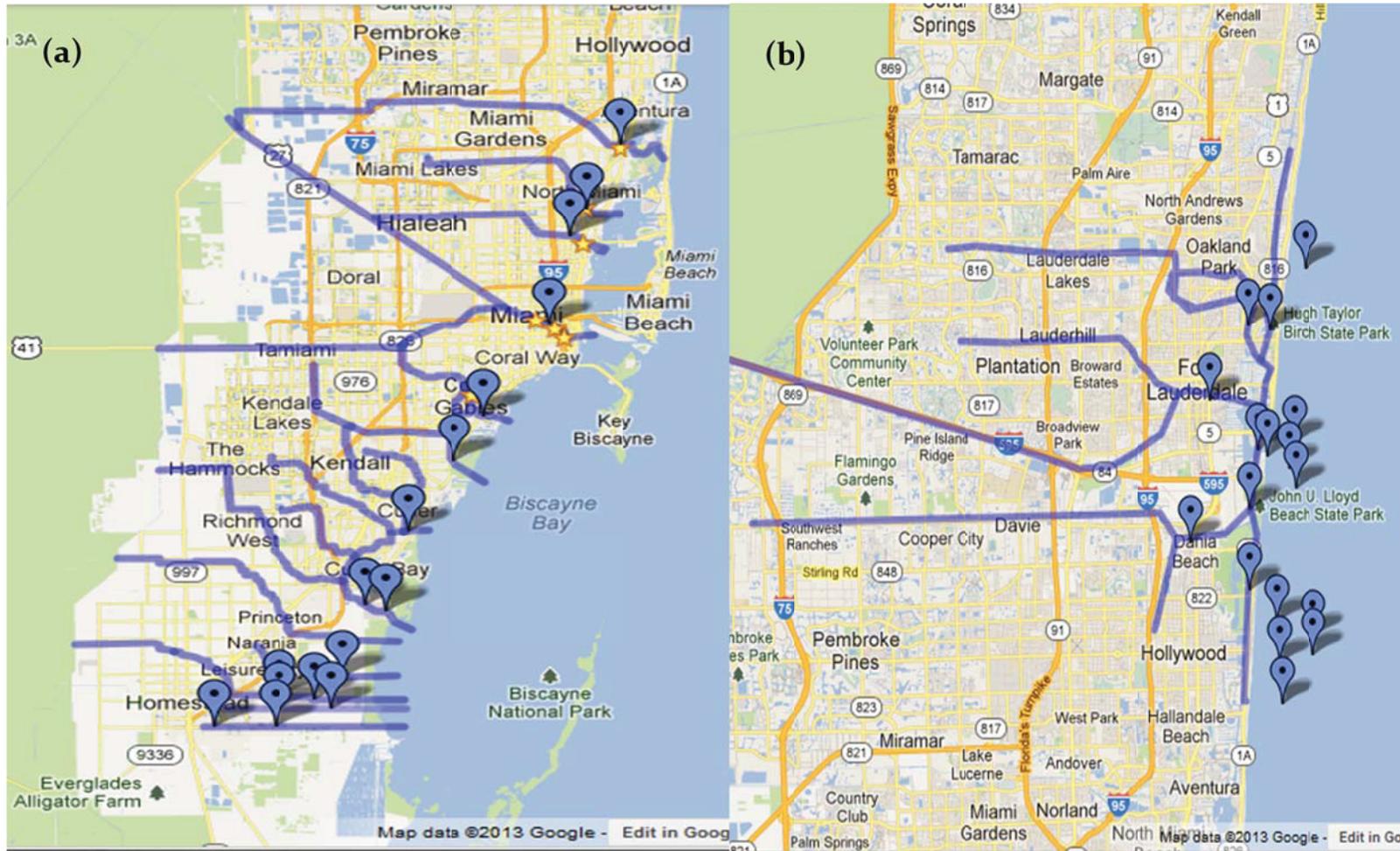
Figure 13. Canals sampled during the study a) from Miami-Dade County b) from Broward County

4.3. Analysis of surface waters

The method described in chapter 2 was applied for the analysis of antibiotics in surface waters. Samples were injected full strength for the determination of antibiotics. As some of the samples were salt waters, the method detection limits were statistically calculated using 7 replicates of salt waters spiked with target analytes in the concentration range of 5.7-150 ng/L.

4.4. Results and Discussion

The MDLs obtained shown in Table 13, were higher compared to the ones in river and reclaimed water and which was expected as the method was not optimized for the salt waters. However, the detection limits obtained were still low enough to measure analytes in environmental waters.

Table 13. Method detection limits of target analytes in salt waters

Antibiotic	MDL in salt water (ng/L)
Sulfadiazine	36.0
Sulfathiazole	56.3
Sulfamerazine	24.4
Sulfamethazine	11.3
Sulfamethizole	33.3
Sulfachlorpyridazine	5.58
Sulfamethoxazole	10.2
Sulfadimethoxine	23.1
Enoxacin	88.1
Ofloxacin	10.6

Norfloxacin	18.8
Ciprofloxacin	7.20
Danofloxacin	26.3
Enrofloxacin	13.8
Sarafloxacin	9.16
Oxytetracycline	40.6
Tetracycline	20.6
Chlortetracycline	13.9
Doxycycline	15.6
Meclocycline	12.4
Spiramycin	65.2
Clindamycin	29.4
Tylosin	36.1
Erythromycin	32.0
Clarithromycin	14.6
Azithromycin	19.4
Roxithromycin	43.7
Lincomycin	7.88
Amoxycillin	56.3
Trimethoprim	13.0
Nalidixic acid	16.5
Average	26.2

A plot of total antibiotic concentrations measured versus sampling locations is shown in Figure 14. Error bars are shown for the sampling locations with multiple samples from the same location. Number of antibiotics detected in each sampling location is shown in parenthesis above the bar. The highest antibiotic concentrations were found in the sample

collected at the Hollywood ocean outfall (HWO1). The other sampling station with high antibiotic concentrations is Miami River and this result shows that the Miami River is clearly under the influence of anthropogenic discharges.
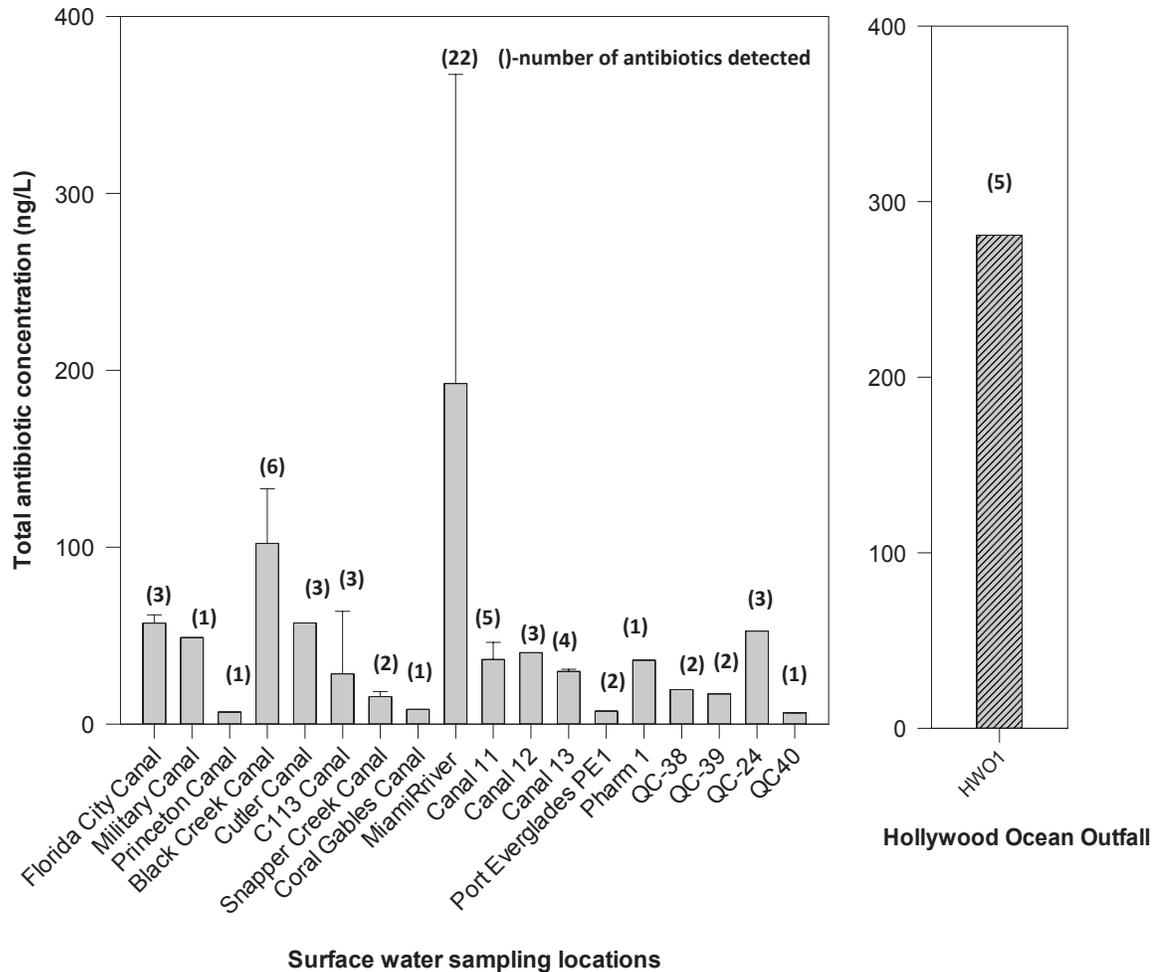
Figure 14. Distribution of antibiotics in major canals from Miami-Dade and Broward counties presented as the total concentration detected. ()- indicate the number of antibiotics detected in the selected sampling location

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To our knowledge this is the first report on the study of antibiotics in an open water body in South Florida. Though there is no direct evident release of wastewater effluents in the area, along with high human activity, the existence of drainage overflows or leaching from landfills during storm events could be a potential source for these observations (Gardinali and Zhao, 2002). The Miami River drains in to Biscayne Bay, which is not only a natural habitat for many estuarine organisms and algal communities but also an important recreational area for the city of Miami. Presence of residual antibiotics in these water bodies is a concern due to the potential risk for proliferation of antibiotic resistant organisms (Daughton and Ternes, 1999). Moderately high antibiotic concentrations found in Black Creek Canal could be due to its close proximity to both landfill and a sewage treatment plant. Canals with no traces of antibiotics include North Canal drive, Mowry Canal, QC-37, QC-10, QC-15, OS1, OS2, OS3, Pharm 2, Pharm 3, Pharm 4, HWO1 and HWO2.

The distribution of antibiotics in the sampled canals is shown as box plots in Figure 15. The blue and black lines in the box plot indicate mean and median of the sample population, respectively. Antibiotics detected at relatively higher concentrations (based on average) include tylosin, sulfadiazine, erythromycin and meclocycline and the antibiotics detected most frequently (19-25%) are erythromycin, sulfadiazine, trimethoprim and sulfamethoxazole. Erythromycin, trimethoprim and sulfamethoxazole were among those detected in all reclaimed waters analyzed Detailed information on the type and concentration of antibiotic detected in each sampling location are shown in Table 14.
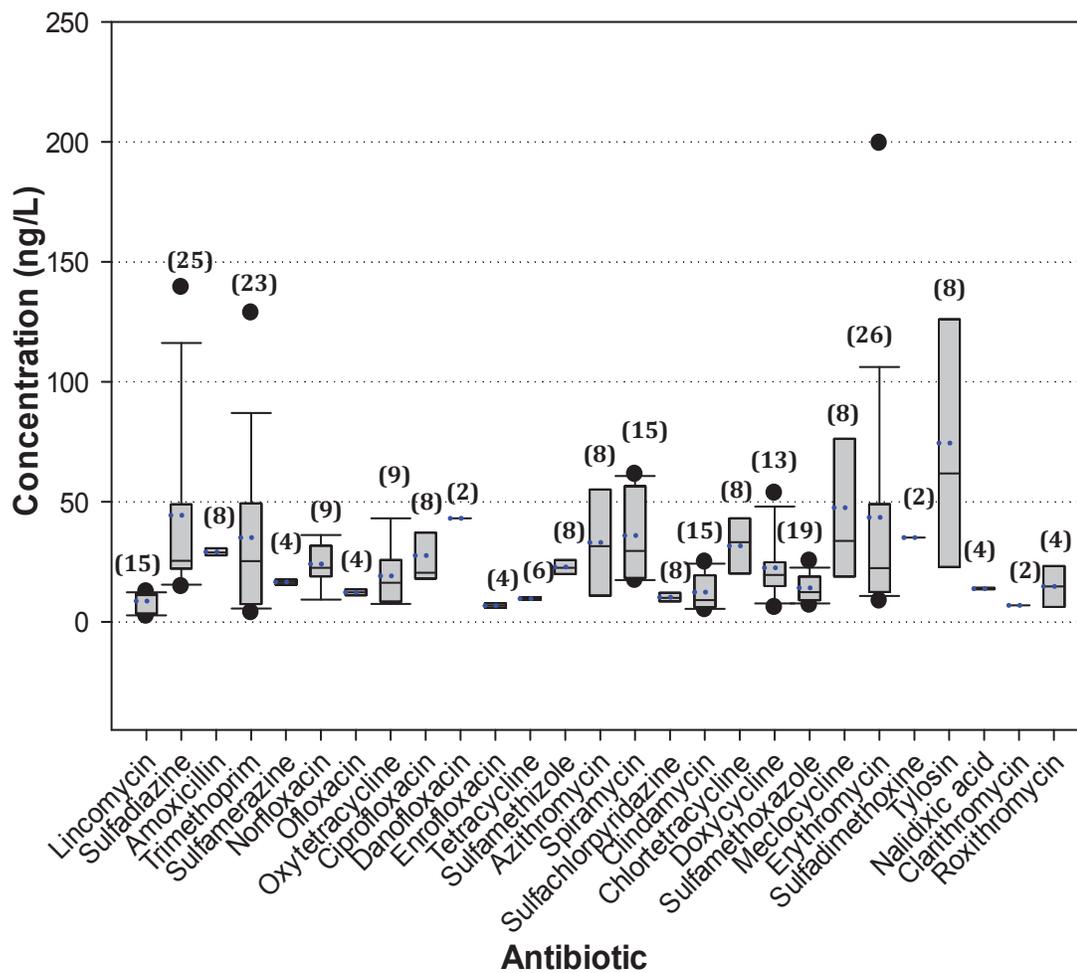
Figure 15. Distribution of antibiotics in surface waters; values in parenthesis indicate percent frequency of detection

Table 14. Distribution of antibiotics in the selected sample locations in ng/L

Antibiotic	Florida City Canal	Military Canal	Princeton Canal	Black Creek Canal	Cutler Canal	C113 Canal	Snapper Creek Canal	Coral Gables Canal	Miami River Canal	Canal 11	Canal 12	Canal 13	QC-38	QC-39	QC-24	QC-40	Port Everglades PE1	Pharm 1	HW01
Lincomycin	---	---	---	---	---	3.30	---	---	10.0	---	---	---	11.1	---	---	---	3.50	---	---
Sulfadiazine	---	---	---	24.2	14.6	42.3	---	---	56.0	---	24.0	---	---	---	---	---	---	---	---
Amoxicillin	---	---	---	---	---	---	---	---	29.2	---	---	---	---	---	---	---	---	---	---
Trimethoprim	---	---	---	---	---	---	---	---	49.6	---	---	---	8.41	8.39	11.3	6.34	3.79	---	129
Sulfamerazine	---	---	---	---	---	---	17.6	---	---	15.4	---	---	---	---	---	---	---	---	---
Norfloxacin	26.4	---	---	22.2	---	---	---	---	---	9.22	---	---	---	---	---	---	---	36.1	---
Ofloxacin	---	---	---	---	---	---	---	---	---	11.0	---	---	---	---	---	---	---	---	13.5
Oxytetracycline	---	---	---	---	---	---	---	---	26.4	---	---	---	---	8.72	---	---	---	---	---
Ciprofloxacin	---	---	---	34.9	22.7	---	---	---	17.8	---	---	---	---	---	---	---	---	---	66.9
Danofloxacin	---	---	---	---	---	---	---	---	43.1	---	---	---	---	---	---	---	---	---	---
Enrofloxacin	---	---	---	---	---	---	---	---	7.7	---	---	---	---	---	---	---	---	---	---
Tetracycline	---	---	---	---	---	---	---	---	43.1	9.43	---	9.76	---	---	---	---	---	---	---
Sulfamethizole	---	---	---	---	---	---	---	---	17.8	---	---	18.4	---	---	---	---	---	---	---
Azithromycin	---	48.9	---	---	---	---	---	---	43.1	---	7.69	---	---	---	---	---	---	---	61.2
Spiramycin	---	---	---	---	---	---	---	---	35.9	---		---	---	---	---	---	---	---	---
Sulfachlorpyridazine	---	---	---	11.3	---	---	---	---	10.6	---	8.65	---	---	---	---	---	---	---	---
Clindamycin	---	---	---	---	---	---	---	---	12.4	---	---	---	---	---	---	---	---	---	---
Chlortetracycline	---	---	---	---	---	---	---	---	32.9	27.9	---	---	---	---	---	---	---	---	---
Doxycycline	---	---	---	---	---	---	---	---	23.7	---	---	14.7	---	---	---	---	---	---	---
Sulfamethoxazole	18.5	---	---	19.7	19.8	---	---	8.35	9.81	---	---	6.89	---	---	---	---	---	---	10.4
Meclocycline	---	---	---	---	---	---	---	---	47.6	---	---	---	---	---	---	---	---	---	---
Erythromycin	12.2	---	---	8.67	---	11.0	---	---	56.5	---	---	---	---	---	---	---	---	---	---
Sulfadimethoxine	---	---	---	---	---	---	---	---	---	---	---	---	---	---	35.2	---	---	---	---
Tylosin	---	---	---	---	---	---	---	---	74.4	---	---	---	---	---	---	---	---	---	141
Nalidixic acid	---	---	---	---	---	---	13.5	---	14.1	---	---	---	---	---	---	---	---	---	---
Clarithromycin	---	---	6.86	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Roxithromycin	---	---	---	---	---	---	---	---	23.2	---	---	---	---	---	6.21	---	---	---	---

4.5. Conclusions

Major canals from Miami-Dade & Broward counties and ocean outfall were analyzed for the presence of antibiotics. Results showed that highest concentrations of antibiotics were detected in ocean outfall followed by Miami River canal and Black Creek canal. High concentrations in Miami River canal might be due to drainage overflows or leaching from landfills whereas detections in Black Creek canal may be due to the influence of a nearby sewage treatment plant and a landfill. The highest frequency of detection for any antibiotic detected in surface waters is 33% (for erythromycin) and the average concentration of antibiotics detected was lower compared to the same in reclaimed waters. Detection of 22 antibiotics (out of 31) in Miami River waters showing that it could be more contaminated in comparison to other canals tested in this study. Detection of antibiotics in surface water indicates the possible contamination of some of the South Florida surface waters with wastewater intrusion.

CHAPTER 5

Drinking water and Ground water analysis

Results from drinking waters (Panditi, V., Batchu, S., Gardinali, P., 2013. Analytical and

Bioanalytical Chemistry, 405 (18), 5953-5964).

5.1. **Introduction**

Antibiotics have been detected in waste waters and surface waters worldwide at concentrations reaching up to few µg/L (Nakata et al., 2005; Yang et al., 2005; Karthikeyan and Meyer, 2006; Segura et al., 2007b; Gulkowska et al., 2008). Previous studies have shown that antibiotics may possibly migrate into ground waters from the contaminated surface waters (Meyer et al., 1999; Heberer et al., 2002). Usage of the contaminated ground waters as sources of drinking water rises concern over the potential for these antibiotics to occur in finished drinking waters and thus, to affect human health through chronic exposure at low levels. In a drinking water treatment plant, ground water will be subjected to coagulation, sedimentation, filtration and chlorination to make it potable. The removal efficiency of antibiotics in the drinking water treatment plant varies both among chemicals and between different processes employed in the treatment plants (EPA, 2013).

To date, limited research had been published on the existence of antibiotics in drinking waters in different countries. In a study conducted by USGS and Centers for Disease Control and Prevention, Stackelberg et al., analyzed raw, settled, filtered and finished drinking water samples from a drinking water treatment plant (DWTP), which is located in a heavily populated and highly urbanized drainage basin for the presence of 106 organic waste-water related contaminants, including 25 antibiotics. Although no traces of antibiotics were found in the finished drinking waters, erythromycin (measured as anhydroerythromycin) and sulfamethoxazole were detected in more than 10% of stream and raw water supplies of the drinking water treatment plant at concentrations reaching as

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high as 1 µg/L (Stackelberg et al., 2004) Only erythromycin was detected (1-2 ng/L) in treated drinking waters from a DWTP located in the Llobregat River (NE Spain) while other antibiotics showed removal rates greater than 99% and thus were not detected (Boleda et al., 2013). In a more recent survey conducted by the Ontario Ministry of the Environment (MOE) in 2011, Kleywegt et al., found tylosin (max: 31 ng/L), tetracycline (max: 15 ng/L), erythromycin (max: 155 ng/L), enrofloxacin (max: 13 ng/L), lincomycin (max: 1413 ng/L), roxithromycin (max: 41 ng/L), sulfamethoxazole (max: 2 ng/L) and trimethoprim (max: 15 ng/L) in majority of drinking water systems collected over a 16 month period, indicating that they survive the conventional water treatment processes and persist in potable-water supplies. Until now, this is the first study reporting the occurrence of antibiotics at such high concentrations in finished drinking waters (Kleywegt et al., 2011).

All these studies highlight the importance of monitoring source waters that could be prone to contamination such as in South Florida. The Biscayne Aquifer often referred to as groundwater or the water table is located just below the surface of the land in South Florida and provides virtually all of the water that is used by South Florida residents, visitors and businesses. Because this drinking water supply is so close to the surface (barely a few feet down in most places), it is especially prone to contamination (MiamiDade) and hence the quality of ground water is of foremost concern in Florida (Barlett, 2012). There is no research on the occurrence of antibiotics in South Florida drinking waters, which is crucial for public health and safety and thus is the objective of this part of the study.

5.2. **Experimental**

5.2.1. **Sample collection and preparation**

Drinking water samples (n=54) were collected from homes located in the Miami-Dade County area. Drinking water protocols were standardized asking the sampler to run the water for at least 5 minutes and rinse the container at least three times with the tap water. After collection, all samples were immediately transported to the laboratory and sequentially filtered through the 0.45 μm glass fiber filter and finally through 0.2 μm membrane filter to minimize any potential for biodegradation. Filtered samples were stored in the dark at -18°C until time of analysis.

Drinking water samples were prepared by transferring 10.5 mL of the sample to a 11-mL vial containing 50μL of 20% optima LC/MS grade formic acid and 50 μL of surrogate mixture in methanol (1 μg/mL of norfloxacin d5, 0.2 μg/mL of sulfamethoxazole d5, 2 μg/mL of sulfadiazine d4, 1 μg/mL of erythromycin ^{13}C d3, 1μg /mL of spiramycin d3 and 2 μg/mL of demeclocycline), the final solution was shaken on a vertex and subsequently analyzed by LC-MS/MS.

Ground water samples (n=8) were collected from Miami-Dade and Broward County areas using peristaltic pump. The tubing was rinsed twice before each sample collection. Field blank samples were also collected in the same sampling locations for each sample. All samples were processed similar to the drinking waters. Second set of drinking waters (n=5) were collected along with ground water samples. During collection, both sampling

container and filed blank container were opened at the same time and filled with sample and deionized water, respectively. The headspace was kept minimal in all bottles.

5.2.2. Optimization of analytical methodology

Previously, antibiotic residues in drinking waters had been extracted using offline SPE (Watkinson et al., 2009) or online SPE (Garcia-Galan et al., 2011) and detected based on single quadrupole (Kolpin et al., 2002) or ion trap or triple quadrupole (Ye et al., 2007; Watkinson et al., 2009; Boleda et al., 2013) or time of flight mass spectrometry (Garcia-Galan et al., 2011). Most of these published methods focus on the detection of pharmaceuticals including antibiotics, whereas the present method was optimized for the detection of antibiotics only in various environmental matrices, including drinking waters. In the major Miami-Dade DWTPs, the conventional treatment process consists of screening, grit removal, primary sedimentation, activated sludge treatment by oxygenation and chlorination (Wang, 2012). Thus, finished drinking water samples contain residual amounts of chlorine from disinfection, which might either alter the stability of antibiotics or interfere with the analysis. Ascorbic acid was efficiently used to remove traces of free chlorine and chloramine from drinking water samples in an offline preconcentration step (Ye et al., 2007). However, the effect of ascorbic acid under the present experimental settings (samples acidified with 20% formic acid and online preconcentration) could be significantly different and hence was examined in the present study.

To check the effect of ascorbic acid on the recovery of selected analytes, the samples were filtered through 0.45μm glass fiber filter, spiked with 30 µL of ascorbic acid (2 g/L), followed by the addition of formic acid and internal standard solution. Compared to the samples with no ascorbic acid, the spiked samples showed less mean recoveries for all antibiotics except lincomycin, clindamycin and amoxicillin (results were not shown). Hence, the drinking water samples were analyzed within seven days of collection without the addition of ascorbic acid.

5.3. **Results and Discussion**

5.3.1. **Occurrence of antibiotics in drinking waters**

Erythromycin was detected in 82% of drinking water samples suggesting the ubiquitous nature of this compound in the environment, and therefore the need of continuous monitoring and stringent guidelines for surface/drinking water. No antibiotics were detected in ground waters. Even though drinking water standards and health advisories were not established for antibiotics (Stackelberg et al., 2004), erythromycin was recently added to USEPA contaminant candidate list 3 for drinking waters (EPA, 2009). Its concentration ranged from not detected (n.d.) to 66 ng/L in the samples measured during the present study. Both norfloxacin and ofloxacin were detected only once at 17 and 37 ng/L. These results indicate that ground water in South Florida could be under the influence of anthropogenic waste water. Spatial distribution of total concentrations (erythromycin, norfloxacin, ofloxacin) in various sampling locations were shown in

Figure 16 with the quantitative variation in total concentrations shown with dots of different colors starting from white (<MDL), green (MDL-10 ng/L), blue (10-20 ng/L), yellow (20-30 ng/L), organe (30-40 ng/L) and red (60-80 ng/L). The figure also shows the locations of two major drinking water treatment plants, Hialeah and John E. Preston plant and The Alexander Orr, Jr. plant. The Hialeah and John E. Preston plant serves most Miami-Dade residents living between the Miami-Dade-Broward County line and SW 8th Street. The Alexander Orr, Jr. water treatment plant, serves most County residents living between SW 8th Street and SW 264th Street. The other drinking water treatment plant not shown in the graph is South Dade Water Supply System, which is comprised of five smaller water treatment plants that serve residents south of SW 264th Street in the unincorporated areas of the County (MiamiDade). The pink lines in the figure define the limits for the areas served by these three drinking water treatment plants.

5.3.2. Statistical analysis

The total antibiotic concentration (for samples with total concentrations greater than MDL only) versus different drinking water treatment plants under study were compared in Figure 17 and the sample size in each plant was shown in paranthesis above the error bar. Note that only three samples were collected from South Dade water supply system. The figure shows that the average concentration of samples from Hialeah and John E.Preston plant and The Alexander Orr, Jr. plant were similar and was lower than the average of South Dade Water Supply System's samples.

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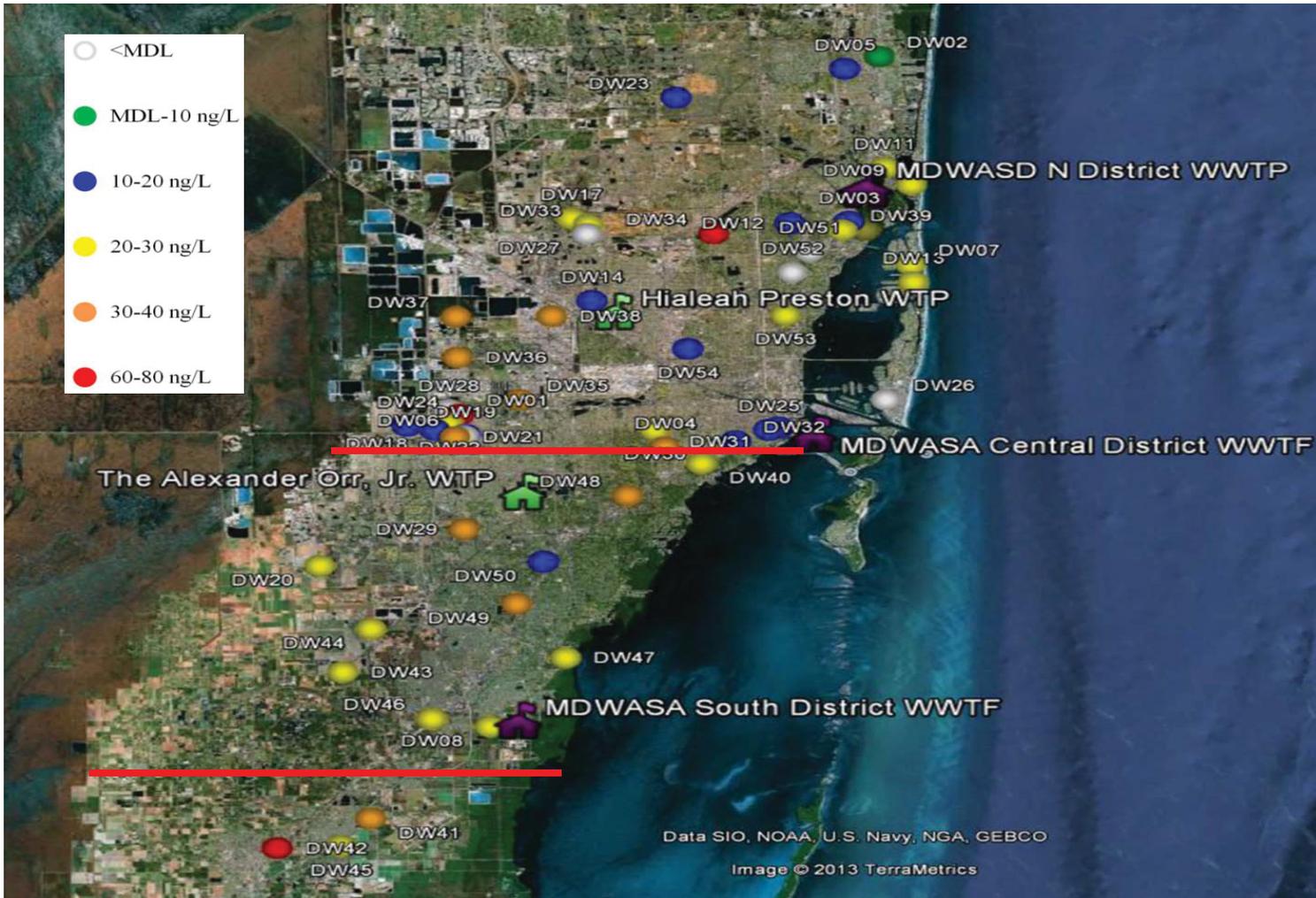
Figure 16. Distribution of total concentration of antibiotics in Miami-Dade County drinking waters

In order to verify if the difference observed in the means among the treatment plants is significantly different, ANOVA (analysis of variance) was performed using Sigmaplot v12 and results showed that the difference in the mean values among the treatment groups is not statistically significant (P=0.118, Table 15).

Antibiotic concentration versus drinking water treatment plants in Miami-Dade
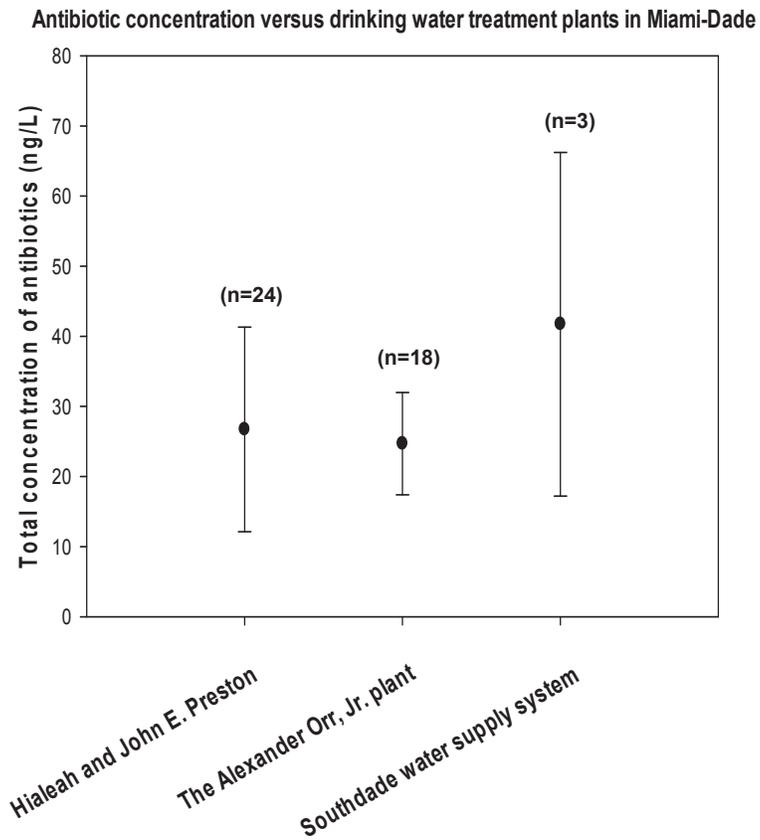
Figure 17. Comparison of samples from the three drinking water treatment plants

Table 15. Results of an ANOVA test using the samples from three major drinking water treatment plants

Group Name	N	Missing	Mean	Std Dev	Standard Error
Hialeah and John E. Preston Plant	24	0	26.727	14.591	2.978
The Alexander Orr, Jr. Plant	18	0	24.684	7.296	1.72
South Dade water supply system	3	0	41.731	24.503	14.147
Source of Variation	DF	SS	MS	F	**P**
Between Groups	2	748.981	374.491	2.246	**0.118**
Residual	42	7002.369	166.723		
Total	44	7751.35			

A plot of the total antibiotic concentration measured at a given sampling site and the location's distance from the drinking water treatment plant for both Hialeah and John E.Preston plant and The Alexander Orr, Jr. plant were shown in Figures 18-19. The plots indicate that there is no correlation between the two factors considered for plot. Assuming that all the three treatment plants were equally efficient in removing the antibiotics from source waters, random distribution of concentrations among the samples may be explained based on the variation in the source water composition on the day of collection as well as the residence time of the finished drinking water in the treatment plant before its distribution (not monitored in the present study). The major source of water for the all treatment plants is Biscayne Aquifer which lies very close to the surface and therefore it is easily prone to contamination by anthropogenic intrusions (MiamiDade). If a treatment plant withdraws water from a well that is under the influence of contamination sources such as waste water treatment plant or agricultural fields, the

finished drinking water from the drinking water treatment plant might show higher concentration compared to the finished waters of other drinking water treatment plants. Absence of significant difference between the concentrations measured among the samples representing different plants might indicate that the source of contamination is random rather than be specific.
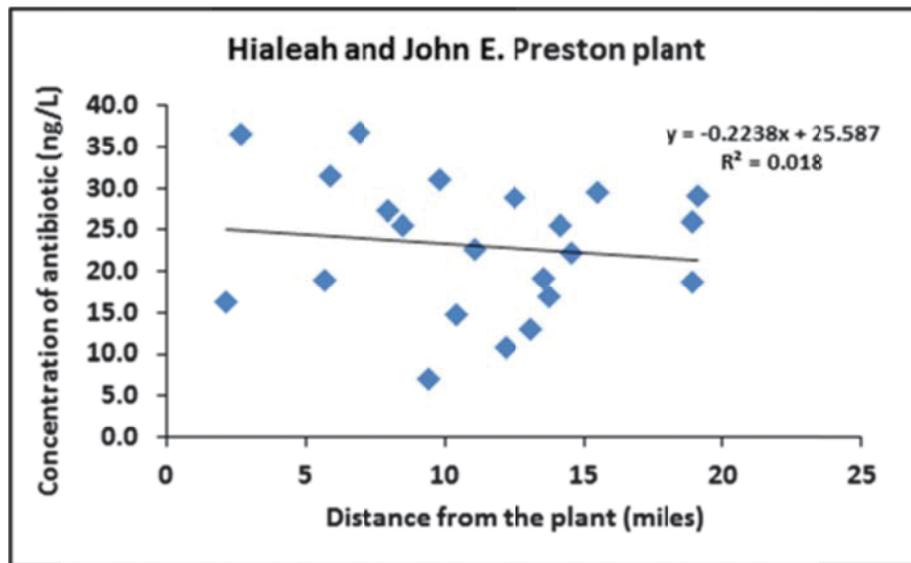
Figure 18. Distribution of samples collected from Hialeah and John E. Preston drinking water treatment plant as a function of sampling location distance from treatment plant
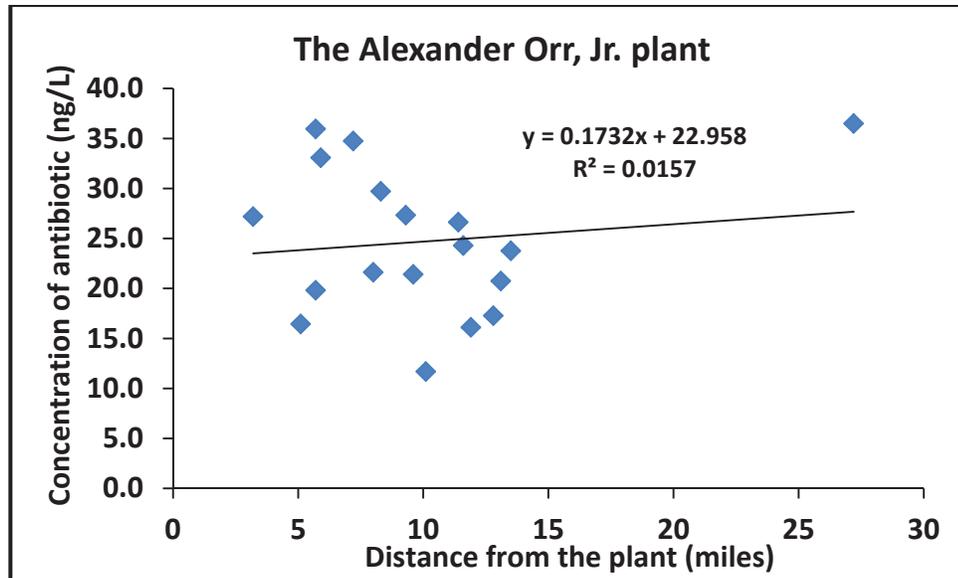
Figure 19. Distribution of samples collected from The Alexander Orr, Jr. drinking water treatment plant as a function of sampling location distance from treatment plant

Ye et al. (Ye et al., 2007) detected erythromycin, roxithromycin, tylosin and sulfamethoxazole in drinking water samples in the concentration range of 1.4 - 4.9 ng/L. Lopez-Serna et al. (Lopez-Serna et al., 2010) also detected macrolides azithromycin, clarithromycin, spiramycin (3.6 - 21 ng/L), sulfonamide sulfamethazine (4.1 ng/L) and fluoroquinolones enoxacin, ofloxacin, ciprofloxacin, enrofloxacin, norfloxacin (13 – 33 ng/L) in the effluents of a drinking water treatment plant. It must be noted that the type of antibiotics detected in drinking waters vary greatly from one study to another and this may be due to the fact that both sources and treatment technologies will greatly differ from one country to another.

There were no antibiotics detected in the second set of drinking water (n=5) and ground water (n=8) samples collected from Miami Dade county and Broward county, the sampling locations were shown in Figure 20.
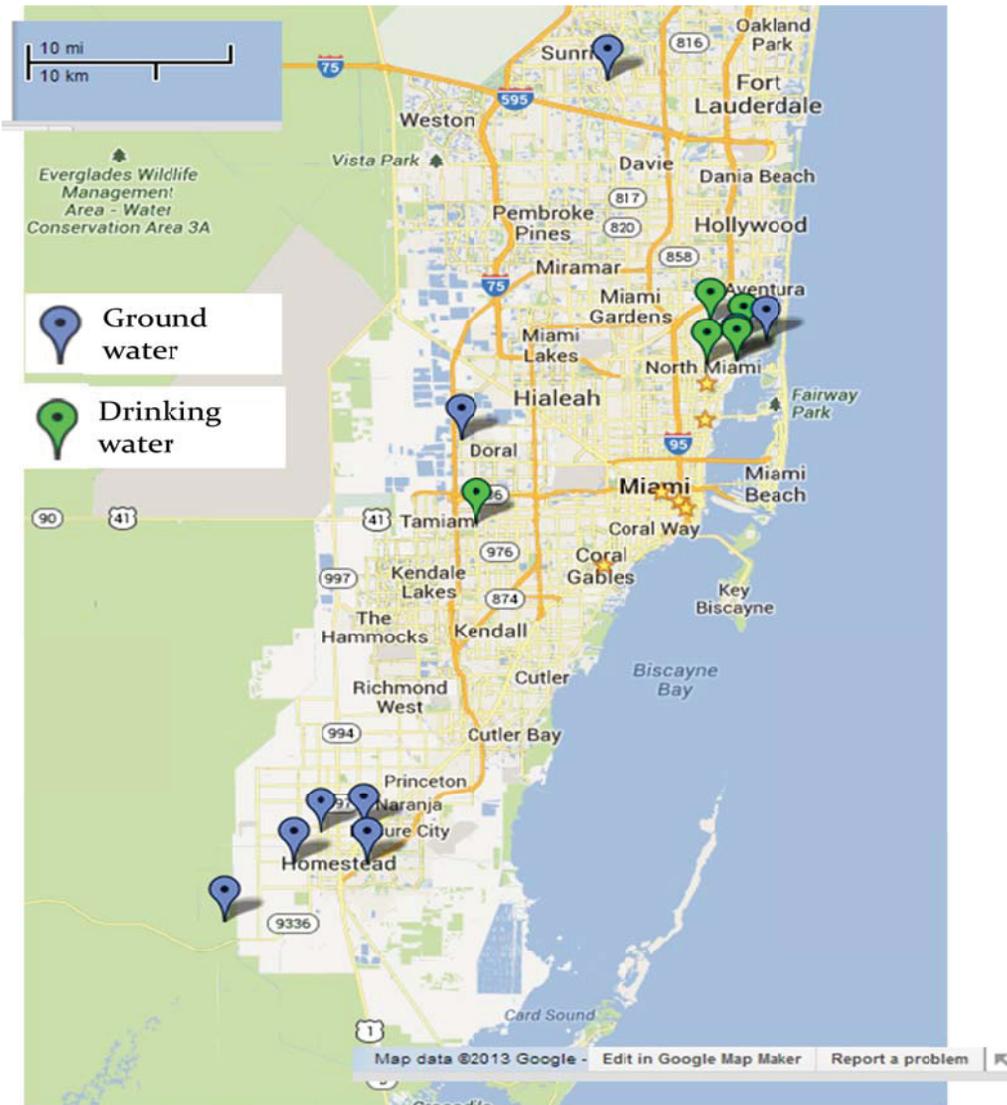
Figure 20. Drinking water and Ground water sampling locations

5.4. **Conclusions**

The Biscayne Aquifer is the primary source of drinking water for Miami-Dade, Broward and southeastern Palm Beach County. It is an unconfined aquifer and lies very close to the surface. Therefore, it interacts directly with natural and man-made bodies of surface water such as streams, canals and reservoirs and thus susceptible to anthropogenic discharges. Initial results from this study shows that both drinking and surface waters are influenced by anthropogenic antibiotic inputs. However no traces of antibiotics were detected when samples (n=5) were collected. Results from this set of samples were not conclusive enough due to limited number of samples compared to first set of samples (n=56). Since detection of antibiotic residues in drinking water raises the public concern over drinking water quality, it is recommended to include multiple sampling locations to continuously monitor the quality of both ground and drinking waters.

CHAPTER 6

Identification of antibiotic metabolites and their transformation products in reclaimed water using high resolution benchtop Orbitrap mass spectrometry

6.1. Introduction

Occurrence and widespread distribution of antibiotic residues in the aquatic environment, has drawn much attention worldwide both in research and public community. Their environmental occurrence is of particular concern due to the potential spread and induction of bacterial resistance (Diaz-Cruz and Barcelo, 2006) and their ability to potentially alter ecosystem functioning (Kummerer, 2009). Hence antibiotics have been included among the group of emerging environmental contaminants of increasing concern (Kolpin et al., 2002). Antibiotics are released into aquatic environment largely through discharge of wastewater treatment plant effluents (WWTPs). After consumption by humans and animals, antibiotics will go through series of metabolic processes and which are often incomplete, so as a result bio-active unchanged antibiotics and their metabolites are excreted and eventually reach WWTPs (McArdell et al., 2003).

Much attention has been paid to unchanged antibiotics compounds while assessing their environmental occurrence and lesser importance to transformation products (Celiz et al., 2009). Here the term transformation product is used in broader sense to include, metabolites formed by biochemical transformation of parent drug in organisms (Gobel et al., 2004) and sewage treatment processes such as chemical, photo and microbial biological oxidation (Homem and Santos, 2011; Bonvin et al., 2012). Drugs or chemicals that enter the human body are cleared mainly by the solo or combination of three mechanisms: metabolism, renal and bile excretion. Metabolism, a primary route of detoxification for most of the drugs, involves series of enzymatic biotransformation reactions to cause chemical alteration or degradation (Liska, 1998). In general the

metabolic reactions can be classified into two phases, phase I and phase II (Murphy, 2001). Phase I metabolic reactions include, oxidation, reduction and hydrolysis, where the parent drug will be transformed into a more polar metabolite by adding or revealing functions groups such as $-OH$, $-NH_2$, $-SH$ and $-COOH$, etc. The liver is the major metabolic site and first intestinal organ that a drug molecule passes through after absorption from the gut (Liska, 1998). The mechanism involves a variety of enzymes such as cytochrome P450 (CYP1, CYP2 and CYP3), that catalyze the oxidation and hydroxylation and NADPH-CYP reductase introducing reactive and polar groups into their substrates (williams, 1947; Liska, 1998; Danielson, 2002; Gu et al., 2003). Phase II metabolism is a conjugative reaction, where the molecule is conjugated with polar groups such as glucuronic acid, glutathione (GSH), sulfate, or glycine catalyzed by group of enzymes called transferases (williams, 1947; Liska, 1998; Holcapek et al., 2008), hence finally converted to more water soluble, hydrophilic species thereby being easily excreted. The metabolite can also be active with the desired therapeutic effect instead of the parent drug when is administered as pro-drug (Guengerich, 2001) one such example is enrofloxacin, which metabolizes to the bioactive ciprofloxacin. The activation of a drug by metabolism can also result in a toxic product (Guengerich, 2011). Hence, it is important to assess the risk of metabolites as well.

Although different analytical instrumentation have been employed in the determination of antibiotics in environmental matrices (Ternes, 2001; Petrovic et al., 2005; Richardson, 2006; Hernandez et al., 2007), liquid chromatography coupled to mass spectrometry, especially tandem mass spectrometry has made impressive progress and is by far the most widely used within this field. With improved selectivity and high sensitivity offered

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by tandem mass spectrometry, the unequivocal detection of antibiotics in complex environmental matrices even at parts-per-trillion level made practically possible.

However many studies considered the detection and quantification of parent drugs, with very little attention given to the potential contributions that metabolites may have. Scarcity of available environmental data on the metabolites excreted into the natural ecosystem is may mainly be attributed to their relatively high polarity and the lack of reference substances, which resulting in difficulty to analyze them (Diaz-Cruz and Barcelo, 2006). Hence, current analysis of targeted metabolites in environmental matrices using tandem mass spectrometry is limited to compounds whose metabolites are well known and the standards are available to confirm their identity (Gobel et al., 2004; Celiz et al., 2009), for example anhydroerythromycin, which is routinely detected in environmental analysis studies (Wang and Gardinali, 2012; Panditi et al., 2013). However, the advent of highly sensitive and powerful analytical instrumentation and increasing number of studies reporting the environmental presence of metabolites is indicating the urge to include the bio-active metabolites as well in routine antibiotic analysis. In wastewater treatment process through physical, chemical, biological means or under certain environmental conditions, excreted metabolites can also be transformed back to the parent compound (Bonvin et al., 2012) and potentially more dangerous than the parent compound (Dantas et al., 2008; Homem and Santos, 2011), one such example is back transformation of acetylsulfamethazine to the active parent compound during the storage of manure (Berger et al., 1986). Erythromycin, the most commonly detected antibiotic in environmental waters is always detected as anhydroerythromycin, a degradation product formed with the loss of one water molecule (Hirsch et al., 1999).

Very little is known about degradation products and epimers of tetracyclines in the aquatic environment. These products are mainly formed through hydrolysis and photolysis reactions yielding relatively more water soluble anhydro-, epi-, and iso- forms of tetracycline (Halling-Sorensen et al., 2002; Diaz-Cruz and Barcelo, 2006). Therefore it is important to include bio-active metabolites as well as transformation products in routine antibiotic analysis to get an overall idea on occurrence, fate and distribution of antibiotics in the environment.

In recent years, the advent of hybrid high resolution mass spectrometers, such as quadrupole time of flight (QTOF), triple quadrupole time of flight (QqTOF), Fourier transform ion cyclotron resonance (FTICR), Orbitrap, and linear ion trap-Orbitrap (LTQ-Orbitrap), with elevated resolution (>40,000) and high mass accuracy (<5ppm) capabilities, has made the conditions ideal for screening and positive confirmation of unknown metabolites and transformation products both in metabolic profiling and environmental studies (Stolker et al., 2004; Gros et al., 2009; Ferrer and Thurman, 2012; Hernandez et al., 2012; Meyer and Maurer, 2012; Qian et al., 2012). Limitations for the use of TOF instruments are less linear dynamic range ($<10^3$) and the need for internal calibration (for each pre and post sample injection) to maintain high mass accuracy (Hernandez et al., 2012). In contrast Orbitrap instruments offer relatively higher sensitivity, and better linear dynamic range. Moreover, external calibration is used to obtain high mass accuracy resulting in simplified operational protocol (Lim et al., 2007).

The main objective of this study is to identify the antibiotic metabolites and transformation products in reclaimed water using a Q Exactive Orbitrap mass spectrometer based on accurate mass measurements in combination with characteristic

MS/MS fragmentation ions in data dependent scan mode and to quantify their relative abundance with respect to the parent antibiotics.

6.2. Experimental

6.2.1. Chemicals and Standards

All the reference standards for parent drug molecules were purchased from Sigma–Aldrich (Oakville, ON and Milwaukee, WI) and isotopically labeled antibiotics (Sulfamethoxazole-d4, spiramycin-d3, sulfadiazine-d4, erythromycin-^{13}C-d3) and demeclocycline were purchased from Toronto Research Chemicals (Toronoto, Canada) CDN Isotope Laboratories (Quebec, Canada) and norfloxacin-d5 from CDN Isotope Laboratories (Quebec, Canada). All isotopically labeled standards presented purity higher than 95% (isotopic purity >99%). Pierce LTQ Velos ESI positive ion and negative ion calibration solutions for Q Exactive Orbitrap mass spectrometer calibration were procured from Thermo Scientific (Rockford, IL). Optima LC/MS grade formic acid, methanol, acetonitrile and water were purchased from Fisher Chemical (Fairlawn, New Jersey, USA). The 0.1% formic acid solution in water was prepared daily before the analysis.

6.2.2. Sample collection, extraction and preparation

Reclaimed water (500 mL) was collected in clear PET plastic bottles (polyethylene terephthalate) directly from reclaimed water sprinklers at Florida International University Biscayne Bay Campus (North Miami, FL). Bottles were rinsed twice with sampling water before collection. Water samples were filtered and stored in dark at below -18°C until the extraction. Sample collection, filtration and storage details were described in detail in Chapter 3.

Samples were extracted using offline solid phase extraction (SPE) procedures for multi residue antibiotic analysis. Two different SPE cartridges with distinct sorbent properties were coupled in tandem to ensure retention of all unknown compounds (Diaz-Cruz and Barcelo, 2006; Seifrtova et al., 2009). In this study an Oasis HLB (3 cc/60 mg, Waters Corp., Milford, MA), was placed on top of a Sep-Pak C18 Plus cartridge (900 mg, Waters Corp., Milford, MA) and conditioned with 6 mL of methanol followed by 6 mL of deionized water. SPE was performed on 12-port vacuum maniforld. Each reclaimed water sample (350 mL) was passed through the cartridges, dried and finally eluted with 4 mL of methanol. The eluents were dried under gentle stream of nitrogen, spiked with the appropriate surrogates and reconstituted to 250 uL with LC─MS grade water.

6.2.3. Liquid Chromatography and High Resolution Mass Spectrometry

Liquid chromatography was performed using a quaternary Accela pump equipped with a Thermo PAL CTC autosampler. Chromatographic separation of analytes was carried out

using a Hypersil Gold C_{18} column (50mm×2.1mm, 1.9 µm) from Thermo Scientific (Bellefonte, PA). Analytes were separated through the column using a mobile phase consisting of 0.1 % formic acid in water (v/v) and pure acetonitle programmed in gradiant cycle (acetonitrile % : 0 min 10%, 2.5 min 10%, 8 min 50%, 11 min 95%, 14 min 95% 15 min 10%, 17 min 10%). The injection volume and mobile phase flow rate were 10 µL and 220 µL respectively.

High resolution mass spectrometric analysis (HRMS) was performed on a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped Ion Max API heated electrospray ionization source (HESI-II) operated in positive ionization mode. Ionization source parameters were set as follows: Sheath gas 35, Auxiliary gas 35, and sweep gas 5 arbitrary units, similarly spray voltage 4 kV, both capillary temperature and vaporization temperature at 300 ºC. The HRMS data was acquired in data-dependent cycle, first in full scan mode, scan range from m/z 75 – m/z 1000 at a resolving power of 70,000 and then three corresponding data-dependent MS/MS scans at resolving power of 35,000. The most abundant precursor ions that surpass the preset threshold of $1*e^{\wedge}6$, triggered data-dependent scanning and were subsequently injected to the C-trap for fragmentation.

The isolation window for MS/MS data-dependent scanning was set at 1 m/z. Normalized collision energy was set to 35 with stepped increment of 25%. High purity nitrogen was used as source gas and argon as collision gas for high pressure induced collision dissociation. Data acquisition and instrumental control was performed using Thermo Scientific Xcalibur software.

6.2.4. Data processing and Interpretation

MetWorks 1.3 (Thermo Scientific, San Jose, CA) software was used for the calculation of exact masses from elemental composition and for tentative identification of potential phase I and phase II metabolites from extracted ion chromatograms (XIC) of the full scan data at high mass accuracy (mass tolerance, 5 ppm). Acquired HRMS data-dependent spectra of the positively identified targeted and unknown compounds were further investigated using Mass Frontier 7.0 software (MF) (Thermo Scientific). The theoretical spectra generated from the assigned chemical structure by the fragment ion search (FISh) feature in MF were compared with those of observed experimental spectra for spectral matching. The similarities between observed and theoretical spectra were considered in assigning molecular structures for the fragments and for positive assessment of metabolite identity.

The instrumental detection of accurate masses was studied by injecting 10 μL of an antibiotic standards mixture at the beginning of analysis and the data is shown in Table 16. The mass accuracy of observed accurate masses was below 1.5 ppm for all antibiotics, excluding spiramycin, shows that the instrument is in excellent condition and ready for analysis.

Table 16. Mass accuracy of selected antibiotics based on exact mass and observed mass measurements

Name	Elemental Composition	Exact Mass (m/z)	Observed accurate Mass (m/z)	Mass Accuracy (ppm)
Erythromycin	$C_{37}H_{67}NO_{13}$	734.4685	734.4692	0.9
Spiramycin	$C_{43}H_{74}N_2O_{14}$	843.5243	843.5217	-3.1
Azithromycin	$C_{38}H_{72}N_2O_{12}$	749.5158	749.5159	0.1
Clarithromycin	$C_{38}H_{69}NO_{13}$	748.4842	748.4846	0.6
Roxithromycin	$C_{41}H_{76}N_2O_{15}$	837.5319	837.5319	0.0
Tylosin	$C_{46}H_{77}NO_{17}$	916.5264	916.5274	1.1
Lincomycin	$C_{18}H_{34}N_2O_6S$	407.2210	407.2214	0.9
Clindamycin	$C_{18}H_{33}ClN_2O_5S$	425.1871	425.1874	0.6
Enoxacin	$C_{15}H_{17}FN_4O_3$	321.1357	321.1359	0.5
Ofloxacin	$C_{18}H_{20}FN_3O_4$	362.1511	362.1511	0.0
Norfloxacin	$C_{16}H_{18}FnN_3O_3$	320.1405	320.1406	0.3
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	332.1405	332.1405	0.0
Danofloxacin	$C_{19}H_{20}FN_3O_3$	358.1561	358.1563	0.4
Enrofloxacin	$C_{19}H_{22}FN_3O_3$	360.1718	360.1720	0.6
Sarafloxacin	$C_{20}H_{17}F_2N_3O_3$	386.1311	386.1314	0.8
Nalidixic acid	$C_{12}H_{12}N_2O_3$	233.0921	233.0924	1.4
Amoxycillin	$C_{16}H_{19}N_3O_5S + CH_3OH$	398.1380	398.1384	0.9
Tetracycline	$C_{22}H_{24}N_2O_8Na$	445.1605	445.1609	0.8
Oxytetracycline	$C_{22}H_24N_2O_9$	461.1555	461.1559	1.0
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	479.1216	479.1221	1.1
Doxycycline	$C_{22}H_{24}N_2O_8$	445.1605	445.1609	0.8
Meclocycline	$C_{22}H_{21}ClN_2O_8$	477.1059	477.1063	0.8
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	254.0594	254.0595	0.4
Trimethoprim	$C_{14}H_{18}N_4O_3$	291.1452	291.1453	0.5
Sulfadiazine	$C_{10}H_{10}N_4O_2S$	251.0597	251.0599	0.7
Sulfamethazine	$C_{12}H_{14}N_4O_2S$	279.0910	279.0912	0.6
Sulfathiazole	$C_9H_9N_3O_2S_2$	256.0209	256.0210	0.4
Sulfamerazine	$C_{11}H_{12}N_4O_2S$	265.0754	265.0756	0.9
Sulfamethizole	$C_9H_{10}N_4O_2S_2$	271.0318	271.0319	0.4
Sulfachlorpyridazine	$C_{10}H_9ClN_4O_2S$	285.0208	285.0209	0.5
Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	311.0809	311.0809	0.2

6.2.4.1. Tentative identification of potential metabolites and transformation products in reclaimed water

After inputting the elemental composition and ionization mode for the parent antibiotics into MetWorks software, XICs for the parent drug along with their metabolites were generated from original data file based on exact mass modifications. For the accurate detection of mass peaks mass tolerance was set at 5 ppm. Tentatively identified antibiotics are shown in Table 17. In general, the metabolites are more water soluble than the corresponding parent drugs, with exception of acetylated transformation products, therefore, while chromatographic separation, earlier retention time is a positive indication for tentative identification of metabolites. Knowing the chromatographic behavior is also useful for the detection of isomeric forms that can't be distinguished by mass spectrometry alone (Ferrer and Thurman, 2012). In this study tetracycline isomers have same exact mass and the potential to follow similar MS/MS fragmentation pattern. However, they show difference in chromatographic retention time.

Table 17. Antibiotics and corresponding tentatively identified metabolites

Parent molicule	Accurate Mass (m/z)	Transformation products	Accurate Mass (m/z)
Sulfachlorpyridazine	285.0208	Acetyl sulfachlorpyridazine	327.0300
Sulfamethazine	279.0910	Acetyl sulfamethazine	321.1019
sulfamethoxazole	254.0594	Acetyl sulfamethoxazole	296.0700
Doxycycline	445.1605	N-desmethyl doxycycline	431.1440
Chlortetracycline	479.1216	N-desmethylchlortetracycline	465.1050
Chlortetracycline	479.1216	didesmethylchlortetracycline	451.0903
Ciprofloxacin	332.1405	Ciprofloxacin N-oxide	348.1340
Nalidixic acid	233.0921	Nalidixic acid N-oxide	249.0860
Nalidixic acid	233.0921	Nalidixic acid glucuronate Anhydroerythromycin	409.1240
Erythromycin	734.4685	Desmethyl-	716.4587
Erythromycin	734.4685	anhydroerythromycin	702.4437
Ofloxacin	362.1511	Desmethyl ofloxacin	348.1340

After the detailed examination of XICs for all potential metabolites in reclaimed water, three transformation products / metabolites were identified, two for erythromycin (anhydroerythromycin, desmethyl-anhydroerythromycin), and one for sulfamethoxazole (acetyl-sulfamethoxazole). In two of the erythromycin transformation products, anhydroerythromycin was the frequently detected antibiotic in environmental waters, whereas the other desmethyl-anhydroerythromycin was detected for the first time.

Regarding erythromycin, an interesting observation reported in scientific literature is that, erythromycin can easily be degraded under experimental conditions ($pH<7$), to anhydroerythromycin (Diaz-Cruz and Barcelo, 2006) by losing one water molecule. Even the same was observed in present study, and discussed in earlier chapter. However, this degradation was observed in extracted spiked samples at neutral pH as well (Hirsch et al., 1999), revealing that the dehydration process takes place in the natural aquatic

environment. Therefore it is difficult to distinguish that the observed transformation was naturally happened or under experimental setup conditions. Hence for the later part of this study, anhydroerythromycin was taken granted as parent molecule and no more discussed as transformation product.

The chromatographic behavior of anhydroerythromycin and its metabolite, desmethyl-anydroerythromycin in reclaimed water shows that the desmethylated- form eluting first at the retention time, 6.14 min, and the parent anhydroerythromycin later at 6.24 min (Figure. 21). For sulfamethoxazole and its metabolite, acetyl-sulfamethoxazole, the parent molecule elutes first at 9.51 min, and the corresponding metabolite later at 9.65 min (Figure. 22), which can be explained by the hydrophobic nature of the acetylated metabolite. Similar type of behavior was also observed for other closely related antibiotics with respect to their metabolites (Gobel et al., 2004; Ferrer and Thurman, 2012; Wang, 2013). This was the first positive sign observed in the process of confirming their identity.
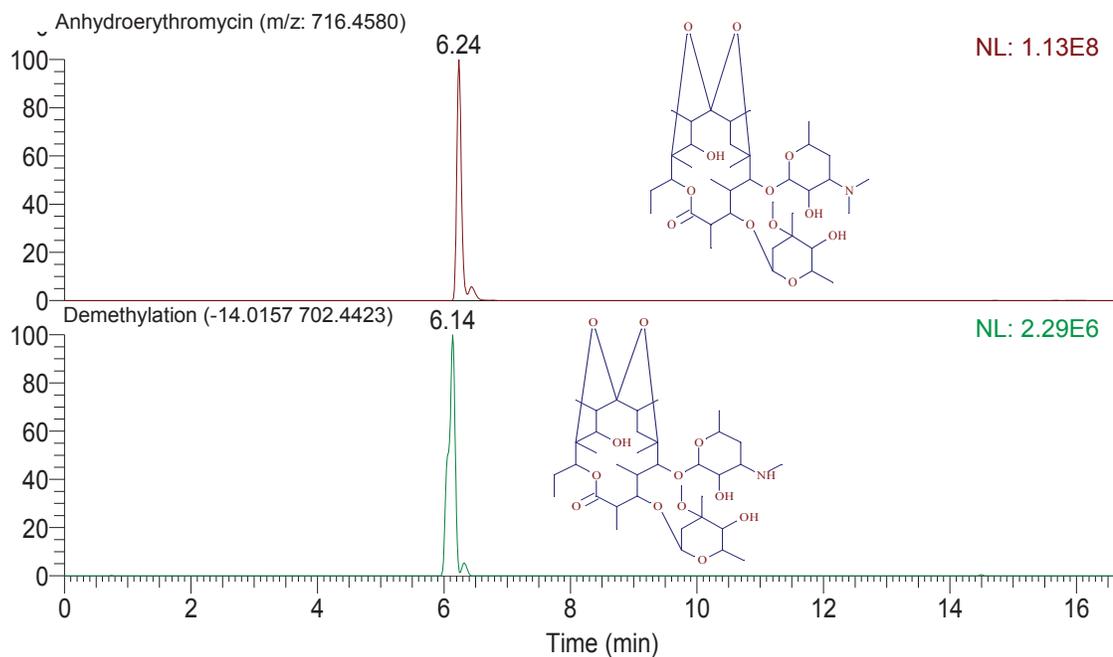
Figure 21. Extracted ion chromatograms of anhydroerythromycin and its metabolite desmethyl-anhydroerythromycin in reclaimed water (Peak settings of mass tolerance 5 ppm, and mass precision 4 decimal places)
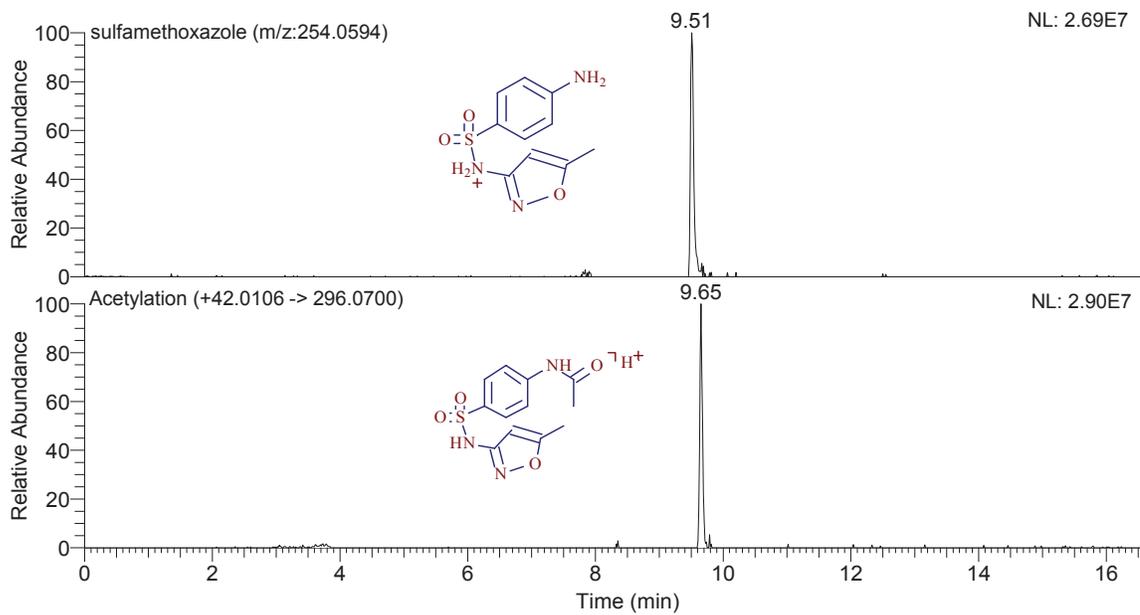
Figure 22. Extracted ion chromatograms of sulfamethoxazole and its metabolite acetyl-sulfamethoxazole in reclaimed water (Peak detection settings of mass tolerance 5 ppm, and mass precision 4 decimal places)

The high resolution mass spectrometry data revealing that the accurate mass of anhydroerythromycin was observed at m/z 716.4587, which is − 0.9 ppm from its exact mass at m/z 716.4580, even the standard spiked sample also eluted at the same retention time with the same measured accurate mass, confirming the positive identity for erythromycin in reclaimed water. At the same time, the accurate mass of sulfamethoxazole was observed at m/z 254.0595, with a mass accuracy of 0.4 ppm, from its exact mass of m/z 254.0594. The retention time was also matched the same with its standard spiked sample, confirming the positive identity for sulfamethoxazole in reclaimed water. Additional confirmation was made by comparing MS/MS spectra of reclaimed water sample with that of standard.

The accurate mass for anhydroerythromycin metabolite, desmethyl-anhydroerythromycin was measured at m/z 702.4437 with mass accuracy 2.0 ppm from its exact mass, m/z 702.4423, calculated based on the elemental composition of the metabolite. This corresponds to the loss of methyl group (14 Da) from its parent, anhydroerythromycin. Similarly the accurate mass of sulfamethoxazole metabolite, acetyl-sulfamethoxazole was measured at 296.0700, with mass accuracy of 0.0 ppm from its exact mass, m/z 296.0700, correspond to the addition of acetyl group (42 Da) to sulfamethoxazole. As the metabolite standards were not readily available while conducting this experiment, an alternative approach to confirm their identity followed was, comparing the fragmentation pattern

between the parent drug and the transformation product, knowing that they both share the same "back bone" in their molecular structures, same approach was also reported in literature (Thurman and Ferrer, 2012).

6.2.5. Identification of Desmethyl-anhydroerythromycin

The high resolution data-dependent MS/MS spectrum of m/z 716.4587, in reclaimed water at retention time 6.24 min was shown in Figure 23b, which was nearly identical with MS/MS spectrum of erythromycin standard spiked sample. The three most abundant fragment ions 158 Da, 116 Da and 98 Da were following the same relative abundance in both the spectra, clearly implying that the ion m/z 716.4587 was anhydroerythromycin. The structures of most abundant fragments were predicted by inputting parent molecule anhydroerythromycin molecular structure in to MF software, where MF generated the possible structures based on the fragmentation rules built into it. All the major fragments observed in MS/MS spectra matched with the MF predicted fragmentation ions, and were shown in red colour. All the labeled fragmentation ions were within 5 ppm accuracy to the proposed structures, and were shown in Figure 23b. Therefore, confirming that the ion m/z 716.4587 was anhydroerythromycin.

The MS/MS spectrum of ion m/z 702.4437 at retention time 6.14 min was shown in Figure 23c, matches with exact mass of proposed desmethyl-anhydroerythromycin, with mass accuracy of 2.0 ppm. The most abundant fragment ions 144 Da, was 14 Da less than 158 Da, the most abundant fragment ion for standard, revealing that the mass loss was corresponding to methyl group with an accuracy of 1.4 ppm. The nominal masses of

other fragment ions, 233 Da and 83 Da, were in good agreement with the MS/MS fragments of anhydroerythromycin standard showing that for sure they both have the similar structural features. The structures for the labeled fragment ions were predicted using MF software and the mass accuracies were within 5 ppm to that of observed MS/MS fragments (Figure 24).
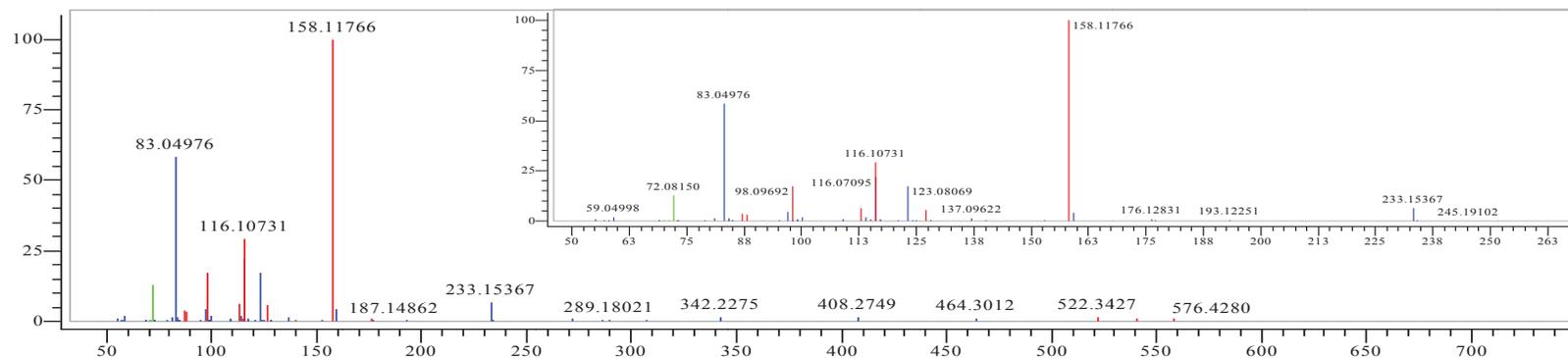
23a
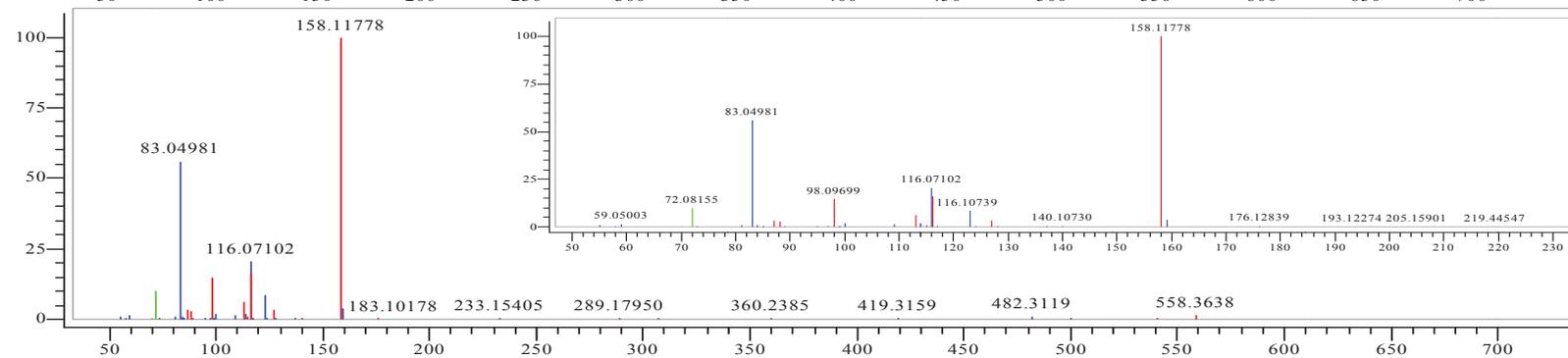
23b
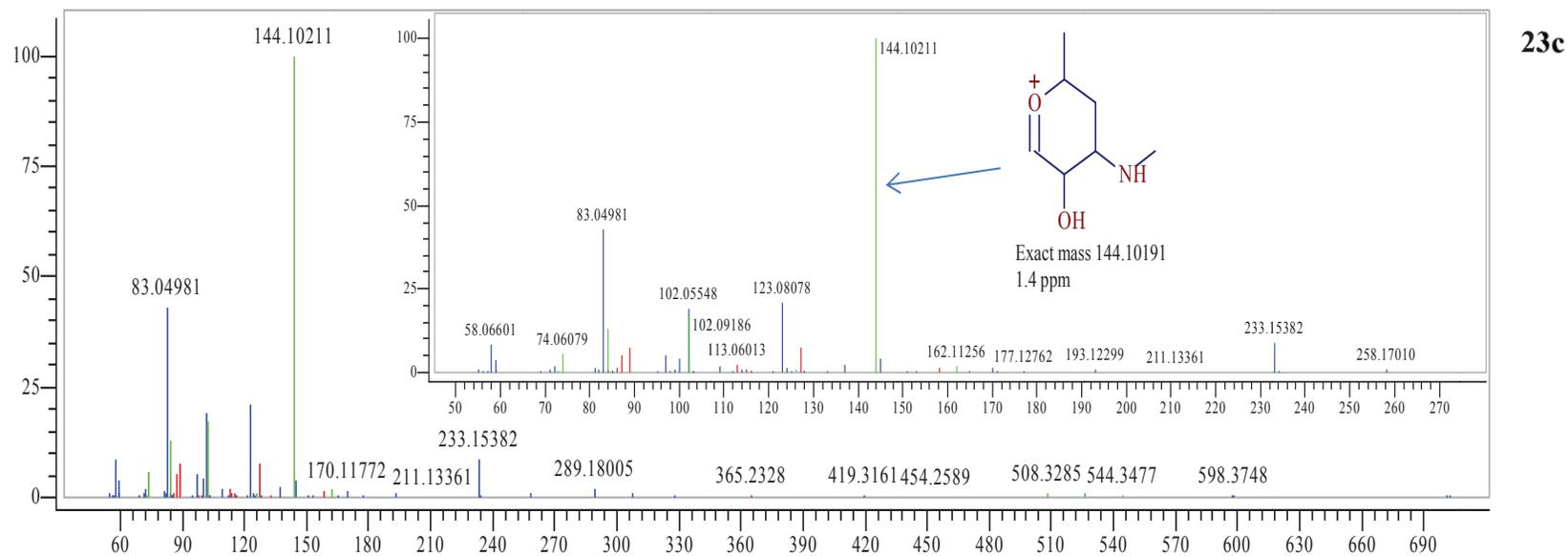
Figure 23. High resolution MS/MS spectra and major fragments for a) erythromycin standard, b) anhydroerythromycin in reclaimed water, and c) desmethyl-anhydroerythromycin in reclaimed water.
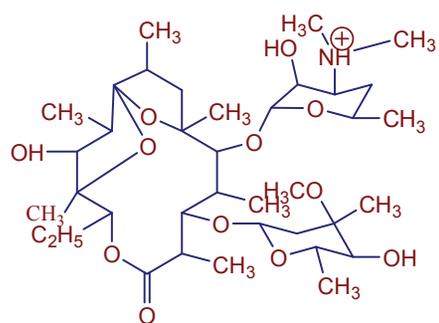
Anhydro erythromycin
$C_{37}H_{66}NO_{12}^+$
Exact Mass: 716.458
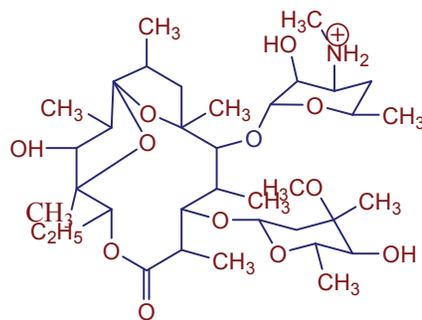
desmethyl anhdro erythromycin
$C_{36}H_{64}NO_{12}^+$
Exact Mass: 702.4423
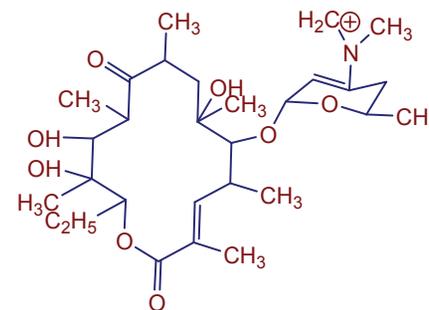
$C_{29}H_{50}NO_8^+$
Exact Mass: 540.3531
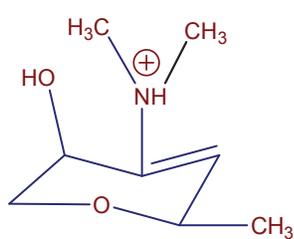
$C_8H_{16}NO_2^+$
Exact Mass: 158.1176
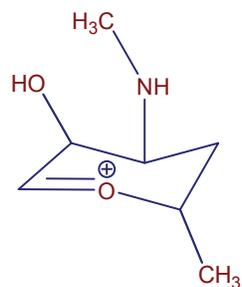
$C_7H_{14}NO_2^+$
Exact Mass: 144.1019
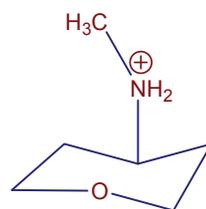
$C_6H_{14}NO^+$
Exact Mass: 116.107
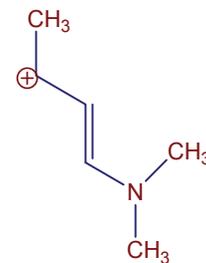
$C_6H_{12}N^+$
Exact Mass: 98.0964
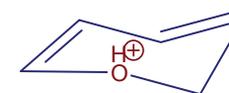
$C_5H_7O^+$
Exact Mass: 83.0491

Figure 24. Major MS/MS fragments for erythromycin and its metabolites.

6.2.6. Identification of acetyl-sulfamethoxazole

The high resolution MS/MS spectrum of ion m/z 254.0595 in reclaimed water at retention time 9.51 min was almost similar to that of sulfamethoxazole standard as shown in Figure. 25. The three major fragment ions 156 Da, 108 Da and 99 Da observed were following the same relative abundance pattern in both the samples and standard, and were also in good agreement with previously reported studies (Sacher et al., 2001; Panditi et al., 2013; Wang, 2013). The structures for the major fragments were predicted using MF software based on the fragmentation rules built into it. All the labeled fragmentation ions were within 5 ppm accuracy to the proposed structures.

The MS/MS spectrum of ion m/z 296.0700 at retention time 9.59 min was shown in Figure 25, matches with exact mass of acetyl-sulfamethoxazole, with mass accuracy of 0.0 ppm. The three major fragment ions 198 Da, 134 Da and 108 Da were in good agreement with those reported in previous studies (Gobel et al., 2004; Wang, 2013). And these fragments matched within 5 ppm to the predicted structures using MF software. The detailed fragmentation pathway was described previously by Wang from the same research group (Wang, 2013). The proposed structures for the fragment ions 134 Da and 198 Da contain acetyl groups evidently showing that these ions were forming from the fragmentation of acetyl-sulfamethoxazole. Therefore, the ion m/z 296.0700 was once again confirmed as acetyl-sulfamethoxazole.
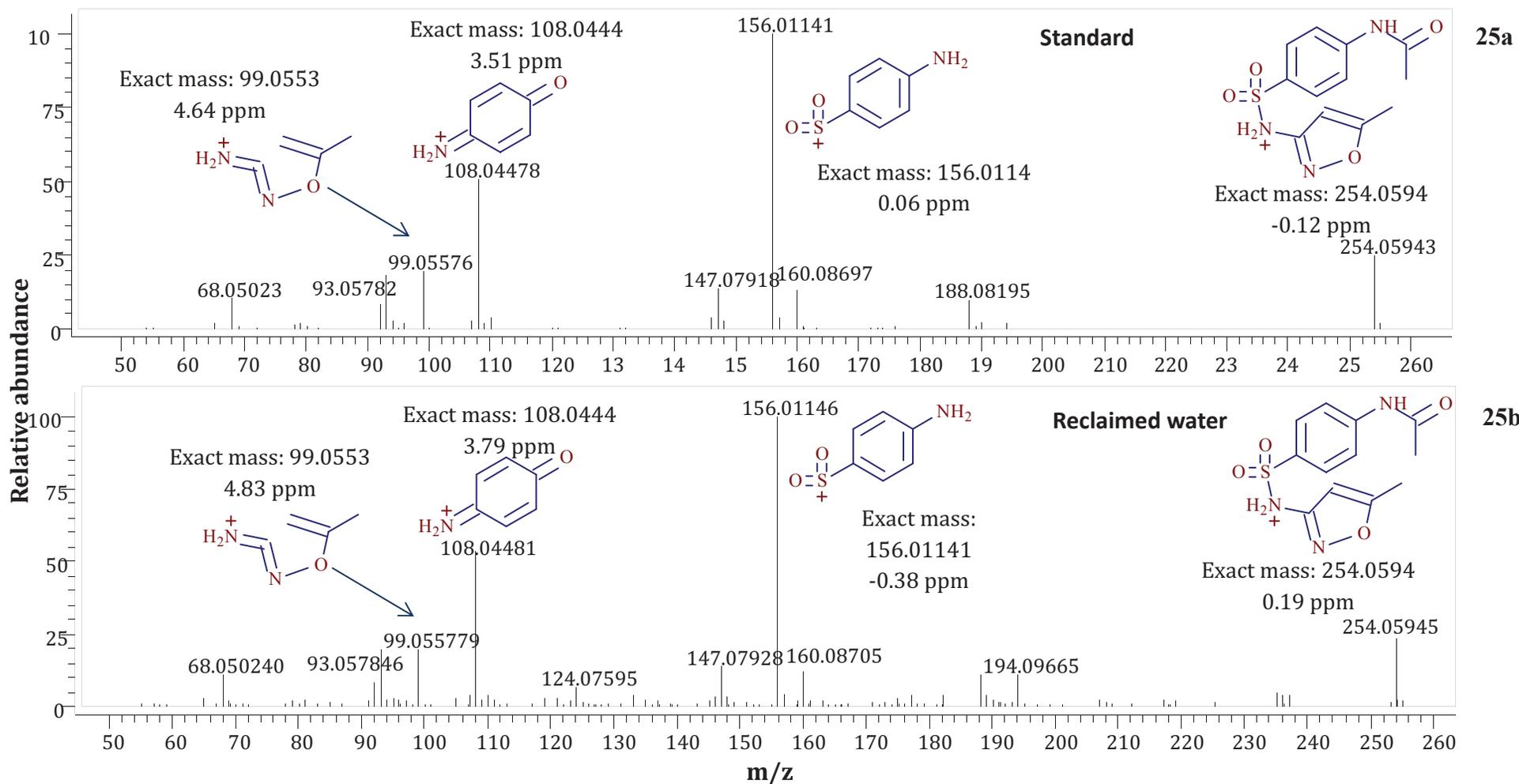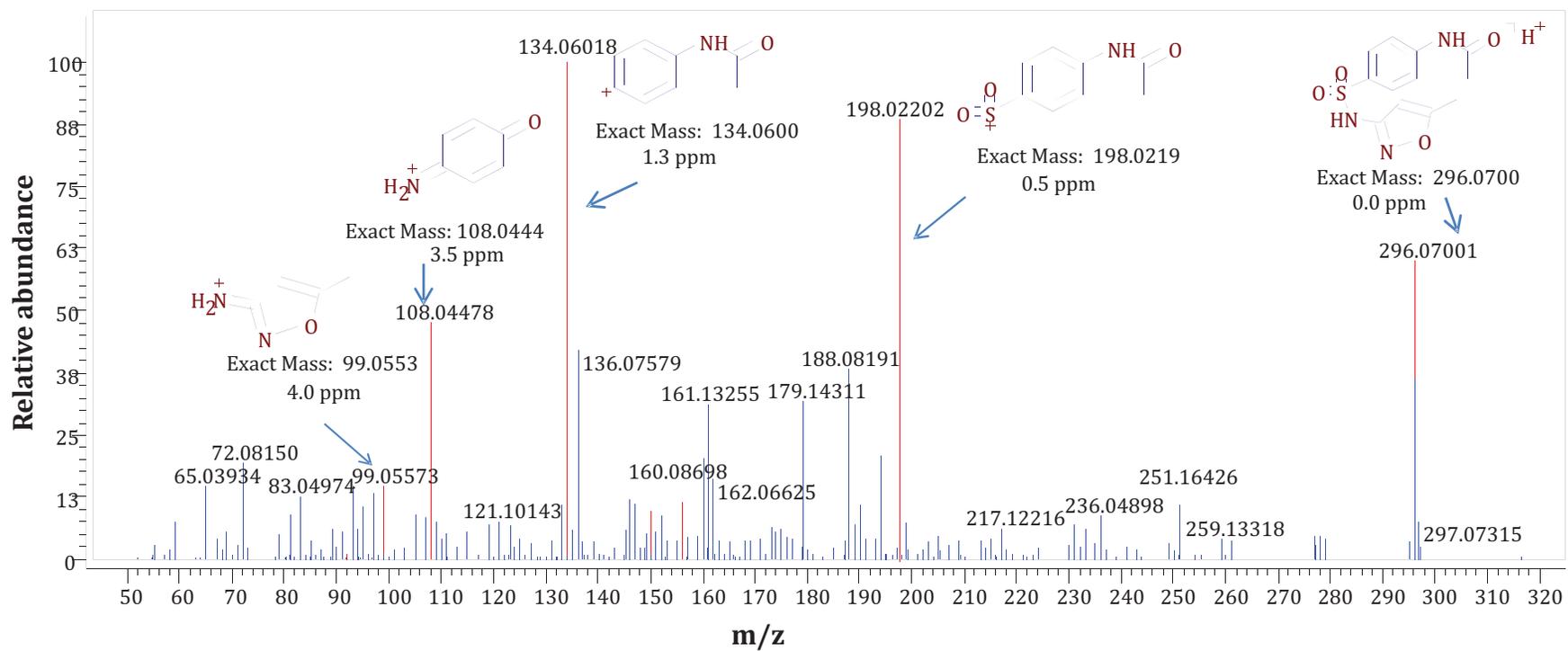
Figure 25. High resolution MS/MS spectra and major fragments for a) sulfamethoxazole standard, b) sulfamethoxazole in reclaimed water, and c) acetyl-sulfamethoxazole in reclaimed water.

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6.2.7. Determination of erythromycin, sulfamethoxazole and their metabolites in reclaimed water

Reclaimed water samples were collected and processed through off-line SPE as described earlier in this chapter. Calibration solutions were prepared by spiking standard solutions of sulfamethoxazole and erythromycin to 4 mL methanol, dried under gentle stream of nitrogen, then 50 µL of internal standards mixture containing sulfamethoxazole-d4 and erythromycin-^{13}C-d3 were added and reconstituted to 250 µL with LC─MS grade water. Matrix spike samples were prepared by spiking second highest calibration solution to reclaimed water and extracted the same way as regular reclaimed waters.

Seven reclaimed water samples were collected over a period of one week from reclaimed water sprinklers at Florida International University Biscayne Bay Campus, North Miami, FL., for the quantitation purposes. Quantitation was performed in MS full scan mode (resolution 70,000) using the peak area of extracted ion chromatogram of the base peak ion by setting the mass tolerance at 5 ppm and mass precision up to four decimal places. Concentrations of metabolites, desmethyl-anhydroerythromycin and acetyl-sulfamethoxazole were tentatively determined based on the response factors obtained for their respective standards. This quantification was performed assuming that structural similarities and closeness in chromatographic retention times of parent antibiotics and their metabolites make them to ionize equally in mass spectrometer, regardless of potential matrix effects. Therefore, retention times and mass precision of parent ions detected were used for their determination.

Only two metabolites along with their parent drugs were consistently detected in reclaimed water suggesting that these are the only two types of antibiotic metabolites out of many metabolites for the selected antibiotics, that can survive intact through waste water treatment process, or alternatively as a matter of fact, there might be many metabolites for single parent drug therefore the concentration of individual metabolites may be very low, in order to trigger MS/MS fragmentation ion scan, hence not detected. The mean concentration of anhydroerythromycin and desmethyl-anhydroerythromycin were calculated as 519 ± 493 ng/L and 76 ± 57 ng/L, respectively (Figure. 26); the metabolite represented 13%, on the basis of mole fraction, of the total erythromycin detected in reclaimed water. At the same time the mean concentration of the other antibiotic, sulfamethoxazole and its metabolite acetyl-sulfamethoxazole were calculated as 552 ± 348 ng/L and 362 ± 92 ng/L, respectively (Figure. 27) and the mole fraction of the metabolite represent 36%, of the cumulative sulfamethoxazole concentration. The other metabolite, sulfamethoxazole glucucuronide was not detected in present study, though it was reported previously from the same research group (Wang, 2013). The possible explanation would be that the glucuronide conjugates were unstable in waste water treatment processes and either could be back transformed as reported previously or used as energy source for microbes (Bonvin, 2012). The preliminary results were showing that some of the metabolites do survive in waste water treatment and can potentially contaminate the other environmental aquatic compartments as well. Once they reach the environment, similar to the parent antibiotics, some of these metabolites were bio-active and can pose risk to untargeted species.
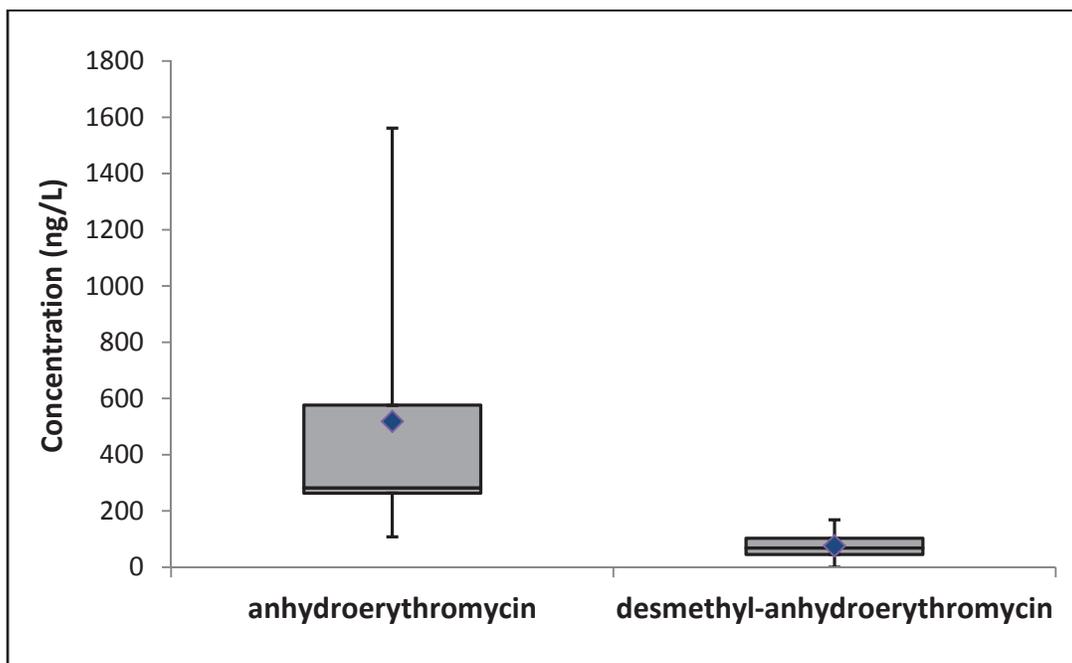
Figure 26. Occurrence of erythromycin and its metabolite desmethyl-erythromycin in reclaimed water (both were measured as anhydro- forms)
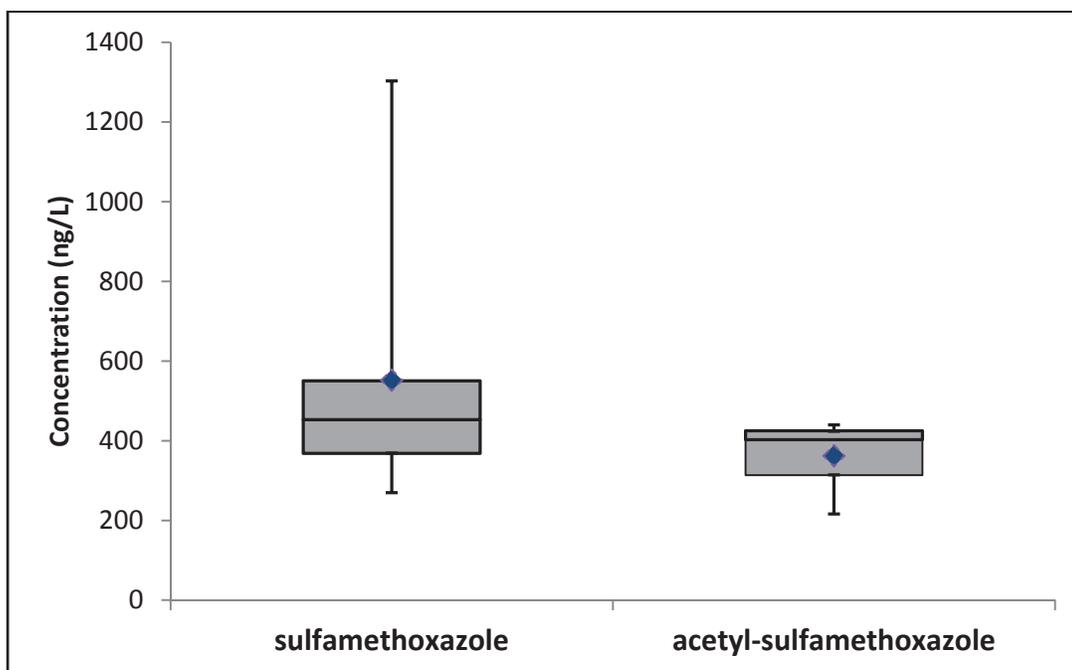
Figure 27. Occurrence of sulfamethoxazole and its metabolite acetyl-sulfamethoxazole in reclaimed water

6.3.Conclusion

High resolution mass spectrometry in combination with metabolic profiling software was successfully applied for identifying the metabolites/ transformation products in reclaimed water. The present study illustrated the use of combination of characteristic ions, MS/MS fragmentation pattern, relative abundance and spectral matching for the positive confirmation of compounds in the condition of no availability of standards. Erythromycin and its bio-active metabolite desmethyl-erythromycin (both observed as anhydro- forms), and sulfamethoxazole and its bio-active metabolite acetyl-sulfamethoxazole were found in reclaimed water. In two of the metabolites determined Desmethyl-erythromycin was identified for the first time in reclaimed waters. Moreover, these identities can help in comprehensive risk and fate assessment of both parent erythromycin, sulfamethoxazole and their respective metabolites in aquatic ecosystems.

CHAPTER 7

Environmental risk assessment of antibiotic residues in reclaimed and surface waters

7.1. Introduction

Antibiotics by their nature are biologically active molecules designed to control disease spreading microorganisms in humans and animals. As described previously, antibiotics reach the domestic sewage systems and landfills through several sources. Many of these substances are not completely mineralized or eliminated in conventional waste water treatment processes applied in WWTPs (Heberer et al., 2002; Batt et al., 2006; Deblonde et al., 2011)and as a result of insufficient removal, antibiotics are finally reaching to surface waters, ground waters, sediments and even to drinking waters (Meyer et al., 1999; Kolpin et al., 2002; Ye et al., 2007; Tamtam et al., 2008; Kummerer, 2009; Panditi et al., 2013). Though the environmental concentrations detected were in ng/L to low µg/L, still they can pose risk to some of the sensitive, non-targeted species (Migliore. L, 1993 ; Halling-Sorensen et al., 2000; Wollenberger et al., 2000; Halling-Sorensen et al., 2002; Kummerer, 2009; Gonzalez-Pleiter et al., 2013). Some of the antibiotics may have same mechanism of action due to their common physico-chemical behavior; especially antibiotics come from same chemical family; in such cases organisms will be at increased risk as a result of additive effects (Kummerer, 2009; Gonzalez-Pleiter et al., 2013). Synergistic effects are also possible due to two or more compounds existing together at the same time, the well-known example is sulfamethoxazole in combination with trimethoprim. In addition, they can also pose risk indirectly by proliferating resistant bacterial strains (Kummerer and Henninger, 2003). As reported previously,, sufficiently

low concentrations of antibiotics could alter community structures that form the basis of food chain (Wollenberger et al., 2000; Kummerer, 2009). Therefore, it is important to evaluate the ecological risk associated with occurrence of antibiotics in the aquatic environment.

Ecological risk assessment is a process that evaluates likelihood that adverse ecological effects may occur or are occurring as a result of exposure to environmental pollutants or stressors (USEPA, 1998). The United States, Food and Drug Administration (FDA) and European Medicines Evaluation Agency (EMEA) described the general principles and guidelines for environmental risk assessment (ERA) for new and existing chemicals, employing similar tiered system. Both the agencies explained the ERA based on the comparison between predicted environmental concentrations (PEC) and worst-case predicted no effect concentrations (PNEC) estimated from standard toxicity assays (EC, 1996; FDA, 1998). Based on EMEA guidelines the PECs in waters are calculated using the following equation (Halling-Sorensen et al., 2000).

$$\text{PEC in water} = \frac{A \times (100-R)}{365 \times P \times V \times D \times 100} \quad \text{.......................... (Equation 4)}$$

Where A is the annual consumption (kg/year), R is the percentage removal in sewage treatment (set to zero when worst-case conditions are assessed or biodegradation in STP is missing), P is the population size, V is the volume of waste water per capita per day and D is the dilution factor in the environment.

Overestimation of most PECs according to these approaches are evident, as they don't include metabolic transformation and natural degradation processes (Hernando et al., 2006). However significant improvements in risk assessment were undertaken recently by introducing assessment factors and using real time exposure data of three or four different species, to reduce the effect of uncertainties and to make the system more reliable (Bound and Voulvoulis, 2004). Environmental risk assessments are commonly based on two types of exposure studies; one is short-term 'acute ecotoxicological studies' and second is long-term 'chronic toxicity screening'. Regulatory concepts of ERA are commonly based on acute toxicological studies conducted in variety of sensitive species (EC_{50} values), by applying assessment factors and evaluating environmental behavior (Hernando et al., 2006). On the other hand, the chronic toxicological screening tests of different variety and appear to be practically tough to achieve for all the drugs selected and may induce deficiencies in screening, as they may not be stable for longer durations (Lange and Dietrich, 2002). Considering the above facts, the toxicity data obtained from the acute ecotoxicity studies with application of risk quotients (RQ) derived from measured environmental concentrations instead of PECs and assessment factors would ensure a more concerted effort in environmental risk assessment.

The aim of the present study was to preliminarily characterize the environmental risk associated with detected antibiotics in reclaimed waters and surface waters by comparing the measured environmental concentrations (MEC) obtained from this study, with available literature on measured effective concentrations (EC_{50} value) obtained from ecotoxicity data.

7.2. Toxicity studies

Most of the selected antibiotics were detected in reclaimed water and surface waters. The maximum and mean concentration of antibiotics in reclaimed and surface waters, data presented in the earlier chapters were used in this part for risk assessment.

Three different organism types (algae, bacteria and invertebrate) commonly used in toxicity studies that represent food chain were selected. Acute toxicological data (EC_{50} value) for all the three organism types in water were compiled from literature (shown in Table 18), and were used to calculate worst-case predicted no effect concentrations for each antibiotic in all the three taxonomic groups. Efforts been made to pool the EC_{50} value for all the antibiotics from single species in a given taxonomic group. However, if the information is not available, then the EC_{50} values obtained from similar species in the same taxonomic group were chosen, assuming that the given antibiotic will have similar mechanism of action and same degree of effect on all the species in single taxonomic group. To account for intra- and inter-species variability standard assessment factor was applied to mean effective concentration. Though, synergistic or antagonistic effects due to combined existence of antibiotics were possible, to aggravate or suppress the possible ecological risk, because of the lack of sufficient combined exposure data (EC50 values) for the selected antibiotics this was not considered in the present study.

Table 18. Antibiotics detected in reclaimed water and their toxicology data from literature

| Antibiotic | EC50 (mg/L) | | | References | Test Organism | | |
	Algae[a]	Bacteria[b]	Invertebrate[c]		Algae[a]	Bacteria[b]	Invertebrate[c]
Naldixic acid	NA	0.206	NA	b (Backhaus and Grimme, 1999)		Vibrio fischeri	
Erythromycin	0.35	0.022	22.45	a,b (Gonzalez-Pleiter et al., 2013) c (Isidori et al., 2005)	Pseudokirckneriella subcapitata	Anabaena sp. CPB4337	Daphnia magna
Sulfadiazine	2.19	0.135	NA	a (Eguchi et al., 2004) b (Lützhøft et al., 1999)	Selenastrum capricornutum	Microcystis aeruginosa	
Clarithromycin	0.002*	100	25.72	b,c (Isidori et al., 2005) a (Zhang et al., 2013)	P. subcapitata	Vibrio fischeri	Daphnia magna
Trimethoprim	110	17.8	92	a,b (Halling-Sorensen et al., 2000) c(Park and Choi, 2008)	Selenastrum capricornutum	activated sludge	Daphnia magna
Sulfamethoxazole	146	84	123.1	a,b (Ferrari et al., 2004) c(Park and Choi, 2008)	Pseudokirchneriella subcapitata	Vibrio fischeri	Daphnia magna
Sulfamerazine	NA	NA	1.056	c (Bartlett et al., 2013)			Hyalella azteca
Azithromycin	NA	NA	120	c (Zhang et al., 2013)			Daphnia magna
Norfloxacin	80	0.29	1449	a (Gonzalez-Pleiter et al., 2013) b (Ando et al., 2007) c (Vazquez-Roig et al., 2012)	Pseudokirchneriella subcapitata	Anabaena flos-aquae ATCC 29413	Daphnia magna
Sulfachlorpyridazine	NA	26.4	233.5	b, c (Kim et al., 2007)		Vibrio fischeri	Daphnia magna

Antibiotic	EC50 (mg/L)				Test Organism		
	Algae[a]	Bacteria[b]	Invertebrate[c]	References	Algae[a]	Bacteria[b]	Invertebrate[c]
Ofloxacin	4.74	0.021	3.13	a (Ferrari et al., 2004) b(Robinson et al., 2005) c (Vazquez-Roig et al., 2012)	Pseudokirchneriella subcapitata	Microcystis aeruginosa	C. dubia
Ciprofloxacin	2.97	0.005	991	a,b (Halling-Sorensen et al., 2000) c (Vazquez-Roig et al., 2012)	Selenastrum capricornutum	Microcystis aeruginosa	Daphnia magna
Spiramycin	2.3	58.5	NA	a (Halling-Sorensen, 2000) b (Liu et al., 2012)	Selenastrum capricornutum	Microcystis aeruginosa	
Tylosin	0.411	0.034	NA	a (Eguchi et al., 2004) b (Halling-Sorensen, 2000)	Selenastrum capricornutum	Microcystis aeruginosa	
Oxytetracycline	0.885	0.39	22.64	a (Munch Christensen et al., 2006)b (Ando et al., 2007) c (Isidori et al., 2005)	Pseudokirchneriella subcapitata	Anabaena flos-aquae ATCC 29413	Daphnia magna
Roxithromycin	NA	NA	7.1	c(Choi et al., 2008)			Daphnia magna
Doxycycline	NA	NA	NA				
Chlortetracycline	NA		225	c. (Park and Choi, 2008)			Daphnia magna
Enrofloxacin	3.1	0.049	56.7	a,b (Robinson et al., 2005 c (Park and Choi, 2008)	Pseudokirchneriella subcapitata	Microcystis aeruginosa	Daphnia magna

Note: Pseudokirckneriella subcapitata was formerly known as Selenastrum capricornutum; * EC50 value obtained from chronic exposure toxicity data

7.2.1. Risk Quotient approach

Risk quotient (RQ) is the basic principle internationally accepted and adopted in the development of environmental risk assessment guidelines (Halling-Sorensen et al., 2000; Hernando et al., 2006). This approach in the present study is the estimation of incidence of the adverse effect occurring in aquatic compartments as a result of an antibiotic at measured environmental concentration. In acute toxicity studies, assessment of whether an antibiotic pose risk to organisms in the environment is done by comparing PEC or MEC with its PNEC to organism, expressed as RQ. Where PNEC represents the worst-case no effect concentration of antibiotic, predicted or derived from acute toxicity data (EC$_{50}$ values) by applying a standard assessment factor of 1000 (EC, 2003; Hernando et al., 2006; Gonzalez-Pleiter et al., 2013). Assessment factor was applied to account for intra- and inter-species variability in sensitivity.

$$\text{PNEC in water} = \frac{\text{EC}_{50} \text{ or LC}_{50}}{1000} \ldots\ldots\ldots\ldots\ldots \text{(Equation 5)}$$

$$\text{RQ} = \frac{\text{MEC}}{\text{PNEC}} \ldots\ldots\ldots\ldots\ldots \text{(Equation 6)}$$

RQ equals or exceeds to one, suggests that an ecological risk is expected for the given antibiotic.

7.3. Results and Discussion-Risk assessment

As shown in Tables 19 & 20 (Figure. 28), PNECs and RQs were calculated for all antibiotics in three selected taxonomic groups based on measured mean environmental concentrations both in reclaimed and surface waters. PNECs were also calculated for highest antibiotic concentrations observed, to represent the least probable highest suspected risk (Table 21 & 22, Figure. 29). Results were interpreted based on RQ ratios following the common criteria used in risk assessment studies (EC, 1996; Hernando et al., 2006), where the RQ values ranged from 0.01 through 0.1 were marked as "low risk", 0.1 through 1 were marked as "medium risk" and equal to or exceeding 1 were marked as "high risk".

The risk quotients obtained for individual antibiotics using mean measured concentrations in reclaimed water (Table 19) showed that erythromycin, clarithromycin, ofloxacin and ciprofloxacin exceeded the RQ value one, in at least one of the three taxonomic groups. This result indicates that the measured concentrations in reclaimed water were relatively high and suspected to induce high ecological risk on representative species of the food chain. Many research studies from different parts of the world also reported similar type of results for erythromycin (Isidori et al., 2005; Hernando et al., 2006; Gonzalez-Pleiter et al., 2013), clarithromycin (Isidori et al., 2005), and ciprofloxacin (Halling-Sorensen et al., 2000) indicating the potential environmental impact of these antibiotic residues. Adding to this, erythromycin has been placed in USEPA environmental contaminant candidate list III for further evaluation (USEPA,

2009). Worst case exposure concentrations (Table 21) (maximum antibiotic concentrations detected in reclaimed water) were showing that, in addition to the above mentioned antibiotics, naldixic acid, sulfadiazine, and tylosin were also suspected to pose potential ecological risk. However, as these concentrations may get diluted when released to other aquatic compartments, the possible hazard may not be the same as suspected.

In order to get more clarity in environmental risk associated with antibiotic exposure, surface water concentrations were used. Surface water results using mean measured concentrations (Table 20) were showing that, for erythromycin, clarithromycin and ciprofloxacin, the respective RQ values were not as high as in reclaimed water but still above one, indicating that they do pose the same level (high) of ecological risk as reclaimed water. Additionally tylosin was also observed as high risk posing antibiotic. Ofloxacin, observed as high risk in reclaimed water, turned as medium risk in surface water due to their low environmental concentrations.

The overall results were showing that the taxonomic group, bacteria were at much higher risk followed by algae. Algae were particularly sensitive to macrolide antibiotics clarithromycin, erythromycin and tylosin. Invertebrates were not under risk for any of the selected antibiotics, at least to the chosen animal model if not for all.

Table 19. PNEC and risk quotients for antibiotics in reclaimed waters using mean antibiotic concentration detected

Antibiotic	MEC (ng/L)_ mean	PNEC (AF=1000)			Risk Quotient		
		Algae	Bacteria	Invertebrate	Algae	Bacteria	Invertebrate
Naldixic acid	176	-	0.000206	-	-	0.85	-
Erythromycin	135	0.00035	0.000022	0.02245	0.39	**6.14**	0.01
Sulfadiazine	128	0.00219	0.000135	-	0.06	0.95	-
Clarithromycin	123	0.000002	0.1	0.02572	**61.5**	0.00	0.00
Trimethoprim	118	0.11	0.0178	0.092	0.00	0.01	0.00
Sulfamethoxazole	112	2.68E-05	0.084	0.1231	0.00	0.00	0.00
Sulfamerazine	94.8	-	-	0.001056	-	-	0.09
Azithromycin	89.7	-	-	0.12	-	-	0.00
Norfloxacin	71.3	0.08	0.00029	1.449	0.00	0.25	0.00
Ofloxacin	68.8	0.00474	0.000021	0.00313	0.01	**3.28**	0.02
Sulfachlorpyridazine	58.9	-	0.0264	0.2335	-	0.00	0.00
Ciprofloxacin	41.6	0.00297	0.000005	0.991	0.01	**8.32**	0.00
Spiramycin	37.3	0.0023	0.0585	-	0.02	0.00	-
Tylosin	22.3	0.000411	0.000034	-	0.05	0.66	-
Oxytetracycline	21.1	0.000885	0.00039	0.02264	0.02	0.05	0.00
Roxithromycin	17.9	-	-	0.0071	-	-	0.00
Doxycycline	17.7	-	-	-	-	-	-
Chlortetracycline	16.9	-	-	0.225	-	-	0.00
Enrofloxacin	16.7	0.0031	0.000049	0.0567	0.01	0.34	0.00

Table 20. PNEC and risk quotients for antibiotics in surface waters using mean antibiotic concentration detected

Antibiotic	MEC (ng/L)_ mean	PNEC (AF=1000)			Risk Quotient		
		Algae	Bacteria	Invertibrate	Algae	Bacteria	Invertibrate
Naldixic acid	14	-	0.000206	-	-	0.07	-
Erythromycin	44	0.00035	0.000022	0.02245	0.12	**1.98**	0.00
Sulfadiazine	44	0.00219	0.000135	-	0.02	-	-
Clarithromycin	7	0.000002	0.1	0.02572	**3.71**	0.00	0.00
Trimethoprim	33	0.11	0.0178	0.092	0.00	0.00	0.00
Sulfamethoxazole	14	2.68E-05	0.084	0.1231	0.00	0.00	0.00
Sulfamerazine	17	-	-	0.001056	-	-	0.02
Azithromycin	29	-	-	0.12	-	-	0.00
Norfloxacin	24	0.08	0.00029	1.449	0.00	0.08	0.00
Ofloxacin	12	0.00474	0.000021	0.00313	0.00	0.58	0.00
Sulfachlorpyridazine	10	-	0.0264	0.2335	-	0.00	0.00
Ciprofloxacin	34	0.00297	0.000005	0.991	0.01	**6.84**	0.00
Spiramycin	36	0.0023	0.0585	-	-	0.00	-
Tylosin	74	0.000411	0.000034	-	-	**2.19**	-
Oxytetracycline	17	0.000885	0.00039	0.02264	0.02	0.04	0.00
Roxithromycin	15	-	-	0.0071	-	-	0.00
Chlortetracycline	32	-	-	0.225	-	-	0.00
Enrofloxacin	6	0.0031	0.000049	0.0567	0.00	0.12	0.00
Lincomycin	9	-	0.1	-	-	0.00	-
Amoxicillin	29	1.5	0.0563	1	0.00	0.00	0.00
Danofloxacin	43	-	-	-	-	-	-
Tetracycline	12	0.00331	0.0000251	-	0.00	0.47	-
Sulfadimethoxine	21	0.0023	0.47	0.248	0.01	0.00	0.00
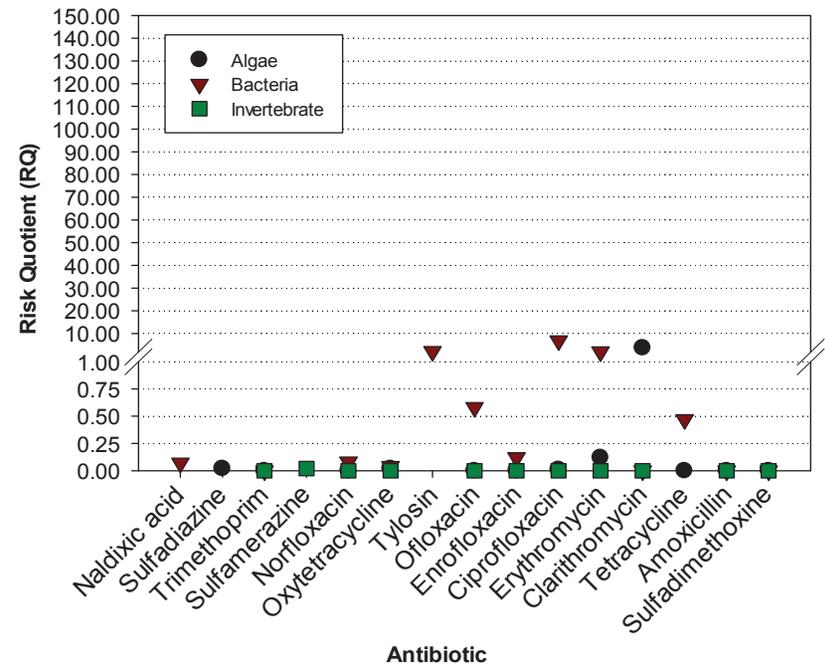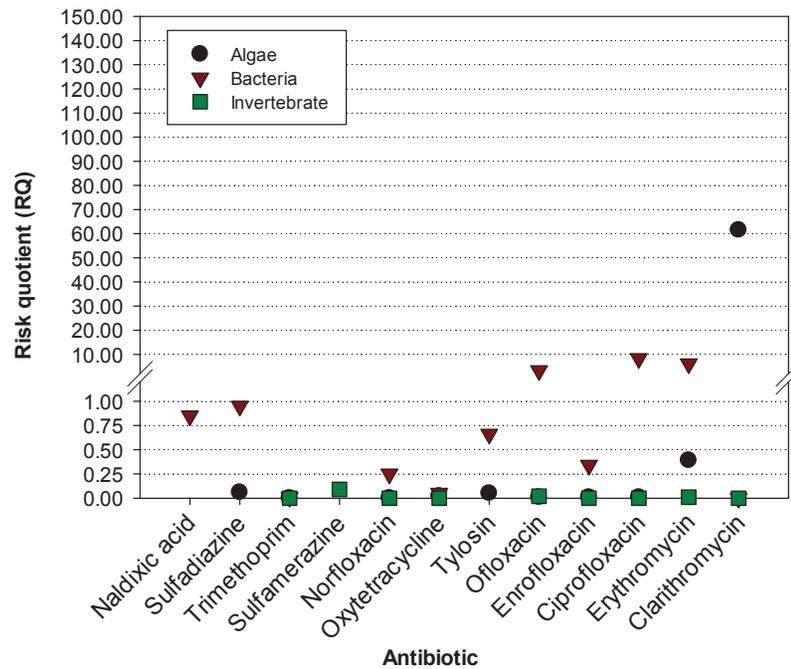
Figure 28. Risk quotients for antibiotics using mean antibiotic concentration detected in reclaimed waters (left) and in surface waters (right). Note that absence of a symbol in the graph indicates lack of ecotoxicological data for the specific antibiotic in the given taxonomic group.

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Table 21. PNEC and risk quotients for antibiotics in reclaimed waters using maximum antibiotic concentration detected

Antibiotic	MEC (ng/L)_ max	PNEC (AF=1000)			Risk Quotient		
		Algae	Bacteria	Invertebrate	Algae	Bacteria	Invertebrate
Naldixic acid	453	-	0.000206	-	-	**2.20**	-
Erythromycin	414	0.00035	0.000022	0.02245	**1.18**	**18.8**	0.02
Sulfadiazine	276	0.00219	0.000135	-	0.13	**2.04**	-
Clarithromycin	284	0.000002	0.1	0.02572	**142**	0.00	0.01
Trimethoprim	605	0.11	0.0178	0.092	0.01	0.03	0.01
Sulfamethoxazole	341	2.68E-05	0.084	0.1231	0.00	0.00	0.00
Sulfamerazine	100	-	-	0.001056	-	-	0.09
Azithromycin	180	-	-	0.12	-	-	0.00
Norfloxacin	71.3	0.08	0.00029	1.449	0.00	0.25	0.00
Ofloxacin	127	0.00474	0.000021	0.00313	0.03	**6.05**	0.04
Sulfachlorpyridazine	66.9	-	0.0264	0.2335	-	0.00	0.00
Ciprofloxacin	68	0.00297	0.000005	0.991	0.02	**13.6**	0.00
Spiramycin	73.7	0.0023	0.0585	-	0.03	0.00	-
Tylosin	36.3	0.000411	0.000034	-	0.09	**1.07**	-
Oxytetracycline	21.1	0.000885	0.00039	0.02264	0.02	0.05	0.00
Roxithromycin	25.1	-	-	0.0071	-	-	0.00
Doxycycline	17.7	-	-	-	-	-	-
Chlortetracycline	16.9	-	-	0.225	-	-	0.00
Enrofloxacin	21.9	0.0031	0.000049	0.0567	0.01	0.45	0.00

Table 22. PNEC and risk quotients for antibiotics in surface waters using maximum antibiotic concentration detected

Antibiotic	MEC (ng/L)_ max	PNEC (AF=1000)			Risk Quotient		
		Algae	Bacteria	Invertebrate	Algae	Bacteria	Invertebrate
Naldixic acid	14	-	0.000206	-	-	0.07	-
Erythromycin	199	0.00035	0.000022	0.02245	0.57	**9.06**	0.01
Sulfadiazine	139	0.00219	0.000135	-	0.06	-	-
Clarithromycin	8	0.000002	0.1	0.02572	**3.99**	0.00	0.00
Trimethoprim	129	0.11	0.0178	0.092	0.00	0.01	0.00
Sulfamethoxazole	25	2.68E-05	0.084	0.1231	0.00	0.00	0.00
Sulfamerazine	18	-	-	0.001056	-	-	0.02
Azithromycin	61	-	-	0.12	-	-	0.00
Norfloxacin	36	0.08	0.00029	1.449	0.00	0.12	0.00
Ofloxacin	14	0.00474	0.000021	0.00313	0.00	0.64	0.00
Sulfachlorpyridazine	13	-	0.0264	0.2335	-	0.00	0.00
Ciprofloxacin	61	0.00297	0.000005	0.991	0.02	**12.2**	0.00
Spiramycin	61	0.0023	0.0585	-	-	0.00	-
Tylosin	152	0.000411	0.000034	-	-	**4.48**	-
Oxytetracycline	43	0.000885	0.00039	0.02264	0.05	0.11	0.00
Roxithromycin	23	-	-	0.0071	-	-	0.00
Chlortetracycline	48	-	-	0.225	-	-	0.00
Enrofloxacin	8	0.0031	0.000049	0.0567	0.00	0.16	0.00
Lincomycin	12	-	0.1	-	-	0.00	-
Amoxicillin	32	1.5	0.0563	1	0.00	0.00	0.00
Danofloxacin	43	-	-	-	-	-	-
Tetracycline	18	0.00331	0.0000251	-	0.01	0.71	-
Sulfadimethoxine	35	0.0023	0.47	0.248	0.02	0.00	0.00
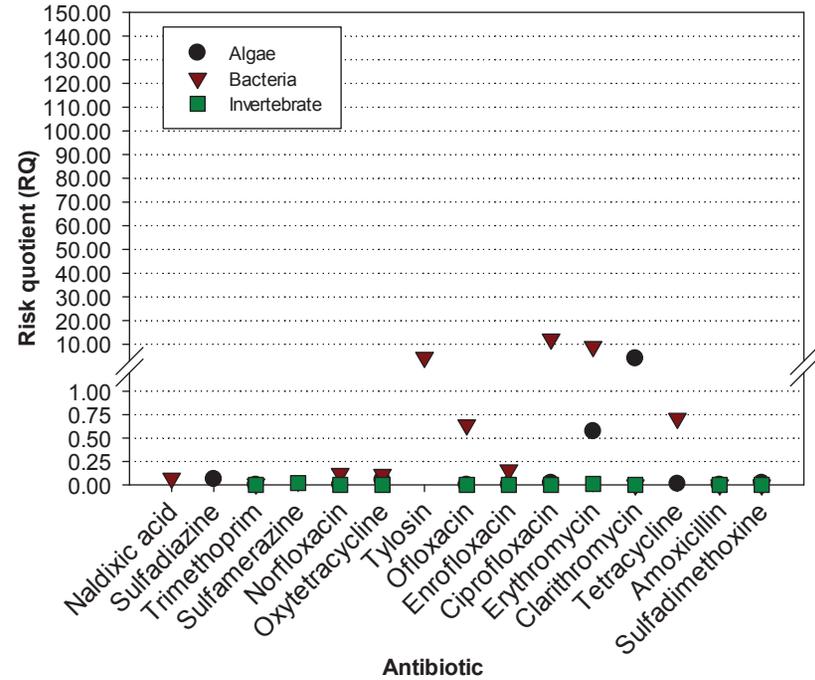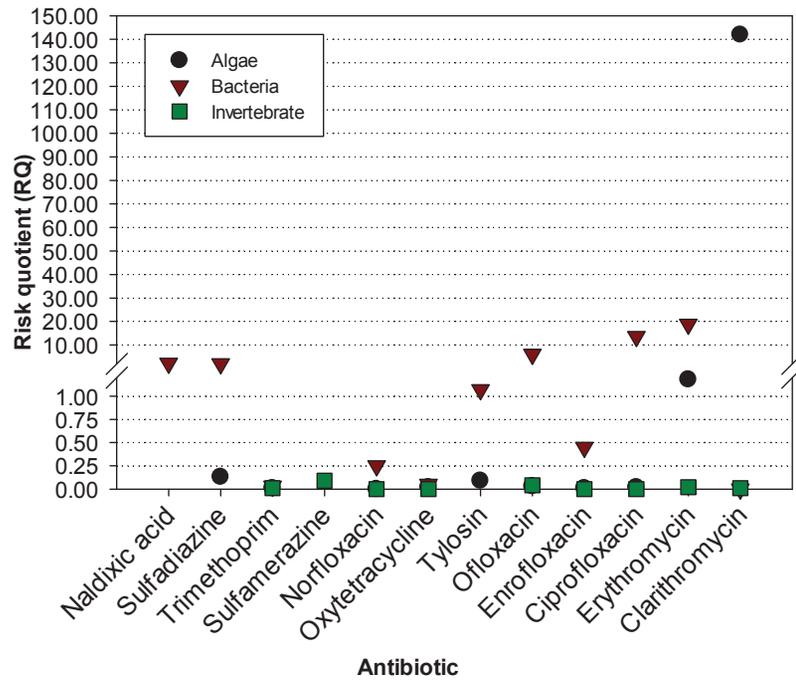
Figure 29. Risk quotients for antibiotics using maximum antibiotic concentration detected in reclaimed waters (left) and in surface waters (right). Note that absence of a symbol in the graph indicates lack of ecotoxicological data for the specific antibiotic in the given taxonomic group.

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7.4. Conclusions

In this study, all the antibiotics detected in reclaimed and surface waters were investigated to assess their potential risk to the aquatic organisms. The surface water antibiotic concentrations that represented the real time exposure conditions revealed that the macrolide antibiotics erythromycin, clarithromycin and tylosin along with quinolone antibiotic, ciprofloxacin were suspected to induce high toxicity to algae and bacteria. Preliminary results showing that, among the antibiotic groups tested, macrolides posed the highest ecological threat, suggesting that they may need to be further evaluated with, long-term exposure studies considering bioaccumulation factors and more number of species selected.

CONCLUSION

An automated on-line Solid phase extraction (SPE) liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS) method was developed for the quantitation of multiple classes of antibiotics in natural waters. The direct coupling of an online SPE to LC-MS/MS was achieved using column switching technique. High sensitivity in the low ng/L range was accomplished by using large volume injections with 10-mL of the sample. This coupling technique increased the sample throughput with a total run time of 20 min. The target analytes were detected in a triple quadrupole equipped with HESI source operated in the positive mode with two selected reaction monitoring (SRM) transitions per antibiotic for positive identity. Quantification was performed by the internal standard approach using isotopically labeled standards, to correct for matrix effects and any losses in the online extraction step. The method yielded MDLs in the range of 1.2-9.7 (except for danofloxacin), 2.2-15, 5.5-63 ng/L in deionized water, surface water and reclaimed waters, respectively and hence has the potential to measure analytes at their environmental concentrations. The method accuracy in spiked river and reclaimed samples ranged from 50-150% for the studied antibiotics. Furthermore, the method was validated in terms of precision, accuracy and linearity. The chosen approach is suitable for both research and monitoring applications.

The method was applied to reclaimed waters (n=56, collected from sprinklers in FIU BBC campus), surface waters (n=43), ground waters (n=8) and drinking waters (n=54) collected from South Florida. In reclaimed waters, nalidixic acid, erythromycin, clarithromycin and azithromycin were detected in all the samples tested (26.9-453.2

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ng/L). Other most frequently detected antibiotics are trimethoprim, sulfamethoxazole and ofloxacin (19.3-604.9 ng/L). Occurrence of antibiotics in reclaimed water suggests that antibiotics can't be completely removed by traditional wastewater treatment process. Based on the levels detected mass loads of antibiotics released into the environment were estimated.

Surface water analysis showed that most of the canals tested were free of antibiotic residues. Among the ones tested positive for the occurrence of antibiotics, highest concentrations were found in Miami River canal (reaching up to 580 ng/L) and reasons could be of anthropogenic origin, drainage overflows and/or landfill leachates. Black Creek canal was also found to contain antibiotic residues at relatively higher concentrations (up to 124 ng/L) and it might be due to its close proximity to a sewage treatment plant and landfill.

Occurrence of antibiotics in both reclaimed and surface waters have prompted to check ground and drinking waters as the major ground water source Biscayne aquifer lies very close to the surface and easily prone to contamination. The number of ground water samples analyzed (n=8), and sampling locations may not be large enough to represent the whole Miami Dade ground water quality. However, the preliminary results showed that there were no traces of antibiotics detected in ground waters tested or may be the levels were lower than the method detection limits. Drinking water samples were collected (n=54) through student sampling and the analysis results showed the presence of erythromycin in 82% of the samples (n.d-66 ng/L). However, no traces of erythromycin were detected in the next set of samples collected using a stringent protocol (n=5).

The non-detection of antibiotics in some of the samples indicates that their concentrations were reduced to levels below method detection limits or that parent molecule has been transformed into metabolites. Benchtop Q-Exactive orbitrap mass spectrometer was used to identify the possible metabolites of antibiotics in reclaimed waters. A phase 1 metabolite of erythromycin was tentatively identified in full scan based on accurate mass measurement. Using extracted ion chromatogram (XIC), high resolution data-dependent MS/MS spectra and metabolic profiling software the metabolite was identified as desmethyl anhydro erythromycin with m/z 702.4423. The proposed structure was confirmed based on MS/MS fragmentation information and published literature. With the knowledge that erythromycin and anhydroerythromycin previously detected in environmental waters, this was the first known report on desmethylated erythromycin metabolite in reclaimed waters.

Finally, all the antibiotics detected in reclaimed and surface waters were investigated to assess the potential risk to the aquatic organisms. The surface water antibiotic concentrations that represented the real time exposure conditions revealed that the macrolide antibiotics, erythromycin, clarithromycin and tylosin along with quinolone antibiotic, ciprofloxacin were suspected to induce high toxicity to algae and bacteria. Preliminary results showing that, among the antibiotic groups tested, macrolides posed the highest ecological threat, and therefore, they may need to be further evaluated with, long-term exposure studies considering bioaccumulation factors and more number of species selected.

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