Prevalence and fate of antibiotics and its derivatives in sewage treatment in Durban and the receiving environment

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ABSTRACT

Antibiotics are released to the environment either directly in an unchanged form or partially metabolized. The discharge is usually through untreated waste or through wastewater treatment effluents. The stable antibiotics in reduced amounts persist through the wastewater treatment processes and end up in receiving waters, where they may impact crops through irrigation or affect drinking water intakes. Antibiotics in the waste and sludge fractions may similarly impact crops and arable land through their use as fertilizers.

Conventional wastewater treatment plants are not designed for the removal of antibiotics but may to a varying extent reduce their concentrations. Their quantitative occurrence within the water matrices depends on the frequency and quantities of use for therapeutic purposes or as growth promoters in animal production. Additional inputs may emanate from individual waste discharges. Antibiotics present in sub-inhibitory concentrations may predicate for resistance among the resident bacteria in the water matrix, biofilms or in humans and animals.

In South Africa antibiotics are extensively used both in human therapy and in animal husbandry without clearly followed regulations and are sometimes readily available. The available studies have focused on the presence of antibiotic resistant bacteria in wastewater influent and effluent but there is a paucity of information relating to these antibiotics as emerging contaminants in South Africa wastewater. In this thesis a rapid and sensitive analytical methodology was initially assessed and applied, based on the use of HPLC/diode array UV detector for six antibiotics (ethionamide (ETI), metronidazole (MET), trimethoprim (TRI), ciprofloxacin (CIP), sulfisoxazole (SUF) and albendazole (ALB). Validation of the method was performed by screening assessment in selected wastewater treatment plants (WWTPs) with the aim of determining the sensitivity of the equipment (Shimadzu 2020), assess the limit of detection, optimize the extraction procedure (solid phase extraction) and screen for the most prevalent

antibiotics. The percentage recovery for the optimized method using wastewater sample was above 65 % for all antibiotics of interest. The limit of detection, which ranges from 0.03 to 0.48 mg L^{-1}, enables the determination of a range of concentration of antibiotics in polluted sample such as the wastewater influent sample.

Furthermore in this thesis, a more advanced, online solid phase extraction – high performance liquid chromatography mass spectrometry (SPE-HPLC-MS) method, was applied to measure the concentration of these and an additional seven antibiotics, norfloxacin (NOR), ofloxacin (OFL), clindamycin (CLI), sulfamethoxazole (SUL), erythromycin (ERY), clarithromycin (CLA), azithromycin (AZI) and roxithromycin (ROX) in ng L^{-1} concentrations. The quantity and occurrence of the selected antibiotics was assessed in untreated wastewater in four wastewater treatment plants in Durban, KwaZulu-Natal (KZN), at different treatment stages and in the effluent and recipient surface water environment. In the influent the additive concentration of the antibiotics associated to the separated sediment fraction through centrifugation and in the supernatant of samples collected were accounted for and analyzed. The limit of detection (LOD) and the limit of quantification (LOQ), ranged from 0.07 – 0.33 ng L^{-1} and 0.23 – 1.09 ng L^{-1}, respectively for the 13 assessed antibiotics and the percentage recovery were in the range of 51 to 111 %. The percentage of antibiotics recovered from the sediment (centrifuged) samples, which would have been lost to filtration if not analyzed in parallel, were in the range of 2.6% – 97% (n = 32), while the frequency of detection in the influent samples for the sampling period ranges from 62.5 – 100 % (n = 32). All the studied antibiotics were detected in the influent of each WWTP and the concentration was in the rage of 1.3 ng L^{-1} (AZI) – 81748 ng L^{-1} (CIP). The antibiotics with the highest concentrations (median) detected in the receiving water (downstream) for each of the four WWTPs in KZN, were TRI (217 ng L^{-1}), SUL (239 ng L^{-1}), CIP (708 ng L^{-1}) and ALB (325 ng L^{-1}) respectively. The overall percentage removal efficiency for the four WWTPs ranged from 21 % - 100 %. The most effective treatment steps were assessed

with the focus on activated sludge filter and trickling filter. Within these, it was actually the sedimentation treatment stages (secondary clarifier), after these steps that played the most vital role in the reduction of antibiotics where > 70 % of the antibiotics was removed. Finally, the impact of post chlorination was analyzed for the effluent of the WWTPs. The presence of transformation product as a result of post chlorination was examined in a parallel study using a controlled experiment and full scale analysis. The efficiency of chlorine in the reduction of antibiotics was more of transformation of antibiotics than degradation. The oxidative ability of chlorine enhances its reaction with antibiotics thereby transforming the antibiotics. The percentage reduction of antibiotics in relation to chlorination was >85 % (pilot experiment) and ranged between 14 % - 97 % in the field experiments. Likewise, UV was effective in the degradation of antibiotics, with longer exposure time producing higher degradation. Future research should focus on determining the toxicological impact of these transformation products. The concentration of the antibiotics in the downstream samples were generally low when compared to their influent concentrations.

Keywords: Antibiotics, wastewater, chlorination, solid-phase extraction, HPLC, UV, MS/MS

DECLARATION BY STUDENT

I declare that this thesis, submitted for Doctor of Philosophy in Chemistry at the Durban University of Technology, is the original work of the author and has not been submitted for a degree at any other University. Where use is made of any author's work, it has been duly acknowledged.

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PUBLICATIONS AND CONFERENCES

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Abstracts of published and In press papers are attached in Appendix 3

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Prevalence and fate of antibiotics and its derivatives in sewage treatment in Durban and the receiving environment

DEDICATION

This thesis is dedicated to my parents Pa and Ma R A Faleye for their words of encouragement, constant prayers and financial support. I would also like to dedicate this thesis to my loving sweet wife Oluwabanke and our gallant boys Oluwadarasimi and Oluwademilade, for their understanding, support and unconditional love.

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LIST OF ABBREVIATIONS

µg L^{-1}	**Microgram per litre**
µL	**Micro litre**
^{0}C	**Degree Celsius**
ACD	**Advanced chemistry development**
ACN	**Acetonitrile**
AHD	**Anti-helminthic drugs**
AIDS	**Acquired immunodeficiency syndrome**
ALB	**Albendazole**
ARB	**Antibiotic resistant bacteria**
AS	**Activated sludge**
AZI	**Azithromycin**
CIP	**Ciprofloxacin**
CLA	**Clarithromycin**
CLI	**Clindamycin**
CLOGP	**Calculation of partial coefficient**
DAD	**Photodiode array detector**
DID	**Define Daily Dose per 1000 Inhabitants per Day**
DLLME	**Dispersive liquid–liquid micro-extraction**
DLLME-HPLC-FD	**Dispersive liquid-liquid micro extraction – high performance liquid chromatography fluorescence detection**
DLLME-LC-UV	**Dispersive liquid-liquid microextraction– liquid chromatography – ultraviolet light detection**

DLLME-UHPLC-MS/MS	**Dispersive liquid-liquid microextraction – ultra high performance liquid chromatography tandem mass spectrometry**
DNA	**Deoxyribonucleic acid**
DWTPs	**Drinking water treatment plants.**
ERY	**Erythromycin**
ESI/APCI	**Electrospray ionization and atmospheric pressure chemical ionization**
ETI	**Ethionamide**
FA	**Formic acid**
FDCT	**Fixed dose combination therapy**
FQ	**Fluoroquinolone**
G	**Grit removal**
g mol^{-1}	**Molecule mass**
HF-LPME	**Hollow-fibre liquid phase micro-extraction**
HF-LPME- HPLC-DAD/FD	**Hollow-fibre liquid phase microextraction – high performance liquid chromatography – diode array detection tandem fluorescence detection**
HF-LPME- HPLC-UV	**Hollow-fibre liquid phase microextraction – high performance liquid chromatography – ultraviolet light detection**
HF-LPME-LC-QqQ-MS/MS	**Hollow-fibre liquid phase microextraction – ultra liquid chromatography triple-quadrupole tandem mass spectrometry**

HF-LPME-UHPLC-MS/MS	**Hollow-fibre liquid phase microextraction – ultra high performance liquid chromatography tandem mass spectrometry**
HIV	**Human immunodeficiency virus**
HLB	**Hydrophilic and lipophilic balance**
HPLC	**High performance liquid chromatography**
HTH	**High Test Hypochloride**
Kow	**n-Octanol-water partition coefficient**
kV	**Kilo volt**
KZN	**KwaZulu-Natal**
LC	**Liquid chromatography**
LC-MS/MS	**Liquid chromatography coupled to tandem mass spectrometry**
LLE	**Liquid-liquid extraction**
LOD	**Limit of detection**
Log P	**Partition coefficient**
LOQ	**Limit of quantification**
LPME	**Liquid-phase micro extraction method**
m/z	**Mass to charge ratio**
MA-DLLME- HPLC-UV	**Microwave assisted dispersive liquid-liquid micro extraction – high performance liquid chromatography – ultraviolet light detection**
MAS	**Microwave assisted extraction**
MDL	**Minimum detection limit**
MDR	**Multidrug resistant**

MeOH	**Methanol**
MET	**Metronidazole**
mg mL^{-1}	**Milligram per millilitre**
mW/cm^2	**Microwatt per square centimetre**
MIC	**Minimal inhibitory concentration**
MIP	**Molecular imprinted polymers**
MIP-HPLC-DAD	**Molecular imprinted polymer high performance liquid chromatography diode array detection**
MIP-HPLC-MS/MS	**Molecular imprinted polymer high performance liquid chromatography tandem mass spectrometry**
MISPE-FI-CL	**Molecularly imprinted polymer solid phase extraction – flow-injection chemiluminescence**
mJ/cm^2	**Millijoule per square centimetre**
ML d^{-1}	**Mega litre per day**
MMHSPE	**Magnetic mixed hemimicelles solid phase extraction**
MMIP	**Magnetic molecular imprinted polymer**
MPa	**Mega Pascal**
MPs	**Magnetic particles**
MSPE	**Magnetic solid phase extraction**
MSPE-HPLC-AD	**Magnetic solid phase extraction – high performance liquid chromatography – amperometric detection**

MSPE-HPLC-UV	**Magnetic solid phase extraction – high performance liquid chromatography – ultraviolet light detection**
NA	**Not available**
NaOH	**Sodium hydroxide**
ND	**Not detected**
ng L^{-1}	**Nanogram per litre**
nm	**nanometre**
NOR	**Norfloxacin**
OFL	**Ofloxacin**
ONLINE-MISPE-LC-FLD	**Online molecular imprinted solid phase extraction – liquid chromatography fluorescence detection**
PDMS	**Polydimethylsiloxane**
pKa	**Acid dissociation constant**
PLE	**Pressurized liquid extraction**
PNEC	**Predicted no effect concentration**
QqQ	**Triple quadrupole mass spectrometer**
RBF	**Rotating biological filters**
ROX	**Roxithromycin**
RP	**Reverse phase**
RT	**Retention time**
S	**Screening**
SCP	**Solvent consumed per sample**
SD	**Standard deviation**

SED	**Sedimentation**
SPE	**Solid phase extraction**
SPE-HPLC-DAD	**Solid phase extraction – high performance liquid chromatography – diode array detection**
SPE-HPLC-MS/MS	**Solid phase extraction – high performance liquid chromatography tandem mass spectrometry**
SPE-LC-MS/MS	**Online solid phase liquid chromatograph tandem mass spectrometry**
SRM	**Selected reaction monitoring**
SRM	**Selected reaction monitoring mode**
SRT	**Solid retention time**
SUF	**Sulfisoxazole**
SUL	**Sulfamethoxazole**
TB	**Tuberculosis**
TDR	**Total drug resistance**
TDS	**Total dissolved solid**
TRI	**Trimethoprim**
UV	**Ultra violet**
WRP	**Wastewater reclamation plant**
WWTPs	**Wastewater treatment plants**
XDR	**Extensively drug resistant**
YSI	**Yellow Spring instrument**

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

The environmental presence of emerging contaminants such as personal care products and pharmaceuticals are of high importance that needs global attention. The occurrence and fate of these contaminants both in the soil and aquatic environments have been the focus of several articles but mainly from developed countries (Peng et al., 2008, Watkinson et al., 2009, Luo et al., 2011, Li and Zhang, 2013, He et al., 2015, Neyestani et al., 2016, Inreiter et al., 2016). These studies include detection of pharmaceuticals in rivers, as well as their occurrence in influent and effluent water from wastewater treatment plants. The presence of these compounds in the environment create a haven for antibiotic resistant organisms (Zhang and Geißen, 2010, Luo et al., 2014, Li, 2014). The development of antibiotics over the past eight decades has been one of the medicinal chemistry's greatest success stories, especially in the curative usage against infectious diseases. While mortality and morbidity due to microbial infections have declined markedly in the developed world, the impact from infections on human health are still common in developing countries (Walsh and Warren, 1980, Müller and Krawinkel, 2005, Karlsson et al., 2014).

The focus of pharmaceuticals in this study will be on antibiotics in water bodies. Among the wide variety of pharmaceutical compounds, antibiotics are of special significance. Antibiotics are consumed to combat bacterial related disease, and a significant fraction of some antibiotics can pass the body unchanged as its active ingredient, which may retain their chemical activity in the environment (Manzetti and Ghisi, 2014). As a result of this most of the antibiotics consumed ends up in sewer systems either directly or indirectly, (Kümmerer, 2010). Unconsumed antibiotics are sometimes discarded directly to the sewer system. In addition, these compounds are released into the environment from other anthropogenic sources in

addition to wastewater treatment plants, which reflect their use among humans, in veterinary medicine and as growth promoters. Their extensive use has contributed to the development of antibiotic resistant bacteria (ARB). Their presence is of major concern and they have repeatedly been reported in many environmental niches, such as natural rivers, hospital effluents, wastewater treatment plants (WWTPs) and lakes around the world including South Africa (Laxminarayan et al., 2013, Odjadjare et al., 2012).

In a global perspective, South Africa has a relatively high consumption of different antibiotics. A report on the mean antibiotics sales per year between 2002 and 2004 are available, with a record of 1 540 tons of active compounds being sold (Henton et al., 2011). The consumption reflects a high prevalence of diseases in the human population among which a large proportion are poor individuals and those living in informal settlements. Here the consumption is also a consequence of secondary diseases, due to a high proportion of HIV infected individuals or other conditions lowering the individual immune response (Klatt et al., 2013). Due to this high consumption a proportion of multidrug resistant organisms have developed. The KwaZulu-Natal region of South Africa with the highest HIV incidence in the country is one of the focus areas in these regards. The antibiotic consumption is assumed to be reflected in their occurrence and quantities in the water of wastewater treatment plants that can function as 'hot-spots' and as means to measure and quantify the occurrence of emerging pollutants. Therefore, selected antibiotics in wastewater treatment plants were the focus for this study as well. The relevance related to different diseases in the region was governing the selection of compounds in focus for this study. Eight antibiotics were primarily of interest for a screening assessment. Out of these, six were further chosen for more in depth studies. These include anti-tuberculosis (ethionamide), respiratory infection antibiotics and generic bacterial candidate antibiotics including some used to treat diarrhoea (trimethoprim, sulfisoxazole, ciprofloxacin) and

antiprotozoal ones (metronidazole and albendazole). Furthermore, with the use of a more sensitive equipment, liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS), (at Umeå University Sweden) seven other antibiotics were added to the list, which includes norfloxacin, ofloxacin, clindamycin, erythromycin, clarithromycin, azithromycin, roxithromycin and sulfamethoxazole to replace sulfisoxazole making thirteen antibiotics altogether. The rationale of selection of the antibiotics is based on the rate of consumption in South Africa (Sarmah et al., 2006, Van Boeckel et al., 2014). Tuberculosis (TB) remains one of the infectious killer worldwide and the leading cause of death among people living with the human immunodeficiency virus (World Health Organisation, 2011, Dye et al., 1999, Kaufmann and Parida, 2008). The growing prevalence of highly resistant strains is the greatest threat of the global TB control. Currently South Africa has the world's third highest burden of TB, with the province of KwaZulu-Natal being the most affected by both drug-susceptible and drug-resistant tuberculosis. Over 100,000 cases of TB are reported every year from this province alone and over 60% are also infected with HIV (K-RITH, 2014). This report shows that higher amounts of anti-tuberculosis drugs are consumed in KwaZulu-Natal than in other parts of South Africa. Pneumonia is also a common illness affecting approximately 450 In South Africa, the morbidity and mortality of pneumonia increased due to the large number of immunocompromised in the population with a more pronounced effect on children below the age of five yrs. Pneumonia ranks as number one in mortality in South Africa (Siemieniuk et al., 2011, Zar et al., 2001). Ampicillin, amoxicillin and erythromycin are among the preferred drugs for the treatment of pneumonia but, resistance to these drugs are developing (Tzialla et al., 2015). In spite of this, they are being used largely. Diarrhoea is common in South Africa, is closely linked to the socio-economic status, and has the most adverse effects in South Africa's impoverished communities. South African children living in poverty are approximately ten times more likely to die from diarrhoea than their more privileged counter-

parts. Poor nutritional status, poor environmental conditions, and illnesses such as HIV/AIDS make children more susceptible to severe diarrhoea. Episodes of persistent diarrhoea also worsen a child's condition and nutritional status. In Africa, the use of metronidazole commonly referred to as Flagyl to combat the bacteria *Clostridum difficile* and parasitic protozoan infections, that causes diarrhoea is on the increase with the population growth (Chola et al., 2015). Albendazole is similar to this drug and is used for the treatment of multicellular parasitic worms (helminths) infection in animals. Considering both the number of animal husbandry farms in the KwaZulu-Natal Province and the reports of antihelmintic resistance in South Africa (Van Wyk et al., 1999) , the probability of having a noticeable concentration of the drugs in wastewater or their receiving water/sediment is high. Information about the presence of these drugs in the South African wastewater environment is scanty (Krogh K A et al., 2008, Brewer et al., 2004). The number of reports that examine or monitor the occurrence and quantities of antibiotics in environmental samples in South Africa are few. Most studies that has been done are focused on the bacterial resistance due to the presence of antibiotics in the effluent receiving river and environment (Lin et al., 2004, Skórczewski et al., 2013). Matongo et al., (2015) and Agunbiade and Moodley, (2014) are the first that have done thorough analysis of pharmaceuticals, antibiotics included, in the recipient river water and sediments in KwaZulu-Natal Province of South Africa (Matongo et al., 2015, Agunbiade and Moodley, 2014).

There are several methods that are used for the determination of antibiotics in wastewater and sediment, which involve solid phase clean up procedures (K'oreje et al., 2012, Dorival-García et al., 2013, He et al., 2015). These methods are largely dependent on the antibiotic types (the chemical composition and structure) involved, the antibiotics nature (hydrophilicity and lipophilicity) and the media (water or sediment) from which the antibiotic is being determined.

Most WWTPs are not built to eliminate antibiotics from wastewater. Instead, the treatment can actually change the nature of the antibiotics by the reaction of the antibiotics with the chemicals used in the treatment of wastewater. The derivatives formed from this reaction may create a more toxic effect than its real natural form (Jelic et al., 2011). In developing countries, one important wastewater treatment process is post-chlorination of final effluent water, which is aimed at disinfecting wastewater of all pathogens. It is usually the last treatment applied in a wastewater treatment plant before the treated effluent is released into the environment. There are other common disinfectants like ozone (O_3) and ultraviolet light, but chlorine is mainly used due to the cheap price and easy availability of chlorine gas (Radjenović et al., 2009, Chamberlain and Adams, 2006). The effect of post chlorination on wastewater is usually to reduce microorganism in the final effluent and not much attention has been given to the generation of chlorinated organic compounds that may be carcinogenic or harmful to the biotic communities in the environment (Watson et al., 2012). Some studies have dealt with the effect of post-chlorination on antibiotics and likely impact on the environment but the information is scarce (Shi et al., 2013, Li and Zhang, 2013, Postigo and Richardson, 2014, Soufan et al., 2012). No studies are currently available in South Africa, to the best of my knowledge. The effect of post-chlorination on antibiotics, including reduction of antibiotics concentration and possible formation of derivates are necessary for further scientific research. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with triple quadrupole (QqQ) analyses is nowadays one of the most advanced technique of choice for trace analysis of pharmaceuticals due to the high selectivity and sensitivity achieved in selected reaction monitoring (SRM) mode. LC-MS/MS has been commonly used for multi-class determination of pharmaceutical compounds, normally including only a few metabolites in the target list of analytes.

The aim of this study is to investigate the prevalence and fate of antibiotics and possible derivatives in sewage treatments and the receiving environment and ensure a correct assessment of influent concentration. This involves the analysis of both the sediment (centrifuged) and supernatant from the centrifugation of influent samples.

To achieve the aim, the research was conducted as follows:

1. A screening assessment for the presence of antibiotics in wastewater treatment plant was done. This was further linked to method validation related to sensitivity and extraction.
2. The quantity and occurrence of the selected antibiotics in untreated wastewater in four wastewater treatment plants in KwaZulu-Natal (KZN) was assessed. The plants represent different treatment configurations and the connected population varies from a socio-economic perspective.
3. The reduction efficacy in removing the studied antibiotics in the wastewater treatment plant accounting for the treatment stages of the wastewater treatment process was examined.
4. Reduction impact of UV and post chlorination on antibiotics in wastewater was examined.
5. Assess the possibility of derivate being formed as a result of chlorination by-products and the spread pattern of antibiotics/derivatives from the wastewater treatment plant (source) to the downstream of the effluents.

The novelty and contribution to the body of knowledge with regards to this research are based on the following reasons,

1. Analysis of antibiotic in wastewater treatment plants, where the choice of the plant partly reflects different treatment configurations and socio-economic strata, has only been performed to a limited extent in Africa.
2. Analysis of antibiotics in wastewater sample, with focus on the liquid part (supernatant) and the semi/liquid part (sediment) of the same sample at the same time has not been researched.
3. Antibiotics loss as a result of sample treatment step (filtration) in most wastewater analysis was elucidated.
4. The efficiency of the application of chlorination towards the removal/reduction of antibiotics in wastewater has not been investigated in Africa.
5. The derivatives formed as a result of post chlorination has not been investigated anywhere in Africa.
6. The use of ultraviolet light for the removal/reduction of antibiotics in wastewater has not been investigated in Africa.

CHAPTER 2: LITERATURE REVIEW

2.1 ANTIBIOTIC RESIDUE IN THE AQUATIC ENVIRONMENT: CURRENT STATUS IN AFRICA

Antibiotics are chemical, which are categorised into three main groups: natural, semi-synthetic and synthetic. They possess either the ability to impede bacterial growth (bacteriostatic) or to kill them (bactericidal) (Korzybski et al., 2013) and are used in prophylaxis and therapeutics of bacterial induced diseases in both humans and animals. In Africa, diseases, such as, tuberculosis, pneumonia and diarrhoea are rampant because of immunocompromised conditions predicated by HIV infection (among others), poor sanitation and general hygiene and drug abuse (Middelkoop et al., 2015, Shisana et al., 2014, Sulieman et al., 2014). An increase in the consumption of antibiotics has occurred in order to cure the diseases (Middelkoop et al., 2015, Byass, 2014, Van Boeckel et al., 2014, Hart and Kariuki, 1998), partly without any physician's prescription. Antibiotics are also used extensively in animal farming to maintain the high demand for animal products (Jechalke et al., 2014, Jobbins and Alexander, 2015, Van Boeckel et al., 2015). The high antibiotic consumption in humans and in farm animals (Van Boeckel et al., 2014, Boyles et al., 2017) result in increasing release of partially metabolized and unchanged active ingredients antibiotics into the aquatic environment through wastewater (Fick et al., 2015). Most of the antibiotics consumed ends up in the sewer system either directly or indirectly, since they are only partially metabolised (Korzybski et al., 2013, Milić et al., 2013, Manzetti and Ghisi, 2014) or broken down with the active component still intact (Kümmerer, 2010). Indirect discharge of antibiotics is as a result of unintentional release (via excretion) of consumed antibiotic into the environment. Unconsumed antibiotics are sometimes discarded directly via the sewer system. They are partially degraded but the active components continue to impact on the environment, which will eventually enhance

resistance in bacteria or may have eco-toxicological effects because of high concentration (Hidalgo, 2001, Zhang et al., 2008, Aiken et al., 2014).

2.2 OCCURRENCE OF ANTIBIOTICS IN THE AQUATIC ENVIRONMENT

Wastewater treatment plants (WWTPs) receive most of the partially metabolised antibiotics from humans via the sewer system, while the rest are either directly dumped into the nearby streams and rivers or escape as seepages from, for example, landfill sites (Tadesse, 2004). General pathways of introduction of antibiotics into the environment are presented in Figure 2.1.

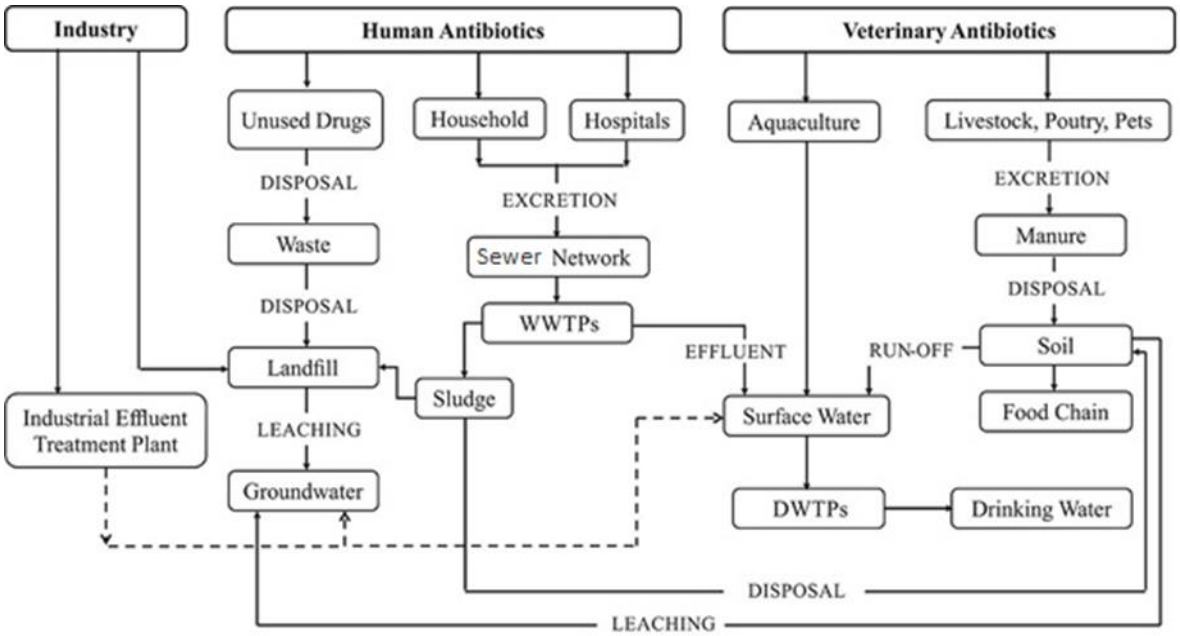

Figure 2.1: Pathways of antibiotics into the environment (Frade et al., 2014)

DWTPs = Drinking water treatment plants.

The figure illustrates the impact of the general society and the eventual contamination of the surface water, which in the end affects both drinking water quality and irrigation of crops. Most WWTPs are not configured to remove antibiotics; hence, the antibiotics are released to the aquatic environment from the final treated effluent, making the WWTPs a main hot spot for environmental impact (Milić et al., 2013, Jelic et al., 2011). In animal husbandry, manure generated by farm animals are frequently reused as organic fertilizers in farm land and may partially end up in environmental waters through runoff. These waste components contain antibiotics (Baguer et al., 2000) and are major sources of increase in the concentration of antibiotics in the aquatic system (Kemper, 2008) with attending public health intricacies.

The presence of antibiotics in the environment, most especially in the aquatic environment, has been confirmed in many reports as emerging contaminants around the world, with majority of reports outside of Africa (Hirsch et al., 1999, Kümmerer, 2009, Milić et al., 2013, Agunbiade and Moodley, 2014, Fick et al., 2015)**.**

2.2.1 Global detections of antibiotics

Globally, antibiotics are detected at varying concentrations in several environmental matrices such as surface waters and sediment. For example, Karthikeyan and Meyer (2006) detected six different antibiotics in raw wastewater in USA. Among these, sulfamethazine was reduced from maximum concentration of 0.21 µg L^{-1} in the influent to below detection limit in the effluents. Sulfamethoxazole with an influent maximum concentration of 1.25 µg L^{-1} was reduced to 0.37 µg L^{-1} in the effluent. The remaining four were tetracycline, ciprofloxacin, erythromycin and trimethoprim, with their concentrations in the influent and effluent ranging between 0.21- 1.30 µg L^{-1} and 0.85 – 0.14 µg L^{-1}, respectively (Karthikeyan and Meyer, 2006). In a wastewater treatment plant in China, Zhou et al. (2013) detected 20 antibiotics in the

influent and 17 in the effluent samples, out of which sulfamethoxazole, norfloxacin, ofloxacin, erythromycin and trimethoprim were the most frequent. The total load of antibiotics per capita in one of the plants investigated ranged from around 500 to 900 μg/d/inhabitant (mean value 670 μg/d/inhabitant) in the influent samples. The corresponding values in the effluent varied from around 130 to 240 μg/d/inhabitant (mean 175 μg/d/inhabitant) (Zhou et al., 2013). Antibiotics have also been detected in drinking water and purified tap water. This is exemplified in a survey of Austrian drinking water in 2014, where sulfamethoxazole was detected at a concentration within a range of 4.4 -8.9 ng L^{-1} among many others (Inreiter et al., 2016). Likewise, the presence of seven sulfonamides, trimethoprim, and four macrolides in 37 rivers in Japan was reported (Murata et al., 2011) and the concentrations were in the range from "not detected" to 630 ng L^{-1} with a median of 7.3 ng L^{-1}. Other reports include different levels of detection of antibiotics in soil and sediment (Jechalke et al., 2014, Krogh K A et al., 2008, Gibs et al., 2013). Five antibiotics were also detected in plant tissues (chlortetracycline, monensin, sulfamethazine, tylosin, and virginiamycin) with concentrations of <10 μg kg^{-1} (Kang et al., 2013).

The adaptation of bacteria to the presence of antibiotics in the environment may lead to emergence of resistance (McEwen and Fedorka-Cray, 2002) as well as selection for antibiotic resistance genes which can be transferred to other bacteria via horizontal gene transfer (Rizzo et al., 2013). This contributes to the increase in the occurrence of antibiotic resistant bacteria (ARB) globally (Pruden et al., 2013) as exposure to sub-lethal concentrations of antibiotics or its derivatives may induce resistance (Gillings, 2013). The occurrence have been reported in wastewater, rivers and drinking water in China (Jiang et al., 2013), Europe (Böckelmann et al., 2009) , Australia (Bruce et al., 2010), America (Brinklov et al., 2009) and Africa (Mitema and Kikuvi, 2004). The concentration of antibiotics in the environment are generally low (in μg L^{-1}) and mostly non-toxic. The degree of toxicity can be determined based on the Predicted no

effect concentrations (PNECs) of the antibiotics on the bacteria in the environment (Pruden et al., 2013). This non-toxic concentration has been identified as an impetus in the development of antibiotics resistance bacteria which is linked to the minimal inhibitory concentration (MIC) (Bengtsson-Palme and Larsson, 2016). It has also been established that concentration of antibiotics below the MICs may aid the development of resistance in bacteria (Gullberg et al., 2014, Gullberg et al., 2011).

Adverse effects of the presence of antibiotics in aquatic environment , most especially on fish have been reported (Corcoran et al., 2010). One of the effects, is the suppression of fish immune system by the presence of tetracycline (0.1 – 50 µg L^{-1}) in environment (Grondel JL et al., 1985). Norfloxacin and sulfamethoxazole at a concentration of 200 µg L^{-1}, has also been reported to have an adverse effect on the growth and reproduction rate of Zebrafish (Yan et al., 2016). There is a need to monitor and study their release and persistence in the environment.

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2.2.2 Removal of antibiotics from wastewater

The presence and fate of antibiotics in raw wastewater and treated sewage effluents (Manzetti and Ghisi, 2014, dos Santos Junior et al., 2014) are sometimes measured in line with national priorities. In Africa, just a few scattered reports are available, despite the assumed high level of consumption of antibiotics because of high prevalence of infectious diseases (Agunbiade and Moodley, 2014, Matongo et al., 2015, Middelkoop et al., 2015, Van Boeckel et al., 2014). Most studies have focused on the prevalence of antibiotic resistant bacteria in sewage effluents, drinking water and water bodies, with no recourse to inducing factors in relation to the presence of antibiotics in such environments (Alonso et al., 2017, Mandomando et al., 2010). This leaves an information gap about antibiotics as a potential inducer of bacterial resistance.

In further parts of this review chapter additional overview on available reports of the concentration of antibiotics in the aquatic environment in Africa, their limitations and the need for continuous monitoring of the concentration of the antibiotics discharged into the aquatic environment in relation to antibiotic resistant pathogens are discussed.

2.2.3 Origin of antibiotics into the environment

Antibiotics in the environment originate from various sources. The human/animal route, socio-demographic route impact and other impacts are described in the following sections.

2.2.3.1 Human/animal health consumption route

The human consumption of antibiotics is directly related to the residuals released through sewage and waste products discarded into the environment. Based on the level of disease resistance in Africa, it was assumed that the level of antibiotic consumption is high (MacKenzie and Gould, 2005).

The information related to the consumption of antibiotics in African countries are scattered and limited. South Africa and Kenya have a partial record of the amount of antibiotics consumed by their population (Boyles et al., 2017, Mitema and Kikuvi, 2004). This record is the most comprehensive in Africa. Other reports from northern and western Africa are scattered (Van Boeckel et al., 2014). A report on the global consumption of veterinary related antibiotics with a view on Africa has been documented, giving detailed information on available data on consumption from Kenya only. The report with reference to Mitema et al., 2001, reveals that between 1995 to 1999, tetracyclines, sulfonamides and trimethoprim were the ones mostly consumed (Mitema et al., 2001). In South Africa the quantities of antibiotics prescribed in the

private health sector was compiled in a Master Thesis (Agyakwa, 2014) with a focus on the consumption of fluoroquinolones. The report represents a view on the high rate of consumption of antibiotics in parallel to the prevalent diseases in South Africa.

Detailed information on the use of antibiotics is important for the assessment of the potential concentration ranges that may be released into the environment. This has also been pointed out by the World Health Organisation (WHO) with emphasis on the measurements and monitoring of the volumes and patterns of use of antibiotics towards controlling their impact on the environment (World Health Organization, 2016).

In a report on the consumption of antibiotics based on the sales (Van Boeckel et al., 2014) the percentage per capita changes in antibiotics consumption between 2000 – 2010 was presented (Figure 2.2). This shows a global increase in antibiotic consumption spear-headed by low and middle income countries and with a major lack of data from major parts of Africa. Western Africa (French speaking) was considered as one unit and the detailed report from Kenya is missing on the map and also in the text of the report.

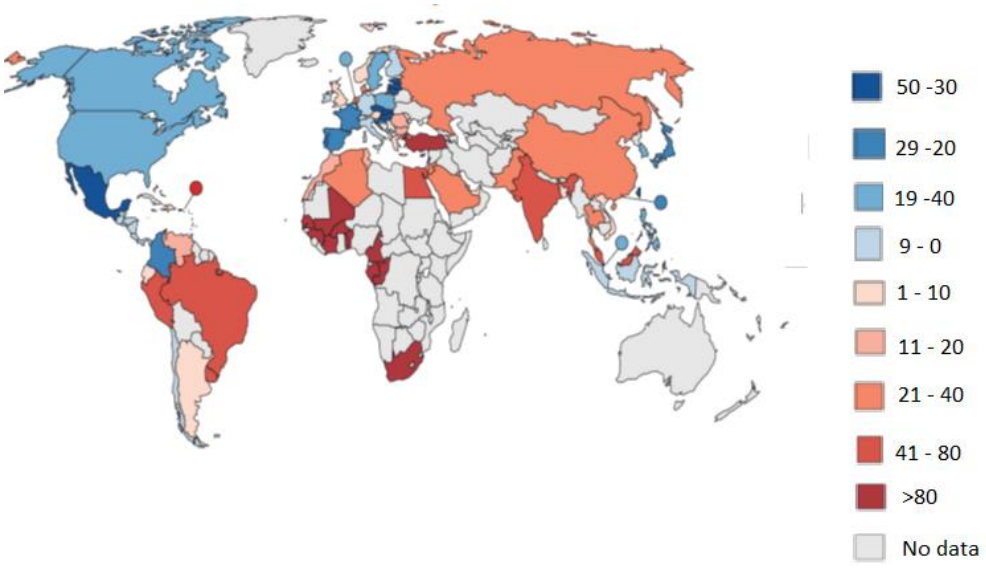

Figure 2.2: Percentage changes in antibiotics consumption per capita 2000 – 2010 by country. The blue colours indicate decreases while the rest are increases. Picture adapted from (Van Boeckel et al., 2014)

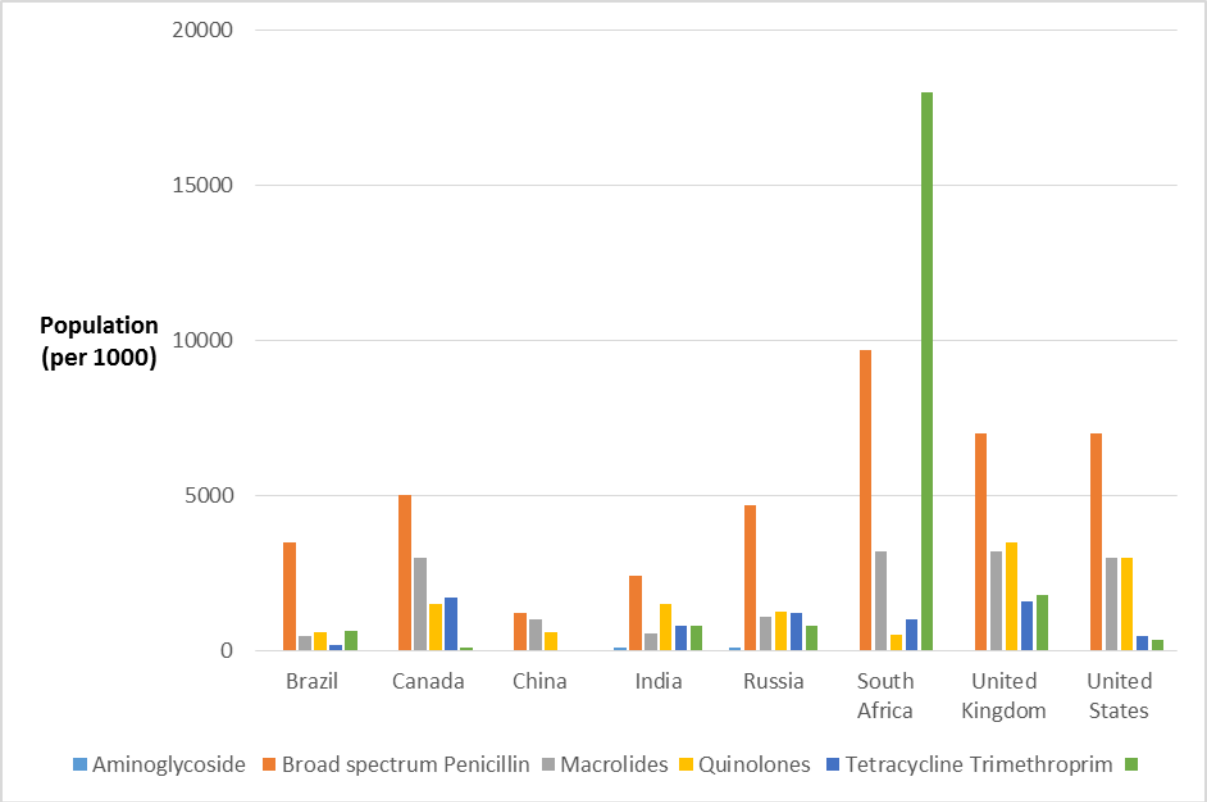

Figure 2.3: Antibiotics used globally in 2010 based on 1000/population (Gelband et al., 2015)

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Sale records of antibiotics used are fairly accurate in South Africa and can be used as an indication of the rate of consumption. The broad-spectrum penicillin are the most sold one, with 18.3 % of the antibiotics market followed by penems and carbapenems with 14% and macrolides with 11.1 % (IMS Health, January 2011.).

A report from IMS by Gelband et al., 2015, as shown in Figure 2.3 summarizes six groups of antibiotics (aminoglycoside, penicillin, macrolides, quinolones, tetracycline and trimethoprim) that are mostly used by humans globally. The use of trimethoprim and penicillin was highest in South Africa and this can be justified based on the pharmacological application of the antibiotics. Penicillin is a broad-spectrum antibiotic while trimethoprim is more specific and is used in the treatment of urinary infection. When trimethoprim is used with sulfamethoxazole, it can be for *Pneumocystis pneumonia* in people with HIV/AIDS (Sulieman et al., 2014), hence

this accounts for the high rate of sale, and assumed high rate of consumption in South Africa. The available published information of the use of antibiotics in veterinary medicine is also sparse or lacking for Africa, including South Africa. A report on the mean antibiotics sales per year between 2002 and 2004 are available (Henton et al., 2011). In South Africa around 1 540 tons of active compounds of five antibiotics were sold. Macrolides and pleuromutilins constituted the major parts of the sales, followed by tetracyclines, sulphonamides and penicillin (Henton et al., 2011).

The dependence on antibiotics for treatment of bacterial infections has increased over the years, while the bacteria are also constantly evolving in order to adapt to these drugs (Davies and Davies, 2010). This evolution of antibiotic resistance is possibly due to induction through exposure to residual or abused antibiotics. This has led to the development of more resistant bacteria associated with human immunodeficiency virus (HIV) (Hughes and Andersson, 2015). The scourge of HIV and tuberculosis (TB) remains of primary concern (Dye et al., 1999, Kaufmann and Parida, 2008, Sismanidis et al., 2014) in all reported regions of Africa (World Health Organization, 2013, Nourzad et al., 2017), with a direct link to the high rate of HIV infection. Due to a rising proportion of immunocompromised systems as a result of HIV prevalence, tuberculosis has dramatically increased the endemicity of the disease (Kaufmann and Parida, 2008). The world's third largest morbidity of TB is currently in South Africa, driven by HIV infection. In particular, the province of KwaZulu-Natal (KZN) alone accounts for more than 30 % of the reported cases. As a comparison the Northern Cape Province just had a little above 2.3 % as the least affected province in South Africa (Middelkoop et al., 2015). Out of the over 100,000 reported cases of TB per year in KZN, well above 60 % of the persons are infected with HIV (Bantubani et al., 2014). Invariably, this is associated with high consumption of anti-tuberculosis drugs such as isoniazid and ethionamide (DID, Define Daily

Dose per 1000 Inhabitants per Day of 2.41) (Agyakwa, 2014), which in parallel is related to the presence in the environment.

Lung infections caused by bacteria such as *Streptococcus pneumoniae* is most common in KwaZulu-Natal (Quinton and Mizgerd, 2015). When it occurs as a secondary infection to HIV, it may sometimes necessitate the use of multiple antibiotics, as single or fixed dose combination therapy (FDCT) to combat the infection. Nasab and Khosravani (2013) reported that approximately 450 million people are infected with pneumonia globally on a yearly basis. This is also a cause of child mortality with close to 2 million deaths yearly (Nasab and Khosravani, 2013). More than half of the global death record, as a result of pneumonia, occurs in Africa and pneumonia ranks as the highest killer infection in South Africa (Siemieniuk et al., 2011, Zar et al., 2001). For atypical polymicrobial pneumonia, several antibiotics are usually taken by patients before reporting into a hospital. Usually a high-dose amoxicillin (four g/day) or amoxicillin-clavulanate (four g/day), or a respiratory fluoroquinolone is the first line of approach (Metersky et al., 2007). However, combinations of antibiotics are used mostly in the treatment for over a minimum period of two weeks (Metersky et al., 2007).

2.2.3.2 Socio-demographic impact

Informal settlement communities in Africa do not always have access to direct potable water and are in many instances exposed to unhygienic domestic water and sanitation conditions. The underlying causes are improper sewage disposal or from on-site sanitation system or from open defecation, leading to contamination of water sources that later are used for domestic purposes. These life conditions, in addition to poor nutritional status, enhance the risk for different types of diarrhoeal infections (Chola et al., 2015). Diarrhoea can be self-regressive after a few days

without treatment (Chhagan et al., 2014) among the immune-competent, but may be persistent among the immunocompromised or immunosuppressed individuals. Diarrhoeal disease is prominent in South Africa among children (Chola et al., 2015) and it is also closely connected to HIV/AIDS. Persistent diarrhoea is usually treated with antibiotics such as metronidazole. The use of this drug is increasing within a growing population that lack basic social amenities (Chola et al., 2015).

The unchanged active ingredients and partially metabolized antibiotics in the environment (Pruden et al., 2013) may increase the development of antibiotics resistant microbes (Baquero et al., 2008, Jiang et al., 2013) and eco-toxic effects (Peltzer et al., 2017). Direct dumping of antibiotics into the sewer system also contributes to their increase in the environment. Hamjinda et al. (2015) reported that most of the oral drugs used by patients in hospitals are discarded into the sewer system, thereby contributing to the concentration of antibiotics in most hospital wastewater effluents (Hamjinda et al., 2015). Likewise, derivatives formed from wastewater treatment reactions may create a more toxic effect than its real natural form (Jelic et al., 2011).

Another important group of antibiotics are the anti-parasitic drugs. These are used to treat infections with multicellular parasitic worms (helminths) and unicellular parasites (protozoans). They are of major importance in both human tropical medicine and in veterinary medicine. The presence of the active component of anti-helminthic drugs (AHD) are well documented in animal tissues and products such as milk, liver, muscle and kidney (Haughey and Baxter, 2006, Luo et al., 2011, R Ballweber and A Baeten, 2012, Dasenaki and Thomaidis, 2015a) but less so from environmental sources.

Considering the high number of animal husbandry farms in many provinces, with reports of AHD resistance rampant in animal farms in South Africa (Van Wyk et al., 1999, Adegoke and Okoh, 2014), the probability of having a high concentration of these drugs in wastewater or

their receiving water/sediment is high. There is a significant risk that anti-helminthic agents may affect freshwater organisms negatively in the environment (Morley, 2009, Yoshimura and Endoh, 2005). These antibiotics are mostly entering the aquatic environment via wash off from grazing farmlands or from where animal manure are applied or stored (Zhang et al., 2014). In promoting the reasons for the widespread use of drugs to control human helminths, Cerami and Warren (1994) suggested that "helminths are less likely to develop resistance or would do so more slowly" compared to other infectious agents because they multiply at a lower rate (Cerami and Warren, 1994). This has certainly not appeared true for helminths in livestock or in the treatment of human helminth infections. It is also estimated that some 1.3 to 2.0 billion people in the world suffer from helminthic infections (Geerts and Gryseels, 2000, Pullan et al., 2014.). Drug resistant helminths may not be a major health issue yet, but may be of possible future environmental concern.

2.3 CURRENT LEVEL OF THREAT ASSOCIATED WITH ANTIBIOTICS' RESISTANCE

The rate of development of Multidrug Resistant (MDR) and Extensively Drug Resistant (XDR) bacteria is alarming and there is a need for a holistic approach towards the management of the crisis (Laxminarayan et al., 2013, Parida et al., 2015). Klopper et al. (2013) showed that tuberculosis bacteria are resistant to the available antibiotics, both with first and second line approaches (Klopper et al., 2013). Likewise, multi drug resistant pneumonia has been identified (Cullinan, 2014). In a recent study from South Africa on MDR tuberculosis, the empiric treatment approach (rudimentary treatment without consideration of the environment) in administration of drugs maybe lead to poor therapeutic outcome, thereby fuelling the

development of XDR (Bantubani et al., 2014). Different approaches towards the elucidation of the cause of the poor performance of the antibiotics in the treatment of tuberculosis seem to have been taken, except the role of residues of antibiotics on microbes in the environment. In relation to this, the possibility of the influence of resistant pathogens from the environment on the pathogens in an immunocompromised patient may be the reasons for the failure of the drug of approach. *Mycobacterium tuberculosis,* which is documented in wastewater, may get exposed to the antibiotic residue and develop resistance (Amha et al., 2017) to form XDR *M. tuberculosis.* Exposure of the XDR bacteria excreted into wastewater, with the wastewater already containing residual antibiotics in sub-lethal concentrations, might have contributed to the emergence of Total Drug Resistance (TDR). Intermittent surveillance of the residual antibiotics in the environment will contribute to the documentation of emergence of resistance and selection for resistance genes (Pruden et al., 2013).

Multiresistant pneumonia-causing bacteria has been reported in South Africa (Cullinan, 2014). Cases of gradual increase in threat level of resistance towards antibiotics in the treatment of different diseases have been reported and are exemplified in Table 2.1. Exposure of pneumonia bacteria to sub-inhibitory concentration of antibiotic residues in the environment may play a vital role in the emergence of the MDR.

Table 2.1: Some bacterial diseases and reported antibiotic resistant statues.

Diseases (causative bacteria)	Current stage of resistance to antibiotics	Drug of approach (percentage resistance)	Study location	References
Pneumonia (*Klebsiella pneumoniae*)	**MDR**	**Amoxicillin (99), Amox/clav (14), Cefuroxime (27), Cotrimoxazole (11)**	**Gauteng, Western cape, Eastern cape, Kwazulu-Natal, Mpumulanga, and Orange free state provinces in South Africa**	**(Liebowitz et al., 2003)**
Pneumonia (*Klebsiella pneumoniae*)	**MDR**	**Ampicillin (98), Cefuroxime (52) Ceftriaxone/cefotaxime (46) Cefepime (44), Co-amoxiclav (52) Piperacillin-tazobactam (40) Ciprofloxacin (31) Levofloxacin (32), Ertapenem (2) Imipenem (0), Meropenem (0)**	**Johannesburg, Pretoria, Durban, Cape Town and Bloemfontein all in South Africa**	**(Brink et al., 2007)**
Pneumonia (*Klebsiella pneumoniae*)	**MDR**	**Cefotaxime (68.3), Carbapenens (4.5), Piperacillin/tazobactam (33.1), Ceftazidime (68.3), Cefepime(68.3), Ciprofloxacin (46.5)**	**Gauteng, KwaZulu-Natal, Free State, Limpopo and Western Cape provinces of South Africa**	**(Perovic et al., 2014)**
Pneumonia (*Streptococcus pneumoniae*)	**MDR**	**Ampicillin (74), Chloramphenicol (55), Cotrimoxazole (66), Gentamicin (16), Chloramphenicol (35), Ampicillin plus gentamicin (14), Penicillin plus gentamicin (11), Chloramphenicol plus ampicillin (29).**	**Maputo Provinces in Southern Mozambique**	**(Mandomando et al., 2010)**

Pneumonia (*Streptococcus pneumoniae*)	**MDR**	**Penicillin (46), amoxicillin (7), Amox/clav (5), Cefuroxime (53), Azithromycin (61), Clarithromycin (61), Cotrimoxazole (51)**	**Gauteng, western cape, Eastern cape, Kwazulu-Natal, Mpumalanga, and Orange free state provinces in South Africa**	**(Liebowitz et al., 2003)**
Pneumonia (*Streptococcus pneumoniae*)	**MDR**	**Chloramphenicol (33), Clindamycin (10), Erythromycin (1.5), Gentamicin (85.2), Oxacillin (18.6), Tetracycline (50.8), Trimethroprime (89.4)**	**Lilongwe district Malawi.**	**(Makoka et al., 2012)**
Shigellosis a and b	**MDR**	**Ampicillin a (66.3), b (85.4) Chloramphenicol a (47.2), b (77.1) Streptomycin a (69.7), b (85.4) Sulfamethoxazole a (91.0) Trimethoprim a (94.4), b (75.0) Tetracycline a (60.7), b (100) Nalidixic Acid a (1.1), b (89.6) Ciprofloxacin a (0), b (0) b-lactamase based a (2.3), b (2.1)**	**All nine provinces in South Africa**	**(Keddy et al., 2012)**
Shigellosis	**MDR**	**Ciprofloxacin(0), Ceftriaxone (0) Co-Trimoxazole (83), Tetracycline (72), Ampicillin (26).**	**Gauteng, South Africa**	**(Agunbiade and Moodley, 2014)**
Shigellosis	**MDR**	**Chloramphenicol (52), Ampicillin (56), Tetracycline (66), Trimethoprim (84), Sulfamethoxazole (84).**	**Southern Mozambique, Mozambique.**	**(Mandomando et al., 2009)**

a = *S. flexneri* type 2a; b = S. *flexneri* type 1b

The resistance to antibiotics used in the treatment of pneumonia was compared between 2007 and 2014 in South Africa (Perovic et al., 2014) and a gradual increase was noticed. Cefotaxime increased from 46 % to 68 %, cefepime from 44 % to 68 % and ciprofloxacin from 31 % to 46

% (Perovic et al., 2014, Brink et al., 2007). This increase may not represent a general trend, but there is in fact an increase in the rate of resistance that should not be overlooked.

A study conducted on children under five years in Mozambique showed a steady build-up of resistance to all antibiotics used in treatment of diarrhoea caused by *Shigella* bacteria (Mandomando et al., 2009) (Table 2.1). In an extensive review on the antibiotic resistance of *Shigella* across Europe, Asia and Africa, it was reported that the rapidly rising trend of antibiotic resistance were similar, with resistance towards the second line of the antibiotics (Eseyin et al., 2016). One of the recommendations made at mitigating these increases in antibiotic resistance is to intensify research towards identification and removal of sub-lethal concentration of antibiotics or their active components from the environment, particularly in wastewater (World Health Organization, 2016).

2.4 PRESENCE OF ANTIBIOTICS IN AQUATIC ENVIRONMENT

Among African countries, South Africa is the country with the highest number of monitoring studies on antibiotics (Table 2.2). Still, antibiotics in the aquatic environment of South Africa has only been addressed in a limited number of studies and not directly been compared to the level of infectious diseases. Likewise globally, such comparisons are few. Only ten antibiotics, out of the numerous antibiotics consumed have been detected in two out of the nine South African provinces. Their concentrations and references are displayed in Table 2.2. The information from other African countries are even more limited. There are only 15 published studies having a limited amount of information, despite generally high sales/consumption on the African continent.

Table 2.2: Antibiotics and their detected maximum concentrations reported in the environment in Africa (the numbers of significant digits have been reduced from what was stated in the respective reference)

Sample sources	Study location	Antibiotics (Class)	Maximum Conc.	Literature
Treated sewage Effluents(µg L^{-1})	South Africa	Fluoroquinolones (Quinolones)	0.12	(Hendricks and Pool, 2012)
		Sulfamethoxazole (Sulfonamide)	0.15	
Surface water (µg L^{-1})	South Africa	Ampicillin (Penicillin)	16	(Agunbiade and Moodley, 2014)
		Ciprofloxacin (Quinolones)	15	
		Nalidixic acid (Quinolones)	23.50	
[a]Sediment (ng g^{-1})	South Africa	Ampicillin (Penicillin)	[a]467	(Agunbiade and Moodley, 2014)
		Ciprofloxacin (Quinolones)	[a]187	
		Nalidixic acid (Quinolones)	[a]104	
Surface water (µg L^{-1})/ [a]Sediment (ng g^{-1})	South Africa	Sulfamethoxazole (Sulfonamide)	7.3/[a]50	(Matongo et al., 2015)
		Erythromycin (Macrolide)	20/[a]16	
		Sulfamethazine (Sulfonamide)	33 / [a]ND	
		Metronidazole (Nitroimidazole)	ND / [a]63	
		Trimethoprim (Trimethoprim)	3.70 / [a]ND	
Surface water (µg L^{-1}) / [a]Sediment (ng g^{-1})	South Africa	Sulfamethoxazole (Sulfonamide)	6 /[a]< MDL	(Matongo et al., 2015)
		Erythromycin (Macrolide)	13.6/[a]<MDL	
		Sulfamethazine (Sulfonamide)	4.6 / [a]ND	
		Metronidazole (Nitroimidazole)	ND / [a]130	
		Trimethoprim (Trimethoprim)	0.80 / [a]92	
River water (µg L^{-1})	South Africa	Nalidixic acid (Quinolones)	3.0	(Gumbi et al., 2017)
Surface water (µg L^{-1})	South Africa	Azithromycin (Macrolide	0.03	(Archer et al., 2017)
		Clarithromycin (Macrolide	0.30	
		Sulfamethoxazole (Sulfonamide)	1.31	
		Sulfasalazine (Sulfonamide)	0.07	
		Trimethoprim (Trimethoprim)	1.20	
River water/ Wastewater (µg L^{-1})	Kenya	Sulfamethoxazole (Sulfonamide)	14 / 3	(Ngumba et al., 2016)
		Ciprofloxacin (Quinolones)	0.50 / 0.07	
		Trimethoprim (Trimethoprim)	2.70 / 0.07	

River water/ Wastewater (µg L^{-1})	**Kenya**	**Chloramphenicol (Amphenicol)**	**0.70 / 0.20**	**(K'oreje et al., 2016)**
		Ciprofloxacin (Quinolones)	**ND / 0.30**	
		Levofloxacin (Quinolones)	**0.04 / 1.60**	
		Metronidazole (Nitroimidazole)	**4 / 3**	
		Nalidixic acid (Quinolones)	**ND / 2.80**	
		Sulfadoxine (Sulfonamide)	**1460 / 3.20**	
		Sulfamethazine (Sulfonamide)	**0.60 / ND**	
		Sulfamethoxazole(Sulfonamide)	**40 / 10**	
		Trimethoprim (Trimethoprim)	**7 / 4**	
River water (µg L^{-1})/[a]Sediment (ng g^{-1})	**Nigeria**	**Chloramphenicol (Amphenicol)**	**0.40 / [a]ND**	**(Olarinmoye et al., 2016)**
		Chlortetracycline (Tetracycline)	**0.10 / [a]ND**	
		Clarithromycin (Macrolide)	**0.10/ [a]43**	
		Doxycycline (Tetracycline)	**0.10 / [a]ND**	
		Erythromycin (Macrolide)	**1 / [a]147**	
		Oxytetracycline (Tetracycline)	**0.10 / [a]ND**	
		Roxithromycin (Macrolide)	**0.02 / [a]10**	
		Sulfadiazine (Sulfonamide)	**0.04**	
		Sulfadimidine (Sulfonamide)	**0.01**	
		Sulfamethoxazole (Sulfonamide)	**1.50/[a]11**	
		Tetracycline (Tetracycline)	**0.10**	
		Trimethoprim (Trimethoprim)	**0.40 / [a]38**	
		Azithromycin (Macrolide)	**ND /[a]10**	
Ground and Surface water (µg L^{-1})	**Nigeria**	**Ciprofloxacin (Quinolones)**	**0.90**	**(Olaitan et al., 2014)**
[+]Wastewater (µg L^{-1})	**Egypt**	**Amoxicillin (Penicillin)**	**99400.0**	**(Abou-Elela and El-Khateeb, 2015)**
		Ampicillin (Penicillin)	**70600.0**	
		Dicloxacillin (Penicillin)	**119400.0**	
[++]Wastewater (µg L^{-1})	**Tunisia**	**Erythromycin (Macrolide)**	**< 0.01**	**(Moslah et al., 2017)**
		Clarithromycin (Macrolide)	**< 0.01**	
		Ofloxacin (Quinolones)	**< 0.01**	
		Ciprofloxacin (Quinolones)	**< 0.01**	
		Sulfamethoxazole (Sulfonamide)	**< 0.01**	

Wastewater / Seawaters (µg L^{-1})	**Tunisia**	**Chloramphenicol (Amphenicol)**	**3.30 / 15.60**	**(Tahrani et al., 2016)**
		Thiamphenicol (Amphenicol)	**1.20 /ND**	
		Florfenicol (Amphenicol)	**3.30 / 18.4**	
		Paromycin (Aminoglycoside)	**4.2 / ND**	
		Kanamycin (Macrolide)	**7.50 / 1.50**	
		Aparamycin (Aminoglycoside)	**1.50 / 1.80**	
		Streptomycin (Aminoglycoside)	**2.70 / 3.40**	
		Amikacin (Aminoglycoside)	**2.30 / 1.20**	
		Sisomycin (Aminoglycoside)	**6.70 / 0.40**	
		Neomycin (Aminoglycoside)	**16.4 / 0.70**	
		Gentamycin (Aminoglycoside)	**1.60 / 1.40**	
Surface water (µg L^{-1})	**Zimbabwe**	**Oxytetracycline (Tetracycline)**	**150.0**	**(Dzomba et al., 2014)**

$^{+}$ = Concentration was originally in mg L^{-1}, $^{++}$ = Concentration was originally in ng L^{-1}, ND = Not detected, ^{a} = sediment sample, MDL = minimum detection limit

An important factor related to the information of antibiotics detected in a country is the sampling coverage area. Analysing a large number of antibiotics from different geographical locations gives more information about the environmental concentration within the country. This is much better than identifying a large number of antibiotics in one sampling spot. For example, a total of 13 antibiotics have been identified in Nigeria representing two sampling areas in the Western region of the country (Olaitan et al., 2014, Olarinmoye et al., 2016). In the first report, only one antibiotics was detected by Olaitan et al., 2014 and in the second report, 12 antibiotic were identified by Olarinmoye et al. (2016) (Olaitan et al., 2014, Olarinmoye et al., 2016). Based on the sampling, only from the Western region, this report cannot represent the level of antibiotics for the whole country. Nigeria is the most populated country in Africa with a large proportion living on below $1 per day (Eseyin et al., 2016). Such low income standards are associated with malnutrition, high disease burden, and correlated with high rate of drug consumption (Abegunde et al., 2007). Hence one would assume that

further reports would be available, based on the influencing factors and accounting for the antibiotics on this large population. Reports on the environmental presence of antibiotics from other West African countries are also largely lacking. From the North African countries, reports are available from Morocco, Tunisia and Egypt (Errayess et al., 2017, Tahrani et al., 2016, Moslah et al., 2017, Abou-Elela and El-Khateeb, 2015). In Egypt and Tunisia, three and sixteen antibiotics were detected respectively in wastewater samples. The first five antibiotics detected in Tunisia (Moslah et al., 2017) were identified in wastewater at µg L^{-1} levels of concentration, while the remaining 11 antibiotics were detected in wastewater/seawater in ng mL^{-1} (Tahrani et al., 2016). The differences in the results of the analysis cannot be compared because, different antibiotics were analysed for. East Africa is represented by the analysis of 10 antibiotics form Kenya, while in Southern Africa, 11 antibiotics were detected (South Africa with 10 antibiotics) and a limited study in Zimbabwe in surface water related to a drinking water intake with 1 antibiotic (See also Table 2.2).

A different range of concentration of antibiotics was reported by United Nation (UN) global surface water assessment for pharmaceuticals, when compared to that reported concentrations in Africa (Weber et al., 2016). For example, sulfamethoxazole, the most reported antibiotic, was found in South Africa at a concentration of 7.3 µg L^{-1} (Matongo et al., 2015), while in Nigeria the reported concentration was 1.5 µg L^{-1} (Olarinmoye et al., 2016). The minimum and the maximum concentrations reported in Africa were from 0.00027 µg L^{-1} in Tunisia and 40 µg L^{-1} in Kenya (K'oreje et al., 2016) respectively. A higher concentration of antibiotics is noticed in river near slum than in wastewater effluent in the Kenya, this can be attributed to direct dumping of fecal matter into the river, a practice that is common in most African countries.

The corresponding minimum and maximum concentration reported by the United Nations were in the range of 0.1 – 29 µg L^{-1} (Weber et al., 2016). Likewise, ciprofloxacin was reported within the range of 19 – 6500 µg L^{-1} by the UN (Weber et al., 2016) and 0.0002 - 15 µg L^{-1} in Africa from Tunisia (Moslah et al., 2017) and South Africa (Agunbiade and Moodley, 2014), respectively. These UN measurement values are in contradiction to the published report stated here from Africa, which may be due to the mode that the UN data was obtained.

Globally, quinolones and flouroquinolones are antibiotics that are frequently used for the treatment of bacterial infections and they are also among the five (β-lactam, macrolides, fluoroquinolones, sulfonamides, and tetracyclines) most commonly detected in the environment (Díaz-Cruz and Barceló, 2006). In a study on a wastewater reclamation plant (WRP) in Beijing, China, (Chen et al., 2013), 0.1 µg L^{-1} of flouroquinolones were detected at the tertiary effluent. The concentration was similar to findings in South Africa by Hendricks and Pool (2012) but much lower than what was found in the surface/sediment analysis (15 µg L^{-1} and 190 µg g^{-1}) by Agunbiade and Moodley (2011).

Further information on the detection of antibiotics within the African environment will be beneficial for several reasons:

1. To answer questions related to correlations of population density and socio-economic strata as compared to consumption of antibiotics and concentrations in surface water and ground water.

2. To relate sewer system and poor method of sewage disposal with different barriers and release routes of residual antibiotic in the wastes. This may also contribute to an assessment of the accumulation of antibiotics in the environment.

3. To promote laboratory capacity which may be essential for future assessment of the environmental presence and impact of pharmaceuticals in parallel to their PNEC.

2.5 IDENTIFICATION OF ANTIBIOTICS IN WASTEWATER: CURRENT STATE OF EXTRACTION PROTOCOL AND FUTURE PERSPECTIVES

This section 2.5 is based on the journal paper A.C Faleye et al. (2017). Identification of antibiotics in wastewater: current state of extraction protocol and future perspectives. Journal of Water and Health: Volume 15 pages 982 -10003 as part of the current thesis.

Antibiotics are released to the aquatic environment through various routes. After administration to humans, they are excreted partly as incompletely metabolized products (Chang et al., 2010) and partly as unchanged active compounds via urine and feces, ending up in the sewer system and are subsequently released into the environment in the effluents after potential wastewater treatment (Kümmerer, 2009). The wastewater treatment plants (WWTPs) have been shown to have low capacity towards the removal of antibiotics from the wastewater (Wei et al., 2010, Seifrtová et al., 2009). Hence the effluents are major contributors of antibiotics into the aquatic environment. The use of sludge, from WWTPs or on-site sanitation facilities, as fertilizers with active antibiotics components also contributes to release of antibiotics to the soil environment and can affect the micro-organisms present therein (Kumar et al., 2005, Baguer et al., 2000). Subsequent run-off of organic fertilizers from agricultural land increases the spread of the antibiotics further into the aquatic environment (Michael et al., 2013, Pruden et al., 2013).

Animal excreta (manure) applied to agricultural fields as fertilizers are a source of contamination, as most of the antibiotics consumed by the animals ends up in their excreta in the active form or modified form (Toumi et al., 2015). Residual antibiotic in animal excreta enters the environment, through run-offs from agricultural land or irrigation and are sometimes taken up by plants and transferable via the consumption of such plants (Venglovsky et al., 2009, Eggen et al., 2011). The active compounds of the antibiotics or their biologically active metabolites, may also percolate from land or leaking sewers to the groundwater (Frey et al., 2015). The presence of these active components in the environment remains a potential risk whether at low (sub-lethal level) or high (toxic level) concentration (Jiang et al., 2013).

Quinolones (ciprofloxacin) are among the most used antibiotics in the world and the wide spread of resistant strains has been well established (Acar and Goldstein, 1997, Rocha et al., 2017). Treatments of lower respiratory tract infections are sometimes not susceptible to ciprofloxacin (Fuller and Low, 2005, Pribul et al., 2017, Pereyre et al., 2016). This trend in bacterial resistance is on the increase, creating a haven of super resistant bacteria to the stronger antibiotics. The same fate of resistance applies to sulphonamide which has led to reduction in its usage (Enne et al., 2001, Alonso et al., 2017). One of the causes of antibiotic resistance is the "misuse of antibiotics" which leads to selection for resistance genes (Lukačišinová and Bollenbach, 2017). The presence of these antibiotics in the water and food we consume (Jones et al., 2005) may lead to its unintentional misuse and further resistance development (Jones et al., 2003).

Several methods have been developed for the identification and quantification of antibiotics in different aquatic regimes, such as sewage water (Lindberg et al., 2005, Li et al., 2007, Dorival-García et al., 2013), drinking water (Fick et al., 2009, Rodríguez et al., 2011, Dzomba et al., 2014) river water (Ngumba et al., 2016, Senta et al., 2008, Agunbiade and Moodley, 2014) and groundwater (Olaitan et al., 2014, Batt and Aga, 2005). Prior to the analysis, the analyte of

interest must be concentrated and extracted from the collected sample matrices using the sample preparation steps which involves filtration and the use of different analyte-sequestering methods such as solvent extraction (Nabais and Cardoso, 1995, Yan et al., 2011) and solid-phase extraction methods (Errayess et al., 2017, Dorival-García et al., 2013).

Environmental detection and quantification of antibiotics is essential and hence the extraction/analysis protocol is a vital part of the chemical risk assessment. The common traditional methods of extraction of antibiotics in wastewater are liquid-liquid extraction (LLE) and solid phase extraction (SPE). These have several shortfalls in their traditional set-up including, cumbersome procedure, use of expensive glassware, low efficiency in sample clean up, low specificity towards the target analyte, and reduced recovery when compared with the related recent advanced methods.

Quinolones and sulphonamides are the two types of antibiotics focused on here as examples. Their selection is based on the rate of use in relation to the prevalent diseases and with reference to available data on the rate of production/sales (Van Boeckel et al., 2014), their stability in the aquatic environment (wastewater) (Gaugain et al., 2013) and the impact of the antibiotics in relation to antibiotic resistance (Rodriguez-Mozaz et al., 2015). Their extraction procedures have a good base for comparison of LLE and SPE as well as the advanced states of the extraction methods that use similar detection instrumentation. These criteria were the basis for the screening of research papers.

2.5.1 Factors influencing sample preparation

All experiments are dependent on the quality of sampling. Sample collection and preparation (preservation, filtration and extraction) are the first steps and an essential part of the analytical

procedure, followed by chromatographic separation, detection and data analysis. Proportionately, 80 % of the analytical time is used for sampling and sample preparation (Buszewski and Szultka, 2012). Sampling generally implies choosing a small fraction of a matrix that is representative of the quality of the whole matrix. Factors such as frequency and time of sampling, temperature, sampling method and sampling equipment must be critically considered in order to have a good sample for antibiotics analysis (Ort et al., 2010).

A good knowledge of the physicochemical properties of the analytes (antibiotics) is an important precondition in sample preparation (Namieśnik et al., 2005). As highlighted in Table 2.3, the acid dissociation constant (pKa) is an indicator of the acid–base property of the antibiotics and determines its ionization rate which enables an effective adjustment of the sample pH for preservation and extraction (Qiang and Adams, 2004). Partition coefficient (log P), is an indicator of the degree of hydrophilicity and hydrophobicity of a substance and measures its solubility in two different phases. Antibiotics with high log P are hydrophobic in nature, whereas a low log P is hydrophilic (Pavlović et al., 2007). Cyclic or ring structures of antibiotics can also influence their solubility as well as the level of complexity (the bond structure within the rings) and number of rings. These parameters are used to guide the selection of solvent and extraction media when choosing the extraction method.

Table 2.3: Interrelatedness of physicochemical properties of some antibiotics (DrugBank, 2017)

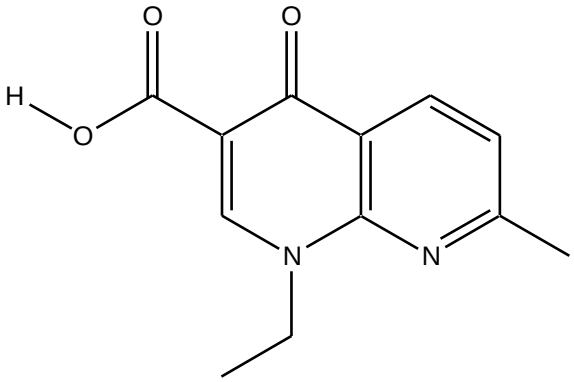
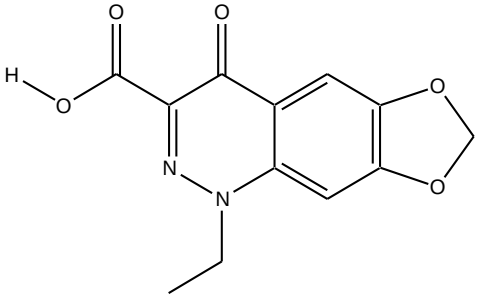
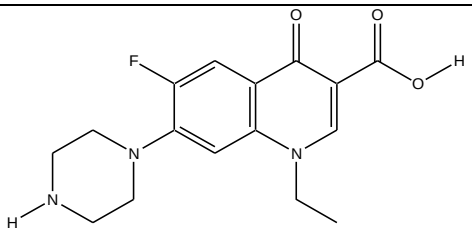
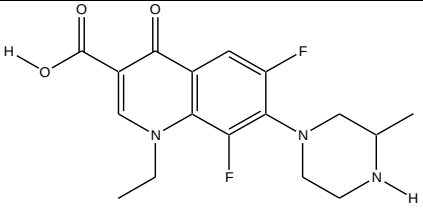
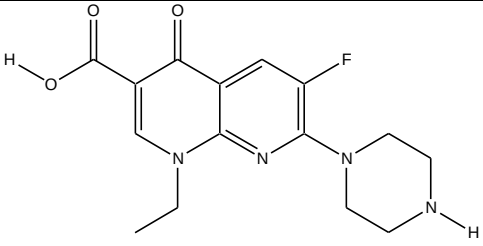
Antibiotics	**Class of antibiotics**	**pKa (acid)**	**Log P**	**Water solubility g L^{-1}**	**Number of rings per antibiotic**
Tetracycline	**Tetracycline**	**-2.20**	**-0.56**	**1.33**	**4**
Doxycycline		**-2.20**	**-0.72**	**0.63**	**4**

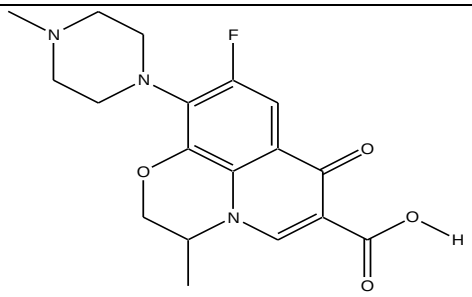
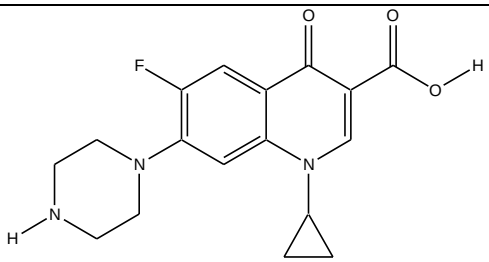
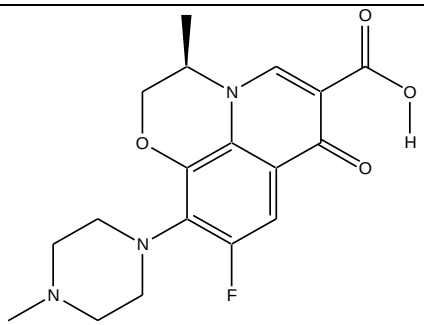
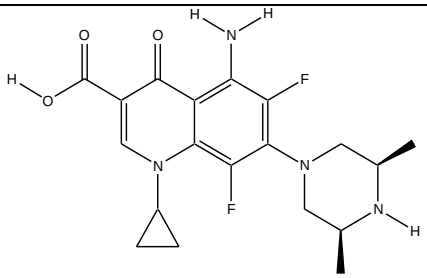
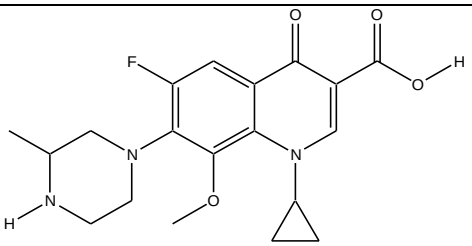
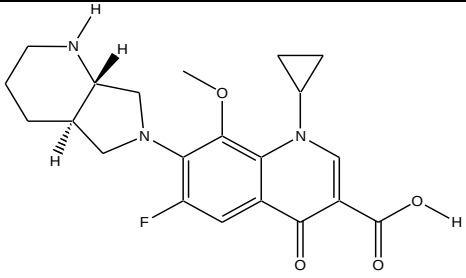
Oxytetracycline		**0.24**	**-0.99**	**1.40**	**4**
Amoxicillin	**Beta lactam**	**3.23**	**0.75**	**0.96**	**3**
Flucloxacillin		**3.75**	**2.69**	**0.06**	**4**
Ceftriaxone		**3.19**	**-0.01**	**0.11**	**4**
Erythromycin	**Macrolide**	**12.44**	**2.37**	**0.46**	**3**
Azithromycin		**12.43**	**3.03**	**0.51**	**3**
Clarithromycin		**12.46**	**3.18**	**0.22**	**3**
Sulfamethoxazole	**Sulphonamides**	**6.16**	**0.79**	**0.46**	**2**
Sulfanilamide		**-0.25**	**-0.16**	**10.4**	**1**
Sulfadiazine		**6.99**	**0.25**	**0.60**	**2**
Metronidazole	**Imidazole**	**15.44**	**-0.15**	**5.92**	**1**
Albendazole	**Benzimidazole**	**9.51**	**3.22**	**0.02**	**2**
Ethambutol	**Antituberculosis**	**14.82**	**-0.12**	**7.54**	**0**
Ethionamide		**11.89**	**1.88**	**0.84**	**1**
Isoniazid		**13.61**	**-0.71**	**34.90**	**1**
Trimethoprim	**Anisoles**	**17.33**	**1.26**	**0.62**	**2**
Ciprofloxacin	**Quinoline carboxylic acids**	**5.76**	**-0.57**	**1.35**	**4**
Norfloxacin		**5.77**	**-0.47**	**1.01**	**3**
Ofloxacin		**5.45**	**-0.02**	**1.44**	**4**
Clindamycin	**Amino acids, peptides, and analogues**	**5.91**	**1.59**	**0.46**	**2**
Sulfamethoxazole	**Benzenesulfonamides**	**6.16**	**0.79**	**0.46**	**2**

Azithromycin	**Carbohydrates and carbohydrate conjugates**	**-3.2**	**3.03**	**0.51**	**3**
Roxithromycin		**12.45**	**2.9**	**0.19**	**2**

It can be inferred that there is a tendency for the solubility of an antibiotic in water to increase with decreasing number of rings in the molecule. Various models for predicting the solubility of drugs exist, based on parameters such as the log P of the drug, molecular weight and fragment pattern (Lipinski et al., 2012, Sanghvi et al., 2003, Knopp et al., 2015). Commercial computational means of predicting the solubility of drugs also exist, such as CLOGP manual (by Daylight Chemical Information Systems) and ACD (Advanced Chemistry Development, Inc). Most of these models require experimental procedures to justify the numerical solubility values assigned to drugs. None of the published models makes use of the number of ring structures to estimate the drug solubility. Data of the different quinolones and sulphonamides as tabulated in Tables 2.4 and 2.5 indicate a relationship between the number of rings and the solubility of the antibiotics. Nalidixic acid (quinolone) and sulfacetamide (sulphonamide) have the smallest number of rings and the highest solubility values in relation to other antibiotics in the table.

Table 2.4: Relating quinolones structure (number of rings) to their solubility in water (DrugBank, 2017)

Groups of quinolones	Class	Structure	Number of rings	Solubility in Water ($g\ L^{-1}$)
Nalidixic acid	**1st generation**		**2**	**2.3**
Cinoxacin			**3**	**0.96**
Norfloxacin	**2nd generation**		**3**	**1.01**
Lomefloxacin			**3**	**0.11**
Enoxacin			**3**	**1.09**

Ofloxacin			**4**	**1.44**
Ciprofloxacin			**4**	**1.35**
Levofloxacin	**3rd generation**		**4**	**1.44**
Sparfloxacin			**4**	**0.11**
Gatifloxacin			**4**	**0.63**
Moxifloxacin			**5**	**0.17**

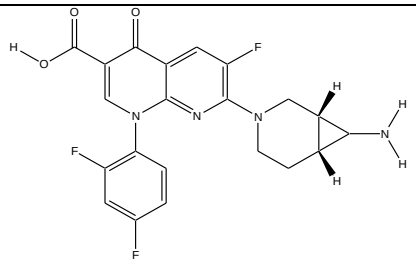
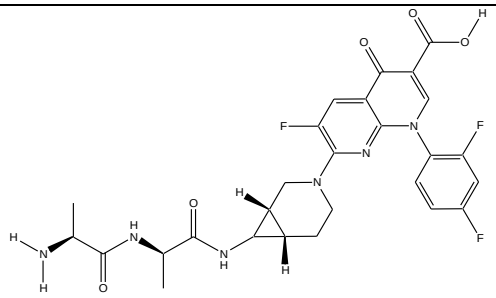
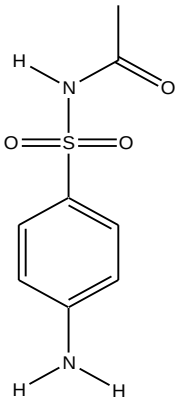
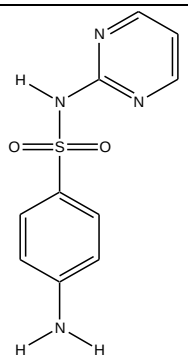
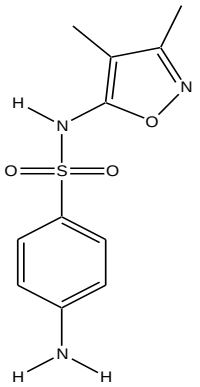
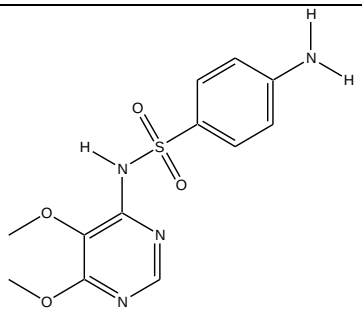
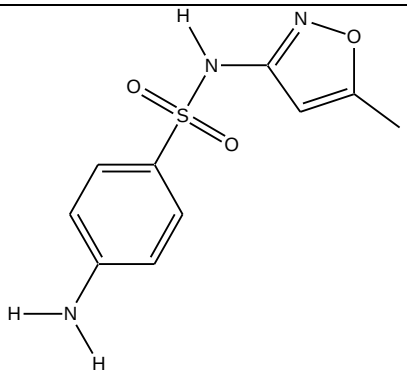
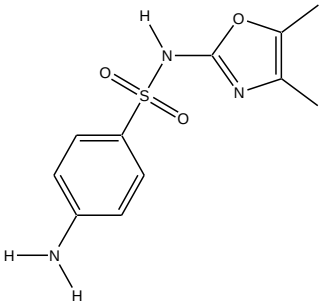
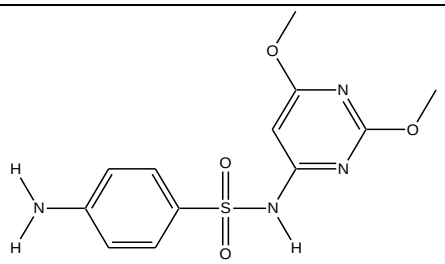
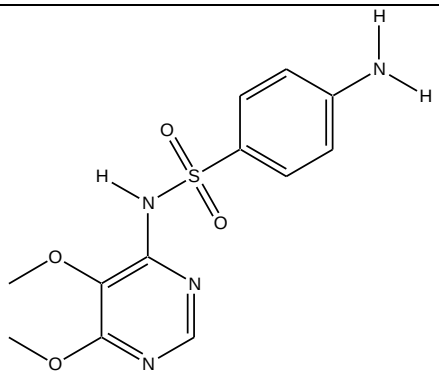
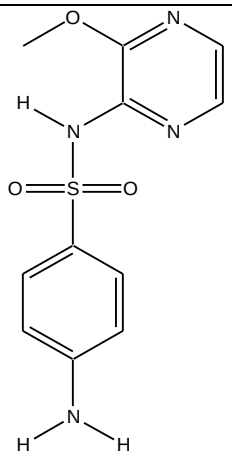
Trovafloxacin	**4th generation**		**5**	
Alatrofloxacin			**5**	**0.04**

Table 2.5: Relating sulphonamide structure (number of rings) to their solubility in water (DrugBank, 2017)

Groups of sulphonamide	Class	Structure	Number of rings	Solubility in water (g L^{-1})
Sulfacetamide	**Short acting**		**1**	**4.21**

Sulfadiazine			**2**	**0.60**
Sulfafurazole			**2**	**0.31**
Sulfadoxine	**Intermediate acting**		**2**	**0.30**
Sulfamethoxazole			**2**	**0.46**
Sulfamoxole			**2**	**0.27**

Sulfadimethoxine	**Long acting**		**2**	**0.28**
Sulfadoxine	**Ultra long acting**		**2**	**0.30**
Sulfametopyrazine			**2**	**0.41**

2.5.2 Sulphonamides and quinolones extraction techniques from wastewater

In the fight against bacterial infection, sulphonamides were the pioneer group of drugs that achieved great success. The simplest and oldest form of sulphonamides is sulphanilamide and all others are its derivatives, while quinolones are derivatives of nalidixic acid (Figures 2.4 and 2.5).

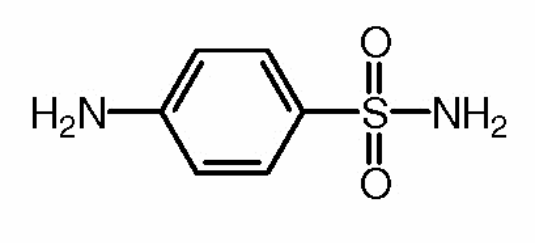


Figure 2.4: Sulphanilamide

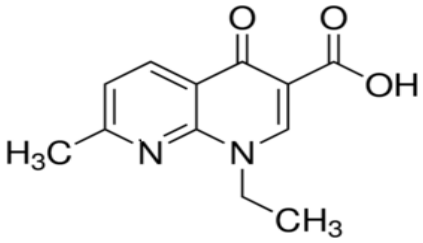


Figure 2.5: Nalidixic acid

Sulphonamide and quinolones are mostly insoluble in water (Tables 2.4 and 2.5) at neutral pH and become more soluble in an acidic environment with high stability (Luo et al., 2011). The frequency of use of these antibiotics and stability accounts especially for their detection in the aquatic environment (Senta et al., 2008). Solid phase extraction (SPE) and liquid–liquid extraction (LLE) are widely used as a pre-concentration step. In SPE, analytes are extracted from liquid samples, based on the polarity of the compound. The analytes are retained on the sorbent, based on their affinity and subsequently eluted for instrumental analysis. SPE makes use of a solid phase and a mobile phase to separate analytes based on their different degrees of affinity for the liquid or solid phase. SPE has been widely used with excellent recoveries (Mutavdžić Pavlović et al., 2010, Płotka-Wasylka et al., 2016, Dorival-García et al., 2013), but is time consuming (conditioning, sample loading washing and elution), especially when the sample volume is large (Płotka-Wasylka et al., 2016).

LLE or solvent extraction is an extraction process that makes use of two immiscible solvents to separate compounds based on their relative solubility (Soniya and Muthuraman, 2015). To the best of my knowledge LLE has not been extensively used for the extraction of both sulphonamide and quinolones in recent years (after 2000). The drawbacks in pre-concentration have mainly been resolved. The use of a small volume of solvent in LLE enhances the efficiency and environmental friendliness (Xing et al., 2015, Gjelstad and Pedersen-Bjergaard,

2013) and, in SPE, time required has been reduced in its further development with magnetic solid phase extraction (MSPE) and molecular imprinted polymers (MIP) (Li et al., 2015). Improvements in the traditional LLE and SPE methods are aimed at enhancing the speed and reducing the costs in the sample pre-concentration and adaptation to the analytical instrument to be used (Wen et al., 2014, Thurman and Snavely, 2000, Ahmad et al., 2015, Hanson, 2013). These advancements are exemplified in relation to efficiency and frequency of use with dispersive liquid–liquid micro-extraction (DLLME) and hollow-fiber liquid phase microextraction (HF-LPME) for the LLE extraction and with MSPE, and MIP for the SPE (Tables 2.6 and 2.7) (Wen et al., 2014, Ebrahimpour et al., 2015). DLLME is a process in which two immiscible solvents (organic and aqueous) are used for the extraction of organic compounds from water samples while HF-LPME involves the use of an acceptor solution concealed in a polypropylene fiber in an organic solvent (Sharifi et al., 2016).

2.5.3 Matrix effect on recovery

Matrix effect is the interference experienced as a result of the presence of other compounds that are not of interest when analyzing for the target substances at instrumental detection point (Matuszewski et al., 2003, Petrović et al., 2005). The source of the samples is a determinant for the constituents where, for example, wastewater from an animal farm will have different constituents compared with wastewater from a pharmaceutical company. Evaluation of the matrix effect is very important to ensure reliable analysis, where co-elution of compounds can lead to signal enhancement or suppression and analytical inaccuracy (Bolong et al., 2009). Several papers on matrix effect evaluation have been published (Hamid and Eskicioglu, 2012, Matuszewski et al., 2003, Lamble and Hill, 1998) and the most reliable with regards to antibiotics in water samples is the one where an isotope-labelled internal standard (surrogate) had been used (Zwiener and Frimmel, 2004). These surrogates are not commercially available

and other method such as an internal calibration method (addition of pure standard of analyte of interest at a fixed concentration) and standard addition method (addition of pure standard of analyte of interest at varying concentration) are used (Gros et al., 2006). In Table 2.6, Dasenaki et al. (2015b) and Ye et al. (2007), compensated for the matrix effects using the standard addition method, while Pedrouzo et al. (2008) compared the use of both internal and external standard and obtained better results with the use of an internal standard. MIP (section 2.5.4.2) posesses high selective properties which enable reduction of the matrix effect significantly via targeted analyte sorbents. Chen et al. (2013) used external calibration to evaluate the reduction of the matrix effect of the MIP extract. Out of the papers reviewed 25 made use of external calibration, while four utilized surrogate standards (Senta et al., 2008; Wang et al., 2010; Dorival-García et al., 2013; Castiglioni et al., 2005) and one (Renew & Huang, 2004) used both surrogate and standard addition method. Despite the different sample sources, the recovery results presented here are reliable based on the use of one form of matrix check for result validation.

Table 2.6: Comparisons between different extraction methods for the determination of sulphonamide in wastewater (extraction method abbreviations are given as a footnote)

Extraction methods	Extraction time(min) per sample volume (mL)	SCP	Limit of detection (ng L^{-1})	Matrices (sample source)	Percentage recovery (%)	References
SPE- HPLC-MS/MS	*\50 mL	12	6.6 – 22	[a.] Wastewater influent [b.] Wastewater effluent	[a.] 38.5 – 78.1 [b.] 42.2 – 79.0	(Dasenaki and Thomaidis, 2015b, Pedrouzo et al., 2008, Ye et al., 2007, Peng et al., 2008, Agunbiade and Moodley, 2014)
SPE-HPLC-MS	8 min/100 mL 20 min/250 mL	9	20	[a.] Wastewater influent [b.] Wastewater effluent	[a.] 25.0 – 56.0 [b.] 27.0 – 53.0	
SPE-HPLC-MS/MS	167 min/500 mL	9	1 – 3	Raw wastewater	23.1 – 87.0	
SPE-HPLC-DAD	100 min/500 mL 30 min/150 mL	29	70 – 80 200 – 250	[a.] Wastewater influent [b.] Wastewater effluent	[a.] 64.0 – 72.0 [b.]65.0 – 71.0	
SPE-HPLC-DAD	100 min/500 mL	28	310	Environmental water (river water)	87.4 – 92.5	
MIP-HPLC-MS/MS	25 min/500 mL	11	380 – 1320	Environmental water (river, lake, sewage water)	76.8 – 32.8	(Chen et al., 2013, Lian et al., 2014, Qin et al., 2012)
MIP-HPLC-DAD	16 min/4 mL	4	50	Sea water	88 – 79.2	
MIP-HPLC-DAD	333 min/500 mL	7	4.1-19.3	Wastewater influent	84.1 – 98.6	

MSPE – HPLC-UV	**15 min/500 mL**	**6.5**	**20 – 30**	**Environmental water (sewage effluent, tap water, lake water)**	**70 – 102**	**(Sun et al., 2009, Li et al., 2007, Tolmacheva et al., 2016)**
MSPE-HPLC-UV	**35 min/150 mL**	**4.5**	**150 – 350**	**River water**	**89 – 113**	
MSPE– HPLC– AD	**10 min/100 mL**	**2.5**	**0.2 -0.3**	**River water**	**84 – 105**	
DLLME-HPLC- DAD	**13 min/5 mL**	**1.94**	**+ 350 – 10500**	**Run off water**	**78 – 117**	**(Herrera-Herrera et al., 2013, Xu et al., 2011, Song et al., 2014)**
DLLME-HPLC– FD	**12 min/10 mL**	**1.05**	**10 – 20**	**River water**	**95 – 110**	
MA-DLLME-HPLC– UV	**1.5 min/2 mL**	****0.1**	**330 – 850**	**Environmental water (tap, pool, lake, river water)**	**75.1 – 115.8**	
HF-LPME-HPLC-DAD/FD	**360 min/50 mL**	**0.05**	**+ 1000 – 15000 for DAD detection 300– 33000 for FD detection**	**Environmental water (wastewater influent, effluent, river water , tap water)**	**36.2 – 101**	**(Payán et al., 2011b, Tao et al., 2009, Yudthavorasit et al., 2011)**
HF-LPME-HPLC-UV	**480 min/4 mL**	**4.03**	**100 – 400**	**Environmental water (fish, duck, pig farm water, river water)**	**82.2 – 103.2**	

HF-LPME-UHPLC-MS/MS	**60 min/20 mL**	**0.02**	**10 -250**	**River water**	**79 – 118**	

***Extraction time was not stated; SCP =Solvent consumed per sample (mL)**

******0.16 g of ionic liquid was dissolved in 0.1 mL of acetonitrile.

[+] Poor detection limit as a result of the use of a less sensitive detector (DAD)

Abbreviations for extraction methods: SPE-HPLC-MS/MS (solid phase extraction – high performance liquid chromatography tandem mass spectrometry); SPE-HPLC-DAD (solid phase extraction – high performance liquid chromatography – diode array detection); MIP-HPLC-MS/MS (molecular imprinted polymer high performance liquid chromatography tandem mass spectrometry); MIP-HPLC-DAD (molecular imprinted polymer high performance liquid chromatography diode array detection); MSPE-HPLC-UV (magnetic solid phase extraction – high performance liquid chromatography – ultraviolet light detection); MSPE-HPLC-AD (magnetic solid phase extraction – high performance liquid chromatography – amperometric detection); DLLME-HPLC-FD (dispersive liquid-liquid microextraction – high performance liquid chromatography – fluorescence detection); MA-DLLME- HPLC-UV (microwave assisted dispersive liquid-liquid microextraction – high performance liquid chromatography – ultraviolet light detection); HF-LPME- HPLC-DAD/FD (hollow-fiber liquid phase microextraction – high performance liquid chromatography – diode array detection tandem fluorescence detection), HF-LPME- HPLC-UV (hollow-fiber liquid phase microextraction – high performance liquid chromatography – ultraviolet light detection); HF-LPME-UHPLC-MS/MS (hollow-fiber liquid phase microextraction – ultra high performance liquid chromatography tandem mass spectrometry); HF-LPME-LC-QqQ-MS/MS (hollow-fiber liquid phase microextraction – ultra liquid chromatography triple-quadrupole tandem mass spectrometry); MISPE-FI-CL (molecularly imprinted polymer solid phase extraction – flow-injection chemiluminescence); ONLINE-MISPE-LC-FLD (online molecular imprinted solid phase extraction – liquid chromatography fluorescence detection); DLLME-LC-UV (dispersive liquid-liquid microextraction – liquid chromatography – ultraviolet light detection); DLLME-UHPLC-MS/MS (dispersive liquid-liquid microextraction – ultra high performance liquid chromatography tandem mass spectrometry).

Table 2.7: Comparative study of different types of extraction methodologies for the determination of fluoroquinolones in water

Extraction methods	Extraction time (min) per sample/volume (mL)	Solvent consumed per sample (mL)	Limit of detection (ng L^{-1})	Matrices (sample source)	Percentage recovery (%)	References
SPE-UPLC-MS/MS	36 min/100 mL	23	20 – 40	Wastewaters (wastewater treatment plant)	98.5 – 103.9	(Senta et al., 2008, Wang et al., 2010b, Dorival-García et al., 2013)
SPE-HPLC-MS/MS	40 min/200 mL	14	6.5 – 13.2	Wastewater (primary effluent)	53 – 60	
SPE-HPLC-MS/MS	250 min/500 mL	12	0.09–0.25	Tap water	61.4 – 91.3	
ONLINE-MISPE-LC-FLD	30 min/25 mL	17.5	1 – 11 1 – 12	Drinking water Fish farm water	62 – 102	(Rodríguez et al., 2011, Luaces et al., 2013, Lian and Wang, 2016)
MISPE-FI-CL	10 min/10 mL	3	270	Environmental water (mineral, tap and river water)	84 – 119	
MISPE-HPLC-FD	20 min/5 mL	2	200	Sea water	75.2 – 112.4	
MSPE-HPLC-DAD	17 min/50 mL	0.5	50 – 120	Environmental water (lake water, reservoir water)	72.0 – 118	(Liu et al., 2016, Huang et al., 2013, Wu et al., 2016a)
MSPE-HPLC-DAD	60 min/50 mL	0.5	200 – 460	Environmental water (lake water, reservoir water, surface water)	52.1 – 104.5	
MSPE-HPLC-UV	9 min/10 mL	0.4	200–1000	Environmental (tap water, river and lake)	83.5 – 103.0	
DLLME-LC-UV	7 min/8 mL	0.6	140 – 810	Wastewater (wastewater from Pharmaceutical factory)	82.7 – 110.9	(Yan et al., 2011, Vázquez et al., 2012, Guan et al., 2016)
DLLME-LC-FD	19 min/10 mL	0.5	0.8 – 13	Ground water	85 – 107	

DLLME-UHPLC-MS/MS	12 min/5 mL	1.2	6 – 9.1	Environmental water (tap water, river water, running water, wastewater)	76.8 – 100	
HF-LPME-UHPLC-MS/MS	60 min/20 mL	0.02	10 – 250	River water	78 – 118	(Yudthavorasit et al., 2011, Payán et al., 2011a, Denadai and Cass, 2015)
HF-LPME-HPLC-DAD-FD	330 min/50 mL	0.05	0.3 – 16	Environmental water (river water, wastewater)	97 – 100	
HF-LPME-LC-QqQ-MS/MS	4.5 min/0.5 mL	0.05	5.3 – 31.8	Environmental water (surface and wastewater)	79.5 – 112	

2.5.4 Solid phase extraction (SPE)

SPE is currently more frequently used for sample clean-up in wastewater than previously due to enhanced ease of use and commercially available solid-phase sorbents with extraction kits including extraction manifolds. Sorbents packed in a cartridge are placed on the manifold, and the appropriate extraction solvent is used in the four-step extraction process. These steps are essentially: 1) conditioning of sorbent; 2) loading of sample; 3) washing of impurities (not in all cases); and 4) elution of the analyte.

Different types of sorbent can be used for the separation. The choice mainly depends on the chemical characteristics (polarity and functional group) of the analyte and the interaction of the functional groups of the analyte with the sorbent (Płotka-Wasylka et al., 2016, Babić et al., 2006, Chen et al., 2010, Mutavdžić Pavlović et al., 2010). Different sorbents have been used for the determination of many organic compounds including different pharmaceuticals in water/wastewater environment (Tong et al., 2009, Jelic et al., 2011, Renew and Huang, 2004). Mainly the polymeric based sorbents (e.g Oasis HLB and strata X) and the silica-based sorbents (e.g Strata C18-E and C8) are preferred in wastewater analysis (D'Archivio et al.,

2007, Mutavdžić Pavlović et al., 2010, Masiá et al., 2014). The polymeric based sorbents are preferred due to their compatibility with most analytes and their ability to perform within a broad pH range (Jiang et al., 2013, Karthikeyan and Meyer, 2006, Cheng et al., 2015, Skendi et al., 2016). The silica-based sorbents are restricted to more specific pH values within the neutral range. The silica in the sorbent is unstable at both low and high pH values where it tends to hydrolyse and thereby reduce the efficiency of the extraction process (Kirkland et al., 1997). Comparative assessments of sorbent efficiency have been carried out for optimal use in relation to the particular analyte of interest (Mutavdžić Pavlović et al., 2010, Mutavdžić et al., 2006, Tayeb et al., 2015). Table 2.8 lists a few examples of the polymeric and silica-based sorbents used in the extraction of sulphonamides and fluoroquinolones from wastewater and aquatic recipients. Hartig et al. (1999) utilized LiChrolut EN SPE cartridges, which are polymeric sorbents, for the extraction of sulphonamides in secondary effluent and obtained a recovery between 77 and 100 %. The polymeric sorbent Oasis hydrophilic-lipophilic (HLB) was used by Tong et al. (2009) for the extraction of 13 antibiotics including sulfamerazine and ofloxacin. They obtained a recovery of over 90 % for sulfamerazine while the efficiency was much lower, 65%, for ofloxacin (Table 2.8). Non-specific/generic extraction methods tend to be advantageous (high recovery) to many antibiotics while some may render low recoveries and require specific treatment as, for example, for ofloxacin (Table 2.8).

The highest recovery on the SPE was generally below 100 % except for Dorival-García et al. (2013) which had a recovery of 103 %. The advanced methods often gave 100 %. The presence of phthalates from the SPE cartridges usually accounts for the above 100 % recovery rates (Table 2.8). Since the choice of sorbent for SPE is based partly on economic consideration further developments of the analytical procedure should account for this as well. The percentage recovery is calculated from the ratio of the experimental concentrations to the theoretical concentration of the antibiotics with reference to matrix effect elimination.

Modification and advancement in SPE (Zhu et al., 2013, Li et al., 2015, Whang et al., 2012, Xu and Lee, 2012) has included sorbent coating to enhance the performance, as exemplified by magnetic coatings (Yu et al., 2013, Zhang and Anderson, 2014) and micro extraction fibers (Bagheri et al., 2012, Pelit et al., 2015). High recoveries have been obtained with magnetic adsorbents (MSPE), such as magnetic molecular imprinted polymer (MMIP) (Mehdinia et al., 2011, Herrero-Latorre et al., 2015). These modifications have partly taken over the traditional use of the SPE in sample pre-treatment.

Table 2.8: Recovery of common solid phase extraction sorbents used for sulphonamide and fluoroquinolones

Sorbent packaging	**Sorbent mass /sample volume**	**Matrices (sample source)**	**Antibiotics**	**Percentage recovery**	**Reference**
Oasis Hydrophilic–lipophilic (HLB) balanced	60 mg/50 mL	Wastewater (swine wastewater)	Sulfamerazine Ofloxacin	93 – 98 % 58 – 63 %	(Tong et al., 2009)
Oasis Hydrophilic–lipophilic (HLB) balanced	60 mg/150 mL	Wastewater (municipal wastewater influent)	Sulfapyridine	66 – 110 %	(Shaaban and Górecki, 2012)
Anion-exchange cartridge (Isolute) + hydrophilic–lipophilic balance (HLB)	500 mg + 500 mg/1000 mL	Wastewater (municipal wastewater effluent)	Ciprofloxacin Oxofloxacin	90 – 154 % 62 – 123 %	(Renew and Huang, 2004)
Oasis Cation exchanger (MCX)	60 mg/500 mL	Wastewater (municipal wastewater effluent)	Ciprofloxacin Oxofloxacin	36 – 28 % 3 – 25 %	(Castiglioni et al., 2005)
Oasis Hydrophilic–lipophilic balanced (HLB)	500 mg/100 mL	Wastewater (municipal wastewater effluent)	Ciprofloxacin Oxofloxacin	99 – 100 % 99 – 102 %	(Dorival-García et al., 2013)
[a] Strata C8 [b] Strata C18-E	200 mg/100 mL	Wastewater (water municipal	Sulfadiazine	[a] 37 – 47 % [b] 28 – 40 % [a]92 – 94 %	(Mutavdžić Pavlović et al., 2010)

		wastewater effluent)	Sulfamerazine	[b]73 – 78 %	
LiChrolut EN SPE cartridges	200 mg/1000 mL	Environmental water (water municipal wastewater effluent)	Sulfathiazole Sulfadiazine Sulfamerazine Sulfapyridine	77 – 100 % 88 – 107 % 83 – 117 % 77 – 97 %	(Hartig et al., 1999)
[a] Strata C8 [b] Strata C18-E	200 mg/100 mL	Wastewater (water municipal wastewater effluent	Norfloxacin Sulfamerazine	[a]54 – 62 % [b]84 – 95 % [a]71 – 74 % [b]97 – 99 %	(Mutavdžić Pavlović et al., 2010)

Note: Analysis utilized LC-MS/MS for the analysis and recovery percentages (ratio of experimental concentration to theoretical concentration).

2.5.4.1 Magnetic solid phase extraction (MSPE)

In the MSPE method, magnetic particles (MPs) are used as adsorbents. They are not packed into cartridges like in the traditional SPE, but are dispersed in the sample solutions to adsorb the analytes. A larger surface area to volume ratio is achieved resulting in higher extraction efficiency. The ease of preparation of the magnetic particle adsorption step encourages its use (Šafaříková and Šafařík, 1999). An external magnet is applied to collect the MPs and separate these from the liquid phase as shown in Figure 2.6. This procedure excludes the need for time consuming centrifugations or filtrations of the sample during the clean-up process. It is a time and labour effective approach, overcoming the setbacks of the traditional SPE procedure, especially the sample loading step (Li et al., 2015). The MPs frequently used are ferrite- or iron-oxide nanoparticles and Fe_3O_4, (crystalline iron oxides of maghemite or magnetite). Other types of MPs include silica-based MPs used in the purification of DNA (Rittich and Španová, 2013), alumina coated nanoparticles used for

metal/metalloid pre-concentration and/or separation (Giakisikli and Anthemidis, 2013), magnetic molecular imprinted polymer (Chen et al., 2010) and also polydimethylsiloxane (PDMS) used for the determination of phthalate diesters in water samples (Jeddi et al., 2014). After completion of the extraction process the extract is subjected to further analysis such as LC-MS/MS in order to detect and determine the analytes quantitatively and qualitatively. The reusability of the magnetic sorbent is ensured by regenerating it through washing with an organic solvent such as methanol (Sarafraz-Yazdi et al., 2015). The separation of MPs depends on the type of MPs (sorbents), and is connected with the interaction of analyte molecules with the surface functional groups of the sorbents, in a similar manner as the traditional extraction in the solid phase (Aguilar-Arteaga et al., 2010).

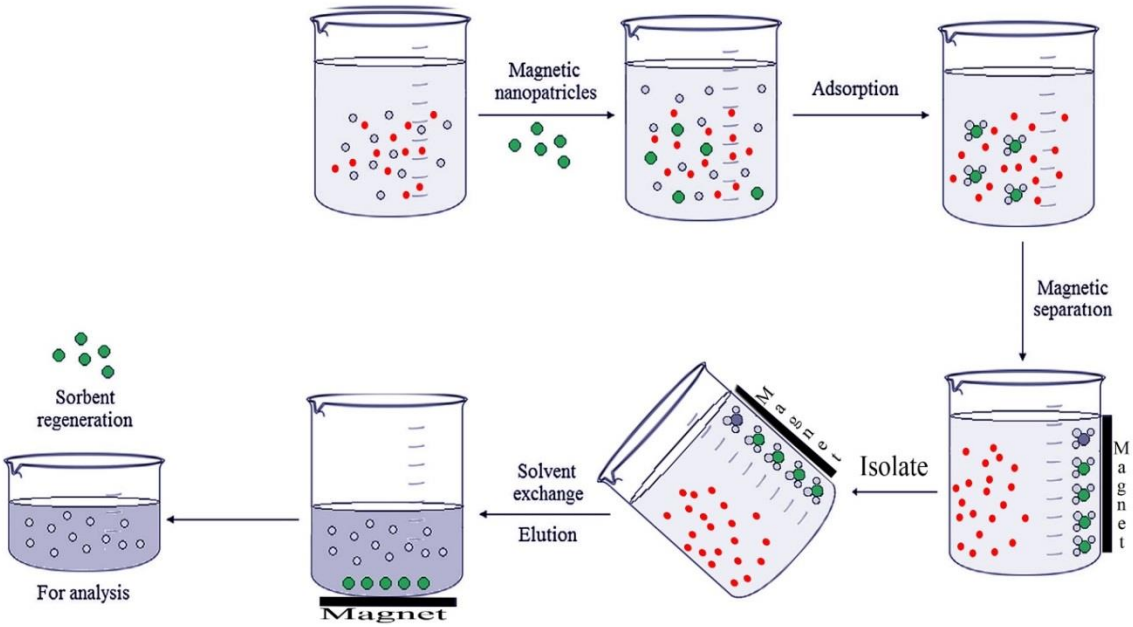


Figure 2.6: Application for enriching analyte as MSPE-NP sorbent (Wierucka and Biziuk, 2014).

The mechanism of the MSPE is based on the different types of interaction between the analyte and the magnetic sorbent; ionic, dipole-dipole, dipole-induced dipole, hydrogen bonding and dispersion forces (Shi and Ye, 2015, Wierucka and Biziuk, 2014). Similar to the traditional

SPE, the reverse-phase sorbent of the MSPE is a weak polar or non-polar compound and the interaction are mostly with hydrophobic compounds through Van der Waal forces. In the normal phase sorbent, polar compounds are used as the stationary phase and the mobile phase are non-polar. The interaction is based on hydrogen bonding, dipole-dipole interactions and π-π interactions. A good knowledge of the analyte of interest such as ionization and solubility will guide in the selection of sorbent for the extraction protocol. These properties would determine the level of affinity for the sorbent and the extraction efficiency of the analyte from the sample solution (Wierucka and Biziuk, 2014).

Another important factor is the choice of the eluting solvent. To ensure effective and quantitative elution, the solvent should have the right elution strength that matches the desired analyte (Sun et al., 2009). The amount of solvent and time used for the MSPE is generally small when compared with other types of SPE and LLE (Tables 2.6 and 2.7). Sun et al. (2009) made use of a magnetic hemimicelles solid phase extraction (MMHSPE) for several sulfonamides from environmental water samples (Sun et al., 2009). The process was time effective, making use of 15 min/500 mL with low solvent consumption of 6.5 mL. In addition a recovery of close to 100 % with low standard deviation (within 1–6 %), was recorded, indicating a better result when compared to other SPE processes used in the same category. In addition to the better yield, the high efficiency of the regenarated MPs makes MSPE economical and unique in a wastewater environmental analyte clean up process (Šafaříková and Šafařík, 1999).

2.5.4.2 Molecular imprinted polymer (MIP)

Another methodological extraction SPE-based alternative is the molecular imprinted polymer (MIP). These are polymers that have been processed using the molecular imprinting technique which leaves cavities in the polymer matrix with an affinity to a chosen ‘template’ molecule

(Figueiredo et al., 2016). MIPs are prepared by forming complexes with a template molecule (target molecule or its derivatives) and a functional monomer(s) that either covalently or non-covalently links with the template molecule followed by co-polymerization in the presence of a cross-linker (Takeuchi and Sunayama, 2014). The efficiency of this method has been proven by many researchers, for example, Luaces et al. (2013) prepared fluoroquinolone-selective MIP beads for the determination of trace amounts of enrofloxacin in environmental waters, and a recovery of 84–119 % was recorded. Other records of the use of MIP in the determination of sulphonamide and quinolones are listed in Tables 2.6 and 2.7.

Magnetic molecularly imprinted polymers (MMIPs) are produced by coating MIPs with magnetic particles such as Fe_3O_4. The MMIPs possess the template-binding property of the MIP and the magnetic property of the MPs, hence making it a multifunctional method of extraction (Zhou et al., 2010, Tan and Tong, 2007). The main difficulty encountered in the use of molecular imprinted polymers is the production, which includes template bleeding and cumbersome synthesis procedures (Figueiredo et al., 2016). Careful laboratory methods (synthesis of selective template) are currently used for the production of templates (Luaces et al., 2013, Lian et al., 2014) but commercial availability of the imprinted polymer would make the use of MIPs much easier and faster. The possibility of making use of an external electrical current to generate the magnetic force needed in this extraction method, with the aim of generating an appropriate force to attract a particular analyte (based on their ionic charges) at a particular time will greatly enhance the speed and efficiency of this method in the near future**.**

2.5.5 Liquid–liquid extraction (LLE)

As a result of the advancement in SPE (e.g., MSPE, MIP), the use of conventional LLE is currently infrequently used, mainly due to three setbacks: 1) time consuming extraction period;

2) cumbersome procedure and use of expensive glassware; 3) large quantity of extraction solvent needed. Due to these setbacks, literature referrals are limited on traditional LLE for clean-up of antibiotics in wastewater, especially for antibiotics like quinolones and sulphonamides. Specifically, after the year 2000, so far as I can ascertain, there is little or no available literature for the extraction of quinolones and sulphonamides in wastewater using LLE.

LLE is however still undergoing further development as demonstrated by Leong et al., (2014) and others in the use of miniaturized pre-concentration techniques, and the use of dispersive liquid and phase extractions (Ahmad et al., 2015, Leong et al., 2014, Bendicho et al., 2015). Micro-extraction techniques are environmentally friendly, less expensive and simple to operate which has renewed the interest in a miniaturized LLE method. The improvements involve the reduction of the acceptor to donor phase ratio, thereby minimizing the solvent use (Sarafraz-Yazdi and Amiri, 2010) in the liquid-phase micro extraction method (LPME). Several alternative methodological approaches exist, hollow-fiber micro-extraction (HF-LPME), dispersive liquid–liquid micro-extraction (DLLME) and pressurized liquid extraction (PLE) are a few example. PLE is an advanced form of LLME, not further detailed here (Li et al., 2013, Vazquez-Roig and Picó, 2015). The extracts from the PLE method are mostly enhanced by further clean-up procedure which govern the recovery (Vazquez-Roig and Picó, 2015).

2.5.5.1 HOLLOW FIBER – LIQUID PHASE MICRO EXTRACTION (HF-LPME)

In HF-LPME a hollow fibre containing an organic solvent is used to prevent direct mixing of acceptor solution with the sample solution (Hu et al., 2013) as illustrated in Figure 2.7, with the vial filled with the aqueous sample. A porous rod (typically made of polypropylene) placed in a glass vial is used as the main components in HF-LPME (Psillakis and Kalogerakis, 2003).

Before extraction, the porous rod (HF) is immersed in an organic solvent to immobilize this in the pores, and excess solvent is removed. A hydrophobic solvent is used as a thin barrier within the wall of the HF to ensure that the organic does not mix with the aqueous sample during the extraction process. Depending on the type of phase involved in HF-LPME, the acceptor solution can either be an 'organic solvent' in the hollow fibre which makes it a two-phase extraction process or an acidic or alkaline 'aqueous solution' which makes it a three-phase extraction process.

In the two-phase extraction process the targeted analytes are extracted from the aqueous sample into the organic solvent (acceptor solution) present both in the porous HF wall and inside the core of the HF. In the three-phase process, the analytes are extracted from the aqueous sample via the organic solvent in HF pores, then into the aqueous acceptor solution in the core of the HF (Sarafraz-Yazdi and Amiri, 2010). The HF-LPME process has proven to be a simple, low cost, small solvent use sample preparation technique. The low solvent consumption is exemplified in the extraction of sulphonamide and quinolones in Tables 2.6 and 2.7. More specifically, Yudthavorasit et al., (2011) made use of 0.02 mL of solvent for the preconcentration of 11 antibiotics, which includes sulphonamides and quinolones, in 20 mL of water samples. With this method an excellent percentage recovery of 79–118 % was recorded. Based on the assesment of the solvent consumed by other methods , HF-LPME uses the least solvent. A prolonged contact time of 60 mins and above between the aqueous sample and HF-LPME must be ensured to achieve maximum extraction of the intended analyte. This makes the process time consuming for the extraction of sulphonamide and quinolones when compared with other methods.

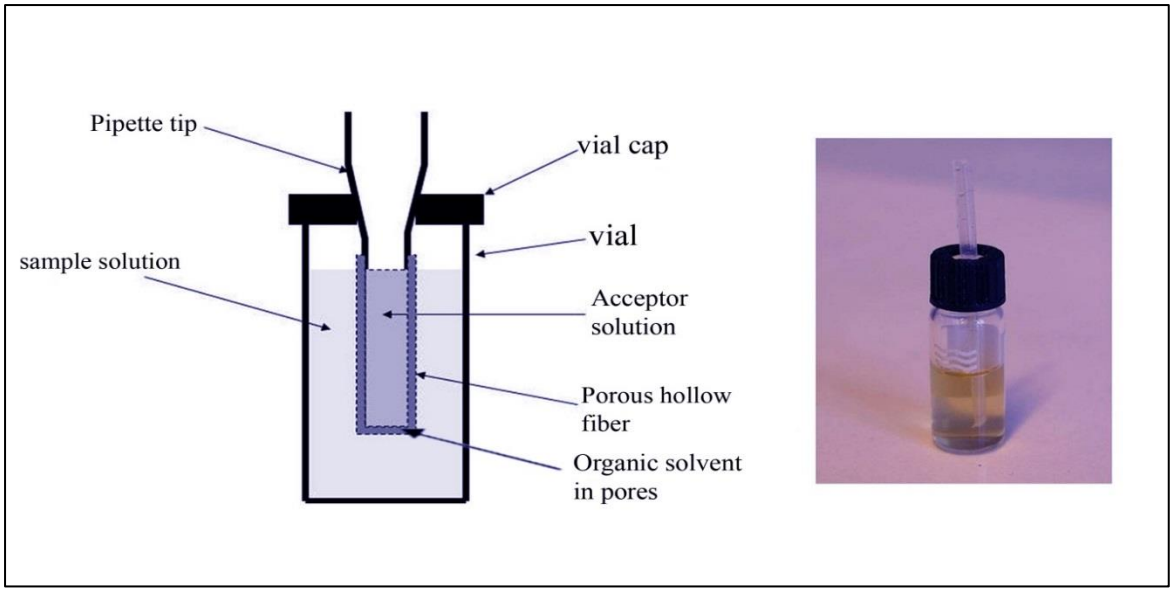


Figure 2.7: Hollow-fibre micro-extraction (HF-LPME) (Gjelstad and Pedersen-Bjergaard, 2013).

2.5.5.2 Dispersive liquid-liquid micro-extraction

The dispersive liquid–liquid micro-extraction technique (DLLME) is generally based on the dispersion of extracting solvent in a sample matrix, and it is composed of a disperser solvent (an amphiphilic compound) that is applied to an aqueous sample to form a turbid solution. Extraction is achieved due to the large contact surface area between the droplets of the extractant solvent and the sample. After, the introduction of the extractant, the sample mixture is centrifuged and the extraction solvent usually sediments at the bottom of the tube after which it is drawn up with a micro syringe for further analysis (Herrera-Herrera et al., 2013). The simple extraction process and low consumption of organic solvent makes the DLLME a good method of extraction. The main drawbacks of DLLME is its inability to extract hydrophilic compounds into the extraction solvent, volume of samples is restricted to the volume of the vial and time spent in centrifugation (Tables 2.6 and 2.7). A maximum volume of 10 mL is mostly used for the analysis (Tables 2.6 and 2.7) and a larger volume would mean longer time of extraction. Current further developments deal with some of these constraints, where the

centrifugation (which prevents automation) is replaced with the use of a process referred to as the ionic liquid-based DLLME, and the hydrophilic limitation is being investigated via the use of ion-pair based emulsification liquid phase micro extraction (IP ELPME) (Ebrahimpour et al., 2015).

2.5.6 Societal impact

The release and persistence of antibiotic residues in the wastewater, receiving water bodies and the environment lead to the emergence of antimicrobial resistance among environmental isolates. This also triggers selection for antibiotic resistant genes. Quantitative and qualitative analysis of antibiotics in the environment is, therefore, vital for effective planning against the resultant of an ecological resistance pool effect and spread to humans through several transmission routes.

Different approaches have been employed by many researchers to quantify the antibiotic residues in the environment, mainly in the aquatic environment. Despite this being an issue of global concern, few reports are available on this study area, particularly from developing countries. This thesis presents a critical assessment of advances in methods of extraction evaluation for antibiotic micro-pollutants in wastewater with a focus on sulphonamide and quinolones. A comparative analysis on these antibiotics in wastewater was done, using the few available reports, with a view to identifying quick and effective ways of extracting and quantifying them in the environment. Structural elucidation of various antibiotics with respect to their physiochemical properties were correlated with their solubilities, towards enhancing the extraction protocols.

CHAPTER 3: DEVELOPMENT AND OPTIMIZATION OF THE METHOD FOR THE DETECTION OF ANTIBIOTICS IN WASTEWATER USING SOLID-PHASE EXTRACTION (SPE) WITH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY–PHOTODIODE ARRAY DETECTOR (HPLC-DAD).

3.1 INTRODUCTION

This chapter describes the development and optimization of a solid phase extraction – liquid chromatography – diode array detection method (SPE-LC-DAD) for the determination of six antibiotics. The six antibiotics are as also stated in Chapter 1, ethionamide (ETI), metronidazole (MET), trimethroprim (TRI), ciprofloxacin (CIP), sulfisoxazole (SUF), albendazole (ALB). These antibiotics are chosen based on their use in animal husbandry and for therapeutic approaches in human medicine. In the method development and optimization, two different SPE cartridges were employed (Strata XL, by Phenomenex and HLB Oasis by Waters). These have been extensively described in Chapter 2. Extraction was carried out with two solvents (methanol and acetonitrile) and instrumental analysis was carried out using a Cortecs C18 column by Waters on a HPLC coupled with a photo diode array detector (DAD), using a gradient elution for the analyte peak separation. The solvent used as the mobile phase was also tested to achieve the best separation of analyte in the chromatograph. Ultra-pure water and wastewater were used as samples for the development of the method. The developed method was validated with samples from the influent of four WWTPs in South Africa.

Table 3.1: Characteristics of the antibiotics of interest (DrugBank, 2017)

Antibiotics	**Log of Kow**	**Molecular weight (g mol^{-1})**	**CAS No.**	**Molecular formula**
Ethionamide **(ETI)**	**0.37**	**166.24**	**536-33-4**	**$C_8H_{10}N_2S$**
Metronidazole **(MET)**	**0.02**	**171.15**	**443-48-1**	**$C_6H_9N_3O_3$**
Trimethoprim **(TRI)**	**0.91**	**290.32**	**738-70-5**	**$C_{14}H_{18}N_4O_3$**
Ciprofloxacin **(CIP)**	**0.28**	**331.35**	**85721-33-1**	**$C_{17}H_{18}FN_3O_3$**
Sulfisoxazole **(SUF)**	**0.89**	**267.30**	**127-69-5**	**$C_{11}H_{13}N_3O_3S$**
Albendazole **(ALB)**	**1.27**	**253.28**	**54965-21-8**	**$C_{12}H_{15}N_3O_2S$**

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

All antibiotics used as standards were purchased from Sigma Aldrich chemicals South Africa. HPLC-grade acetonitrile (ACN) and methanol (MeOH), 98.9 % formic acid (FA), sulphuric acid, and sodium hydroxide were used for the analysis. Ultra-pure water was produced by using AquaMax Ultra, water purification unit system (Ultra 370 series) by Younglin, Japan. Formic acid and sodium hydroxide (NaOH) were used for pH adjustment.

The individual stock solutions of antibiotics were prepared by dissolving accurate quantities of the powdered standard in different solvents according to their solubility and then made up in ultra-pure water. Ethionamide, trimethoprim and sulfisoxazole were dissolved in 1.25 mL of ACN, ciprofloxacin and albendazole were dissolved in 0.1 mL formic acid. Metronidazole was only prepared in ultrapure water because of its solubility in water. All stock solutions, 100 mg L^{-1}, (1 mg/10 mL), were prepared and stored in the dark in graduated glass bottle at 4 ^{0}C. Working solutions were made by dissolving the stock solution according to the required concentration in 10 % ACN/Ultra-pure water. All working solutions were freshly prepared before analysis.

3.2.2 Sample pre-treatment

Frozen samples were thawed and 100 ml of wastewater sample was centrifuged for 15 min at 30,000 rpm at 20 ^{0}C. The supernatants were decanted and filtered through 0.45 µm syringe filters. This was to ensure that the samples were free of any particulate matter that might block the HPLC tubing. The sediment from the centrifugation was kept in an amber bottle and frozen at – 80 ^{0}C for further analysis. The filtered water samples were acidified with 0.1 % of formic acid to a pH of 3.4 prior to SPE extractions. The results of the SPE method

development showing percentage recovery at different pH ranges (where the best recovery was at pH 3) are tabulated included in table 3.2.

3.3 THE SPE PROCEDURES

The development and optimization of the SPE procedure was carried out by using 100 mL of ultra-pure water and wastewater, respectively. The samples were acidified with 0.1 % formic acid and spiked with 0.5 mL of stock solution mixture (100 mg L^{-1}) to produce a concentration of 0.5 mg L^{-1}. The spiking was necessary to enable the identification of the difference in analytical responses in the samples due to matrix effect. Same concentration of antibiotics was spiked and the ultra-pure water contains no matrix, hence, it will give the exact value of the concentration of the antibiotics while the wastewater, will produce values similar to the ultra-pure water but influenced by the matrix. From these, the efficiency of the SPE procedure and the instrumental response (PDA) can be calculated.

The antibiotics were extracted on a 24 position SPE manifold. Prior to the samples extraction, the two SPE cartridges (HLB and Strata XL) were preconditioned with 5 mL methanol followed by 5 mL of 0.1 % acidified ultra-pure water (pH 3). The samples were loaded on the activated cartridge at a flow rate of 2 mL min^{-1}. The cartridges were washed with ultrapure water and dried under vacuum for 5 -10 min. The loaded cartridges were eluted with 5 mL of different solvents. Three different solvents were tested for the elution step, namely, 100 % methanol, 100 % acetonitrile and 50/50 (v/v) of 100 % acetonitrile and methanol. An effective elution of the retained analyte was achieved using 50/50 (v/v) of 100 % acetonitrile and methanol solvent, for both cartridges, at a flow rate of 1 mL min^{-1}. Eluted samples were dried under pressurised air and then reconstituted in 1 mL of 5 % acetonitrile solvent in water. Un-spiked samples were extracted using the same procedure as the control. All analysis was done in triplicate.

3.3.1 Results of the SPE procedure.

The results of the pre-tests are summarised as follows (further used below):

With analysis conducted on ultrapure water, the result with Strata XL had a maximum average recovery of 96 % and a minimum of 69 %, while the corresponding results with Oasis HLB were 91 % for the higher recovery and 64 % for the lower. In wastewater samples, the percentage recovery using Strata XL was on an average below 70 %, while that of Oasis HLB was above 70 % for most antibiotics except ETI that was 64 %. Based on these results Oasis HLB was used in the subsequent extraction of antibiotics from wastewater samples (Table 3.2).

Table 3.2: Percentage recovery (R) for 6 antibiotics using Oasis HLB and Strata XL cartridges in wastewater and ultra-pure water sample at different pH

Antibiotics	**ETI**	**MET**	**TRI**	**CIP**	**SUF**	**ALB**
% R (SD)+ Oasis HLB pH3	**64.3 ± 2.4**	**84.9 ± 3.2**	**123.9 ± 18.1**	**93.2 ± 0.2**	**110.2 ± 21.1**	**74.9 ± 0.1**
% R (SD)+ Strata XL pH3	**65.5 ± 14.6**	**63.3 ± 11.0**	**67.7 ± 4.2**	**64.7 ± 9.7**	**69 ± 4.4**	**68.7 ± 3.5**
% R (SD)* Oasis HLB at pH3	**72.7 ± 2.2**	**86.6 ± 0.6**	**92.2± 3.9**	**64.2 ± 0.3**	**80.0 ± 3.5**	**90.3 ± 1.2**
% R (SD)* Oasis HLB at pH10	**46.7 ± 2.5**	**31.1 ± 5.3**	**22.7 ± 2.3**	**40.4 ± 1.9**	**64.4 ± 2.9**	**42.5 ± 1.9**
% R (SD)* Oasis HLB at pH7	**24.7 ± 2.5**	**58.5 ± 1.5**	**46.8 ± 3.8**	**44.1 ± 2.6**	**60.2 ± 1.8**	**45.7 ± 0.8**

+ = wastewater, * = ultra-pure water

SD: standard deviation

The percentage recovery was calculated by comparing the amount of analyte obtained after SPE extraction with the concentration before the extraction.

3.4 CHROMATOGRAPHIC SEPARATIONS

Initially investigations were directed towards high-performance liquid chromatography (HPLC) analysis performed using Shimadzu LCMS 2020 equipped with, a diode array detector (DAD), and a mass spectrometer MS, with a quadrupole mass analyser *(m/z* range: 0-2000; ionization modes: ESI/APCI) with optimizations reported in this chapter 3. Later during the thesis work (see Chapter 4) an online SPE-LC-MS/MS was used for enhanced sensitivity where automation gave better results (Chapters 4 and 5). The HPLC column temperature was set at 25^0C and the injection volume was 5 µL. The analysis was performed using a Cortecs C18 column (non-polar) by Waters, with internal diameter of 2.1 mm and length of 150 mm. The gradient elution method was optimised for the separation of the antibiotics mixture. For this method a combination of two solvents of different polarity, such as acetonitrile and water was used with variation of volume and time. The polarity of the antibiotics determines the elution profile. The assessment parameters to optimize the method is shown in Table 3.3. The optimized gradient consist of 95 % (acetonitrile) – 50 % (water balanced) over a range of time (38.01 min). The water used contains 0.1 % (v/v) formic acid, while the acetonitrile was 100 %.

Table 3.3: Optimized gradient elution parameter used

Solvent B % volume (acetonitrile)	**Solvent A % volume (0.1 % formic acid in water)**	**Time (min)**
5	**95**	**0.01**
5	**95**	**2.00**
50	**50**	**10.00**
50	**50**	**12.00**
90	**10**	**15.00**
90	**10**	**20.00**
5	**95**	**28.00**
5	**95**	**38.00**
	STOP	**38.01**

The flow rate of 0.3 mL min^{-1}, column temperature of 25 ^{0}C and a maximum pressure of 40 MPa was maintained. The DAD was set to run at 180 nm – 400 nm range with detection focused at 254 nm after comparing with other wavelengths. The peaks were confirmed at their characteristic wavelength based on information from literature (Qin et al., 2012, Babić et al., 2006, Lian and Wang, 2016, Pedrouzo et al., 2008) and the retention times were noted (Figure 3.1). The chromatogram (also Figure 3.1) shows the separated peaks for the six antibiotics mixture (standard) at a concentration of 0.5 mg L^{-1}.

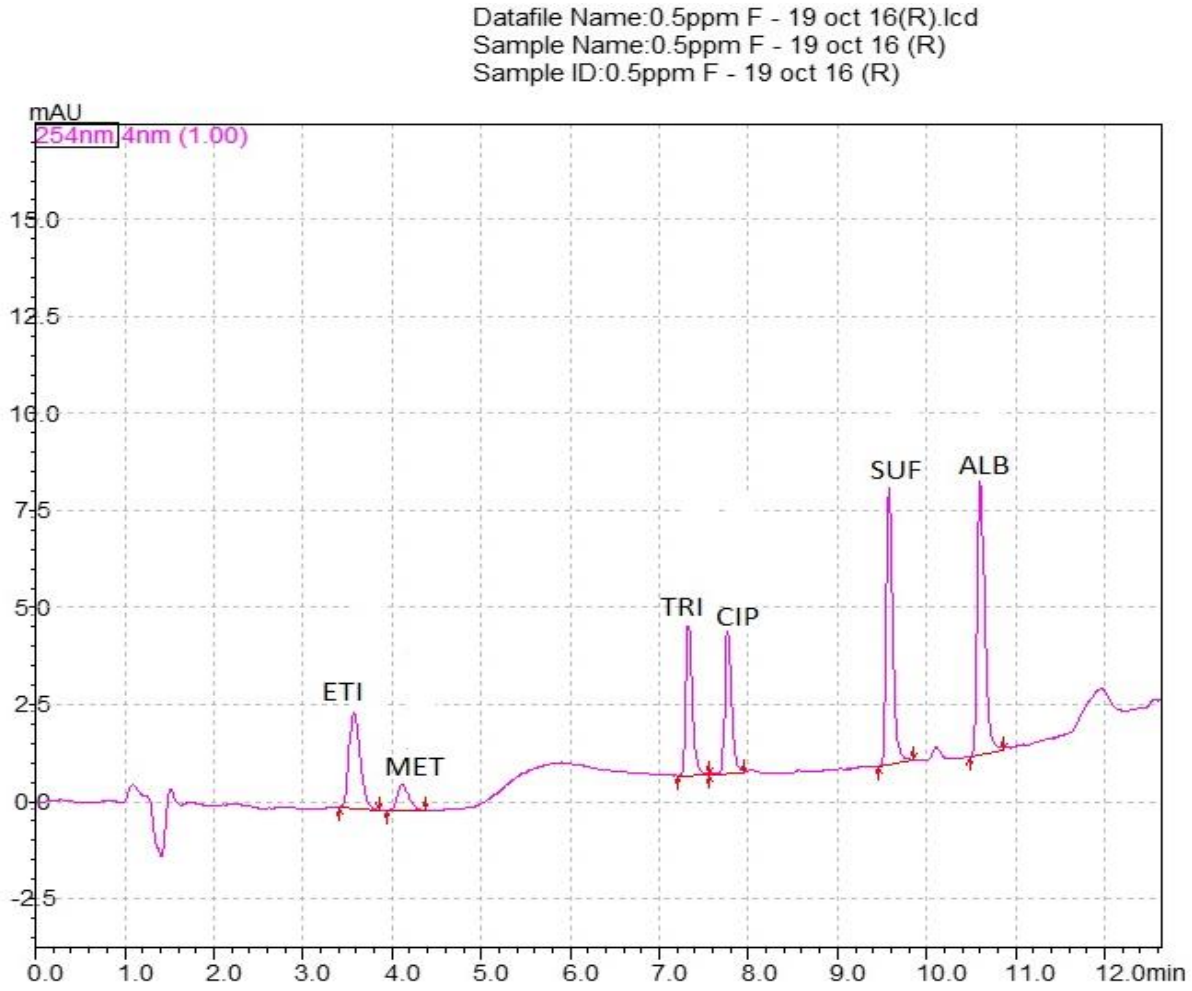


Figure 3.1: Chromatogram for the six antibiotics of interest. Ethionamide (ETI), Metronidazole (MET), Trimethroprim (TRI), Ciprofloxacin (CIP), Sulfisoxazole (SUF), Albendazole (ALB).

The gradient program was 38 min but all peaks were eluted within 12 min. The remaining time was used for equilibration and interference elution

3.5 METHOD VALIDATION

The method for the determination of the six antibiotics was validated for limit of detection (LOD) and limit of quantification (LOQ). Individual antibiotics were identified using the retention time (Rt) and their respective UV spectra pattern. The LOD and LOQ was determined using a calibration curve, after the optimization of the LC/DAD. The calibration curve for each antibiotic was prepared in a mixture of the six antibiotics using five calibration concentrations in the range from 0.025 to 0.5 mg L^{-1}. The peak area of the antibiotics was plotted against each concentration using the linear regression in Excel to generate the slope and standard error. The LOD was calculated using (3 X standard error) / slope, while the LOQ was calculated using (10 X Standard deviation) / Slope. The results are tabulated in Table 3.4.

Table 3.4: LOD and LOQ for six antibiotics

ANTIBIOTIC	**ETI**	**MET**	**TRI**	**CIP**	**SUF**	**ALB**
LOD (mg L^{-1})	**0.04**	**0.14**	**0.05**	**0.09**	**0.04**	**0.03**
LOQ (mg L^{-1})	**0.13**	**0.48**	**0.16**	**0.30**	**0.12**	**0.11**

3.6 RESULTS AND DISCUSSION

3.6.1 Optimization of HPLC–DAD analysis

Analysis of antibiotics, using HPLC – DAD, usually involves the tuning of the instrument to produce a well separated chromatographic peak of the desired analyte. This is performed by changing the mobile phase, column temperature, and rate of flow of injected sample, to suit the chosen analyte. The choice of column for this analysis and the appropriate mobile phase for column elution, were based on the properties of the analyte of interest (Table 3.1). The gradient elution used was obtained from the modification of individual analyte results, from previous experiments (Qin et al., 2012, Babić et al., 2006, Lian and Wang, 2016, Pedrouzo et al., 2008).

The aim of the chromatographic separation (section 3.4) was to regulate the acetonitrile and water balance to produce the desired peak separation. Prior to this, methanol was substituted for acetonitrile to reduce expenses, however, this resulted in an overlapping peaks which was not recorded. The optimized acetonitrile/water solvent (Table 3.3), produced well separated peaks (Figure 3.1), and this was adopted for further experiments. The retention time for ETI was the shortest (3.46 min), followed by MET (4.04 min), TRI (7.17 min), CIP (7.68), SUF (9.98 min) and ALB (10.57 min). The UV spectrum view of individual analyte was used in combination with the retention time for accurate identification.

3.6.2 SPE PROCEDURE

Instrument sensitivity plays a major role in determining the trace concentration of antibiotics in environmental samples (Kümmerer, 2008) . The photodiode array detector (DAD) used for this analysis, is of lesser sensitivity for the analyte of interest (LOD = 0.14 mg L^{-1}– 0.03 mg L^{-1}) Table 3.4. Hence there is a need for sample pre-concentration to allow for appropriate detection. The method for determining the best choice of sorbents (SPE cartridges) for a set of analyte has been discussed extensively in section 2.5. However, two different reverse phase (RP) cartridges (Oasis HLB and Strata XL), were tested to determine the most effective. These cartridges are capable of polar and non-polar interaction and also with a wide range of pH stability, which are suitable for complex samples such as wastewaters (Lin et al., 2005). Standards of antibiotics were prepared in ultrapure water and were extracted, using the cartridges, at different pH. Wastewater samples were spiked with antibiotics at known concentration and subjected to the same extraction procedure (Section 3.3). For the two cartridges, pH of 3 gave the best recovery (Table 3.2) at a flow rate of 2 mL min^{-1}, and was adopted for further extractions. The pH of the functional group of the antibiotics can be adjusted to enhance extraction process. The ionisable functional group of the selected antibiotics (NH_4^+,R_3 NH+, Phenol) with a pKa range of 9.3 – 10 (DrugBank, 2017), this ionization state

affects the retention characteristics on the SPE cartridge. At neutral form the functional group becomes more hydrophobic and gets adsorbed easily on the reverse phase sorbent. This will enable the elimination of co-adsorbed interference prior to the elution. In the ionized form of the functional group, it becomes more polar and are not adsorbed easily on the sorbent. Hence the pH of 3 is aimed at creating a neutral functional group that will enable easy adsorption by the SPE sorbent. This was evident in the results of the SPE method development in table 3.2, where the percentage recovery at different pH has been included, with the pH 3 as the best result.

The percentage recovery for the cartridges was above 60 % in both ultrapure water samples and wastewater samples, however, Oasis HLB, gave a better recovery (64 % - 124 %) in wastewater samples (Table 3.2). The different sizes of the cartridges may be the reason for the slight variation in the extraction efficiency. Oasis HLB and Strata have the same weight of 200 mg, but different volumes of 6 mL and 3 mL, respectively. The bigger diameter of Oasis HLB provided a reverse phase (RP) with a larger surface area for more interaction, thereby retaining more analyte.

3.6.3 Application of the method on wastewater sample.

The developed method was applied for the determination of the six antibiotics in four influent samples from four wastewater treatment plants. The composite sampling procedures (as described in detail in section 4.2.3) was used from the same wastewater treatment plants (also specified in 4.2.3). The samples were subjected to the aforementioned sample treatment procedure. The retention time and the UV spectra were used to confirm the presence of the target compound in line with the SANTE/SANCO validation guidelines (European Commission, 2016). External calibration was used to eliminate matrix effect. Table 3.5 shows the various concentrations of the six antibiotics as detected in the wastewater influent.

Table 3.5: Concentration of antibiotics in the influent of four WWTPs

WWTPs	Antibiotics (mg L^{-1})					
	ETI	**MET**	**TRI**	**CIP**	**SUF**	**ALB**
I	**9.86**	**ND**	**ND**	**ND**	**0.02**	**0.09**
K	**3.02**	**ND**	**ND**	**ND**	**1.75**	**1.87**
P	**1.82**	**0.29**	**3.67**	**ND**	**ND**	**ND**
S	**4.30**	**ND**	**9.60**	**1.17**	**ND**	**ND**

ND: Not detected

An intensive sampling was carried out on these four WWTPs and the analysis was performed on a more sensitive instrument. The analysis and results are discussed in chapter four and five (chapter 4 section 4.2 and 4.3 and chapter 5 section 5.2 and 5.3).

3.7 CONCLUSION

The optimised instrumental parameter was applied for the analysis of six antibiotics in both ultrapure water and wastewater samples. The percentage recovery for the optimized method using wastewater sample was above 65 % for all antibiotics of interest. The limit of detection, which ranges from 0.03 to 0.14 mg L^{-1} enables the determination of low concentration of antibiotics in polluted sample such has the wastewater influent sample. This method can be used for the routine analysis of antibiotics in an environmental sample, provided that the optimized protocol is followed systematically. However, the use of a mass spectrometer (MS) detector will give a better refined resolution. A further refinement and enhanced ability to separate peaks as applied with the mass spectrometer (MS) detector, will enable the detection of more antibiotics in wastewater and has been applied further in chapters four and five.

CHAPTER 4: ANALYSIS OF 13 ANTIBIOTICS IN WASTEWATER SAMPLES IN SOUTH AFRICA

4.1 INTRODUCTION

The continuous introduction of antibiotics into the aquatic environment via the wastewater treatment plant discharges is of major public concern (Kümmerer, 2008) . Their metabolic fate in the body will lead to different concentrations in the excreta (faeces and/or urine) depending on the type of antibiotic which subsequently will be reflected in the environment. The prevalence of antibiotics in the inlet raw wastewater to WWTPs, can therefore be related to the level of consumption as most applied antibiotics ends up in the sewer system (Borghi and Palma, 2014, Alexy et al., 2004).

The objectives of the investigations presented in this Chapter 4 were to:

(1) Identify and quantify 13 antibiotics from four WWTPs in Durban, KwaZulu-Natal, South Africa.

(2) Investigate the efficiency of the WWTPs treatment steps in relation to the removal of antibiotics.

(3) Evaluate the impact of post chlorination on the antibiotics and their concentrations in the downstream sampling point.

(4) Compare the occurrence of the different antibiotics in the four WWTPs in relation to the sources of the influent.

The choices of the WWTPs were based on the different origin of the influent with respect to the ethic community composition and related socio-economic factors. In addition the WWTPs were selected based on their performance record and green drop certification status (Greendrop).

Most wastewater treatment plants are designed to remove solid particles and biodegradable substances through the filtration and biological treatment processes (Sonune and Ghate, 2004). Substances that biodegrade slowly may require additional treatment steps. The use of oxidation process such as ozone, hydrogen peroxide (Zheng et al., 2010, Kim and Tanaka, 2010, Oller et al., 2011, Oturan and Aaron, 2014) and ultra violet light (de Souza Santos et al., 2015) has partly successfully been adopted for the removal of pharmaceuticals including antibiotics. However, very few WWPTs, especially in developing countries have documented the presence and levels of antibiotics and neither considered the use of these advanced processes of wastewater treatment, due to their high cost of maintenance. Consequently, large amounts of antibiotics ends up in the effluent receiving water bodies, which may affect drinking water, raw water intakes, down-streams wastewater and effluent discharge points (Inreiter et al., 2016, Watkinson et al., 2009).

4.2 MATERIALS AND METHODS

The chemical compounds, instrument, methods and sampling protocol used for this analysis are described in this section.

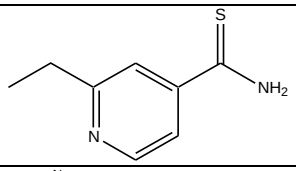
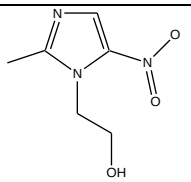
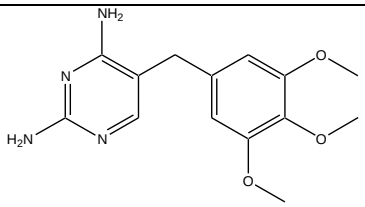
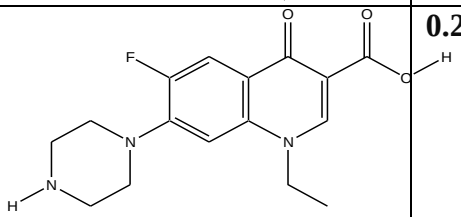
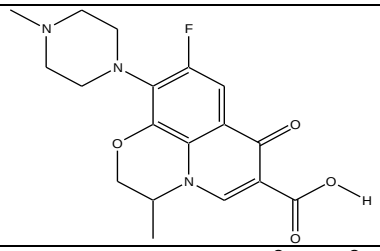
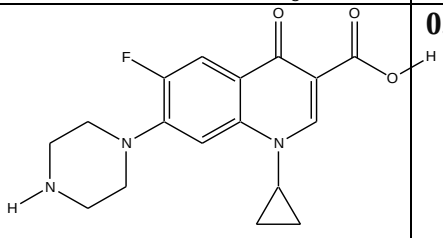
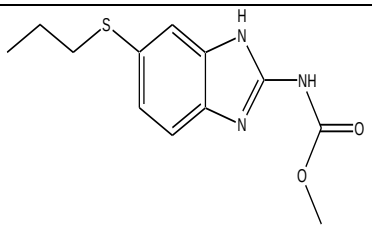
4.2.1 Chemicals

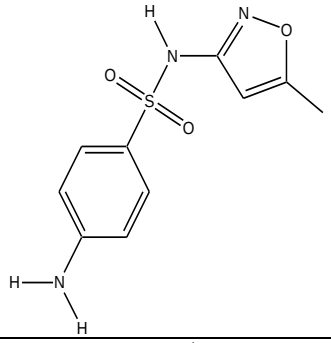
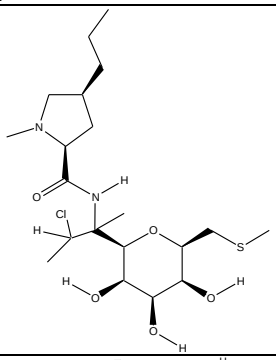
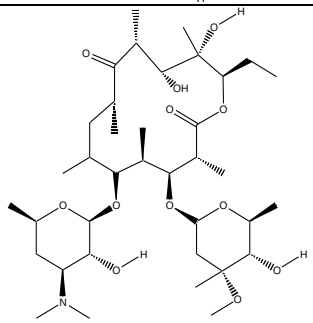
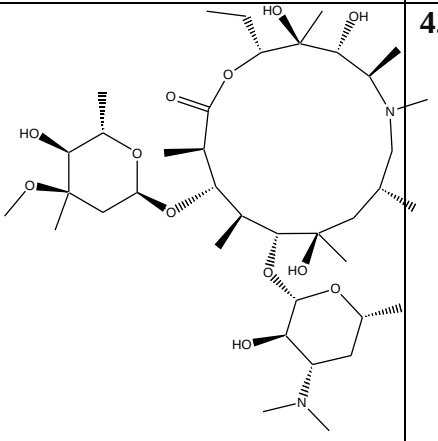
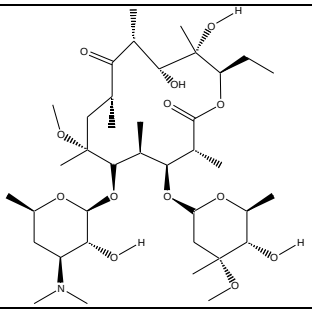
Standards of ethionamide, metronidazole, norfloxacin, ciprofloxacin, ofloxacin, trimethoprim, sulfamethoxazole, clindamycin, albendazole, erythromycin, clarithromycin, azithromycin and roxithromycin, were supplied from Sigma-Aldrich Steinheim, Germany. These were all high-performance liquid chromatography (HPLC) grade 98 % drug standards. Methanol and formic acid (HPLC grade) were obtained from JT Baker and HPLC grade acetonitrile was obtained from Merck Darmstadt, Germany. Purified water (current, 18.2 $MVcm^{-1}$) was prepared by an Elga Maxima HPLC ultrapure water system, equipped with an ultraviolet radiation source. Carbon labelled Tramadol 12C D3, Risperidone D4, Carbamazepin D10, 13C3-trimethoprim

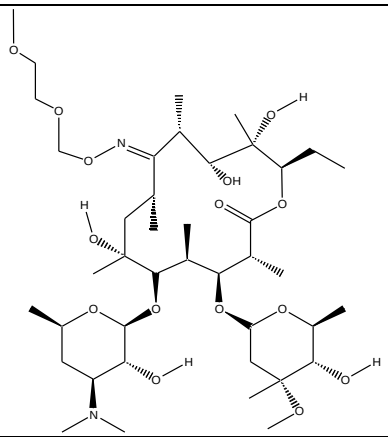
were purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and used as internal standards.

The basic characteristics of the tested antibiotics are tabulated in Table 4.1 including structures, molecular masses and basic uses. The Log Kow is the partition coefficient of water to octanol, and is an indication of the lipophilicity and hydrophilicity of the analyte**.**

Table 4.1: Antibiotics assessed, chemical structure, abbreviation, molecular weight and uses (DRUGBANK, 2017)

Antibiotics common name (Abbreviation)	Chemical structure	Log Kow	Molecular weight (g mol^{-1})	Treatment application (examples)
1. Ethionamide (ETI)		**0.37**	**166.24**	**Multidrug resistant tuberculosis**
2. Metronidazole (MET)		**0.02**	**171.15**	**Antiprotozoal medication**
3. Trimethoprim (TRI)		**0.91**	**290.32**	**Bladder infection**
4. Norfloxacin (NOR)		**0.28**	**319.33**	**Urinary tract infections, gynecological infections and gonorrhea**
5. Ofloxacin (OFL)		**0.39**	**361.37**	**Pneumonia and urinary tract infections.**
6. Ciprofloxacin (CIP)		**0.28**	**331.35**	**Bacterial infections (broad spectrum)**
7. Albendazole (ALB)		**1.27**	**265.33**	**Infections with a variety of parasitic helminths**

8. Sulfamethoxazole (SUL)		**0.89**	**253.28**	**Urinary tract infections,bronchitis, and prostatitis**
9. Clindamycin (CLI)		**2.16**	**424.98**	**Middle ear infections, pelvic inflammatory disease, streptococcal throat infections and pneumonia**
10. Erythromycin (ERY)		**3.06**	**733.94**	**Respiratory tract infections, chlamydia infections, pelvic inflammatory disease, and syphilis**
11. Azithromycin (AZI)		**4.02**	**748.98**	**Middle ear infections, throat infection and pneumonia**
12. Clarithromycin (CLA)		**3.16**	**747.95**	**Pneumonia**

13. Roxithromycin (ROX)		**0.89**	**837.05**	**Respiratory tract and urinary infections**

4.2.2 Standard stock solution

The individual antibiotics were prepared by dissolving 2 mg in 5 mL of pure methanol, except for ciprofloxacin, ofloxacin and norfloxacin, where 20 µL of formic acid (FA) was also added to increase their solubility in the pure methanol. The stock solution concentrations are within the range of 432 - 575.8 µg mL^{-1} for the respective antibiotics and were prepared with references to their individual molecular weight. A mixture of the individual antibiotics was prepared in one stock solution and stored in the dark at -18 ^{0}C. Five concentrations ranging between 10000 – 500000 ng L^{-1} were prepared from the mixture for the calibration curve for the liquid chromatography (LC) separately. Likewise, 10 000 ng L^{-1} mixtures were prepared from the same stock solution of standards for the online SPE analysis. The concentrations for the calibration curve ranges between 10 – 1000 ng L^{-1} for the online SPE/LC. Mixed solution 3030 000 ng L^{-1} of internal standards were prepared. For the recovery experiment, a 9 mL mixture of effluent water sample were spiked with 1 mL of 10,000 ng L^{-1} mixed standard to give a concentration of 1000 ng L^{-1} and analysed with the online SPE/LC (section 4.2.4). The same sample was analysed with direct injection for liquid chromatography (LC) analysis (without the SPE). The chromatographic setting used for this analysis was a ready-made protocol by (Fick et al., 2015) . The protocol for the analysis was explained in section 4.2.4 and 4.2.5

4.2.3 Sample collection and preparation

Out of the 35 wastewater treatment plants in the Durban metropolitan area, four were selected based on the following criteria.

1. The level of efficiency of the WWTPs in relation to the green drop certification (Greendrop) of the Water and Sanitation Department of South Africa;

2. The ethnic composition of the connected community to the WWTP (Black African, Indian, White and Coloured/others);

3. Transportation distance between the WWTP and the University laboratory for ease of access.

The states of operation of the selected WWTPs plants during the past one year were in compliance with green drop certification status. The WWTPs treatment methods, the population served, the sewage source and the operating and discharge capacities, are summarised in Table 4.2.

Composite grab samples of 2.5 L were used for all analysis and collected as 200 mL subsamples every 5 min over a period of 1 hr and pooled in amber glass bottles. Samplings were done during the peak flow period, between 9 am and 12pm. Samples were collect twice a month for the 4 months' period of Feb to May 2017. Figure 4.1 and 4.2 illustrates the treatment steps and the sampling points.

(1) Raw untreated wastewater after the screening step (influent samples)
(2) The activated sludge samples were collected immediately after the deep shaft aeration unit and the trickling filter samples were collected shortly after the rotating biological filtration unit (pre-chlorination samples)
(3) The pre-chlorination samples were collected after the secondary settlement tank and the clarifier unit before the chlorination step (Effluent).

(4) The post chlorination samples were collected five meters from the chlorination point before the final sedimentation.

The downstream samples were collected at approximately 100 meters from the point of impact of the effluent from the WWTPs on the receiving water bodies, at a depth of 1 meter below the water surface. Post-chlorination and downstream effluent samples were collected 4 – 5 hours after the influent peak period. Upstream samples were collected only for "I" WWTP because there is a direct link of the upstream with the effluent from the WWTP and there were no interference from other water bodies. Other WWTPs had several interferences (streams), which were unreachable, connecting to the final effluent as they navigate to the downstream, hence these samples were not collected.

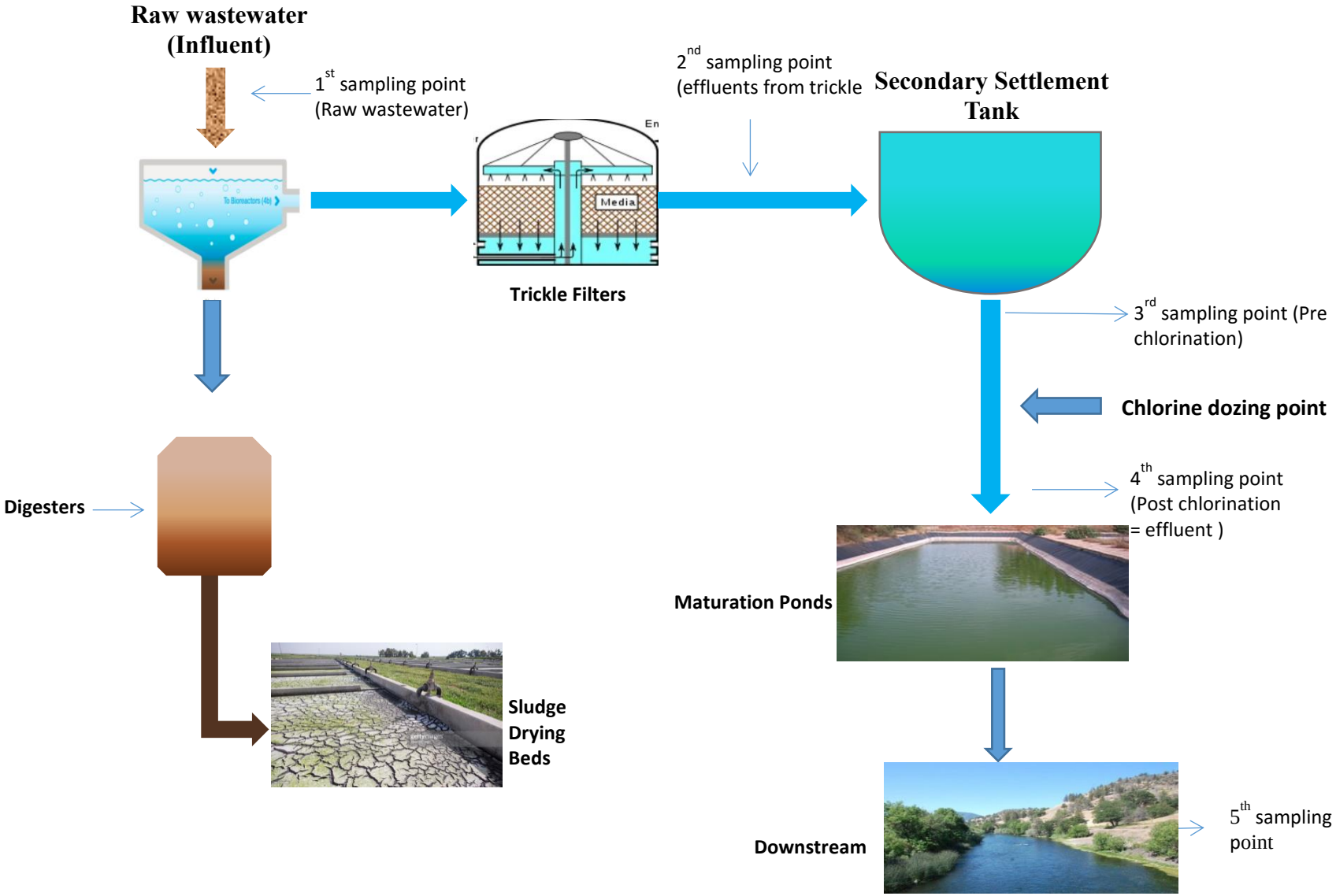


Figure 4.1: Flow diagram of WWTP using trickling filter treatment method showing the various sampling points

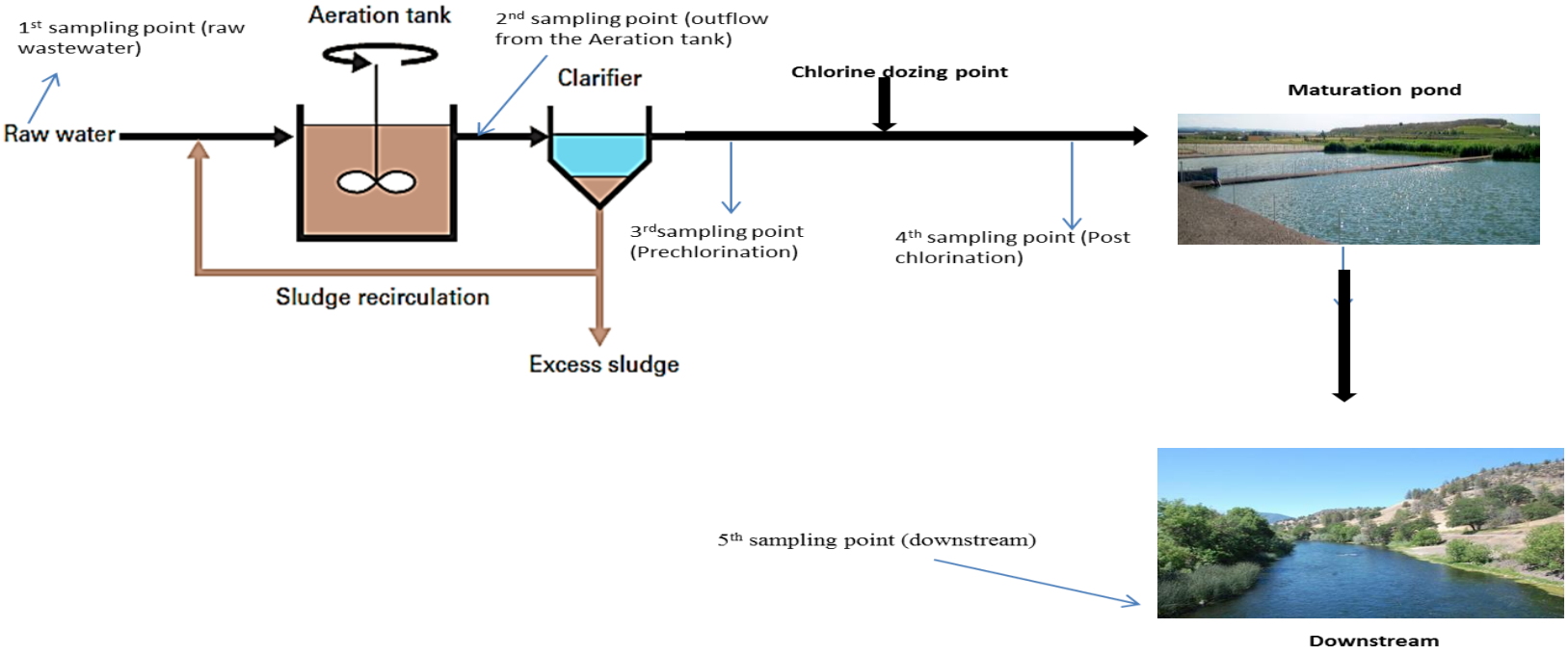


Figure 4.2: Flow diagram of WWTP using activated sludge treatment method showing the various sampling points

Samples were transported to the laboratory at a temperature of 4 ^{0}C. Oxygen demand, pH and Total Dissolved Solid (TDS) analysis were then measured and recorded. The samples were divided into two parts, where one portion was frozen at -80 ^{0}C reserved for later analysis, while the other were processed directly. The Influent and aeration tank samples (150 mL portions) were centrifuged at 10 500 revolutions per minute (rmp) for 5 min and then filtered through 0.45 µm syringe filters. The sediments from the centrifuge process were kept separately from the supernatant (100 mL) in a 10 mL centrifuge bottle for further analysis. The sediment (centrifuged) and supernatant samples were stored in the dark at -80 ^{0}C and later transported to the Environmental Chemistry Department at Umeå University, Sweden for further analysis.

Table 4.2: Characteristics of the four investigated wastewater treatment plants.

WWTPS (with virtual name) /dominating race	**'P'/ Indians**	**'I'/ Blacks**	**'K'/ Blacks**	**'S' /Indians**
POPULATION OF INHABITANT SERVED (Approx.)	**109,000**	**74,000**	**26,000**	**56,000**
SEWAGE SOURCE	**Domestic**	**Domestic**	**Domestic/ Industrial (20%)**	**Domestic**
PRIMARY TREATMENT PROCESS	**S ,AS, Deep shaft and step aeration, RBF, SED**	**S, G, RBF ,trickling filter, SED**	**S, AS, Deep shaft and step aeration, RBF,SED**	**S, AS, Deep shaft and step aeration, RBF, SED**
FINAL TREATMENT PROCESS	**Chlorination**	**Chlorination**	**Chlorination**	**Chlorination**
OPERATING / DISCHARGE CAPACITY (ML/d)	**22.00 / 39.02**	**10.98 / 30.61**	**4.69 / 11.07**	**10.08 / NA**

S, Screening; G, Grit Removal; SED, Sedimentation; AS Activated Sludge; NA Not Available; RBF rotating biological filters

4.2.4 Online SPE LC-MS/MS

Online SPE analysis was done at the Chemistry Department, Umeå University Sweden. Samples were thawed and filtered using 0.45 µm cellulose acetate syringe filters. Prior to the filtration, the samples were centrifuged at 10,500 rpm for 5 minutes; the sediments stored at -80 ^{0}C and supernatants were further filtered to ensure that it was free of particles. Exactly 10 mL of filtered samples were spiked with 30 µL of internal standard and analysed using an online SPE-LC-LC/MS. An online Oasis SPE column was used as the extraction column. The analysis was performed by using a column switching system which consists of 6-ports and 10-ports switching valves manufactured by Thermos Scientific (Figure 4.3)**.** The flow direction is indicated by the various arrows (with different colours indicating different positions of the instrument) in Fig 4.3; with 1.1 mL of sample injected into the 1 mL loop to avoid void volume in the loop. This was further loaded on the SPE using the surveyor pump slowly for a period of 2 min. The divert valve changes the load position to injection position after 2 min of loading the samples on the SPE column. The analytes of interest retained on the SPE were eluted using the Accela pump and further loaded on the analytical column for chromatographic separation and detection. (Khan et al., 2012)

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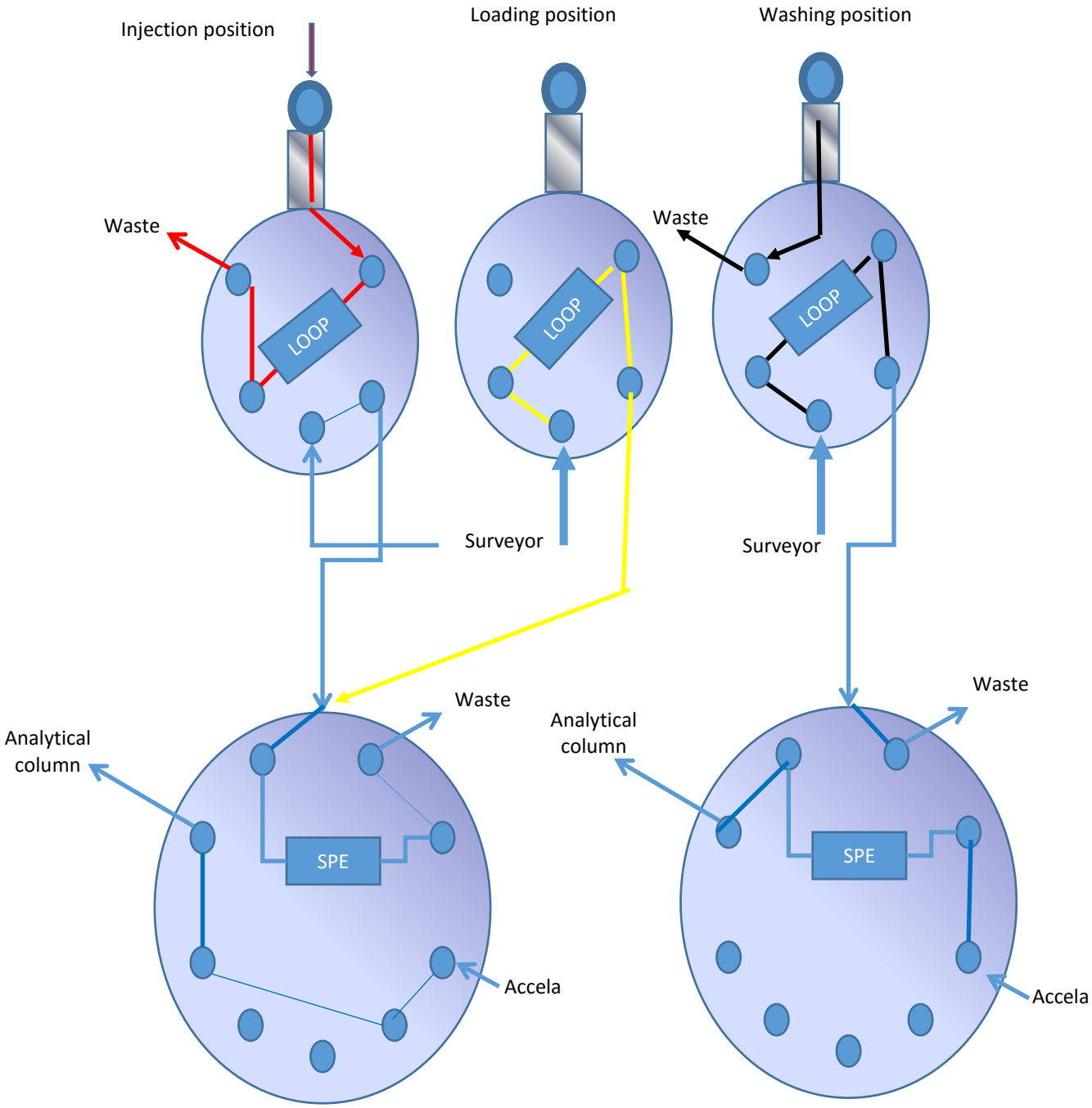


Figure 4.3: Online SPE setup

4.2.5 Method validation

The identification of each standard was based on the retention time, the precursor and product ion generated in tandem mass spectrometry. A calibration curve ranging from 10 – 1000 ng L^{-1} was used to monitor the identification. Analyte in the samples must match the standard in the calibration curve in terms of retention time and transition ions range (Table 4.3) in line with the SANTE/SANCO validation guidelines (European Commission, 2016). The accuracy of the analysis was further improved upon by making use of the internal standard method.

An internal standard was added to the samples, blanks and standard solutions at a constant amount (30 µL), to compensate for both systematic and random errors.

Four internal standards were used;

(1) 13C3-trimethoprim for the identification of ethionamide, trimethoprim and sulfamethoxazole.
(2) Tramadol 12CD3 for metronidazole, norfloxacin, clindamycin, ofloxacin and ciprofloxacin.
(3) Risperidone D4 for albendazole, clarithromycin and roxithromycin
(4) Carbamazepin D10 for erythromycin and azithromycin.

Relative recovery was performed by spiking a specific concentration of antibiotics (1000 ng L^{-1}) in a composite (1 mL taken from each sample) of all wastewater from individual WWTPs and analysed through the online SPE, while unspiked samples of the same composite were analysed with the offline LC. Analysis was done in triplicate. The following calculation was used to calculate the percentage recovery; [(spiked wastewater - unspiked wastewater) X 100]/spiked concentration. The limit of detection was calculated from the regression analysis using the calibration curve, in ng L^{-1}. The results are shown in Table 4.3.

Table 4.3: Antibiotics retention time, limit of detection (LOD), limit of quantification and precursor ions

Name of Antibiotics	Retention time (min)	Parent ion (m/z)	LOD (ng L^{-1})	LOQ (ng L^{-1})	Percentage recovery	Daughter ions (m/z)
Ethionamide	**2.56**	**167.00**	**0.30**	**0.99**	**69.60**	**107.28 - 140.20**
Metronidazole	**2.64**	**172.00**	**0.17**	**0.56**	**86.00**	**82.30 - 128.20**
Trimethoprim	**4.69**	**291.00**	**0.11**	**0.36**	**94.70**	**123.20 - 230.10**
Norfloxacin	**5.16**	**320.00**	**0.22**	**0.73**	**53.70**	**230.10 - 302.10**
Ofloxacin	**5.03**	**362.05**	**0.22**	**0.73**	**51.40**	**261.10 - 318.20**
Ciprofloxacin	**5.24**	**332.00**	**0.33**	**1.09**	**56.20**	**23.10 - 288.20**
Albendazole	**7.22**	**266.00**	**0.16**	**0.53**	**111.10**	**191.20 - 234.20**
Sulfamethoxazole	**5.37**	**254.00**	**0.15**	**0.50**	**75.40**	**108.20 - 156.00**
Clindamycin	**6.61**	**425.10**	**0.07**	**0.23**	**100.60**	**126.20 - 377.30**
Erythromycin	**7.26**	**734.30**	**0.14**	**0.46**	**84.70**	**558.50 - 576.60**
Azithromycin	**7.78**	**749.40**	**0.11**	**0.36**	**54.30**	**158.20 - 591.60**
Clarithromycin	**7.79**	**748.40**	**0.12**	**0.40**	**95.40**	**158.10 - 590.50**
Roxithromycin	**7.85**	**837.40**	**0.09**	**0.30**	**93.10**	**158.10– 769.60**

Lower recovery < 60% were obtained for some of the antibiotics, which may be as a result of low sensitivity for the particular antibiotics or due to poor extraction. However, the acceptable percentage recovery range for low concentration (ng L^{-1}) is 50% - 120% (European Commission, 2016)

4.2.6 Microwave Assisted Extraction of Sediment Samples

This method was adopted and modified from (Dorival-García et al., 2013). Sediments samples of influents and outflow from aeration tank before clarifier (Fig 4.2) of the wastewater treatment plants, were extracted in a microwave assisted extraction (MAS) unit. A sub-volume (0.5 g) of each sediment (centrifuged) sample was diluted with ultra-pure water (5 mL) and placed in a microwave vessel with a Teflon cover. Ten mL of McIlvaine's buffer (pH 3) and methanol (50:50 v/v) was used as the extraction solvent. McILvaine's buffer was prepared by dissolving 28.38 g of the disodium phosphate powder in 100 mL of ultra-pure water and made up to 1 L. Citric acid (0.2 M) was prepared by dissolving 19.21 g powder in 1 L of ultra-pure water. The McIlvaine's buffer was finally produced by mixing 0.41 mL of sodium phosphate and 15.89 mL of citric acid. This was later mixed with methanol at 50:50 v/v to produce the extraction solvent.

The extraction was performed at 87^0C at 1000 W for 17 min with a holding time of 5 min in the microwave extractor. Four samples were processed simultaneously. The samples were allowed to cool to room temperature after the microwave exposure and was thereafter centrifuged for 5 min at 6500 rpm at 25^0C (Dorival-García et al., 2013). The supernatant was filtered and subjected to online SPE as described earlier under the subsection online SPE LC-MS/MS.

4.3 RESULTS AND DISCUSSION

The details of the results of analysis for this research are described in this section.

4.3.1 Antibiotics in sediment of centrifuge samples versus in the supernatant phase

One of the major obstacles in the analysis of high matrix samples such as WWTPs influent is the loss of analyte during the sample filtration step. Filtration involves the use of filters for the elimination of particles, usually organic matters, that can block the analytical tubing and cause reduction of instrument sensitivity (Pavlović et al., 2007). Analyte of interest attached to particles or sediment are like-wise eliminated during the filtration process. Consequently, the results of an analysis, which involves filtration, will only give the liquid fraction concentration of the analyte of interest in the samples. To avoid this error, the applied protocol used centrifugation of the influent samples. The sediments of the centrifuged samples were extracted as stated in section 4.2.6, using the MAE, while the liquid sample (supernatant) was analysed directly using the online SPE method described under 4.2.4 "online SPE-MS/MS". The total concentration of antibiotics in the sediments and supernatants in the influent samples of the four WWTPs composite samples for a period of four months, (bi-weekly samples) were used for the calculation of the percentage of antibiotics in sediments, supernatant and total concentration as shown in Table 4.4. The influent samples were collected 8 times (twice a month for 4 months) making a total of 32 samples. A composite which composes of 10 mL of each sample was used for the analysis.

Table 4.4: Percentage of antibiotics in sediments and supernatant of centrifuged influent samples (n = 32)

Antibiotics	%Antibiotic in sediment (*)	%Antibiotic in supernatant (SD)	Total antibiotics (conc) µg L^{-1} (SD)
ETI	**55.2**	**44.8 (17.5)**	**0.2 (0.1)**
MET	**43.4**	**56.6 (34.4)**	**41.6 (71.4)**
TRI	**28.3**	**71.7 (20.9)**	**8.6 (4.9)**
NOR	**93.1**	**6.9 (1.6)**	**0.6 (0.3)**
OFL	**96.6**	**3.4 (2.3)**	**22.4 (13.6)**
CIP	**97.3**	**2.8 (1.2)**	**493.2 (187.5)**
ALB	**77.1**	**22.9 (38.8)**	**719 (509.3)**
SUL	**19.8**	**80.2 (21.6)**	**16.5 (11.8)**
CLI	**72.5**	**27.5 (15.8)**	**0.2 (0.1)**
ERY	**2.6**	**9.4 (3.3)**	**0.3 (0.2)**
AZI	**90.9**	**9.1 (8.5)**	**0.4 (0.3)**
CLA	**46.1**	**53.9 (39.4)**	**8.4 (8.5)**
ROX	**45.0**	**55.0 (39.2)**	**3.7 (4)**

SD = standard deviation from the four WWTPs, * SD for the sediment and supernatants are same, hence not stated

The total concentration on this table (Table 4.4) is made up of the sum of the concentration of the antibiotics in the supernatant and sediment after centrifugation of the samples. This two different phases (liquid and semi-liquid) of the same sample contains different concentration of the antibiotics. Likewise, large variation was observed in the concentration of the antibiotics in the 32 samples due to the variation in the samples taken; these variations are well documented in the attached appendix 1. Details of the individual concentrations of antibiotics from the four WWTPs are available in a supporting document (Appendix 1).These percentages were calculated using the following calculation step; (100 X Sedi.Con) / (Supn.Con + Sedi.Con), where Sedi.Con is the concentration of antibiotics in the sediments and Supn.Con is the concentration of the antibiotics in the supernatants. The average values for each antibiotic among the four WWTPs are tabulated in Table 4.4

The two different portions (sediment and supernatant) of the influent sample contain varying concentrations of antibiotics. This can be exemplified by NOR, OFL, CIP, AZI where more than 90% of the total antibiotics concentration were found in the sediment portion. A dominance of sediment bound antibiotics also occurred for ALB and CLI (more than 70% in the sediment) (Table 4.4). The adsorption properties of these antibiotics accounts for the higher occurrence in the sediment portion of the samples. NOR, OFL, and CIP belongs to the fluoroquinolone group of antibiotics, which mostly express a high adsorption to soil and sediment (Leal et al., 2013, Zhou et al., 2013). Likewise, the macrolides AZI, CLA, ROX, tends to have high adsorption to sediment/soil with the exception of ERY (Gibs et al., 2013). Sulphonamide (e.g. SUL) has lower adsorption than fluoroquinolones and are generally unlikely to be stable in sediment of WWTPs (Thiele-Bruhn et al., 2004, Radke et al., 2009). There are several factors (beyond the scope of this study) that may influence the adsorption of SUL and ERY to sediment, such as, the pH of the sediment, hydrophobicity and hydrogen bonding, just to mention a few (Wegst-Uhrich et al., 2014). Neither ALB nor antibiotics used in relation to animal husbandry have been investigated in relation to their occurrence in sediments. This is based on non-availability of reports on this aforementioned topic. However, high concentration of a antibiotics similar to ALB (Thaibendazole) has been recorded in soil and sediment samples with high adsorption (Snow et al., 2016, PubChem, 2016). The similarity in structure between Thaibendazole and ALB may explain the relatively high binding proportion (77 %) of the latter in the sediment samples. More than half of ETI (55 %) was quantified from the sediments while MET and TRI were present at above 40 % (Table 4.4). This percentage contributes significantly to the overall concentration of the antibiotics in the influent samples. The lowest percentage quantified in the sediment analysis is 2.6 % for ERY with a standard deviation of 3.5 %. Other low concentration was TRI (28 %) and SUL (19.8 %). These low percentages are due to the low adsorption properties of these antibiotics to

sediments. Thus analysis of the liquid portion of influent samples does not give the total concentration of the antibiotics present in the sample.

4.3.2 Frequency of Detection of Antibiotics

As summarised in Table 4.5, the selected antibiotics were detected in all the WWTPs influent at least once out of the many sampling periods (median and max concentrations are detailed in the following section 4.3.4). For example at "K" and "P" WWTPs, ETI was absent only once during the sampling period (8), twice at "I" WWTPs, and was present in all the samples taken at "S". ETI is one of the least detected antibiotics but was still present moderately in all the samples taken. This low occurrence can be related to the metabolism of ETI leading to the excretion of less than 1 % of ETI as parent compound (Wishart et al., 2006, DrugBank, 2017). ETI, is however a constantly used drug in the second line approach for the treatment of resistant TB (DeBarber et al., 2000). Tuberculosis is a prevalent disease in South Africa and with high incidence in KwaZulu-Natal (KZN), where the samples for this study were taken from (Bantubani et al., 2014). "K" WWTP had the highest number of antibiotic compounds detected among the four plants, with all the antibiotics detected at 100% except for ETI which had a frequency of 85.7%. This was closely followed by "S" WWTP, where AZI, SUL and CLA had a frequency of detection of 75 %, 87.5 %, 87.5 % in the influent samples respectively, while the rest were detected at 100%. Thirdly is "P" WWTP, where ETI, ERY and AZI were detected at 87.5 % and ROX was detected at 75 % of the samples. "I" WWTP has the least frequency of occurrence for most of the antibiotics, with only TRI, NOR, OFL and CIP occurring at 100 % and rest below 100 %. AZI was the least detected antibiotics at 35.7 % frequency of occurrence. The instability of AZI in the environment or low consumption rate may be the reason for the low detection. AZI has a long half-life which encourages the use of small quantity (once daily dose) (McMullan and Mostaghim, 2015), which in parallel, will result in

a low concentration of the drug in the body system. The excretion rate, which is less than 0.1 % of the administered drug, also contributes to the low frequency of detection. Approximately 86 % of CLI is excreted as bio-inactive metabolites (DrugBank, 2017). TRI, NOR, OFL, and CIP were detected in all the 32 influent samples taken from the 4 WWTPs at 100% frequency. This is an indication of the ubiquitous nature of these compounds which can be directly related to its frequency of use. These four antibiotics are used in the treatment of Tuberculosis (TB). Three of these antibiotics are fluoroquinolones (NOR, OFL, CIP) which are frequently used in the treatment of non-resistant/mild cases of TB (Bantubani et al., 2014). Their excretion of unchanged active antibiotics is approximately 40 – 80 % of the administered drug. Likewise, TRI are used mostly for the treatment of resistant cases of TB and their excretion rate is between 50 – 60 % (Gandhi et al., 2006, DrugBank, 2017). Hence the endemic nature of tuberculosis in KwaZulu-Natal (Bantubani et al., 2014) and the high quantity of excreted active components accounts for the high frequency of detection of the aforementioned antibiotics in the wastewater influent. Similar frequency of detection of fluoroquinolones were recorded by (Agunbiade and Moodley, 2014) from sample taken from within the KZN region.

Table 4.5: Frequency and concentration of antibiotics detection in four wastewater treatment plants from influent samples.

	"I"		"K"		"S"		"P"	
	Min-Max Conc (ng L^{-1})	**F (%) n=8**	**Min-Max Conc (ng L^{-1})**	**F (%) n=8**	**Min-Max Conc (ng L^{-1})**	**F(%) n=8**	**Min-Max Conc (ng L^{-1})**	**F (%) n=8**
ETI	**6.4 - 38.6**	**62.5**	**0.1 – 49.5**	**87.5**	**0.3 - 194.7**	**100**	**3.3 - 87.4**	**87.5**
MET	**1.0 - 48.0**	**62.5**	**10.0 – 86.8**	**100**	**10.3 - 164264.4**	**100**	**11.8 - 462.4**	**100**
TRI	**13.3-7649.9**	**100**	**92.5 – 1055.8**	**100**	**78.4 - 8815.2**	**100**	**54.9 -1260.8**	**100**
NOR	**8.0 - 194.0**	**100**	**9.8 – 206.5**	**100**	**24.3 - 261.6**	**100**	**18.4 - 82.1**	**100**
OFL	**1336.2-4646.6**	**100**	**3191.4 – 9662.4**	**100**	**200.9 - 8229.5**	**100**	**760.3 - 2750.0**	**100**
CIP	**16702.1-66941.2**	**100**	**50769.1-113144.8**	**100**	**411.5 - 501575.5**	**100**	**12601.3 - 83630.2**	**100**
ALB	**0.5 - 156796.1**	**87.5**	**5917.2 - 84838.1**	**100**	**6063.2 - 763610.9**	**100**	**278.1 - 266007.2**	**100**
SUL	**215.7-2949.9**	**100**	**1116.2 - 8456.9**	**100**	**26.9 - 4018.6**	**87.5**	**40.6 - 9054.4**	**100**
CLI	**3.1 - 28.2**	**87.5**	**8.5 - 87.1**	**100**	**0.2 - 72.7**	**100**	**2.7 - 15.6**	**100**
ERY	**0.6 - 201.9**	**87.5**	**10.8 - 61.5**	**100**	**0.9 - 71.4**	**100**	**0.8 - 20.1**	**87.5**
AZI	**0.8 - 122.9**	**37.5**	**10.9 - 288.9**	**100**	**2.6 - 527.7**	**75**	**0.5 - 69.8**	**87.5**
CLA	**0.3 - 1743.0**	**62.5**	**289.2 - 1727.9**	**100**	**3.7 - 340.2**	**87.5**	**22.4 - 7515.3**	**100**
ROX	**3.0 - 2514.9**	**75**	**23.3 - 145.1**	**100**	**0.0 - 77.2**	**100**	**0.1 6012.8**	**75**
n = number of sampling, where 8/8 = 100%, F = frequency								

4.3.3 Concentration of Antibiotics in Influent Samples of WWTPs

The median and maximum concentration of the antibiotics in the influent samples of the four WWTPs for the sampling period (number of sample per antibiotics, per WWTP = 8), was used as representative concentration of the individual antibiotics (Table 4.6). This depends on the large variation in the concentration of antibiotics in the samples taken (details of the individual concentration are available in Appendix 1 attached). Of the four WWTPs, the level of antibiotics in "S" WWTP was relatively higher than in the other plants based on the maximum concentration of the individual antibiotics. The highest concentration for all antibiotics was recorded in "S" WWTP, except for ERY and ROX. Some of the recorded maximum concentrations were calculated to be outliers, of the measurement of interquartile range (Q1 and Q2) (Tukey, 1977). Q1 and Q2 were calculated from the median concentration values of the antibiotics of the second quartile and ordinary median value, respectively. For example, the concentration of ALB in 'I',' K' and 'S' WWTPs and CLI in 'K' WWTP were calculated to be outliers. Details of the calculations for all individual values are presented in Appendix 2. The influent concentration (median) was generally below the PNEC except for OFL and CIP which were higher in all the WWTPs. This is an indication of the non-toxic concentration of the individual concentration of the antibiotics, but this does not mean that this concentration cannot initiate antibiotic resistance. The comparison was made based on Bengtsson-Palme and Larsson, 2016, result and no actuall analysis of the inhibitory capacity of the concentration was performed.

Table 4.6: Concentration of the Antibiotics in the Influent Sample WWTPs with the predicted no effect concentration (PNEC)

	Influent (I)		Influent (K)		Influent (S)		Influent (P)		PNEC
Conc (ng L^{-1})	Median	Max	Median	Max	Median	Max	Median	Max	
ETI	8.1	38.6	17.8	49.5	19.7	194.7	21.6	87.4	-
MET	8.8	48	22.4	86.8	76.7	164264.4	51.9	462.4	125
TRI	721.2	7649.9	650	1055.8	295.4	8815.2	269.8	1260.8	500
NOR	33.8	194	40.8	206.5	130.2	261.6	42.5	82.1	500
OFL	1695.6	4646.6	4388.235	9662.412	648.7	8229.5	1805.7	2750	500
CIP	30704.5	66941.2	81748	113144.8	18495.6	501575.5	40959.9	83630.2	64
ALB	41544.5	156796.1	13064.3	84838.1	16956.2	763610.9	65525.5	266007.2	-
SUL	577.8	2949.9	4669.6	8456.9	304.6	4018.6	716.7	9054.4	16000
CLI	9.1	28.2	41.9	87.1	7.3	72.7	7.5	15.5	1000
ERY	37.1	201.9	39.3	61.5	17.6	71.4	2.9	20.1	1000
AZI	0	122.9	81.3	288.9	4.3	527.7	1.3	69.7	250
CLA	26.6	1743	680.4	1727.9	29.4	340.2	1822.4	7515.2	250
ROX	50.5	2514.9	58	145.1	3.4	77.2	471.2	6012.8	1000

***PNEC is sourced from (Bengtsson-Palme and Larsson, 2016), - values not available**

Besides the high concentration of ALB and OFL, the data pattern of ETI, NOR, CLI and all the macrolides' (CLA, ERY, AZI and ROX) showed small variations in all the four WWTPs within a median concentration range of 0 – 1822.4 ng L^{-1}. Similar concentration of macrolide (ERY) of 1130 ng L^{-1} was detected in a WWTP within the KZN province (Matongo et al., 2015). The concentration of the remaining four antibiotics (MET, TRI, CIP and ALB) varied largely, which cannot be explained based on the constant usage of these antibiotics. For example CIP and OFL are also generally abundant in high concentrations in all four WWTPs and can be related to the frequent use of the drug(s) in the first line of approach in the treatment of respiratory infection, which are related to polymicrobial pneumonia (Metersky et al., 2007). Pneumonia ranks as the highest killer infection in South Africa (Siemieniuk et al., 2011). CIP in 'K' WWTP had the highest median concentration of 81748 ngL^{-1}within the range of

64000 ng L^{-1} – 101000 ng L^{-1} for all the recorded concentration (Appendix 1). This result indicates a skewed dataset, which tends towards a higher concentration. This suggests that higher concentrations of CIP were detected above the median level in most of the samples taken. For example, CIP had a relatively higher concentration (median values) in three out of the four WWTPs; 18496 ng L^{-1}, 30704.5 ng L^{-1} and 40495 ng L^{-1} in S, I and P WWTPs, respectively. Similar concentration of CIP (27000 ng L^{-1} ± 1200 ng L^{-1}) was recorded from a WWTP in KZN (Agunbiade and Moodley, 2016). A lower concentration range, 37.21 ng L^{-1} to 2935.40 ng L^{-1} , was documented in a similar analysis in China (Hu et al., 2018). Generally, the concentrations recorded for all the antibiotics in this analysis are higher than those recorded in most published research work globally. This is because, the total concentration of the antibiotics in the sample was recorded with minimal loss of the analyte, and this is based on the use of the already described centrifugation method.

4.3.4 Trickling filter treatment process on antibiotics reduction in "I" WWTP

The removal or degradation of pollutants in wastewater involves the general physical processes of filtration, adsorption and absorption. Typically a trickling filter consists of layers of rocks or other similar materials packed closely to enable the removal of pollutants. This layer also contains layers of microorganism (biofilms) which aids in the adsorption of organic matter from the wastewater. Wastewater is sprinkled over the layered surface and the filtered water trickles through the layers, which are then collected in the clarifier for further treatment in the process. It is a biological process without the use of any chemical (Kasprzyk-Hordern et al., 2009). Organic and inorganic pollutants are removed by adsorption and absorption into the developed biofilm of the solid surfaces, within which microbial conversion and degradation may take place. For the assessment of the trickling filter efficiency, samples were taking from the "I" WWTP at three specific points which are, the influent point, trickling filter and sedimentation stage (pre-chlorination). The sampling points are illustrated in Figure 4.2.

The median values were used for data analysis since a large variation in the concentrations from the taken samples occurred, as shown by the difference between the median/maximum values and the average and large standard deviation (Table 4.7).

All thirteen antibiotics were detected in all the treatment stages of "I" WWTP (Table 4.7). A reduction of the concentration (median concentration) is noted over the specific treatment steps. However, an increase in MET and CLA for the median values between the influent and the trickling filter stages was observed which resulted into a negative reduction value of -653.4% and -12.4 % respectively. This increment can be attributed to the accumulation of the antibiotics over time in trickling filter chamber, resulting into seepage of the accumulated antibiotics (Hörsing et al., 2011) and reformation of the clarithromycin metabolite (des-methyl clarithromycin) which is constantly detected in WWTP effluents (Ibáñez et al., 2017). Aside from this, the remaining twelve antibiotics where effectively reduced at this treatment step

(Influent/trickling filter) as shown in Table 4.7. The efficiency of this treatment step is very crucial in ensuring an overall effective treatment process. Further reduction occurred at the sedimentation step (before chlorination) where three antibiotics NOR, OFL and ROX were completely removed (See also Table 4.7). Particle that are attached to the sludge from the trickling filter is expected to remain in the sedimentation chamber, while an antibiotic free effluent flows to the next treatment stage. The high total reduction of the antibiotics in "I" WWTP proved the efficiency of the trickling filter unit treatment process. A similar efficiency result was achieved at WWTP in Taiwan using a similar treatment method, were CLA, SUL and ERY were removed at 99 %, 66 % and 56 %, respectively (Lin et al., 2010).

Table 4.7: Median, maximum and average concentration with standard deviation of investigated antibiotics through "I" WWTP with related percentage reduction

"I"	**Trickling filters Conc (** ng L^{-1}**)**			**Pre-chlorination Conc (** ng L^{-1}**)**			**Percentage reduction**	
Analyte	**Median**	**Max**	**Average (SD)**	**Median**	**Max**	**Average (SD)**	**% reduction (A - B) median**	**% reduction (B–C) median**
ETI	**1.6**	**54081.3**	**6762.3 (17885)**	**0.2**	**20.8**	**4.2 (6.9)**	**80.2**	**87.5**
MET	**66.3**	**14651.5**	**1947.1 (4804.4)**	**55.3**	**440.3**	**111.7 (140.4)**	**-653.4**	**16.6**
TRI	**505.2**	**2927.6**	**743.9 (859.2)**	**645**	**1655.6**	**782.7 (557.9)**	**30**	**-27.7**
NOR	**0.5**	**16.7**	**4.7 (7)**	**0**	**31.3**	**5.2 (10.5)**	**98.5**	**100**
OFL	**1.6**	**293.6**	**52.7 (98.6)**	**0**	**285.7**	**40.6 (93.5)**	**99.9**	**100**
CIP	**59.1**	**3393.8**	**658.0 (1095.9)**	**351.5**	**5153.6**	**1142.9 (1662.6)**	**99.8**	**-494.8**
ALB	**11.9**	**3570.1**	**551.5 (1234)**	**0.7**	**978.5**	**146.3 (318.9)**	**100**	**94.1**
SUL	**2.6**	**475.4**	**84.5 (156.1)**	**8.4**	**1650.3**	**292.3 (540.6)**	**99.6**	**-223.1**
CLI	**1.6**	**11.3**	**4.2 (4.8)**	**0.5**	**9.0**	**2.4 (3.6)**	**82.4**	**68.8**
ERY	**22.7**	**265.1**	**87.4 (108.6)**	**72.6**	**908.4**	**258.8 (329.8)**	**38.8**	**-219.8**
AZI	**0**	**22.6**	**3.4 (7.5)**	**1.8**	**32.6**	**8.9 (12.6)**	**0**	**0**
CLA	**29.9**	**88.3**	**28.8 (28.4)**	**8.3**	**65.3**	**19.3 (23.6)**	**-12.4**	**72.2**
ROX	**3.9**	**35.0**	**6.8 (11)**	**0**	**68.9**	**8.8 (22.8)**	**92.3**	**100**

A - B = percentage reduction between Influent and trickling filter, B - C = Percentage reduction between trickling filter and pre chlorination (sedimentation)

4.3.5 Activated sludge treatment

Activated sludge (AS) system uses aeration and biological floc for the sewage treatment (Henze, 1992). The system uses oxygen for aeration to oxidize organic compounds and the biological floc to reduce the organic content of the sewage. The main wastewater treatment unit processes in "K" "S" and "P" WWTPs (Table 4.2) are based on the use of activated sludge, and chlorination, as the final treatment step. Similar to the trickling filter analysis, the median values of the antibiotics concentrations were used for the calculation of the results. Due to the large variation in the concentration of the antibiotics from the collected samples, which are well documented in the attached appendix 1, the median values was introduce to harmonize the results. The percentage reduction was calculated using the following formula;

$$\text{Percentage reduction} = [(A - B) \times 100)] / A$$

A = Influent concentration; B = Activated sludge; A and B are interchanged for B and C (sedimentation) to calculate the percentage reduction for the sedimentation stage.

$$\text{Percentage reduction for the sedimentation stage} = [(B - C) \times 100)] / B$$

All investigated antibiotics were detected at varying concentrations at all the treatment stages of the three WWTPs (Table 4.8, 4.9, and 4.10). Remarkable reduction of > 95 % was recorded in the concentration of ALB for the three WWTPs, which shows that the AS system is an efficient method for the removal of ALB in wastewater. Information is limited in relation to the presence of antihelmintic drugs (ALB) in biological wastewater treatment processes and the aquatic environment in general, especially in Africa. The negative impact of the sub-lethal concentration on earthworms has been reported (Oh et al., 2006); hence the removal efficiency of antihelmintics may play an important role in sustaining a balanced ecosystem.

Low percentage reduction was recorded for all antibiotics in the samples collected immediately after the activated sludge, when compared to the concentration after the sedimentation from the three WWTPs. The impact of the AS treatment system, which involves biodegradation of the antibiotics (Li and Zhang, 2010) was only significant at the sedimentation stage. For example, the percentage reduction of NOR, OFL, CIP were negative at the aeration stage and > 80 % at the sedimentation stage for all the WWTPs. Similar reduction pattern were recorded for most of the antibiotics in all the WWTPs, except for ROX, TRI, CLA and ERY in the "P" WWTP and also ERY in "S" WWTP. These few exceptions can be as a result of gas turbulence (produced by anaerobic/aerobic microbes) in the sediment, leading to the release of adsorbed analyte. Judging from these results, it can be deduced that the AS system efficiency is largely dependent on the sedimentation step. Similar reduction efficiency was recorded in a more detailed experiment (Sipma et al., 2010) and in Finland, >80 % reduction was recorded for fluoroquinolones in a sewage treatment plant that utilizes mainly activated sludge treatment method (Vieno et al., 2007).

Table 4.8: Median concentration of investigated antibiotics through "K" WWTP with related percentage reduction

'K'	**Influent Conc (ng L^{-1}) (A)**	**Activated Sludge Conc (ng L^{-1}) (B)**	***Sedimentation Stage (ng L^{-1}) (C)**	**Percentage reduction**	
Analyte	**Median**	**Median**	**Median**	**% reduction (A - B) median**	**% reduction (B – C) median**
ETI	**17.8**	**37.8**	**0.9**	**-112.4**	**97.6**
MET	**22.4**	**154.8**	**4.2**	**-591.1**	**97.3**
TRI	**650.0**	**93.7**	**5.8**	**85.6**	**93.8**
NOR	**40.8**	**188.5**	**0.2**	**-362**	**99.9**
OFL	**4388.2**	**14531.9**	**84.6**	**-231.2**	**99.4**
CIP	**81748.0**	**209755.6**	**350.6**	**-156.6**	**99.8**
ALB	**13064.3**	**26267.5**	**ND**	**-101.1**	**100**
SUL	**4669.6**	**850.4**	**186.7**	**81.8**	**78**
CLI	**41.9**	**88**	**13.7**	**-110**	**84.4**
ERY	**39.3**	**0.3**	**2.4**	**99.2**	**-700**
AZI	**81.3**	**72.4**	**ND**	**10.9**	**100**
CLA	**680.4**	**300**	**38.1**	**55.9**	**87.3**
ROX	**58.0**	**14.6**	**2.7**	**74.8**	**81.5**

A - B = percentage reduction between Influent and activated sludge, B - C = percentage reduction between activated sludge and pre chlorination(sedimentation), * = Pre-chlorination stage.

Table 4.9: Median concentration of investigated antibiotics through "S" WWTP with related percentage reduction

'S'	**Influent Conc** (ng L^{-1}) **(A)**	**Activated Sludge Conc** (ng L^{-1}) **(B)**	***Sedimentation Stage** (ng L^{-1}) **(C)**	**Percentage reduction**	
Analyte	**Median**	**Median**	**Median**	**% reduction (A - B) median**	**% reduction (B – C) median**
ETI	**19.7**	**14.2**	**0.1**	**27.9**	**99.3**
MET	**76.8**	**51.4**	**23.9**	**33.1**	**53.5**
TRI	**295.4**	**155.3**	**29.3**	**47.4**	**81.1**
NOR	**130.2**	**359.1**	**8.7**	**-175.8**	**97.6**
OFL	**648.7**	**8389**	**50.3**	**-1193.2**	**99.4**
CIP	**18495.7**	**200979.1**	**810.2**	**-986.6**	**99.6**
ALB	**16956.3**	**11146.5**	**50.1**	**34.3**	**99.6**
SUL	**304.7**	**424.4**	**159.1**	**-39.3**	**62.5**
CLI	**7.4**	**53.1**	**7.1**	**-617.6**	**86.6**
ERY	**17.6**	**22.8**	**31.7**	**-29.5**	**-39**
AZI	**4.4**	**260**	**1.5**	**-5809.1**	**99.4**
CLA	**29.4**	**130.2**	**20.7**	**-342.9**	**84.1**
ROX	**3.4**	**2.4**	**0**	**29.4**	**100**

A - B = percentage reduction between Influent and activated sludge, B - C = percentage reduction between activated sludge and pre chlorination (sedimentation), * = Pre-chlorination stage.

Table 4.10: Median concentration of investigated antibiotics through "P" WWTP with related percentage reduction

'P'	**Influent Conc (ng L^{-1}) (A)**	**Activated Sludge Conc (ng L^{-1}) (B)**	***Sedimentation Stage (ng L^{-1}) (C)**	**Percentage reduction**	
Analyte	**Median**	**Median**	**Median**	**% reduction (A - B) median**	**% reduction (B – C) median**
ETI	**21.7**	**49.5**	**14.5**	**-128.1**	**70.7**
MET	**52**	**400.5**	**36.4**	**-670.2**	**90.9**
TRI	**269.8**	**88.9**	**48**	**67**	**46**
NOR	**42.5**	**261.3**	**0.8**	**-514.8**	**99.7**
OFL	**1805.7**	**7957**	**26.2**	**-340.7**	**99.7**
CIP	**40960**	**138755.8**	**330.8**	**-238.8**	**99.8**
ALB	**65525.6**	**23094.9**	**855**	**64.8**	**96.3**
SUL	**716.7**	**398.7**	**208.2**	**44.4**	**47.8**
CLI	**7.6**	**64.8**	**1.6**	**-752.6**	**97.5**
ERY	**2.9**	**0.2**	**0.1**	**93.1**	**50**
AZI	**1.4**	**7.3**	**0.9**	**-421.4**	**87.7**
CLA	**1822.5**	**84.2**	**9**	**95.4**	**89.3**
ROX	**471.2**	**0.5**	**1**	**99.9**	**-100**

A - B = percentage reduction between Influent and activated sludge, B - C = percentage reduction between activated sludge and pre chlorination (sedimentation), * = Pre-chlorination stage.

4.3.6 Comparative analysis of the efficiency of wastewater treatment methods

The efficiency of the two basic treatment methods is compared in this section. The percentage reduction previously calculated (Table 4.8 – Table 4.10) between the trickling filter and the sedimentation step was used for the comparison with regards to the trickling filter process. Likewise, AS and sedimentation step percentage reduction, was used for the AS process. Table 4.11 shows a comparison of the percentage differences in concentration of the antibiotics between the trickling filter and the activated sludge treatment stage for the four WWTPs. The highest reduction in the antibiotics concentration was recorded at the sedimentation step, after the two main stages (Trickling filter and Activated sludge) for each WWTP. In general, the proportion of antibiotics removed using the activated AS was higher than with the trickling filter process (Table 4.11).

A low reduction percentage was recorded for MET in the trickling filter when compared with the AS treatment processes (Table 4.11). This can be related to the lack of oxidation to enhance their removal of most especially MET. This can be proven from the low removal efficiency of MET via adsorption only, which has been demonstrated in other studies (Kummerer et al., 2000, Prados-Joya et al., 2011). Likewise an oxidation process has been shown to be more effective in removing MET from wastewater (Shemer et al., 2006). Furthermore, the effect of oxidation on MET resulted in 16.6 % removal in the trickling filter and above 50 % for all the AS processes. High percentage removal of above 90% was recorded for NOR, OFL and ALB, for the two treatment process. The reason for the negative reduction designated as zero in Table 4.11, cannot be explained, however, the possibility of emerging gases from the sediment, triggering the release of adsorbed antibiotics may be a possible inference. Likewise, this may be due to metabolic reactions such as, cleavage, glucuronation or hydroxylation, reverting back the parent active ingredient and making it seems that there is an increase of that parent compound in WWTPs (Díaz-Cruz and Barceló, 2005).

Both treatment methods has proven over time to be effective in the removal of antibiotics and other organic compounds from wastewater (Kasprzyk-Hordern et al., 2009, Henze, 1992, Sipma et al., 2010, Li and Zhang, 2010, Abou-Elela and El-Khateeb, 2015).

Table 4.11: Comparison of trickling filter and activated sludge system in wastewater treatment

Analyte	Trickling filter ("I" WWTP)(% reduction)	Activated sludge ("K" WWTP) (% reduction)	Activated sludge ("S" WWTP) (% reduction)	Activated sludge ("P" WWTP) (% reduction)
ETI	**87.5**	**97.6**	**99.3**	**70.7**
MET	**16.6**	**97.3**	**53.5**	**90.9**
TRI	**NR**	**93.8**	**81.1**	**46**
NOR	**100**	**99.9**	**97.6**	**99.7**
OFL	**100**	**99.4**	**99.4**	**99.7**
CIP	**NR**	**99.8**	**99.6**	**99.8**
ALB	**94.1**	**100**	**99.6**	**96.3**
SUL	**NR**	**78**	**62.5**	**47.8**
CLI	**68.8**	**84.4**	**86.6**	**97.5**
ERY	**NR**	**NR**	**NR**	**50**
AZI	**NR**	**100**	**99.4**	**87.7**
CLA	**72.2**	**87.3**	**84.1**	**89.3**
ROX	**100**	**81.5**	**100**	**NR**

Negative reduction or No reduction = NR

4.3.7 Post chlorination effect on antibiotics in wastewater treatment

The percentage reduction at the post-chlorination step was calculated using the median concentration of the antibiotics at the pre-chlorination (sedimentation stage) with the following formula; [(100 (a-b))/a], where "a" is the concentration of the antibiotics at the pre-chlorination stage and "b" is the average concentration of the antibiotics at the post-chlorination stage. The four WWTPs utilize chlorine in the form of hypochlorite for the treatment of their final effluent. This concentration was inconsistent (2mg L to 75 mg L), because all the WWTPs chlorinate directly, using an improvised bucket where the hypochlorite is mixed with water constantly, and released into the wastewater effluent in drops (10 -30 drops per min). Chlorine in the form of calcium hypochlorite $Ca(ClO)_2$ is used as at the time of sampling at the WWTPs. $Ca(ClO)_2$ reacts with water to produce a weak acid, hypochlorous acid as the main chlorine species;

$$ClO^- + H_2O \longrightarrow HOCl + OH^+$$

which further dissociates into hypochlorite

$$HOCl \rightleftharpoons ClO^- + H^+$$

The hypochlorous acid reacts with most of the organic and inorganic compound during post chlorination of effluent water.

Hypochlorous acid (HOCl) is an oxidizing agent with pathogen disinfectant properties (Williams and Braun-Howland, 2003). It has been demonstrated that HOCl is a powerful oxidant to certain organic functional group such as tertiary amino groups and phenolic moieties (Deborde and Von Gunten, 2008). This oxidative property is carried out through oxidation reaction, addition reaction, and nucleophilic substitution reaction with organic compounds. For example, TRI (mass of 290.32 g mol-1) with the molecular formula $C_{14}H_{18}N_4O_3$ has been reported to react with chlorine to form several reaction products (Wu et al., 2016b). The increase in the molecular weight is as a result of the additional chlorine molecule attached to

TRI. Many antibiotics possess these functional groups in their chemical structures. Hence, HOCl will oxidize a large number of antibiotics. Amino group is present in the following antibiotics: ETI, MET, TRI, NOR, OFL, CIP, ALB, and SUL, while phenolic group is present in CLI, ERY, AZI, CLA and ROX. Therefore, all the antibiotics in this research are expected to be oxidized to a certain extent by HOCL (Deborde and Von Gunten, 2008). The formation of derivates from this reaction were analysed (See Chapter 5). The effect of chlorine on the three fluoroquinolones (NOR, OFL, CIP) in this study, was inconsistent. Only CIP was removed in "I" WWTP at 81.8%, similarly, NOR alone, was removed in "S" WWTP at 70.1 %, while OFL (21.5 % in "K", 41.2 % in "P") and CIP (66.8 % in "K", 20.6 in "P") were removed, respectively (Table 4.12). The average residual chlorine at the point of sampling for the four WWTPs ("I", "K", "S", "P") were 0.3, 0.2, 0.2, and 0.3 mg L^{-1}, respectively, while the average total dissolved solid (TDS) was 400 mg L^{-1}. Li and Zhang (2013) reported a removal efficiency for fluoroquinolones within the range of 51 % – 85 %, with a higher residual chlorine of 5 mg L^{-1} – 15 mg L^{-1}. Concentration of the residual chlorine plays an important role in the removal efficency antibiotics (Li and Zhang, 2013) and this explains the low removal/inconsistency recorded for floroquinolones in this analysis. In addition to this, the high removal rate can be related to the low concentration of antibiotics (n gL^{-1}) in the effluent.

Table 4.12: Percentage reduction of antibiotics in wastewater at post chlorination stages

	"I"WWTP	"K" WWTP	"S" WWTP	"P" WWTP
Antibiotics	**% Reduction**	**% Reduction**	**% Reduction**	**% Reduction**
ETI	**NR**	**88.9**	**NR**	**35.9**
MET	**96.6**	**35.7**	**13.9**	**64.0**
TRI	**53.3**	**NR**	**NR**	**74.8**
NOR	**NR**	**NR**	**70.1**	**NR**
OFL	**NR**	**21.5**	**NR**	**41.2**
CIP	**81.8**	**66.8**	**NR**	**20.6**
ALB	**100**	**NR**	**NR**	**20.1**
SUL	**100**	**NR**	**NR**	**NR**
CLI	**100**	**93.4**	**97.1**	**37.5**
ERY	**NR**	**8.3**	**30.6**	**NR**
AZI	**100**	**NR**	**78.6**	**12.5**
CLA	**100**	**20.7**	**NR**	**97.8**
ROX	**NR**	**70.4**	**NR**	**100**

NR = no reduction or negative reduction

The macrolides (CLA, ERY, AZI, and ROX) and the remaining antibiotics exhibited similar low and irregular percentage removal with post chlorination (Table 4.12). These erratic records can be linked to the difference in the characteristics of the wastewater (presence of organic compound, ammonia compounds and suspended solids (Li and Zhang, 2013) and also the low chlorine residual. However, the low residual chlorine had impact on some of the antibiotics as follows, 100 % apparent removal was recorded for five antibiotics; ALB, SUL, CLI, AZI and CLA in "S" WWTP, >70 % removal of ETI, CLI and ROX in "K" WWTP, >70 % removal of NOR, CLI and AZI in "S" WWTP and >60 % of MET, TRI, CLA and ROX in "P" WWTP (Table 4.12). References for comparison of the impact of chlorine on antibiotics in wastewater are limited. Most analysis are based on controlled laboratory experiments and this allows for a significant level of bias in the result (Wang et al., 2010a).

4.3.8 Overall removal of antibiotics in WWTPs

The overall percentage removal of antibiotics was calculated as the difference between the median concentrations of antibiotics in the total influent and effluent (post-chlorination stage) samples taken from the four WWTPs (Table 4.13). The percentage removal of antibiotics is mostly affected by, the efficiency of treatment method employed by the WWTP and the chemical characteristics of the antibiotics in concern. The result in Table 4.13 show that "K" WWTP (which employed AS method) had the highest removal of all the antibiotics. The percentage removal was > 90 % for all antibiotics except for MET with 88 %. The proportion of antibiotics removed was the lowest at "S" WWTP (with same AS method), where the percentage removal of TRI (21 %), CLA (26 %) and ROX (39 %) was the least among the four WWTPs. The reason for the difference in the removal efficiency for "S" WWTP, among the three WWTPs that makes use of AS is largely unknown, but may be based on the higher concentration of antibiotics recorded at the influent level (Table 4.6). Other factors such as the process loading, solids retention time (SRT), nitrogen removal and dilution of the raw sewage may also have affected the removal (Vieno et al., 2007).

"I"WWTP which employs the trickling filter treatment, had a very good removal for all the antibiotics except for TRI with 58 % removal and a negative removal value for ERY. The percentage reduction at "P" WWTP was generally above 50 % except for AZI which was 47 %. Generally, the percentage reduction for all the antibiotics in the four plants was judged as high except for ERY. This indicates that both the AS and trickling filter method are effective in removing antibiotics from wastewater. The results obtained in this study is in line with earlier reports (Sipma et al., 2010, Bernhard et al., 2006, Kasprzyk-Hordern et al., 2009).

Chemical properties of antibiotics linked to adsorption play an important role in the removal efficiency. One of the macrolide (ERY) has the lowest recovery among all the antibiotics,

which is as a result of its low adsorption properties to biofilms of trickling filters (Gibs et al., 2013). This has also been proven in an early analysis (see section 4.3), where 3 % of ERY was detected in the sediment sample and 97 % in the supernatant (Table 4.4). However the high percentage removal of ERY in "K" WWTP cannot be explained. The remaining three macrolide (AZI, CLA and ROX) tends to have higher affinity for biological sludge (Table 4.4) than ERY, and in parallel the percentage removal was relatively high. The percentage removal of all fluoroquinolones (CIP, OFL and NOR) was > 90 % except for OFL at "S" WWTP with a relatively high percentage removal of 86 %. This high removal efficiency from the aqueous phase can be associated with documented high adsorption properties to sediment/biological sludge (Golet et al., 2003). A Similar result has been reported in Sweden (Lindberg et al., 2005) and USA (Karthikeyan and Meyer, 2006). ALB exhibited very high removal efficiency, between 99 % and 100 % in the four WWTPs. This is basically due to the high rate of adsorption (section 4.3 of this study).

Aside from the high concentration of SUL recorded in the influent of "S" WWTP, which accounts for the low removal percentage, the transformation of SUL may be another contributory factor to the low removal efficiency. During secondary wastewater treatment (biological treatment), acetylsulfamethoxazole, has been reported to be transformed back into the parent compound sulfamethoxazole (Göbel et al., 2007, Joss et al., 2006). This may lead to variation in the removal efficiency of SUL if acetylsulfamethoxazole is present in higher concentration than the parent compound in the influent samples. This re-transformation has not been accounted for as part of the present investigations. However, the large variation in the removal of SUL may be attributed to this phenomenon. TRI has been reported to have a low adsorption property to sludge (Castiglioni et al., 2006, Lindberg et al., 2006), which is also evident from its low percentage values in the sediment analysis (suspended matter collected at the bottom of the centrifuge tube) (Table 4.4). This property accounts for the 21 % and 58 %

removal in “S” and “I” WWTPs, respectively, while the higher values of 96 % and 99 % removal in “P” and “K”, respectively may be attributed the effect of chlorination. CLA exhibits high adsorption property to soil/sediment (Kodešová et al., 2016), likewise, this has been proven by the 72 % concentration in sediment (suspended matter at the bottom of the centrifuge tube) analysis in Table 4.4. This enables the antibiotics to adhere to the sludge/biofilm on the trickling filter and hence lower concentrations are available in the effluent liquid sample. ETI and MET adsorption rate are 55 % and 43 %, respectively (Table 4.4). The adsorption of ETI has not to my knowledge been discussed in the literature but the adsorption properties of MET has been proven to be low (Zhang et al., 2013, Rabølle and Spliid, 2000).

Generally, the removal of antibiotics from wastewater generally is due to the plant process and the properties of the antibiotics. The WWTP operation processes aids the separation of the antibiotics from the water body, without which the antibiotics will still be in the sewer; that is why direct dumping of untreated faecal matter is not encouraged because the antibiotics cannot be removed by the mere adsorption properties of the antibiotics. However, the adsorption properties of the antibiotics are also involved in the separation process, but it is enhanced by the wastewater treatment process.

Table 4.13: Overall Percentage removal of antibiotics

WWTPS	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
P (%)	57	75	96	97	99	99	99	51	87	55	47	100	100
S (%)	88	73	21	98	85	94	100	30	97	-25	93	26	39
K (%)	100	88	99	94	98	100	100	95	98	94	100	96	99
I (%)	86	79	58	100	100	100	100	100	100	-193	ND	100	100

4.3.9 Comparison of antibiotics in the final effluent (post-chlorination) and the receiving water body

The first contact of effluents from the WWTP is the nearby stream or river. These receiving water bodies are also linked with other sources of discharge, such as drainage water and wash off from farmlands. Drainages may be polluted with high concentration of antibiotics and other pharmaceuticals as a result of direct dumping of unconsumed pharmaceuticals, hence the concentration of antibiotics in the downstream of WWTP does not only represent the WWTPs effluent concentration. In order to assess the impact of antibiotics on the downstream, samples were taken approximately 100 meter away from the WWTPs discharge, thereby allowing the mixing of the effluent from the WWTPs with the receiving water body.

The highest concentration (median) of antibiotics for both post chlorination and downstream samples was recorded for CIP with a concentration of 1142.6 ng L^{-1} and 707.6 ng L^{-1}, respectively at "S" WWTP. CIP was identified in all the samples taken from both the final effluent (Post-chlorination) and downstream of "S", "P", "K" WWTPs except form the downstream of "I" WWTP. In a similar analysis, Agunbiade and Moodley (2014) reported a much higher CIP concentration in the range of 5660 - 16900 ng L^{-1} in an effluent receiving river in Durban (Agunbiade and Moodley, 2014) which may be as a result of the river flow rate. In Kenya, a higher concentration in a river water (509 ng L^{-1}) than in the WWTP effluent

(67 ng L^{-1}) was reported (Ngumba et al., 2016) potentially due to leaching of untreated excreta into the river. Likewise in Nigeria, CIP was reported at a concentration of 860 ng L^{-1} in surface water (Olaitan et al., 2014). These reports are evidence of the ubiquitous nature and frequent use of CIP. An extremely high concentration of 6,500,00 ng L^{-1} was reported in a lake in India (Fick et al., 2009) as a result of the close proximity of the lake to a pharmaceutical industry and the stagnancy of the water body and the environmental stability of CIP.

The other fluoroquinolones (NOR and OFL), were detected in the final effluent and downstream samples of all the WWTPs except for, "I" WWTP final effluent and downstream, where NOR, was not detected at the downstream of "K" WWTP. The maximum downstream concentration of 0.8 ng L^{-1} was detected for NOR at "P" WWTP, while OFL (65.6 ng L^{-1}) at "K" WWTP. Hendricks and Pool, (2012), reported fluoroquinolone concentration of

120 ng L^{-1} from a wastewater impacted receiving water body, at Eastern Cape, South Africa, while in Tunisia a concentration of 868 ng L^{-1} was recorded (Moslah et al., 2017). In China a concentration of 18.8 ng$L^{-1}$was likewise reported (Zhang et al., 2017).

The difference in the concentration of the final effluent and the receiving water body will be affected by the volume of the receiving water body and the flow rate, thereby reducing the concentration. Another factor that may affect the concentration of antibiotics in the receiving water body includes additional sources of contamination downstream and before additional sampling points. However, the concentration of ETI (3.5 ng L^{-1}), ALB (117 ng L^{-1}) and CLI (0.5 ng L^{-1}) at "S" WWTP was higher than the final effluent concentration. Similarly, at "P" WWTP, CLA (2.8 ng L^{-1}) was higher, likewise at "I" WWTP, MET (4.9 ng L^{-1}), SUL (0.1 ng L^{-1}), CLA (3.6 ng L^{-1}). All concentration of the antibiotics were high at "K" except for MET (1.2 ng L^{-1}) and OFL (65.6 ng L^{-1}). These higher concentrations are as a result of other sources

of antibiotics which are not related to the WWTP effluent; such as, direct defecation, run-off from farm lands and dumping of unconsumed antibiotics into the downstream (Karthikeyan and Meyer, 2006, Kümmerer, 2009). The highest concentrations of Macrolides (ERY, AZI, CLA) at the downstream sites were 63.3 ng L^{-1} at "I" WWTP, 0.1 ng L^{-1} at "P" WWTP, 37.7 ng L^{-1} at "K" WWTP, respectively, while ROX was not detected in all the downstream samples. A higher concentration of 2023 ng L^{-1} of macrolide was reported by (Matongo et al., 2015) in surface water that is not directly connected to a WWTP, with the assumption that other sources may have influenced the high concentration.

The concentration of antibiotics at the upstream is essential towards establishing a basis for the environmental contamination of the receiving water body without the influence of the wastewater effluent. Samples were collected from the upstream of "I" WWTP (Table 4.14). Relevant upstream sampling sites of the other WWTPs were not directly accessible and hence were not analysed. TRI (114.3 ng L^{-1}) was detected in high concentrations at the upstream, and this concentration is also reflected at the downstream site, with 217.2 ng L^{-1} (Table 4.14) The high concentration of TRI cannot be directly explained, but may be as a result of direct dumping of excreta. A total of 415.3 ng L^{-1} (summation of the concentration of post chlorination and upstream) could result at the downstream site without mixing and variation, but several factors like adsorption and photo degradation may led to reduction in concentration. Other antibiotics that were detected upstream were similarly affected. In general, the concentration of antibiotic detected at the downstream for all the WWTPs , were below the predicted no effect concentration (PNEC) of the bacterial inhibitory concentration in Table 4.6 except for CIP which has a relatively high value (EMEA, 2006, Bengtsson-Palme and Larsson, 2016).

Table 4.14: Concentration of antibiotics in downstream, upstream and post chlorination point.

		ETI (ng L^{-1})	MET (ng L^{-1})	TRI (ng L^{-1})	NOR (ng L^{-1})	OFL (ng L^{-1})	CIP (ng L^{-1})	ALB (ng L^{-1})	SUL (ng L^{-1})	CLI (ng L^{-1})	ERY (ng L^{-1})	AZI (ng L^{-1})	CLA (ng L^{-1})	ROX (ng L^{-1})
"S"	**Post chlorination**	**2.3**	**20.5**	**232.4**	**2.6**	**94.1**	**1142.6**	**50**	**212.8**	**0.2**	**22**	**0.3**	**21.8**	**2.1**
	Downstream	**3.5**	**17.9**	**161.8**	**0.5**	**43.5**	**707.6**	**117**	**124.9**	**0.5**	**18.2**	**0**	**13.2**	**0**
"P"	**Post chlorination**	**9.3**	**13.1**	**12.1**	**1.1**	**15.4**	**262.6**	**682.7**	**353.9**	**1**	**1.3**	**0.7**	**0.2**	**0**
	Downstream	**4.9**	**6.9**	**9.6**	**0.8**	**9.3**	**61.2**	**324.9**	**59.3**	**1**	**0.1**	**0.1**	**2.8**	**0**
"K"	**Post chlorination**	**0.1**	**2.7**	**7**	**2.4**	**66.4**	**116.5**	**0**	**236.8**	**0.9**	**2.2**	**0**	**30.2**	**0.8**
	Downstream	**0.2**	**1.2**	**9.9**	**0**	**65.6**	**211.6**	**0**	**238.9**	**8.1**	**3.8**	**0**	**37.7**	**0**
"I"	**Post chlorination**	**1.2**	**1.9**	**301**	**0**	**0**	**64.1**	**0**	**0**	**0**	**108.6**	**0**	**0**	**0**
	Downstream	**0.3**	**4.9**	**217.2**	**0**	**0**	**0**	**0**	**0.1**	**0**	**63.3**	**0**	**3.6**	**0**
	Upstream	**1**	**9.6**	**114.3**	**0**	**0**	**28.4**	**7.4**	**0**	**0**	**10.6**	**0**	**9.7**	**0.3**

There are two logical assumption for the high concentration recorded in this table (Table 4.14). 1. The impact of the upstream on the downstream; because the upstream could not be assessed this assumption could not the ascertained, most especially for "S","P" and "K" WWTPS. 2. Direct dumping of fecal matter into the downstream. Human activities are seen along the downstream, such as washing, farming and this may lead to direct dumping of fecal matters into the downstream.

4.3.10 Conclusion

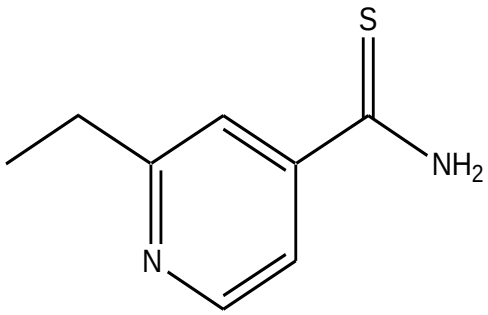
This investigation ensures a minimal loss of analyte through the use of centrifugation instead of just the conventional filtration method. The analysis of antibiotics in the supernatant and sediment (suspended matter at the bottom of the centrifuge tube) samples improves the level of detection. The 13 antibiotics chosen were constantly detected throughout the four WWTPs investigated in this study. “S” WWTP samples were found to have the highest level of antibiotics load. The concentrations of the antibiotics in the influent were greatly reduced by the WWTP process, where the main treatment processes (trickling filter and activated sludge) plays a significant role in reducing the concentration of the antibiotics. Post-chlorination likewise reduces the concentration of the antibiotics even though it may be a form of transformation of the antibiotics that may be of adverse effect in the environment. The rationale of relating the concentration of antibiotics in influent sample to the rate of infection within the community may differ. Factors such as the appropriate use of the drug and method of sewage discharge will impact. The impact of antibiotics concentration from the WWTPs effluent on the downstream is low and can be regarded as insignificant based on dilution factor of the large water bodies. When a comparison of the predicted no-effect concentration (PNEC) values of the antibiotics on bacteria is made with the detected concentration of the antibiotics in the downstream samples, none of the antibiotics concentration values pose any immediate environmental risk.

CHAPTER 5: IMPACT OF THE APPLICATION OF ULTRAVIOLET RADIATION AND HYPOCHLORIDE ON ETHIONAMIDE AND METRONIDAZOLE

5.1 INTRODUCTION

This study was set up to (1) investigate the reduction of antibiotics in wastewater as compared with pure water at different times of exposure to UV (254 nm) radiation and concentrations of chlorine, (2) identify if possible transformation products was formed based on chlorination and (3) examine the impact of chlorination on antibiotics in full scale wastewater treatment plants. Ethionamide (ETI) and metronidazole (MET) was used and are antibiotics used in the treatment of tuberculosis and protozoan infection, respectively (Nourzad et al., 2017, Çelik and Aras Ateş, 2006). ETI (Figure 5.1a) belongs to the thioamide group and is administered in combination with other anti-tuberculosis drug such as ethambutol and isoniazid to treat multi-drug resistance (MDR) tuberculosis (Jenner et al., 1984, Malinga et al., 2016). The World Health Organisation (WHO) are listing ETI as one of the essential medicines needed in a health system (World Health Organization, 2016). ETI is administered orally and metabolized by the liver, with approx. 5% of the drug being excreted through urine in an unchanged form (Ferraz et al., 2016). A high quantity of ETI is assumed to be present in the environment through wastewater discharges (detailed discussion in Chapter 2). MET (Figure 5.1b) commonly known as Flagyl is used in the treatment of protozoan infections in both human and animals. The choice of MET for this analysis, is as a result of its uses and prevalence in the environment, which has also been discussed in Chapter 2.

[a]



[b]

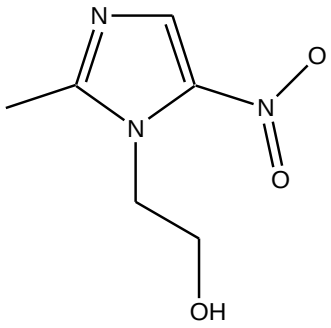


Figure 5.1: [a] Chemical structure of Ethionamide; [b] Chemical structure of Metronidazole

MET are excreted via both urine and faeces either relatively unchanged or as metabolites which may retain chemical activity in the environment (Sarmah et al., 2006). Treatment methods in WWTPs makes use of a variety of oxidation techniques, such as, the use of ozone and ultraviolet light for the reduction of micropollutants (including antibiotics) in wastewater. These methods have proven to be effective for the removal of several antibiotics (Gao et al., 2017, Qin et al., 2014, Wu et al., 2016b) . The use of chlorine as a disinfectant in WWTPs is commonly practiced in a majority of conventional WWTPs in developing countries (Dong et al., 2017). The possibility of chlorine or UV reacting with antibiotics in wastewater to form more hazardous product cannot be dismissed. The reaction pathways with chlorine or UV are not specific and can vary with changes in temperature and pH. Several reaction products/pathways of different antibiotics, has been proposed and investigated (Deborde and Von Gunten, 2008). However, insufficient information is available for the UV and chlorine reactions with ethionamide and metronidazole. The selection of these two antibiotics is based on prevalent diseases associated with their uses that as a consequence will lead to assumed high concentration in the environment.

5.2 MATERIALS AND METHODS

The two antibiotic standards and sodium thiosulphate ($Na_2S_2O_3$) were purchased from Sigma Aldrich Chemicals. The chlorine solution used in wastewater treatment, High Test Hypochloride (HTH) were obtained from Kingsburgh wastewater treatment plant. The wavelength of Ultraviolet light unit is 254 nm. Pure water was produced by using AquaMax Ultra, water purification unit system (Ultra 370 series, Younglin, Japan). All antibiotic standards were prepared in pure methanol by weighing 2 mg of antibiotic powder and dissolving it in 5 mL of pure methanol.

5.2.1 Experiential procedures

The separate effect of UV and chlorine on antibiotics was determined by comparing the concentration of antibiotics before and after exposure to UV and chlorine, respectively. The experiment was modified from the analysis by (Qin et al., 2014) and (Wu et al., 2016b). The pH and total dissolved solid of the samples (pure water and wastewater) were measured after filtration (filtered through 0.45 µm membrane filters) and before spiking with the respective antibiotics. The pH was 7.6 and 6.8 and the total dissolved solid (TDS) of the pure water was 35 mg L^{-1} and wastewater was

250 mg L^{-1}, respectively. Hundred mL portions of the samples (in transparent glass bottles) were spiked with 1.5 mg L^{-1}, 4 mg L^{-1}, 9 mg L^{-1} and 13 mg L^{-1} of the two antibiotics combined (ETI and MET). The pH of the samples changed to 7.8 in pure water and to 8.1 in wastewater due to the addition of the antibiotics.

Experimental set-up for UV: The samples were placed in a reactor equipped with a water bath, with the temperature set at 25 ^{0}C and subjected to UV radiation (254 nm) in a dark room at varying duration of time (15, 30, 45, and 60 min). The distance between the samples and the UV light were kept constant at 10 cm above the samples. The calculated UV dose in mJ/cm^2 (UV dose = UV intensity X time mJ/ cm^2; intensity = 520 microW/cm^2) for 15, 30, 45 and 60 min was 468, 936, 1404 and 1,872 mJ/cm^2, respectively.

Samples were taken intermittently at the above time intervals with a syringe (Elmolla and Chaudhuri, 2010). These samples were kept in HPLC vials covered with a foil to prevent light

penetration. The antibiotic concentration was determined using a liquid chromatography – tandem mass spectroscopy (LC-MS/MS) instrument by Thermos Scientific, Waltham, MA.

For the chlorine exposure pilot experiments 100 mL portions of pure water and wastewater were chlorinated using HTH (High test hypochlorite 65% chlorine) at concentration of 1 mg L^{-1}, 2 mg L^{-1}, 5 mg L^{-1} and 10 mg L^{-1}, which were calculated for the HTH percentage of chlorine concentration. This chlorinated pure water and wastewater were spiked with 1.5 mg L^{-1} of the above antibiotics and exposed in the dark during the stated time intervals (15 min, 30 min, 45 min, and 60 min). At each time interval, samples were collected and the chlorine reaction was quenched with sodium thiosulphate ($Na_2S_2O_3$) at a concentration ratio of $Na_2S_2O_3$ to chlorine of 4:1 (Elmolla and Chaudhuri, 2010, Qin et al., 2014). For the chlorination pilot experiment, wastewater samples were subjected to a solid phase extraction (SPE) to extract the antibiotics from the sample using the described protocol (Dorival-García et al., 2013). The waste extract from the offline SPE, which is expected to be free from the two antibiotics being investigated, were used. Likewise, pure water was used for comparative analysis.

Hundred mL of the samples above (pure water and wastewater) were used and the concentrations of antibiotics that may be present were measured before spiking. A blank sample of pure water and wastewater were each later spiked with 1.5 mg L^{-1} of antibiotics.

In parallel to the chlorine analysis, 100 mL (pure water and wastewater) of each chlorine concentration (1, 2, 5, and 10 mg L^{-1}) were spiked with 1.5 mg L^{-1} of antibiotics and directly tested at various time intervals (15, 30, 45, and 60 min) for chlorine residual. An iodometric method was used for the residual chlorine test. The initial concentration of the chlorine from the HTH (granules) was tested in parallel using the same method. The results of the chlorine concentration over the time range up to 60 min, gave a negligible reduction (< 0.01 mg L^{-1}). For the full scale

WWTPs post chlorination analysis, colorimetric method was used to measure the chlorine concentration and the TDS was measured using the Yellow Spring instrument (YSI). Samples from the wastewater treatment plants were collected twice a month, for four months in 2017 (Feb, Mar, Apr and May). Analysis of the antibiotics concentration was conducted in triplicate using similar instrument and the method described earlier.

5.2.2 Analytical method

The concentrations of ETI and MET were determined by a liquid chromatography–tandem mass spectrometry (LC–MS) system (Thermo Fisher, Waltham, MA).

This was equipped with a PAL HTC auto sampler (CTC Analytics AG, Zwingen, Switzerland) and an Accela LC pump (Thermo Fisher Scientific, San Jose, CA, USA).

Hypersil GOLD C18 column (50 mm×2.1 mm ID. Thermo Fisher Scientific, San Jose, CA, USA) with a guard C18 column (2 mm×2 mm ID. Thermo Fisher Scientific, San Jose, CA, USA) were used as the column and guard for the analysis.

The analysis was conducted in triplicate using the protocol setup by (Khan et al., 2012). The mobile phase consisted of 0.1 % formic acid (FA) in acetonitrile as the organic phase (B) and 0.1 % FA in purified water as the aqueous phase (A). The program for the gradient elution was made up of 95 % of A to 5 % of B with a 14 min run time. The injection volume was 20 µL at a flow rate of 1.5 µL min^{-1}. Argon was used as the collision gas at a flow rate of 1.5 mL min^{-1}. A constant source voltage of 3.0 kV was maintained for the analysis. Electrospray ionization tandem mass spectrometer (ESI-MS/MS) in the positive mode was used as the detector. Internal standard (IS) calibration was used for quantification. 13C3-trimethoprim and tramadol 12CD3 was used as IS for both ETI and MET, respectively. The limits of detection for ETI and MET were 0.12 ng mL^{-1}

and 0.14 ng mL^{-1}, respectively. Analytes were determined based on the retention time, mass to charge ratio and similarity of the precursor ion of the standard compounds.

5.3 RESULTS AND DISCUSSION

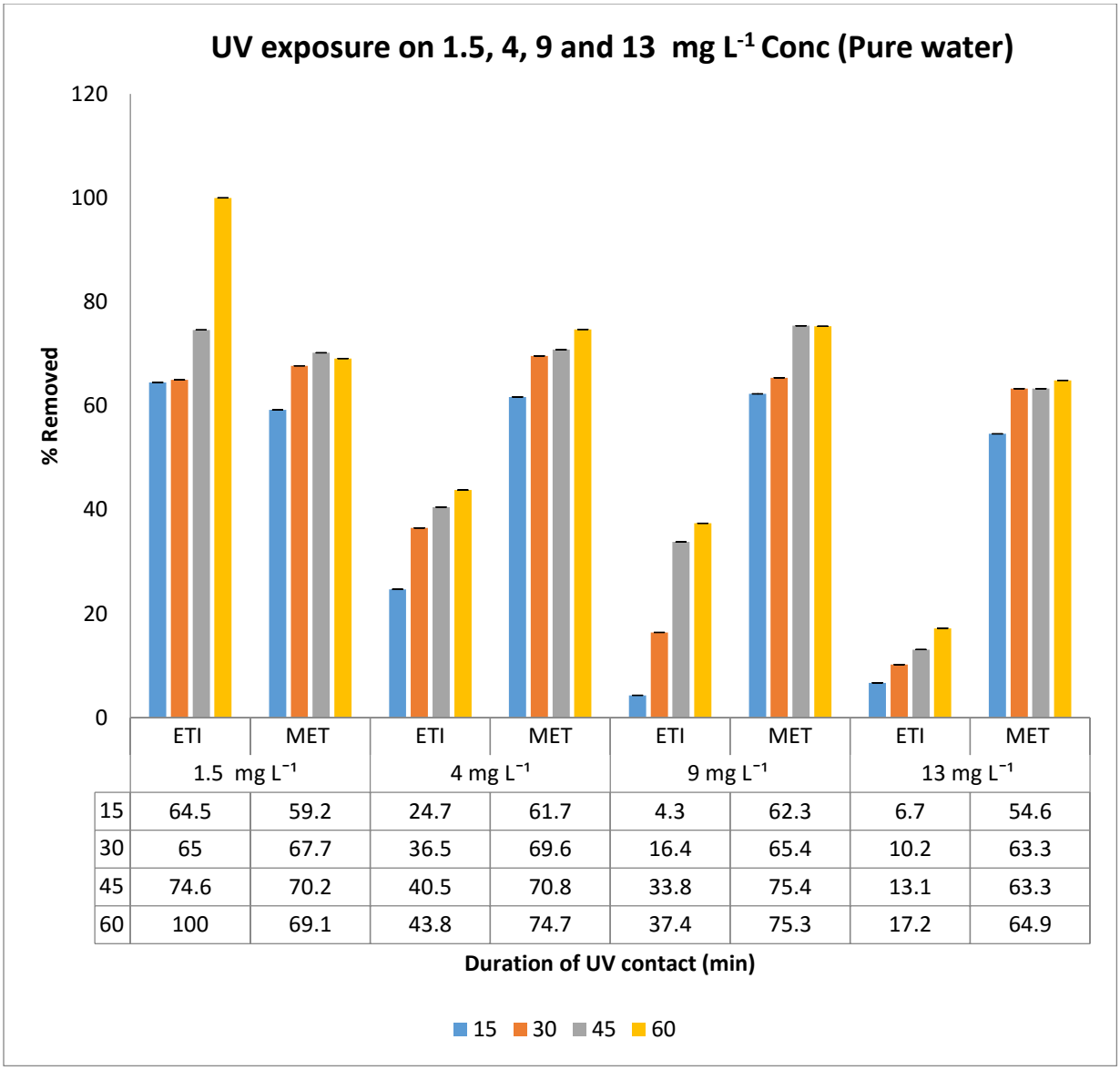
5.3.1 The effect of UV radiation on the antibiotics Ethionamide and Metronidazole

The reductions of the two antibiotics, ethionamide and metronidazole at different concentrations in the pure water and in wastewater after exposure to UV radiation during different times are presented in Figures 5.2 and 5.3. A reduction occurred, with a greater reduction at the lower concentration of antibiotics. The proportional effect of the radiation was reduced as the concentration of the antibiotics increases. ETI at 1.5 mg L^{-1} in the pure water (Figure 5.2) was reduced to a higher extent than in the wastewater (Figure 5.3). A higher percentage removal occurred with increasing radiation time. A time of 15, 30, 45 and 60 min resulted in the corresponding reduction values of 64.5, 65, 74.6 and 100 % respectively.

The lowest reduction was 4.3% for ETI at 9 mg L^{-1} concentration influenced by time (15 min) and concentration. The negative values (Figure 5.3) maybe related to instrumental error. The duration of exposure played a significant role in the reduction, as reflected by the maximum reduction (100 %) of ETI in the pure water which occurred after 60 min exposure time.

The UV radiation resulted in an insignificant change regardless of the concentration or the time of exposure. The percentage removal of MET were always in the ranges between 64 % and 70 %. In a similar experiment, Qin et al (2014) were assessing, 5 µM (1 mg L^{-1}) of ronidazole (RNZ) and exposed it to 3.02 mJ/cm^2 at varying pH, with a reduction of 37.8 % to 95.8 % (Qin et al., 2014).

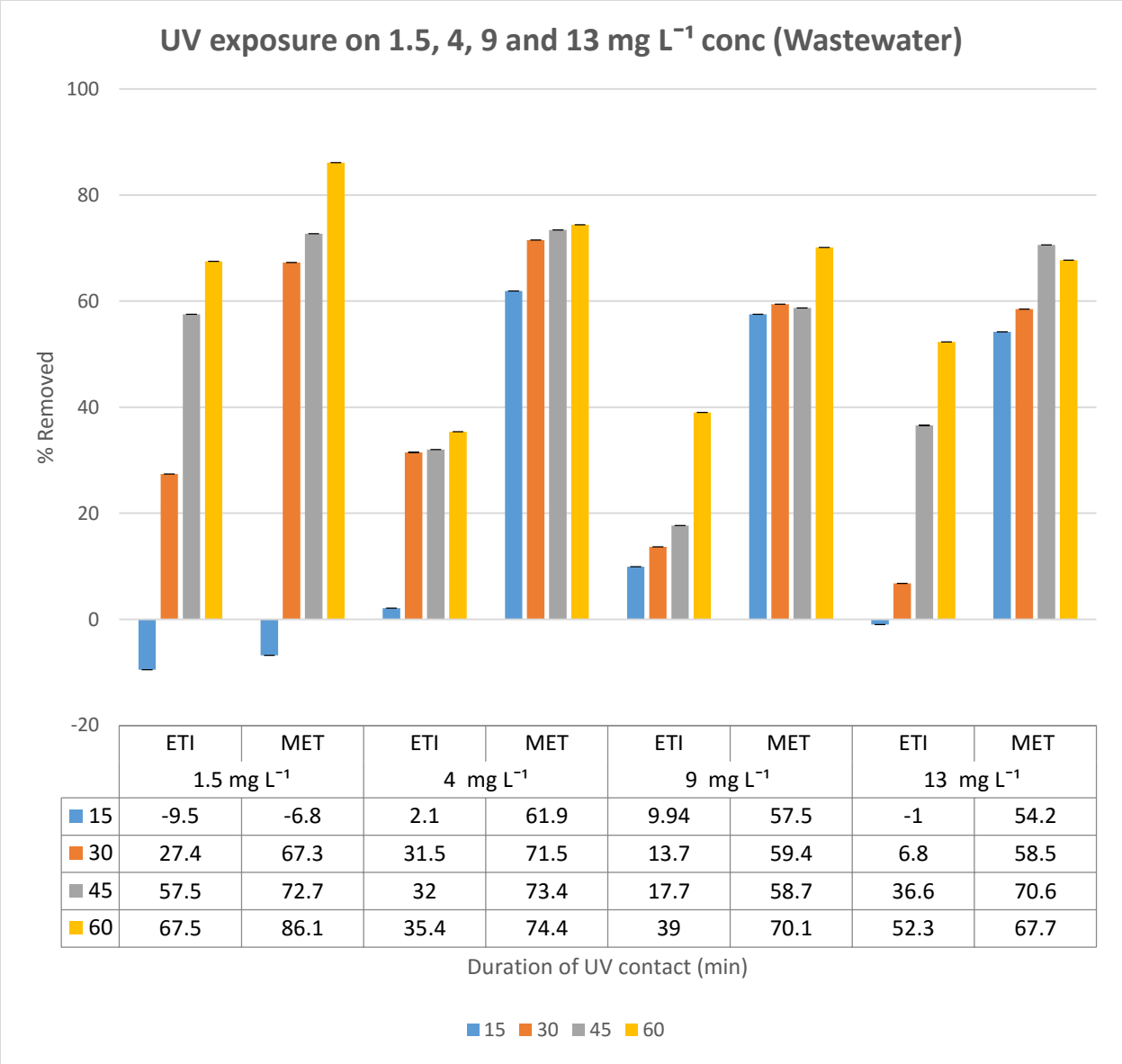
For the wastewater samples, the duration of UV exposure played an important role in the reduction. For example, a low to marginal reduction effect was noticed within 15 min of UV exposure for ETI at the different concentrations as shown in Figure 5.3. With an extended exposure time (from 30 – 60 min) or for the various concentrations the reduction increased gradually. (Figure 5.3). This may be a result of the higher TDS value of 250 mg L^{-1} of the wastewater sample as compared to the 35 mg L^{-1} of the pure water. The high TDS value is assumed to prevent the proper penetration of the UV light (intensity 468 mJ/cm^2) within a short exposure time (Karanfil et al., 2005). The reduction efficiency increases with longer period of exposure, but is not as effective as in the pure water with a lower TDS value. MET was affected slightly by time of exposure (Figure 5.3). However, a similar pattern of removal occurred in the pure water as compared with the wastewater, where the concentration of the antibiotics and time of exposure did not influence the reduction efficiency. The zero reduction effect of 15 min UV impact on MET at 1.5 mg L^{-1} may be as a result of the TDS value of the wastewater (Figure 5.3)**.**



	ETI	MET	ETI	MET	ETI	MET	ETI	MET
	1.5 mg L⁻¹		4 mg L⁻¹		9 mg L⁻¹		13 mg L⁻¹	
15	64.5	59.2	24.7	61.7	4.3	62.3	6.7	54.6
30	65	67.7	36.5	69.6	16.4	65.4	10.2	63.3
45	74.6	70.2	40.5	70.8	33.8	75.4	13.1	63.3
60	100	69.1	43.8	74.7	37.4	75.3	17.2	64.9

Figure 5.2: Antibiotics degradation in pure water during UV radiation (n = 3)

(SD of the % removal was very low = > 0.01% as indicated in the error bars.)



	ETI	MET	ETI	MET	ETI	MET	ETI	MET
	1.5 mg L^{-1}		4 mg L^{-1}		9 mg L^{-1}		13 mg L^{-1}	
15	-9.5	-6.8	2.1	61.9	9.94	57.5	-1	54.2
30	27.4	67.3	31.5	71.5	13.7	59.4	6.8	58.5
45	57.5	72.7	32	73.4	17.7	58.7	36.6	70.6
60	67.5	86.1	35.4	74.4	39	70.1	52.3	67.7

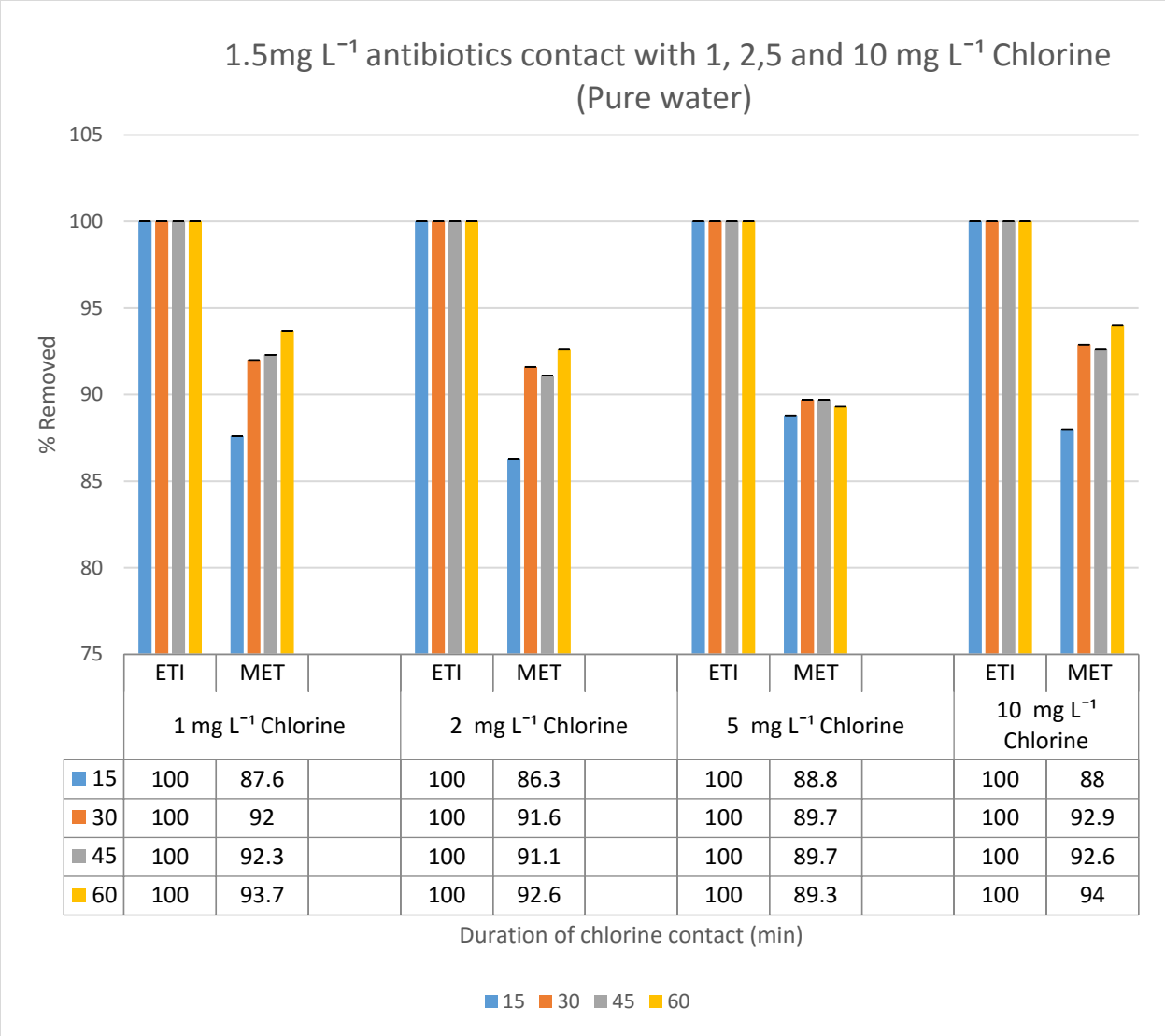
Figure 5.3: Antibiotics degradation in wastewater during UV radiation. (n = 3)

(SD of the % removal was very low = > 0.01% as indicated in the error bars.)

5.3.2 The effect of chlorination on the antibiotics Ethionamide and Metronidazole

The effect of chlorination on ETI concentration (1.5 mg L^{-1}) was remarkable (Figures 5.4 and 5.5). A total removal of ETI was recorded in samples regardless of time and chlorine concentration. In wastewater, the reduction was slightly less with 90 % reduction in the presence of 1 mgL^{-1} chlorine (Figure 5.5). At higher concentrations, the reduction was again 100 %. MET reduction again was independent of chlorine concentration. A longer time of exposure resulted in a slightly higher reduction. For MET, the percentage removal was within the same range as for the pure water and in the wastewater samples of 86-94 % and 80-93 %, respectively.

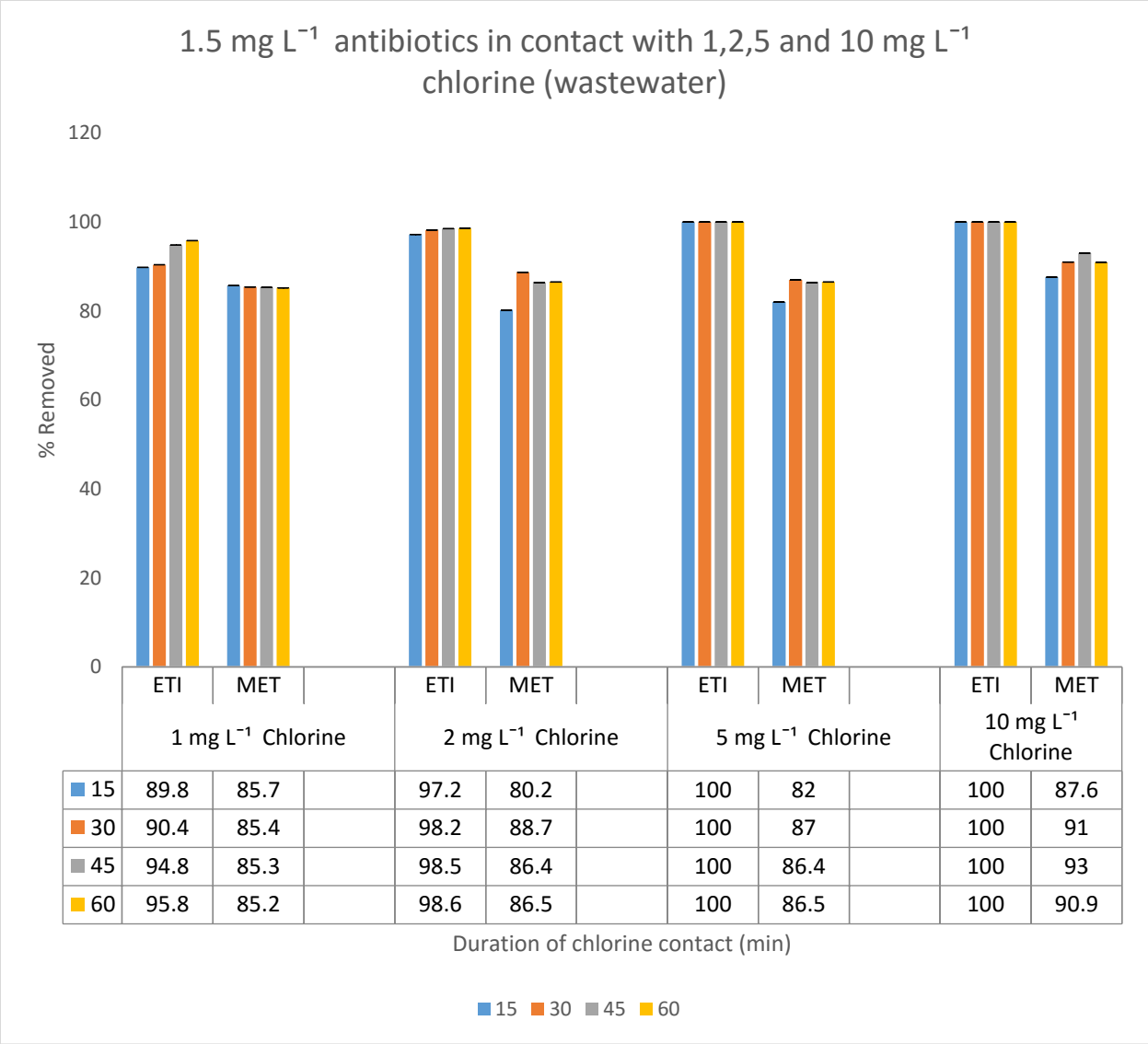
Chlorine is a powerful oxidant (Fuqua, 2010) with high potential to affect many emerging pollutants through its replacement ability (Deborde and Von Gunten, 2008) . The 100 % removal of ETI may be the well-established substitution reaction of the sulphur in ETI with chlorine (Deborde and Von Gunten, 2008). This substitution reaction is in line with non-metal reactivity and has previously been reported for some other contaminants, where the sulphur moieties of compounds in water has been substituted (Deborde and Von Gunten, 2008). A by-product compound is formed with a different mass to charge ratio. In this case the mass of ETI is 166.24 g mol^{-1} while the proposed chlorine substituted derivative mass is 169.24 g mol^{-1}. However, in our experiments the chromatogram shows a derivate formed with a mass to charge ratio, similar to the parent compound but with a different retention time (Figure 5.7). Figure 5.6 shows the chromatogram of the standard ETI.



	1 mg L⁻¹ Chlorine			2 mg L⁻¹ Chlorine			5 mg L⁻¹ Chlorine			10 mg L⁻¹ Chlorine	
	ETI	MET		ETI	MET		ETI	MET		ETI	MET
15	100	87.6		100	86.3		100	88.8		100	88
30	100	92		100	91.6		100	89.7		100	92.9
45	100	92.3		100	91.1		100	89.7		100	92.6
60	100	93.7		100	92.6		100	89.3		100	94

Figure 5.4: Reduction of antibiotics by chlorine in pure water (n = 3)

(SD of the % removal was very low = > 0.01% as indicated in the error bars.)



	1 mg L⁻¹ Chlorine			2 mg L⁻¹ Chlorine			5 mg L⁻¹ Chlorine			10 mg L⁻¹ Chlorine	
	ETI	MET		ETI	MET		ETI	MET		ETI	MET
15	89.8	85.7		97.2	80.2		100	82		100	87.6
30	90.4	85.4		98.2	88.7		100	87		100	91
45	94.8	85.3		98.5	86.4		100	86.4		100	93
60	95.8	85.2		98.6	86.5		100	86.5		100	90.9

Figure 5.5: Reduction of antibiotics by chlorine in wastewater (n = 3)

(SD of the % removal was very low = > 0.01% as indicated in the error bars.)

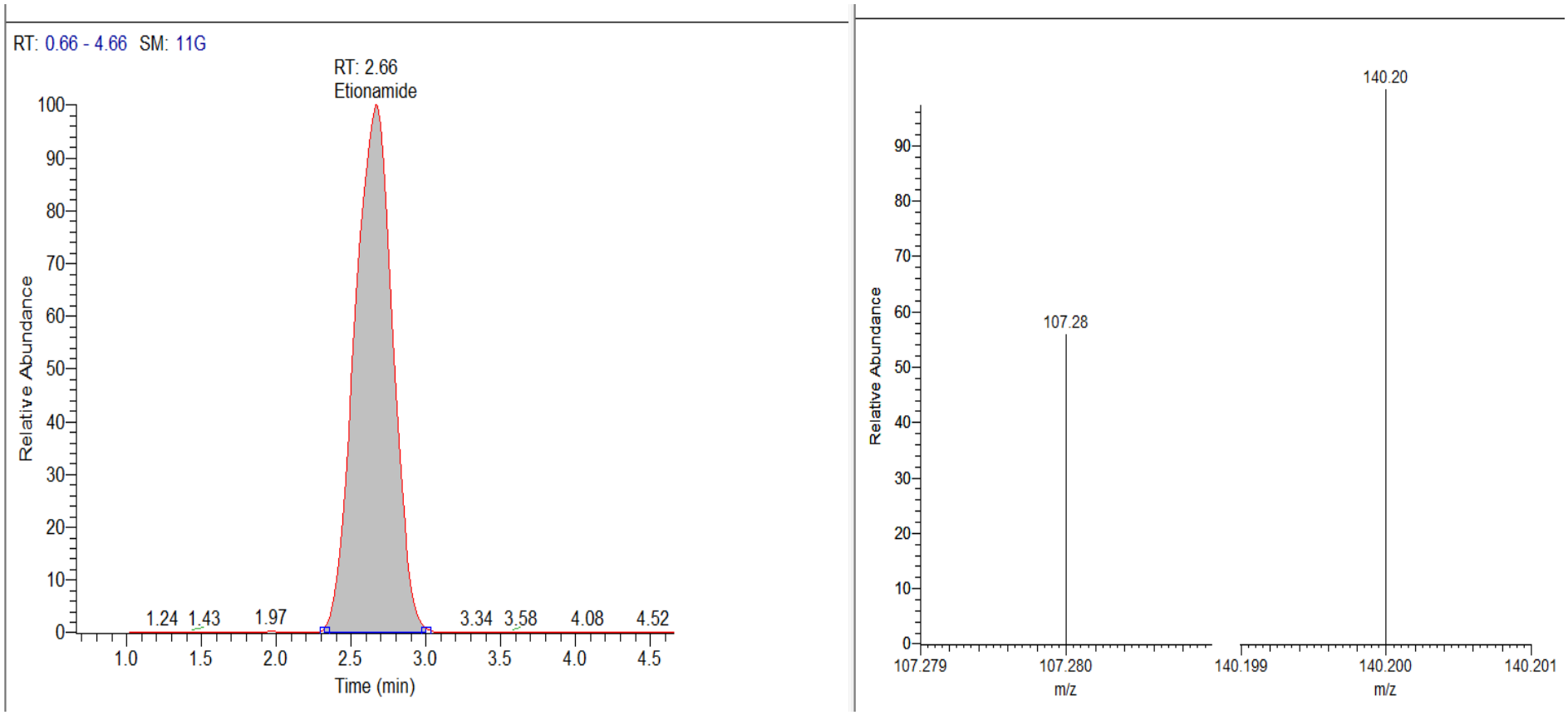


Figure 5.6: Standard chromatogram of ETI showing the retention time and the spectrum plot on the right side.

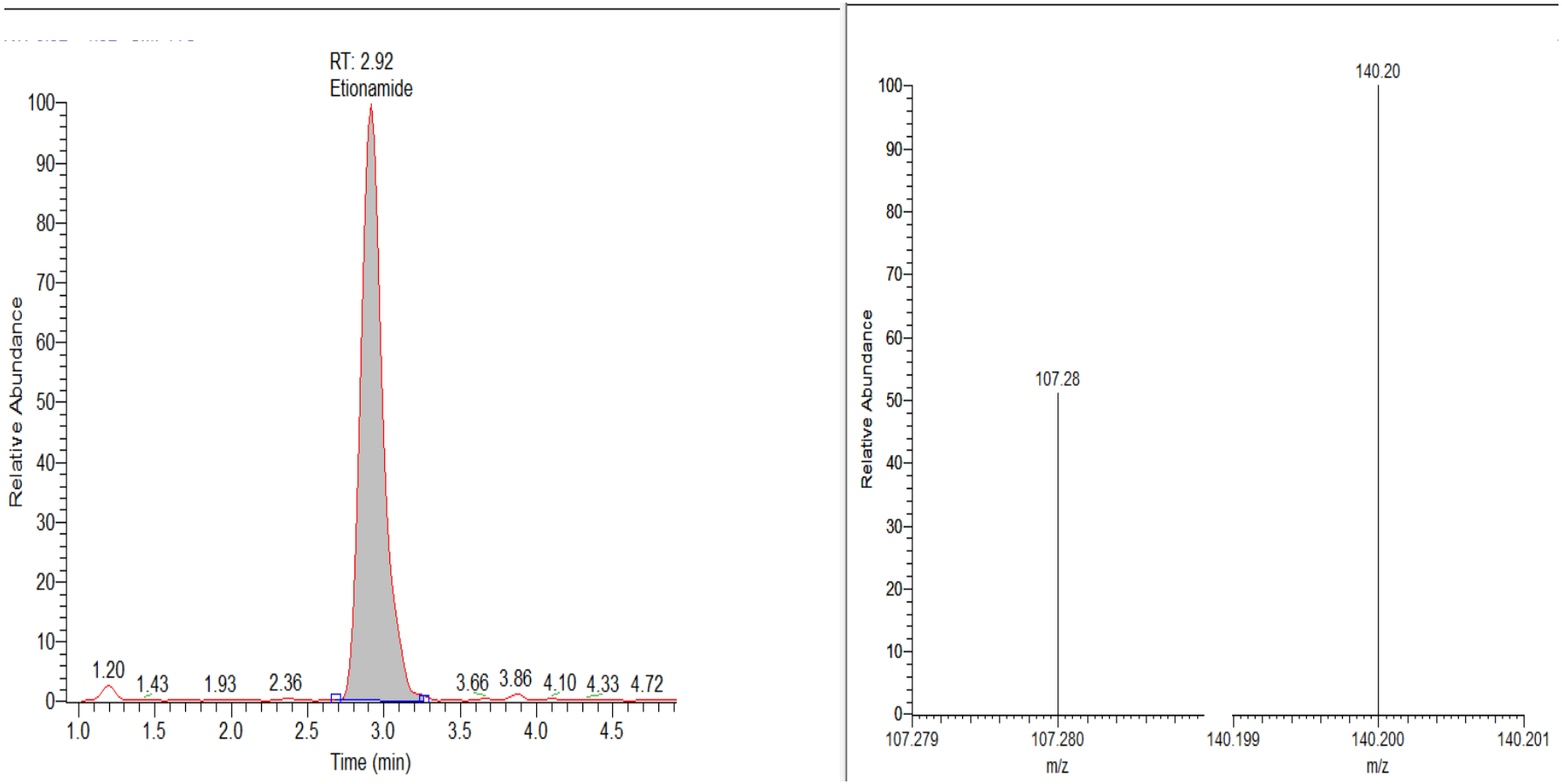


Figure 5.7: Chromatogram of possible ETI derivate showing a different retention time and spectrum plot from the standard ETI.

The possibility that this peak is a derivate of ETI is high but since information in the literature is lacking a more detailed fragment elucidating experiment will be required to confirm this assumption. Independently, it can be deduced that the removal efficiency regarding ETI and chlorine is more of a transformational nature. UV radiation was also effective in the reduction of ETI as indicated in Figure 5.5. Degradation pathways of ronidazole (RNZ) by chlorine and UV was proposed by Qin et al. (2014). Four structures where identified with the UV impact on RNZ. These structures were identified with different *m/z* of 217, 233, 173 and 157, respectively (Qin et al., 2014). This compound, (RNZ), can be related to MET being that MET and RNZ belong to the same class of antibiotics (Nidazole) and also have similar chemical structure. Hence, a proposed m/z for MET derivatives (172 and 205 m/z) was generated by substituting H_2COH and one hydrogen atom on the MET compound with chlorine respectively. This proposed compound was searched for in the scan mode of the LCMS results. The search gave no indication of any compound with such m/z ratio. Therefore, the proposed m/z ratio may be incorrect or a fragmentation reaction as a result of the ionization has transformed the compound. The derivate formed has a similar mass to charge ratio with the parent compound (Figure 5.9) and similar for MET. Figure 5.8 shows the chromatogram of the MET standard. It may be speculated that the presence of other compounds in wastewater may affect the number and structure of the potential fragments and that a more detailed analysis will be needed to elucidate and predict the presence of such compound.

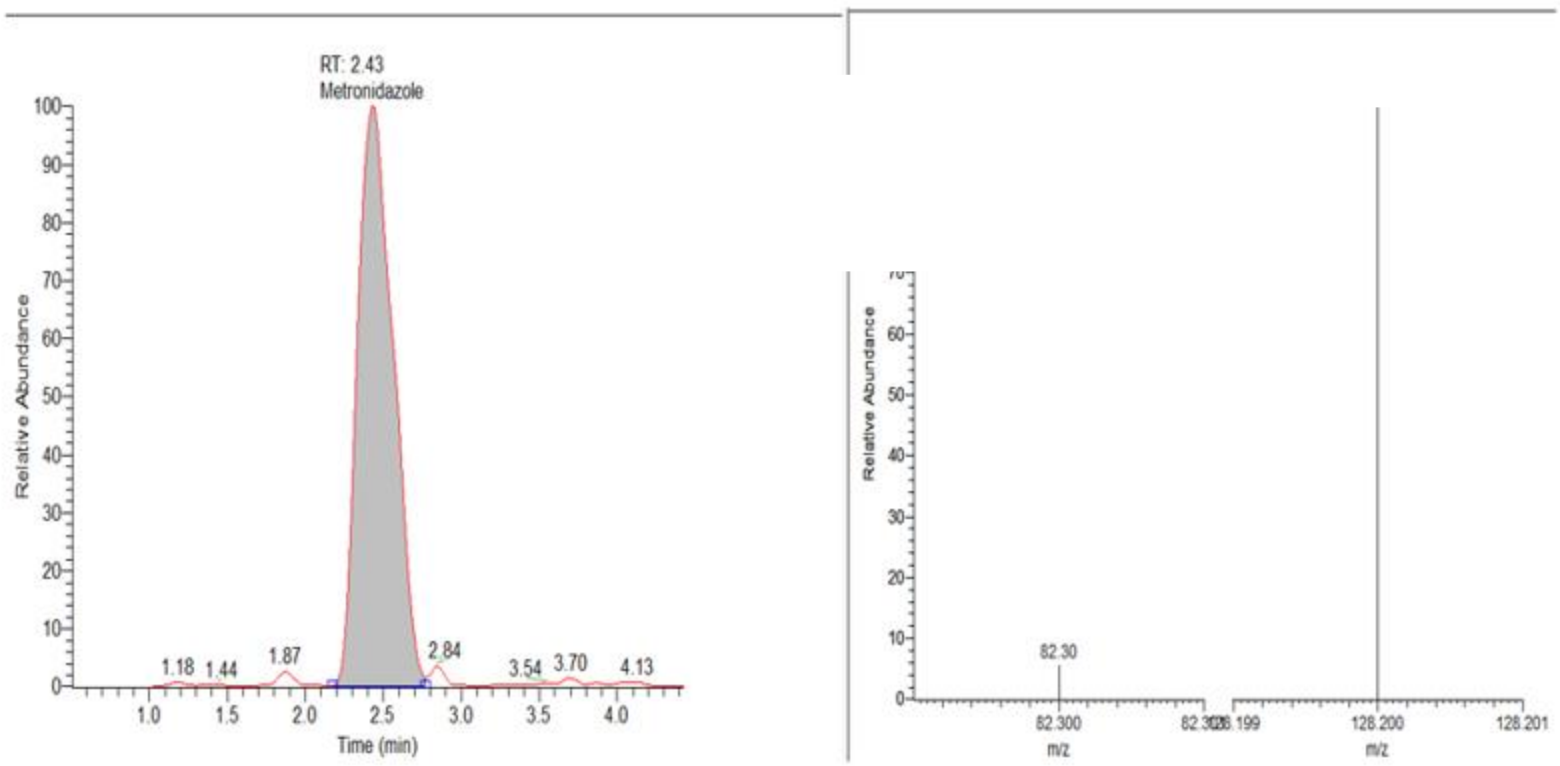


Figure 5.8: Standard chromatogram of MET showing the retention time and the spectrum plot on the right side.

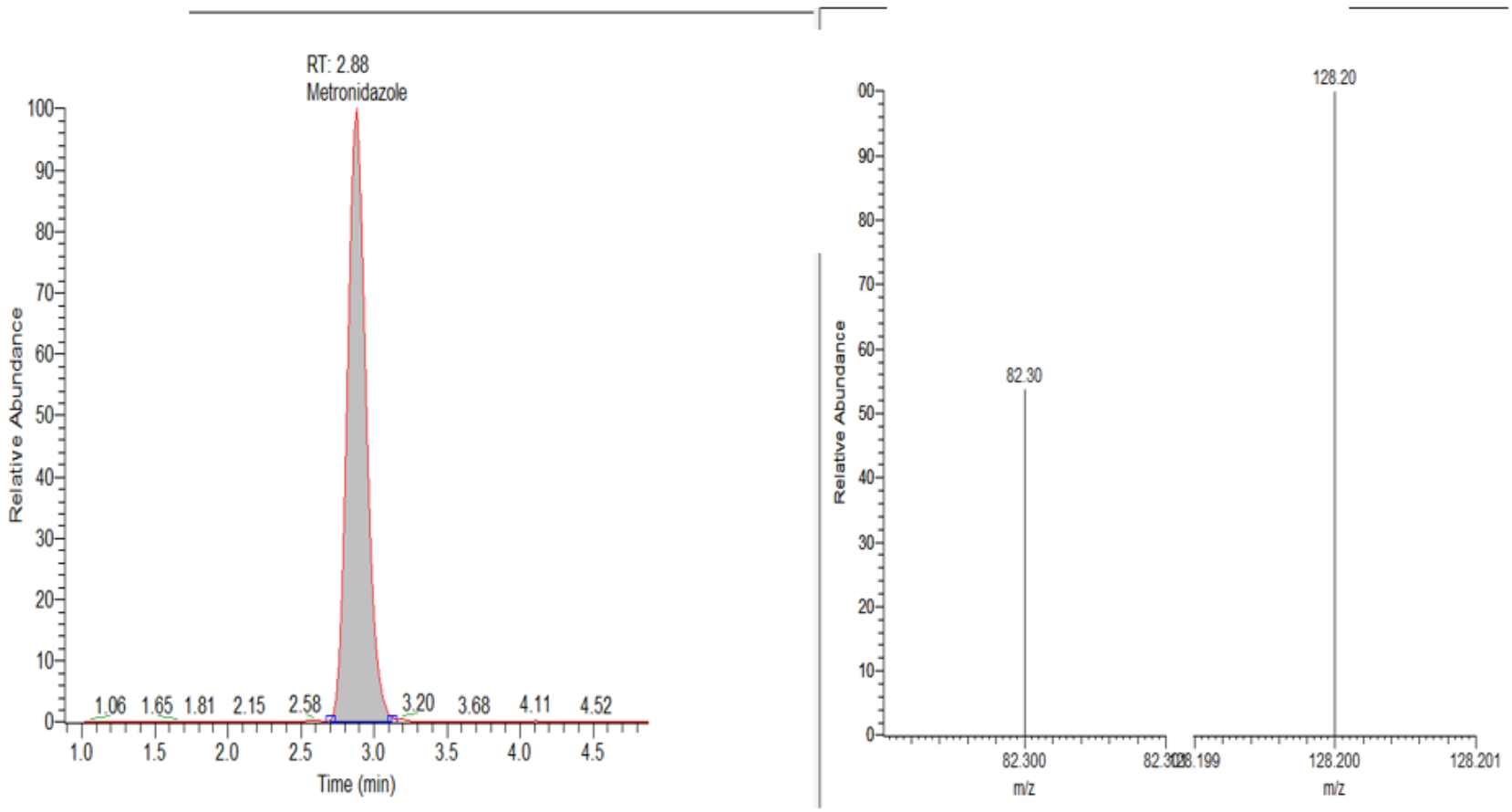


Figure 5.9: Chromatogram of possible met derivate showing a different retention time and spectrum plot from the standard MET

5.3.3 Impact of chlorination in four WWTPs

The concentration of the antibiotics in the pre-chlorination and post chlorination stages in four WWTPs ("I", "K", "S"," P") was compared with the laboratory results. The average residual chorine at the point of sampling for the four WWTPs were, 0.3, 0.2, 0.2, and 0.3 mg L^{-1}, respectively, while the average total dissolved solid (TDS) was 400 mgL^{-1}. The median, maximum and average concentrations for ETI and MET in the post-chlorination and pre-chlorination samples of the four WWTPs for the sampling period (number of sample per antibiotics, per WWTP = 8), are presented in Table 5.1. As a result of the large variation (as indicated by the SD and Max values) in the concentration values, the median values were used to calculate the percentage reduction of the chlorination impact.

The percentage reduction (median) was calculated using the following formula;

[(Conc of Pre Cl_2 – Conc of Post Cl_2) X 100]/Conc of Pre Cl_2

Table 5.1: Concentration of antibiotics in Pre and Post-chlorination samples and the percentage reduction in 4 WWTPs

Antibiotics (WWTP)	**Pre-chlorination (n = 8)**			**Post –chlorination (n = 8)**			**% Reduction Median**
	Median (ng L^{-1})	**Max (ng L^{-1})**	**Average(SD) (ng L^{-1})**	**Median (ng L^{-1})**	**Max (ng L^{-1})**	**Average(SD) (ng L^{-1})**	
ETI ("I")	**0.2**	**20.8**	**4.2 (6.9)**	**1.2**	**2.9**	**1.3 (1.4)**	**0**
MET ("I")	**55.3**	**440.3**	**111.7 (140.4)**	**1.9**	**98.4**	**19.5 (32.1)**	**96.6**
ETI ("K")	**0.9**	**8.5**	**1.8 (2.7)**	**0.1**	**1**	**0.4 (0.5)**	**88.9**
MET ("K")	**4.2**	**304**	**41.7 (99.2)**	**2.7**	**899.2**	**115.7 (296.2)**	**35.7**
ETI ("S")	**0.1**	**52**	**7.3 (17)**	**2.4**	**12.7**	**3.1 (3.9)**	**0**
MET ("S")	**23.9**	**188**	**47.2 (61.5)**	**20.5**	**67.4**	**25.2 (22.2)**	**13.9**
ETI ("P")	**14.5**	**41.7**	**18.6 (17)**	**9.4**	**51.7**	**13.5 (15.5)**	**35.2**
MET ("P")	**36.4**	**206.9**	**49.6 (64)**	**13.1**	**72.6**	**18.9 (21.7)**	**64.0**

n = number of samples, 0 values represent negative reduction, Max= maximum

The reduction pattern cannot be related to the laboratory analysis, because the concentration of the dosed chlorine was inconsistent (2 mg L – 75 mg L) and therefore cannot be used as base for the direct comparison. The concentration of the initial dose plays a major role in the reaction, however, the percentage reduction of antibiotics in the WWTPs showed a reduction within the range of 35.2 % – 88.9 % for ETI and 13.9 % - 96.9 % for MET in the four wastewater treatment plant (Table 5.1). A similar laboratory analysis was performed by (Chamberlain and Adams, 2006) , with the concentration of the dose chlorine similar to the residual chlorine at the WWTPs (in this thesis). The result of the analysis showed a complete removal (100 %) of carbadox (0.5 mg L^{-1}) within one minute of contact time, at a chlorine concentration of 0.1 mg L^{-1}. Also, 69 % of all the macrolide where removed with a chlorine concentration 1 mg L^{-1} in surface water, while with 0.1 mg L^{-1} of chlorine the reduction was 28 % in deionized water (Chamberlain and Adams, 2006). Thus, the residual chlorine at the WWTPs (in this thesis) might have also had an impact on the reduction of concentration of the antibiotics.

5.4 CONCLUSION AND RECOMMENDATION

The separate impact of chlorine and ultraviolet radiation on antibiotics in the water environment leads to an overall reduction in the concentration of antibiotics in the controlled experiment, while the effect of chlorine on the full scale WWTPs played an important role at reducing the concentration of antibiotics.

- The impact of the UV radiation towards the degradation of antibiotic was affected by the TDS value, where the higher TDS value was impacted less by the UV.
- Chlorination was not affected by the TDS value especially in the controlled experiment, hence chlorination is a more reliable method for the removal of antibiotics in wastewater based on the laboratory experiment and partially of the full scale analysis. This effect will reduce antibiotic contact with microbes and consequently reduce the creation of antibiotic resistance pathogens. This reduction can be regarded as a form of transformation of the antibiotics rather than degradation.

It is recommended that health hazard implication of such products should be investigated as they may be more dangerous than the parent compound.

CHAPTER 6: SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 SUMMARY

The occurrence of antibiotics in the aquatic environment is now recognized as pollutants of major concern in many countries. This has led to extensive research towards identification and quantification, facilitated by the use of advanced chromatographic separation technique and sophisticated detection instruments like the photodiode array detector (DAD) and mass spectrometry. However, the paucity of this research area in Africa is a call for concern.

Chapter 1, entails the general overview of the thesis topic "Prevalence and fate of antibiotics and its derivatives in sewage treatment and the receiving aquatic environment". It presents parts of the global and local knowledge that is currently available with regards to the presence of antibiotics in aquatic environment, most especially in wastewater. The effects of post-chlorination in wastewater treatment plant, are exemplified in relation to the potential creation of derivatives of antibiotics generated. The impact of antibiotic resistant microorganisms to human health with a focus on South Africa are further elucidated in the chapter. Finally, the aims of the research are highlighted and method of achieving the aims listed. In parallel to this, the novelty of the research are accounted for.

6.1.1 Current status of research on antibiotics residue in Africa and extraction protocol

An understanding and summation of the current status of the research for the analysis of antibiotic in the aquatic environment in Africa and most especially in South Africa is essential. The information, on the presence of antibiotics in the aquatic environment, is sparse for all types of water in Africa, including groundwater, surface water, effluent of WWTPs and municipal potable water. Hence, in Chapter 2, a review on the published analysis on the occurrence of antibiotics in the aquatic environment in each country in Africa was compiled. Table 2.2 in Chapter 2, summarize the details of the different antibiotics and their detected maximum concentrations reported in the aquatic environment from different countries in Africa (Faleye et al., 2018). In general, sulfamethoxazole was the most commonly detected antibiotics in African surface water (with 8 reports from 4 countries) and with a concentration range of 0.000265 µg L^{-1} - 38.85 µg L^{-1}. It is assumed that the sales of antibiotics can be used to predict its concentration in the environment, in parallel to the related diseases. The issue is discussed in the Chapter 2. With the relatively high sales of different antibiotics, to treat infectious diseases in the human population of Africa, the residual of the antibiotics is bound to be released through excretion via urine or fecal matter in parallel to the high sales. Figure 2.3 in Chapter 2 presents the antibiotics used globally in 2010. The use of trimethoprim and penicillin was highest in South Africa (IMS Health, January 2011.). Cases of gradual increase in threat level of resistance towards antibiotics in the treatment of different diseases are reported and exemplified in Table 2.1 Chapter 2. This is of importance because of the part of the population of immuno-deficient African residents ravaged by HIV/AIDS, poor nutrition and less efficient sanitation system. This review in Chapter 2 provided the rationale in the choice of the antibiotics that was analysed in Chapter 3 and 4. The analysis of antibiotics in aquatic environment (wastewater environment) needs a systematic approach towards achieving reliable results. In this regard, Chapter 2 also presents an assessment of the advancement in

methods for extraction of antibiotics with solid phase extraction (SPE) and further refer to literature on liquid-liquid extraction (LLE) methods applied in different aquatic environmental media. This assessment has been published as an output for the thesis (Faleye et al., 2017). These advanced methods do enhance specificity, and also exhibit high accuracy and recovery. The aim of this was to assess the pros and cons of the methods of extraction towards identification of quinolones and sulphonamides as examples of relevant antibiotics in wastewater. Table 2.6 and 2.7 of chapter 2, presented some comparisons between different extraction methods for the determination of sulphonamide and fluoroquinolones, respectively in wastewater. The challenges associated with the improvements were also examined with a view to providing potential perspectives for better extraction and identification protocols in the near future. Tabulated recoveries of some common solid phase extraction sorbents used for sulphonamide and fluoroquinolones are presented in Table 2.8 of Chapter 2 and created a base for the protocol and sorbent used for the analysis of antibiotics in Chapters 3 and 4.

6.1.2 Development and optimization method for antibiotics in wastewater using solid-phase extraction (SPE) with high performance liquid chromatography–photodiode array detector (LC-DAD)

Different methods for the analysis of antibiotics in the wastewater environment needs to be optimized for the analyte of interest and the instrument to be used (extensively addressed in Chapter 2). It is therefore essential to develop a method that will be appropriate for the instruments available (LC-DAD), in parallel to the antibiotics to be analysed. This was done in relation to the instrument sensitivity and extraction protocol. Based on the review in Chapter 2, solid phase extraction (SPE) was selected for the extraction protocol for this research. However, extracting small concentrations (parts per million/billion) of analyte from a polluted sample such as wastewater requires a systematic approach with the use of appropriate sorbents

(SPE cartridges) in order to sequester the analyte of interest present in the sample. Oasis HLB produced by Waters and Strata XL by Phenomenex, were tested for the adsorption of the antibiotics, from pure water and wastewater samples, respectively. Both cartridges showed a good recovery in pure water, but Oasis HLB had a better recovery in wastewater samples than Strata XL, as shown in Table 3.2 Chapter 3 (Details of the experimental procedure is available in section 3.3 of Chapter 3). The instrument used for this study, a high-performance liquid chromatography (HPLC), LCMS 2020 by Shimadzu, equipped with, a diode array detector (DAD), and a mass spectrometer MS (m/z range: 0-2000; ionization modes: ESI/APCI), was optimized using gradient "5A" for the chromatographic separation of the selected antibiotics as shown in Table 3.3 Chapter 3 (details in section 3.4 of Chapter 3). The optimized method was used for the analysis of influent samples taken from 4 wastewater treatment plants. The selected six antibiotics were identified at different concentrations (Table 3.5 of Chapter 3). The limit of detection and quantification were also determined for the individual antibiotics (reference to Table 3.4 of Chapter 3) in fulfilment of objective 1 of the study. In parallel, the capacity of the instrument used, (HPLC at Food Technology Department, DUT) was determined (instrument can only determine analyte concentration ≥ 0.01 mg L^{-1}). Analyte of interest may be at concentrations far less than 0.01 mg L^{-1} (ng L^{-1}), hence a higher resolution instrument was needed to improve the result of the analysis. This constituted a major part of the studies and is presented in Chapter 4, where a high sensitive instrument, online solid phase extraction – high performance liquid chromatography mass spectrometry (SPE-HPLC-MS), at the Environmental Chemistry Department, Umeå University, Sweden, was utilized for the analysis.

6.1.3 Analysis of 13 antibiotics in wastewater sample in South Africa

All the shortcomings observed during the optimisation process (Chapter 3), were further addressed in Chapter 4. This included loss of analyte as a result of the filtration process and the sensitivity of the analytical instrument. To achieve a better sample homogeneity grab samples were avoided and composite samples (each represented by multiple subsamples during 1-2 hours) were collected twice per month for a period of four months (Feb, March, April and May 2017) from the selected four WWTPs in Durban, South Africa. The selection criteria are detailed in section 4.2.3 of Chapter 4, while the characteristics of the WWTPs are tabulated in Table 4.2. Collected samples were centrifuged to prevent the loss of analyte to filtration. Seven new antibiotics were added, which resulted in a total of 13 antibiotics in the Chapter 4 study. The names, chemical structure, abbrevation, molecular weight and uses of all the antibiotics are tabulated in Table 4.1 of Chapter 4. Analysis of both supernatants and sediments to determine their concentrations in the inlet treatment were carried out. Details of the protocol employed are specified in section 4.2.4. An online solid phase extraction – high performance liquid chromatography mass spectrometry (SPE-HPLC-MS) was used to measure the concentration in ng L^{-1}. The limit of detection (LOD) ranged from 0.07 – 0.33 ng L^{-1} for the 13 assessed antibiotics and the percentage recovery was in the range of 51 to 111 % (reference to Table 4.3). The percentage of antibiotics recovered from the sediment samples, which would otherwise have been lost to filtration were in the range of 3 % – 97 % (n = 32) (reference to Table 4.4). The frequency of detection in the influent samples for the sampling period ranges from 65 – 100 % (n = 32) (Table 4.5). The concentration of antibiotics (median value) in the influent samples ranges from 1.3 ng L^{-1} (AZI) to 81748 ng L^{-1} (CIP) for the four WWTPs. Analysis of the antibiotics in the supernatant and sediment samples improves the concentration of detection. This method is an improvement on the optimization method in chapter 3. The advantages of the method improvement are the reduction in the loss of analyte of interest that

would have occurred if a sample filtration step was the only one used for sample preparation. The 13 antibiotics chosen were constantly detected throughout the 4 WWTPs investigated in this study. Azithromycin (AZI) was the least detected. The overall removal efficiency for the 4 WWTPs ranges from 21 % - 100 %. (Table 4.13). The antibiotic removal process in WWTPs depends on various treatment steps and factors. Activated sludge and trickling filter method are the core biological methods used by most WWTPs in Durban. The result of the analysis of the WWTP stages shows that, antibiotics reduction occurs in the trickling filter chamber, for the trickling filter method, while the main reduction in the assessed activated sludge treatments occurs at the secondary clarifying sedimentation stage thereafter. (Reference to Tab 4.7, 4.8, 4.9 and 4.10).

The impact of antibiotics concentration from the WWTPs effluent on the downstream sampling point was low (Table 4.14 of Chapter 4) and can be regarded as insignificant based on dilution factor of the receiving water bodies. Based on the comparison of the Predicted no-effect concentration (PNEC) values of the antibiotics (to the inhibitory effect on bacteria) with the detected concentration of the antibiotics in the downstream samples, none of the antibiotics concentration values pose any immediate environmental risk. However, a prolong exposure may be speculated to have an effect on aquatic organism. The impact of chlorine in this study was judged as due to transformational changes of the antibiotics rather than degradation based on the chromatogram examination in Chapter 5 (Figure 5.6 and 5.7). A laboratory experiment was set up to examine the transformation process. A pilot experiment based on ethionamide and metronidazole as standard antibiotics; and pure water and wastewater were used as samples for the experiment.

6.1.4 Impact of the application of ultraviolet radiation and hypochloride on ethionamide and metronidazole

The main aim of Chapter 5 was to elucidate the impact of post chlorination on antibiotics reduction assessed in full scale in Chapter 4 and in parallel, examine the effects of ultra violet light in a laboratory set-up. Ethionamide and metronidazole were used as representatives of antibiotics, while high test hypochloride (HTH) was used as the source of chlorine. A laboratory experiment was set up to (1) investigate the level of degradation of the selected antibiotics in wastewater and pure water in parallel to the different time of exposure of UV (254 nm) radiation and chlorine, (2) identify possible transformation products formed during the reaction in objective (1).

The investigation compared the impact of UV and chlorine on pure water and wastewater samples. Details of the experimental procedure are available in section 5.2.1 of Chapter 5. The percentage reduction of the two antibiotics was influenced by its concentration in pure water for both the UV and the chlorine process. Lower reduction were recorded for concentration of antibiotics > 1.5 mg L^{-1} during the UV degradation in the ultra-pure water (reference to Figure 5.2 Chapter 5). The total dissolved solid (TDS) of the samples (pure water and wastewater) may have an influence on the reduction effect of the UV. Antibiotics in pure water with a TDS value of 35 mg L^{-1} were degraded more than in the wastewater with a TDS value of 250 mg L^{-1} The impact of chlorine removal for both pure water and wastewater was within the range of 80 – 90 % for ETI and MET and was not affected by the TDS value. Generally, it was speculated that the TDS value played a significant role on the impact of UV, but a more detailed analysis which involves different TDS levels will elucidate this speculation. The chromatographic results of the scan mode show a constant strong peak at 2.94 min retention time in almost all cases of chlorine impact on the antibiotics (Figure 5.7).

Experiments performed using pure water and wastewater under controlled condition showed a remarkable results, although a more intensive analysis is needed to justify this claim. All (100 %) of an initial concentration of 1.5 mgL^{-1} of ETI was removed by chlorine concentration within the range of 1-10 mg L^{-1}. A lesser reduction within the range of 86 – 94 % occurred for MET under similar condition (Fig 5.4 and 5.5 of Chapter 5). Analysis of antibiotics in WWTPs, full scale plant (with % reduction), showed a reduction within the range of 28-80 % for ETI at a pre-chlorination concentration range of 13-168 ng L^{-1} and, 47-82 % of MET (concentration of 288- 906 ng L^{-1}) (Table 5.1 of Chapter 5). The residual chlorine was within the range of 0.2 - 0.3 mg L^{-1} and might have an impact on antibiotics reduction

The degradation of the antibiotics may be regarded as a transformation rather than a degradation particularly from the impact of chlorine. The impact on antibiotics from UV and chlorination differed in this study. The concentration of the pre-chlorination and post chlorination stages in four WWTPs in Chapter 4 was compared with the laboratory study (section 5.3.4 Chapter 5). The reduction pattern can be related to the laboratory analysis, in parallel to the residual chlorine concentrations and TDS (reference Table 5.1 Chapter 5). The scan mode chromatogram result in an unknown constant peak (Figure 5.8 and 5.9) and gives a confirmation of the formation of derivatives of the antibiotics with chlorine.

6.2 CONCLUSIONS AND RECOMMENDATIONS

The major conclusions based on the objectives of this thesis are;

1. The screening assessment for the presence of antibiotics in four wastewater treatment plant was able to identify six antibiotics at the influent of the four WWTP. Also a validated method was developed using the LC-DAD
2. An intensive and extensive analysis was used to quantify 13 antibiotics in the selected four WWTP. The analysis was performed on an online SPE - LC-MSMS instrument at Umeå University in Sweden. The analysis was comprehensive in its approach by analysing the influent sample without the loss of analyte through filtration. The quantities of the identified antibiotics were compared, which is further linked to the community the WWTP is connected to and different treatment configuration.
3. The methods of wastewater treatment at the four WWTPs were examined. Two major types of system were utilized in the treatment method, which are the “Trickling filter” and “activated sludge” systems. Antibiotics were mostly retained in the trickling filter chamber, while the sedimentation stage was mostly responsible for the reduction of antibiotics with the activated sludge system. Further analysis of the antibiotics at the downstream section of the WWTPs, indicated that the concentration poses no immediate environmental risk.
4. The impact of post chlorination was examined for the four WWTPs which were related to the reduction of the antibiotics MET and ETI. The varying concentration of the antibiotics did not influence the reduction effect of chlorine. The impact of chlorination on antibiotics was more related to transformation than reduction. The chromatogram results revealed the presence of a derivate of the antibiotics.

Recommendations

1. Regular analysis of waste water treatment effluents and the receiving environment for antibiotics will provide useful information on environmental monitoring.
2. Utilization of advance treatment method will improve the removal of antibiotics pollutant. Hence it is recommended that most WWTPs should include such treatment methods in the existing treatment system in the nearest future.
3. Frequent monitoring of the antibiotics in the WWTPs influent, will indicate (partially) the rate of consumption of antibiotics in parallel to associated prevalent diseases.
4. Analysis of the derivate formed as a result of the impact of chlorination of the effluent should be examined in relation to its environmental impact in nearest future.

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APPENDICES

APPENDIX 1. INDIVIDUAL CONCENTRATION OF SAMPLES AT EACH WWTP WITH SAMPLING DATE

"I" WWTP SAMPLES RESULTS

"I" WWTP	**FEB, 06/02/17 I1**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	9.8	1.0	594.0	32.6	1829.8	31177.3	44741.6	2515.3	27.3	45.8	0.0	871.0	626.1
Pre-chlorination Sample	0.2	55.6	194.0	10.4	37.0	1354.6	28.5	525.6	9.0	82.4	3.2	22.6	0.0
Post-chlorination Sample	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	170.4	0.0	0.0	0.0
Down stream Sample	0	0	125.3	0	0	0	0	0	0	522.1	129.3	0	0
Up stream Sample	47	54.1	53.1	31.2	2927.8	48927.2	13594.9	0	11.5	0	0	19.4	9.1
"I" WWTP	**FEB, 24/02/17 I7**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	6.4	22.8	387.8	34.8	1544.8	33146.1	66.0	603.6	4.1	17.0	0.8	1743.0	2514.9

Pre-chlorination Sample	8.0	8.0	1627.6	31.3	285.7	5153.6	978.5	1650.3	0.8	62.8	27.6	49.6	68.9
Post-chlorination Sample	2.6	98.4	879.4	2.2	47.7	948.0	23.1	315.9	8.1	219.3	5.5	97.6	6.1
Down stream Sample	25.6	53.5	493.3	5.7	68.8	268.4	9.2	0.0	1.2	82.7	0.0	14.6	0.0
Up stream Sample	2.8	67.7	782.2	5.9	0.0	386.1	387.0	0.0	0.0	59.1	0.0	70.9	0.0
"I" WWTP	**MAR, 07/03/17 I13**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	19.3	16.6	4608.6	8.0	2009.0	25306.4	156796.1	1576.0	26.2	122.5	12.1	52.9	3.0
Pre-chlorination Sample	0.1	54.9	329.7	0.0	0.0	1932.3	1.4	0.0	8.0	55.4	7.4	5.8	0.0
Post-chlorination Sample	2.9	36.5	261.3	4.2	47.6	277.0	213.2	0.0	0.0	59.5	4.7	67.2	0.0
Down stream Sample	1545.8	851.9	89.1	0.0	0.0	0.0	189.4	0.0	0.0	1.5	0.0	7.2	0.0
Up stream Sample	0.1	15.1	101.4	0.0	22.9	245.9	54.6	44.5	0.0	64.4	10.5	32.2	0.7
"I" WWTP	**MAR, 23/03/17 I19**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	0.0	0.0	7649.9	13.2	1392.6	16702.1	0.5	215.7	8.7	0.6	122.9	1312.9	96.9

Pre-chlorination Sample	0.1	82.6	814.9	0.0	0.0	658.7	0.0	16.9	0.0	251.3	32.6	65.3	0.4
Post-chlorination Sample	2.3	1.6	682.4	0.0	22.4	128.3	0.0	101.8	0.1	157.7	11.2	46.9	0.4
Down stream Sample	0.5	9.5	351.6	1.0	17.0	0.0	0.0	8.0	0.0	0.0	9.0	40.4	0.2
Up stream Sample	0.8	1.7	136.3	0.0	0.0	54.3	14.7	3.6	0.4	6.3	0.0	10.8	6.6
"I" WWTP	**APR, 06/04/17 I25**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	13.76523	20.31932	164.4307	194.0432	1561.443	23303.33	68104.68	485.0639	9.549148	52.60307	0	0.266859	0
Pre-chlorination Sample	20.8	440.3	475.1	0.1	1.8	44.3	162.4	145.6	0.3	0.6	0.5	10.8	0.7
Post-chlorination Sample	2.7	16.4	136.6	0.0	0.0	335.3	4.6	25.5	0.2	0.0	0.0	0.0	0.1
Down stream Sample	0.0	180.7	221.3	1.1	14.5	0.0	2.7	38.2	109.0	43.9	2.0	46.4	6.6
Up stream Sample	1.1	37.8	41.7	0.0	0.0	2.4	0.0	14.4	23.0	4.1	0.0	8.6	2.8
"I" WWTP	**APR, 20/04/17**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	0	0	1148.766	137.2327	4646.619	66941.21	0	2949.938	28.15899	201.8897	0	0	491.6044

Pre-chlorination Sample	0.0	11.4	1655.6	0	0	0.0	0	0	0	709.6	0	0	0
Post-chlorination Sample	0.0	0.3	476	0	0	0	0	0	0	427.6	0	0	0
Down stream Sample	0.2	0	213.1	0	0	0	0	0.2	0	120.5	0	0	0
Up stream Sample	0.8	0.3	127.2	0	0	0	0	0	0	37.6	0	0	0
"I" WWTP	**MAY, 04/05/17**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	38.6	0.0	13.3	32.8	1336.2	30231.6	45486.6	552.1	3.1	28.3	0.0	0.0	4.1
Pre-chlorination Sample	0.0	223.8	204.2	0	0	0.0	0	0	0.8	0	0	0	0
Post-chlorination Sample	0.0	1	340.7	0	0	0	0	0	0	0	0	0	0
Down stream Sample	0.6	0.4	180.4	0	0	0	0	1.1	0	106.8	0	0	0
Up stream Sample	100.4	0	22.1	0	0	0	0	0	0	14.8	0	0	0
"I" WWTP	**MAY, 16/05/17**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	0.0	48.0	848.4	57.3	2305.7	55920.7	38347.4	476.9	0.0	0.0	0.0	0.0	0.0

Pre-chlorination Sample	4.2	16.9	960.4	0	0.0	0.0	0	0	0	908.4	0	0	0
Post-chlorination Sample	0.0	2.1	208.3	0	0	0	0	0	0	0	0	0	0
Down stream Sample	0.0	0	366.8	0	0	0	0	0	0	0	0	0	0
Up stream Sample	0.2	4	559.4	0	0	0	0	0	0	0	0	0	0

“K” WWTP SAMPLES RESULTS

“K” WWTP													
	FEB, 09/02/2017												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	47.0	21.7	855.2	9.8	3925.1	66677.8	5917.2	8456.9	46.9	48.2	68.0	1175.5	145.1
Aeration samples	0.1	2.3	8.1	2.1	161.0	1473.1	144.0	991.7	9.6	0.0	2.1	42.5	0.0
Prechlorination samples	1.0	5.1	6.7	0.8	87.0	719.3	0.0	0.0	7.9	4.6	0.4	3.3	0.0
Post chlorination samples	0.0	0.0	4.4	3.1	56.6	0.0	0.0	297.1	0.0	2.3	0.0	28.0	0.0
Down stream samples	0.7	0.6	38.4	0.0	80.7	0.0	0.0	316.6	2.5	5.0	0.0	26.7	0.0
“K” WWTP													
	FEB, 24/02/2017												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	7.5	23.2	788.7	52.0	7802.8	71324.5	22124.6	6401.5	42.3	37.4	94.5	1727.9	120.0
Aeration samples	1.2	0.0	403.3	49.2	594.1	9523.0	0.0	1050.0	7.8	0.0	5.6	222.3	11.0
Prechlorination samples	0.0	0.7	6.8	0.0	108.1	489.2	0.0	0.0	13.8	0.0	0.0	9.4	0.1
Post chlorination samples	1.0	2.5	9.6	0.0	34.7	0.0	0.0	0.0	0.8	0.3	0.0	8.7	1.5
Down stream samples	0.0	0.5	10.6	0.0	68.9	200.4	0.0	14.7	8.2	11.8	0.0	12.2	0.0
“K” WWTP	**Mar, 07/03/17**												

	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	0.1	36.2	92.5	31.2	3191.4	50769.1	13107.1	1116.2	17.3	35.8	10.9	345.6	23.3
Aeration samples	2.8	18.9	771.8	1.1	191.4	2712.2	0.0	2079.2	6.6	11.5	31.6	0.0	0.0
Prechlorination samples	0.9	13.1	9.2	3.0	297.8	1950.2	0.0	1892.6	25.7	7.7	0.0	157.1	3.4
Post chlorination samples	0.1	1.6	10.1	10.1	77.2	515.6	81.8	159.4	0.5	0.7	4.2	32.5	0.4
Down stream samples	0.3	2.3	9.1	5.3	62.3	415.4	0.0	290.4	17.2	4.7	0.0	59.4	0.0
“K” WWTP	**Mar, 22/03/17**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	42.1	17.7	351.1	87.4	8901.8	113144.8	9635.5	5868.5	37.3	61.5	288.9	550.3	40.5
Aeration samples	0.3	0.4	14.3	0.0	0.0	192.2	0.0	417.4	3.4	0.0	9.6	1046.9	0.0
Prechlorination samples	0.0	0.7	0.0	0.0	82.1	212.0	0.0	345.2	13.6	14.8	5.8	23.4	10.2
Post chlorination samples	0.0	0.2	2.9	0.0	35.3	0.0	165.0	522.9	3.4	5.4	3.1	8.4	0.0
Down stream samples	0.0	1.8	4.0	0.0	31.9	222.8	0.0	0.0	11.7	2.9	0.0	63.1	0.0
“K” WWTP	**Apr, 06/04/17**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	0.0	37.2	511.4	33.2	4243.6	55233.0	11150.3	4441.4	41.6	61.4	61.5	776.6	66.3

Aeration samples	0.0	1.1	2.3	0.0	106.9	0.0	22.2	407.3	16.7	9.4	0.0	99.4	1.8
Prechlorination samples	0.0	304.0	469.7	0.0	11.8	7.9	1.8	28.1	165.3	156.7	2.3	170.7	2.0
Post chlorination samples	0.8	899.2	874.4	6.3	84.8	313.4	60.5	176.5	677.7	539.0	128.8	339.2	13.2
Down stream samples	0.0	0.8	17.5	0.0	38.9	346.8	0.0	516.5	5.0	22.3	2.9	44.9	0.0
“K” WWTP	**Apr, 20/04/17**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	28.1383	86.76237	1055.785	48.47829	3673.864	92171.55	13021.38	4897.802	8.48099	26.32761	127.2397	584.2629	76.93134
Aeration samples	0.0	0.8	0.0	4.1	73.4	0.0	155.3	1653.5	2.7	0.2	0.0	88.7	3.9
Prechlorination samples	1.7	1.2	4.1	0.3	53.3	128.7	0.0	0.0	2.5	0.1	0.0	52.0	5.2
Post chlorination samples	0.0	3.8	2.6	0.5	11.3	0.0	0.0	46.4	1.0	2.0	0.0	36.9	0.0
Down stream samples	1.5	4.2	9.2	0.0	41.4	0.0	0.0	0.0	7.9	0.6	0.0	30.5	4.3
“K” WWTP	**May, 04/05/17**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	49.47577	10.03852	860.126	206.5321	4532.852	98736.75	84838.1	2709.8	48.20438	10.76627	11.12204	855.7845	40.84269
Aeration samples	0.3	1.9	4.4	1.2	40.7	308.4	55.1	357.5	3.5	0.3	0.0	47.6	3.8
Prechlorination samples	8.5	5.7	5.0	0.0	59.1	0.0	63.2	452.4	6.1	0.0	0.1	43.2	4.0

Post chlorination samples	1.0	3.0	0.1	1.6	76.2	232.9	0.0	725.0	0.0	1.8	0.0	38.7	11.0
Down stream samples	7.5	1.6	0.1	4.1	114.7	0.0	0.0	771.1	4.6	1.3	0.0	21.9	4.6
"K" WWTP	**May, 16/05/17**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	3.1	14.1	206.5	24.2	9662.4	105799.7	20414.3	2661.9	87.1	41.2	154.3	289.2	49.7
Aeration samples	0.4	10.1	3.5	1.3	288.0	1151.0	0.0	494.2	22.1	7.6	0.0	72.5	0.0
Prechlorination samples	2.6	3.3	4.7	4.9	152.7	983.6	0.0	2013.9	53.9	0.0	0.0	33.0	0.0
Post chlorination samples	0.0	15.5	10.9	8.0	170.7	348.6	0.0	485.4	11.2	17.4	0.0	22.3	1.2
Down stream samples	0.0	0.8	18.5	0.0	91.9	404.1	0.0	187.3	29.5	0.0	7.3	50.3	0.0

"S" WWTP SAMPLES RESULTS

"S" WWTP	FEB, 06/02/17												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	0.3	74.4	986.8	261.6	603.2	23063.5	552843.5	1419.1	6	22	0	340.2	37.3
Aeration samples	94.7	444.4	182	577.4	8030.4	201990.2	10854.4	856.9	38.8	5.4	0	23.8	3.4
Prechlorination samples	51.9	101.4	26.5	1.4	14.8	199	470	1358.4	5.5	0	0.2	8.6	0
Post chlorination samples	12.7	67.4	252	1.7	20	112.7	339.1	367.4	0.2	2.1	1.7	9.5	0.7
Down stream samples	19.5	20.5	346.9	0	37.8	427.5	829.9	2357	0.3	3.1	0	8.9	0
"S" WWTP	**FEB, 21/02/17**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	12.4	652.6	421.3	183.7	8229.5	116089	7682.1	571.8	72.7	4.4	527.7	53.2	0
Aeration samples	20.5	54.1	71.8	45.7	392.6	21224.2	6781.1	455.6	4.6	6.9	3.6	120.7	9.4
Prechlorination samples	3.6	36.2	26.4	12.6	41.7	1683.2	192.9	268.5	6.2	15.6	0	5.8	0
Post chlorination samples	0.2	21	402.7	1.9	41.4	1686.4	293.9	316.4	0.2	8.4	12.7	2.1	0
Down stream samples	4.3	6.7	270.5	1	32.6	752.4	209.3	180.7	3.8	13.6	0	11.9	0
"S" WWTP	**MAR, 10/03/17**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX

Influent samples	194.7	164264.4	8815.2	253.9	1341.7	501575.5	10298.3	4018.6	8.6	2.2	182.7	115.5	4.3
Aeration samples	7.9	29476.6	1005.1	343.2	12175.1	199968.1	13866.2	1227.2	157.6	16.4	678	200.6	5.1
Prechlorination samples	0	0	0	0	0	0	0	0	8	23.1	1.9	15.4	0
Post chlorination samples	3.8	54.6	378.4	30.2	109.8	1917.3	84.2	161.7	0	25.9	0.6	34.5	44.6
Down stream samples	2.6	3.6	154.5	17.4	27.7	662.7	434.5	3.9	0.7	24.4	4.5	47.3	0
"S" WWTP	**MAR, 22/03/17**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	27	17.2	199.7	55.4	634.6	12714.7	6063.2	0	9.5	13.1	2.6	3.6	0
Aeration samples	0	3.4	5.3	8.3	14.3	154.8	184.4	23.7	0	7.5	4.6	91.8	2.4
Prechlorination samples	0.1	1.1	32	1.7	50.4	0	0	162.7	7.8	31.7	1	84.7	11
Post chlorination samples	0.1	0	152.8	0	79.5	0	15.8	228.1	3.8	18.1	15	26.9	3.4
Down stream samples	10.1	64	0	0	0	0	0	0	0	22.9	0	0.3	0
"S" WWTP	**APR, 06/04/17**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	0.9	10.3	120.5	76.4	262.7	2964.1	763610.9	220.1	0.4	29.2	3.8	10.6	2.4
Aeration samples	0	61.5	145.2	0	13882.8	358066.3	7845.3	393.2	100	71	2.8	37.6	0

Prechlorination samples	0	20.1	212.7	8.1	49.5	0	98.5	0	0	48.5	76	25.8	5.8
Post chlorination samples	2.7	7.8	150.8	3.3	184.2	598.9	0	8.3	12.3	6	0	9.5	0
Down stream samples	11.3	135.8	154.4	50.4	249.3	4103.1	24.7	178.1	0	0	0	0	0
"S" WWTP	**APR, 21/04/17**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	6.5	128.5	78.4	76.6	200.9	411.5	11499.4	26.9	0.2	0.9	0	34.7	0
Aeration samples	45.5	0	162.8	0	8743.2	213102.9	0	69.3	57.5	29.2	8.3	139.6	2.4
Prechlorination samples	0	1.6	13.2	9.3	50.2	762.6	0	75.1	22	71	4.5	33.9	0
Post chlorination samples	2.4	20	212.7	4.7	49.5	1387.3	4.7	58	0	68.8	0	25.9	5.8
Down stream samples	1.8	0.6	169.1	0	49.2	413.6	0	71.8	0.2	13.4	0	14.5	0
"S" WWTP	**MAY, 05/05/17**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	34.6	22.5	391	24.3	692.5	33348.7	22413	116.8	3.8	42.2	5.7	0	68.9
Aeration samples	0.5	5.9	173.4	401.5	8478.9	269643.5	11438.6	724.9	46.2	67.2	0	170.8	1.5
Prechlorination samples	0.1	27.6	10.7	3.1	118.1	1386.7	1.6	155.4	10.1	38.1	0	85.8	0

Post chlorination samples	2.3	22.7	551.2	0	120.5	897.9	196.8	197.4	0.3	34.6	0	76.9	0
Down stream samples	2.3	17.8	465.9	0	102.7	1262	716.9	421.9	1.7	28.3	0	45.6	1.9
"S" WWTP	**MAY, 15/05/17**												
	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	30.3	79.1	191.6	215.6	662.8	13927.7	116941.9	389.1	18.3	71.4	4.9	24	77.2
Aeration samples	99.4	40.8	147.8	439.6	7965.4	167312.7	9625.9	214.9	52.2	34.9	577.1	52.5	0.9
Prechlorination samples	0	2	577.8	24	224.9	857.6	0	713.5	1.4	4.1	37.9	0	0
Post chlorination samples	0.6	8	38.3	9.3	108.8	2995.1	0	557	12	49.4	0	17.7	3.5
Down stream samples	0.9	17.9	110.3	25.9	205.5	2327.4	0	48.3	3.3	49.7	6.6	15.8	0
"K" WWTP													

"P" WWTP SAMPLES RESULTS

"P" WWTP	**FEB, 09/02/2017**												
Conc (ngL-1)	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	177.4	406.4	92.4	266.2	8980.7	154846.2	24208.9	713	85.2	0.4	10.8	71.8	0
Aeration samples	21.5	30.2	42.3	1.6	12.6	460.2	655.2	713	0.2	0.2	0.6	1.2	0
Pre-chlorination Sample	15.4	21.7	13.6	0.7	18.4	122	368.8	532.5	0.8	0	0	2.5	0.2
Post-chlorination Sample	18.3	25.6	8.9	1.3	23	421.6	601.1	400.3	2.2	0	0.5	3.5	0
Down stream Sample	21.2	29.8	13.1	0.9	15.8	259.8	438	425.6	4.5	0	0	15	0
"P" WWTP	**FEB, 21/02/2017**												
Conc (ngL-1)	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	87.4	97.9	321.8	30	1538.3	20421.2	9624.3	893.3	4	4.5	0.5	27.6	5.4
Aeration samples	69.6	403.4	85.4	240.1	7209.4	127492.1	25261.9	0	55.2	4.6	11.4	99	1
Pre-chlorination Sample	13.5	206.9	28.8	0.4	10.5	140.7	224.9	283.7	1.2	0	0	0	3.4
Post-chlorination Sample	51.7	72.6	29.6	0.6	10.2	152.1	499.8	515.7	0.2	2.9	0	0.4	0.1
Down stream Sample	8	11.3	236.9	2	19	157.5	364.4	116	3.3	1.9	1	53.3	3.1
"P" WWTP	**Mar, 10/03/17**												
Conc (ngL-1)	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	26.5	35.2	106.4	48.6	2426.4	47425.7	10011.1	86.1	15.5	1.3	0	35.3	0.1
Aeration samples	214.5	526.4	627.5	206.1	6544.5	138061.9	25097.8	2989.2	60.1	0	1	6.1	11.4

Pre-chlorination Sample	36.4	51.1	49.8	1.3	30	564.5	819.8	737.1	1.9	4.7	0.8	9.7	0
Post-chlorination Sample	6	8.4	11.7	1.3	22.7	253.9	540	230.2	1.2	0	0.9	0	0.6
Down stream Sample	4.3	6	8.9	1.7	15.4	154.4	285.4	165	0.7	3.5	0.8	5.7	0
"P" WWTP	**Mar, 23/03/17**												
Conc (ngL-1)	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	19.5	11.8	54.8	18.4	760.3	12601.3	278.1	961.5	2.7	4.9	4.1	22.4	0
Aeration samples	1.6	535.5	78.3	421.6	12024.5	197891.5	15017.6	337.6	70.5	13.8	0.1	147	0
Pre-chlorination Sample	0	0	73.8	4082	5283.4	0	20690.6	0	0	0.1	0.2	0.1	1
Post-chlorination Sample	0	0	14.1	6.9	15.1	271.3	712025.4	0	0.8	2.7	0	0	0
Down stream Sample	23.9	33.5	13	0.3	7.8	65.4	562.9	42.1	1.6	0	0.5	2.7	0
"P" WWTP	**Apr, 04/04/17**												
Conc (ngL-1)	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	37	83.7	217.7	18.7	771.1	16426.6	243812.5	138.9	5.8	1	69.7	7103.6	1476.6
Aeration samples	29.4	244.2	120.4	342.7	11323.5	171654.9	16415	32.6	69.5	0	741.9	271.6	60
Pre-chlorination Sample	39.9	56.1	46.1	0.8	23.5	407	2138.1	0	3.2	0	3.1	8.3	0.9
Post-chlorination Sample	11.1	15.6	5.6	0.5	0.5	1.8	201156.6	14.9	1.9	0	0.2	2455.7	0
Down stream Sample	0	0	0	0	0	0	0	0	0	0	0	0	0

“P” WWTP	**Apr, 21/04/17**												
Conc (ngL-1)	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	23.8	46.4	622.7	50.8	1815.3	34494.1	192856	40.6	6.9	0.8	2.7	7515.2	6012.8
Aeration samples	8	102.8	80.9	261.2	8097.2	139449.7	21980.8	459.9	45.9	0.1	1.8	74.4	0.1
Pre-chlorination Sample	41.7	58.5	8.6	0.2	25.4	483.7	1412.4	202.9	4.5	0.2	0.9	15.6	4.5
Post-chlorination Sample	11.3	15.8	18.6	1.2	18.1	452.9	1771.8	1905.3	0.8	2.6	1.9	0	0
Down stream Sample	0	0	2	0.2	2.8	12.8	405.1	41.8	0	0	0	0	0
“P” WWTP	**May, 05/05/17**												
Conc (ngL-1)	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	8.6	27.6	497.1	36.3	1796	54234.7	266007.2	9054.4	9.8	20.1	0.7	3609.5	1772.7
Aeration samples	57	397.6	265.3	200.3	5170.1	111125.1	17120.9	9756	15.9	23.2	3.9	93.9	0.7
Pre-chlorination Sample	1.5	2.1	99.4	0.7	36.2	431	890.1	0	3	0	0.8	98.7	1.1
Post-chlorination Sample	7.5	10.6	12.5	1	7.5	155.8	120.1	489.1	0.4	0	1.7	0	0
Down stream Sample	0.2	0.3	10.2	0.8	6.5	0	35	76.5	0.1	0.3	0	2.9	0
“P” WWTP	**May, 15/05/17**												
Conc (ngL-1)	ETI	MET	TRI	NOR	OFL	CIP	ALB	SUL	CLI	ERY	AZI	CLA	ROX
Influent samples	3.3	57.5	118.5	77.6	2750	83630.2	7076.3	540.1	8.1	11.2	2	26.6	0
Aeration samples	41.9	109.5	69.7	261.5	7816.7	133932.3	28413.4	333.8	72.8	0	15.2	26.2	0.2

Pre-chlorination Sample	0.4	0.6	55.6	0	26.9	254.5	326.3	213.3	0.6	2.3	4.9	24.3	0.1
Post-chlorination Sample	1.9	2.7	10.1	0.9	15.7	349.6	764.3	307.6	2.9	3.7	1.2	13.5	5
Down stream Sample	5.5	7.7	5	2.6	10.7	57	169.7	8.4	1.4	0.2	0.2	1.2	0

APPENDIX 2. OUTLIERS FOR THE INFLUENT SAMPLES

INFLUENT	Max		"I"			
Conc (ngL-1)	**Max**	**IQR**	**Low L**		**Upper L**	**Upper outliers**
ETI	38.6	15.2	-22.8		38	38.6
MET	48	20.9	-31.35		52.25	
TRI	7649.9	1681.8	-2190.8		4536.4	7649.9
NOR	194	49.6	-46.7		151.7	194
OFL	4646.6	576.4	642.1		2947.7	
CIP	66941.2	14034.1	3754.55		59890.95	
ALB	156796.1	51091.6	-76587.8		127778.6	156796.1
SUL	2949.9	1327.8	-1508.7		3802.5	
CLI	28.2	22.7	-30.25		60.55	
ERY	201.9	57.2	-72.9		155.9	201.9
AZI	122.9	3.6	-5.4		9	122.9
CLA	1743	981.5	-1472.25		2453.75	
ROX	2514.9	523	-782.3		1309.7	2514.9
			"K"	INFLUENT		
	Max					
ETI	49.5	41	-59.1		104.9	
MET	86.8	19.7	-12.75		66.05	86.8
TRI	1055.8	541.5	-497.25		1668.75	
NOR	206.5	31.4	-17.7		107.9	206
OFL	9662.412	4215.221	-2460.53		14400.35	
CIP	113144.8	36685.9	8787.75		155531.4	
ALB	84838.1	10070.3	-4333.85		35947.35	84838.1
SUL	8456.9	3303.9	-2258.05		10957.55	
CLI	87.1	14.9	9.95	8.5	69.55	87.1
ERY	61.5	18.1	6.25		78.65	
AZI	288.9	85.1	-78.75		261.65	
CLA	1727.9	436.6	-155.8		1590.6	1727.9
ROX	145.1	46.9	-29.55		158.05	
			"S"	INFLUENT		
	Max					
ETI	194.7	26.3	-34.35		70.85	194.7
MET	164264.4	238.4	-336.5		617.1	164264.4
TRI	8815.2	388.9	-409.55		1146.05	8815.2

NOR	261.6	154.1	-160.05		456.35	
OFL	8229.5	336.8	12.8		1360	8229.5
CIP	501575.5	43756.8	-55358.2		119669	501575.5
ALB	763610.9	216273.1	-314765		550327	763610.9
SUL	4018.6	689.3	-939.65		1817.55	4018.6
CLI	72.7	8.7	-10.05		24.75	72.7
ERY	71.4	28.5	-38.85		75.15	
AZI	527.7	48	-70.1		121.9	527.7
CLA	340.2	59.9	-80.95		158.65	340.2
ROX	77.2	45.2	-67.8		113	
			"P"	INFLUENT		
	Max					
ETI	87.4	21.8	-25.4		61.8	87.4
MET	462.4	53.9	-47.55		168.05	462.4
TRI	1260.8	413	-504		1148	1260.8
NOR	82.1	30.3	-18.25		102.95	
OFL	2750	1144	-369.5		4206.5	
CIP	83630.2	36950.5	-36003.3		111798.8	
ALB	266007.2	196607.8	-285924		500506.8	
SUL	9054.4	1029.6	-1418.7		2699.7	9054.4
CLI	15.5	5.6	-3.1		19.3	
ERY	20.1	5.5	-7.35		14.65	20.1
AZI	69.7	2.5	-3.25		6.75	69.7
CLA	7515.2	4930.4	-7368.3		12353.3	
ROX	6012.8	1550.5	-2325.65		3876.35	6012.8

I, K, S, P = Names of WWTPs.

Max = maximum (the highest concentration of the antibiotics in the 32 samples)

IQR = interquartile range; (Q1 –Q3); Lower L = Lower limit [(Q1 – (1.5 X IQR)]

Upper L = Upper limit = [Q3 + (1.5 x IQR)].

Any value outside the lower L and upper L is an outlier. (Tukey, 1977)

APPENDIX 3. ABSTRACTS OF PUBLISHED AND IN-PRESS PAPERS

1. IDENTIFICATION OF ANTIBIOTICS IN WASTEWATER: CURRENT STATE OF EXTRACTION PROTOCOL AND FUTURE PERSPECTIVES

A.C Faleye[1,2]*, A.A Adegoke[1], K. Ramluckan[2], Faizal Bux[1] and T. A Stenström[1]

Abstract

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The release and occurrence of antibiotics in the aquatic environment has generated increased attention in the past few decades. The residual antibiotic in wastewater is important in the selection for antimicrobial resistance among microorganisms and the possibility of forming toxic derivatives. This review presents an assessment of the advancement in methods for extraction of antibiotics with solid phase extraction and liquid–liquid extraction methods applied in different aquatic environmental media. These advanced methods do enhance specificity, and also exhibit high accuracy and recovery. The aim of this review is to assess the pros and cons of the methods of extraction towards identification of quinolones and sulphonamides as examples of relevant antibiotics in wastewater. The challenges associated with the improvements are also examined with a view of providing potential perspectives for better extraction and identification protocols in the near future. From the context of this review, magnetic molecular imprinted polymer is superior over the remaining extraction methods (with the availability of commercial templates and monomers), is based on less cumbersome extraction procedures, uses less solvent and has the advantage of its reusable magnetic phase.

Keywords: | Antibiotics; Extraction Protocol; Quinolones; Sulphonamides; Wastewaters

2. ANTIBIOTIC RESIDUE IN THE AQUATIC ENVIRONMENT: STATUS IN AFRICA.

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Abstract

Information on the presence of antibiotics is sparse for all types of water in Africa, including groundwater, surface water, effluent of wastewater treatment plants (WWTPs) and municipal potable water. With the relatively high sales of different antibiotics to treat infectious diseases in the human population of Africa, the residual of the antibiotics is bound to be released through excretion via urine or fecal matter in parallel to the high sales. This article reviews the published analysis on the occurrence of antibiotics in the environment particularly in the aquatic environment in each country in Africa. In general, sulfamethoxazole was the most commonly detected in Africa surface water (with eight reports from four countries) at a concentration range of 0.00027 – 39 μgL^{-1}. Wastewater analysis is believed to give an early warning for preventing epidemics. Thus, we discuss the associated level of antibiotic resistance to some prevalent diseases in Africa whose aetiological agents can develop antibiotic resistance due to exposure to antibiotic residue in water. This is important because of rising population of immuno-deficient African residents ravaged by HIV/AIDS, poor nutrition and less efficient sanitation systems.

Keywords: Antibiotic; Environment; Release; Antibiotic resistance

3. ANTIBIOTIC RESISTANT SUPERBUGS: ASSESSMENT OF THE INTERRELATIONSHIP OF OCCURRENCE IN CLINICAL SETTINGS AND ENVIRONMENTAL NICHES

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Abstract

The increasing threat to global health posed by antibiotic resistance remains of serious concern. Human health remains at higher risk due to several reported therapeutic failures to many life threatening drug resistant microbial infections. The resultant effects have been prolonged hospital stay, higher cost of alternative therapy, increased mortality, etc. This opinionated review considers the two main concerns in integrated human health risk assessment (i.e., residual antibiotics and antibiotic resistant genes) in various compartments of human environment, as well as clinical dynamics associated with the development and transfer of antibiotic resistance (AR). Contributions of quorum sensing, biofilms, enzyme production, and small colony variants in bacteria, among other factors in soil, water, animal farm and clinical settings were also considered. Every potential factor in environmental and clinical settings that brings about AR needs to be identified for the summative effects in overall resistance. There is a need to embrace coordinated multi-locational approaches and interrelationships to track the emergence of resistance in different niches in soil and water versus the hospital environment. The further integration with advocacy, legislation, enforcement, technological innovations and further research input and recourse to WHO guidelines on antibiotic policy would be advantageous towards addressing the emergence of antibiotic resistant superbugs.

Keywords:

Residual Antibiotics; Antimicrobial Resistance; Total Antibiotic Resistance; Critical Control Point; Superbug; Exposure; Health Risk Assessment

4. RESIDUAL ANTIBIOTICS, ANTIBIOTIC RESISTANT SUPERBUGS AND ANTIBIOTIC RESISTANCE GENES IN SURFACE WATER CATCHMENTS: PUBLIC HEALTH IMPACT

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Abstract

Antibiotics are released to the surface water through different routes, like for example the wastewater treatment plants, from human and animal metabolic waste, agriculture run off, industrial antibiotic waste. The release of the antibiotics to the water catchment and/or the environments in sub-lethal concentrations for the microorganisms lead to the emergence of antibiotic resistance (AR) and selection for antibiotic resistance genes (ARGs). The bacteria utilize their quorum sensing to form biofilm within which ARGs are transferred from antibiotic resistant bacteria (ARB) to the susceptible strains, conferring resistance on them. This has contributed substantially to the growing trend of resistance from multiple antibiotic resistance to extended spectrum resistance, extreme resistance and recently to total antibiotic resistance. The antibiotics, ARB, ARGs are sometimes internalized into the crops irrigated with the surface water returning the bacteria to human in a difficult to control form. While quorum quenching strategy is being advocated during treatment of wastewater to disrupt biofilm as well as the spread of resistance, intermittent check for effectiveness of treatment of wastewater before release into receiving water bodies is hereby advocated. To achieve this, there is the need for better measurements, surveillance and follow-up and thereby the further needs to incorporate more integrative (multidisciplinary) approaches and state of the art tools, for appropriate detection and action. This presentation is to critically review the effect of antibiotic release, ARGs, ARB in water catchment on other water related applications in Southern African countries in relation to other part of the world.

Keywords:

Antibiotics; Resistance Genes; Total Antibiotic Resistance; Quorum Quenching; Internalization; Integrative Approach