



**SURVEILLANCE OF ANTIBIOTIC RESISTANCE DETERMINANTS IN GABORONE
AND PALAPYE WASTEWATER TREATMENT FACILITIES.**

By

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CERTIFICATION

The undersigned certifies that he has read and hereby recommends for acceptance by the College of Science a dissertation/thesis titled: Surveillance of antibiotic resistance determinants in Gaborone and Palapye wastewater treatment facilities, in fulfilment of the requirements for the degree of Master of Science in Biology and Biotechnological Sciences of the BIUST.



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TABLE OF CONTENTS

DECLARATION AND COPYRIGHT	ii
CERTIFICATION	iii
TABLE OF CONTENTS	iv
ACKNOWLEDGEMENT	vi
DEDICATION	vii
ABSTRACT	viii
LIST OF FIGURES	ix
LIST OF TABLES	xi
CHAPTER 1 INTRODUCTIONS AND OBJECTIVES	1
1.1. Introduction.....	1
1.2. Statement of the problem.....	3
1.3. Objectives	4
1.4. Research question	5
1.5. Significance of study	6
CHAPTER 2 LITERATURE REVIEW	7
2.1. Wastewater treatment plants	7
2.2. Antibiotics in wastewater treatment plants	9
2.3. ARB in wastewater treatment plants	11
2.4. ARGs in wastewater treatment plants.....	14
2.5. Water quality indicators in treated wastewater	16
CHAPTER 3 METHODOLOGY	17
3.1. Description of study area	17
3.2. Sample collection.....	20
3.3. Bacterial isolation and quantification	21
3.3.1. Statistical analysis	21
3.4. Antibiotic resistance characterization	23
3.4.1. Statistical analysis	23
3.5. Molecular characterization of antibiotic resistance	24
3.5.1. DNA extraction	24

3.5.2. ARGs analysis	24
3.6. Metagenomics analysis	28
3.6.1. Sample preparation.....	28
3.6.2. DNA sequencing and analysis.....	28
CHAPTER 4 RESULTS.....	30
4.1. Bacterial quantification.....	30
4.2. Bacterial isolation	32
4.3. Antibiotic resistant bacteria	33
4.3.1. Multidrug resistance	45
4.5. Antibiotic resistance genes	46
4.6. Microbial diversity in PWWTF downstream environment	53
CHAPTER 5 DISCUSSION	57
CHAPTER 6 CONCLUSION	60
6.1. Recommendations.....	61
REFERENCES	62
APPENDIX A	73
APPENDIX B.....	74
APPENDIX C.....	76
APPENDIX D	78
APPENDIX E	82

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DEDICATION

I would like to dedicate this thesis to my late mother, my family who stood by me every day and made me who I am today.

ABSTRACT

Antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB) have been globally considered emerging environmental contaminants that pose a serious concern to human health and the ecosystem. The ubiquitous nature of ARB and ARGs among pathogens from wastewater effluents, indicate that wastewater treatment facilities are major reservoirs for antibiotic resistance determinants, thus contributing to the widespread dissemination of antibiotic resistance. The primary objective of the study was to characterize the antibiotic resistance phenotypic and genotypic characteristics of bacterial isolates from Palapye wastewater treatment facility (PWWTF), Gaborone wastewater treatment plant (GWWTP) and the downstream environments receiving effluent wastewater. A detailed analysis was conducted with respect to the characterization and quantification of bacterial populations (including ARB) in wastewater influent, effluent and downstream environment. Culture dependent and independent approaches were used to determine occurrence, abundance and diversity of the ARB and ARGs in samples of wastewater influent, effluent and downstream environment. The bacterial concentration ranged between 2.1×10^2 CFU/mL- 4.2×10^{11} CFU/mL in PWWTF and downstream samples, and between 1.00×10^1 CFU/mL- 1.4×10^8 CFU/mL in GWWTP and downstream samples. The study also detected higher frequencies of potentially pathogenic ARB; *Staphylococcus* species, *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas* species, *Brucella* species, *Salmonella* species, *Listeria* species and *Campylobacter* species, and ARGs to clinically relevant antibiotics; *tetA* (tetracycline), *mphA* (macrolides), *strB* (streptomycin), *sul1* (sulphonamide), *dfr* (trimethoprim) and *int1* (mobile ARG cassette) in the samples. Metagenomic analysis of downstream environment of PWWTF also revealed diversity and abundance of bacterial populations belonging to major phyla Cyanobacteria (37%), Proteobacteria (35%), Firmicutes (14%) and the Bacteroidetes (11%). Conclusively, GWWTP had more occurrence and diversity of ARB and ARGs than PWWTF. Taken together the results suggest accumulation of antibiotic resistance determinants in wastewater treatment facilities and subsequent release into the downstream environment, particularly water sources. This research is critical in the development of new water quality monitoring schemes and implementation of policies on the use of effluent and contaminated water particularly for irrigation purposes in Botswana.

LIST OF FIGURES

• Figure 2.1. Mechanism of horizontal gene transfer (HGF) in bacteria.....	13
• Figure 2.2. Mechanism of antibiotic resistance.....	13
• Figure 3.1. The wastewater treatment process in GWWTP and the downstream environment.....	18
• Figure 3.2. The wastewater treatment process in PWWTF and the downstream environment.....	19
• Figure 3.3. Google view of PWWTF and downstream showing the sampling sites.....	20
• Figure 3.4. Google view of GWWTP showing the sampling sites.....	21
• Figure 3.6.2. Ion 16S Metagenomics workflow.....	29
• Figure 4.1. Quantification of bacterial species in the GWWTP, PWWTF and downstream environment.....	32
• Figure 4.2. Number of bacterial isolates collected from the influent, effluent and downstream environment of GWWTP and PWWTF.....	33
• Figure 4.3.1. Percentage occurrence of antibiotic resistant bacteria and in GWWTP and PWWTF study areas.....	33
• Figure 4.3.2. Percentage occurrence of antibiotic resistant bacteria isolated from different sources (influent, effluent and downstream environment) in GWWTP and PWWTF study areas.....	34
• Figure 4.3.3. Bacterial isolates resistant against different antibiotics from Influent, effluent and downstream environment of PWWTF and GWWTP.....	36
• Figure 4.3.4. Antibiotic resistance in <i>Staphylococcus</i> and <i>Pseudomonas</i> species from PWWTF and GWWTP.....	43
• Figure 4.3.5. Antibiotic resistance in <i>Campylobacter</i> and <i>Salmonella</i> species from GWWTP and PWWTF.....	44
• Figure 4.3.6. Antibiotic resistance in <i>Listeria</i> and <i>Brucella</i> species from GWWTP and PWWTF.....	44
• Figure 4.3.7. Antibiotic resistance in <i>Escherichia coli</i> and <i>Enterobacter aerogenes</i> from PWWTF and GWWTP.....	45
• Figure 4.3.8. Multidrug resistant isolates in the treatment plants.....	46
• Figure 4.5.1. Summary of positive amplification of PCR target genes.....	48
• Figure 4.5.2. Antibiotic resistance genes in GWWTP and PWWTF.....	49

- **Figure 4.6.1.** Visualization of bacterial diversity at the level of phylum.....53
- **Figure 4.6.2.** Visualization of bacterial diversity at the level of class.....54
- **Figure 4.6.3.** Visualization of bacterial diversity at the level of order (a) and family (b).....55
- **Figure 4.6.4.** Visualization of bacterial diversity at genus level.....56
- **Figure A1.** Different uses of final effluent from the treatment plants.....73
- **Figure D2.** Positive amplification of PCR target genes in selected bacterial isolates.....80

LIST OF TABLES

• Table 2.1. Classes of antibiotics and their antimicrobial properties.....	10
• Table 3.3. Different selective media that were used in the study with their targeted bacterial species and the pathogenicity.....	22
• Table 3.4. Different classes of antibiotics and their minimum inhibitory concentration (MIC).....	23
• Table 3.5.1. Primers and their annealing temperature used in the study.....	26
• Table 4.5.1.48 multiple resistant bacterial isolates that were selected for detection of ARGs.....	47
• Table 4.5.2. ARGs present in GWWTP.....	51
• Table 4.5.3. ARGs present in PWWTF.....	52
• Table B1. Quantification of bacterial isolates in GWWTP and PWWTF.....	74
• Table C1. Total isolated bacteria from the treatment facilities.....	76
• Table C2. Total antibiotic resistant bacteria from the treatment facilities.....	76
• Table C3. Total antibiotic resistant bacteria according to antibiotics.....	76
• Table C4. Multidrug resistant bacteria.....	77
• Table D1. Presence of antibiotic resistance genes in the selected samples.....	78
• Table E1. Antibiotic resistance in bacterial species from PWWTF.....	82
• Table E2. Antibiotic resistance in bacterial species from PWWTF.....	84

CHAPTER 1 INTRODUCTIONS AND OBJECTIVES

1.1. Introduction

The world health sector faces a major challenge caused by continuing cases of uncontrollable diseases, increase in mortality and threats of antibiotic resistance which put certain human activities in the mainstream of human concern (Amisu *et al.*, 2003). The misuse of antibiotics has recently been characterized as one of the major causes of antibiotic resistance. Initially, antibiotics were developed for treatment of bacterial infections and improving human, animal and plant health but recently their uses have been extended to growth promotion in animal husbandry and fodder additives (Kummerer, 2009). This indiscriminate use has widely increased consumption of antibiotics in many parts of the world (Aminov, 2009).

Nearly all consumed antibiotics are usually excreted unchanged into the environment because the body partially metabolizes them. This together with disposal of unused antibiotics has led to the frequent detection of their residues in different environments such as wastewater treatment plants (WWTPs) (Jelic *et al.*, 2011; Rosal *et al.*, 2010).

WWTPs receives all sorts of discharges from hospitals, industries and domestic waste which make them an interface between different environments and, therefore, provide an opportunity for antibiotics in wastewater to select for resistant bacteria and promote the transfer of resistance genes in mobile genetic elements (e.g. plasmids, integrons) among the microbial communities (Szczepanowski *et al.*, 2007). Presence of antibiotic residues in the ecosystem can lead to chemical pollution, which impacts microbial community structure. It may also induce the development of antibiotic resistance genes (ARGs) which can be acquired by bacterial populations in various environments (Kummerer, 2009). This is demonstrated in a study by Gao *et al.*, (2012), that showed a positive correlations between some ARGs and their corresponding antibiotics in a WWTP. ARGs can be disseminated among bacterial species through horizontal gene transfer (Du *et al.*, 2015; Luo *et al.*, 2014).

Horizontal gene transfer (HGT) is the transfer of genetic material (DNA) between bacteria of the same species or different species through three mechanisms namely; transduction, transformation and conjugation (Todar, 2009). Conjugation occurs when there is a direct cell-cell contact between two related or unrelated bacteria, transferring plasmids. Transformation occurs through the uptake of DNA in the external environment from other

lysed or dead bacteria (Todar, 2009). Lastly, transduction is the transfer of DNA through bacteriophages between closely related bacteria (Todar, 2009).

ARGs have been considered as emerging contaminants because of their recent and frequent detection in the environment (Bouki *et al.*, 2013; Negreanu *et al.*, 2012).

Development and dissemination of ARGs is a serious problem in the treatment of infectious diseases (Goossens, 2005). Human impacted and naturally aquatic environments can serve as a reservoir of ARGs as stated by Baquero *et al.*, (2008) that ARGs have been found widely in aquatic environments such as WWTPs. The statement is further supported by many other studies indicating that WWTPs are recognised as reservoirs for ARGs that are associated with human pathogens (Rizzo *et al.*, 2013; Forsberg *et al.*, 2012; LaPara & Burch., 2011; Rahube & Yost, 2010; D'costa *et al.*, 2006).

Pathogens together with other bacteria carrying resistance genes from the wastewater might pose a threat to public health by migrating into groundwater or travelling off-site into surface water, soil and plants through the discharge of effluent wastewater and irrigation using wastewater. Different methods of waste treatment such as activated sludge and trickling filters have been developed for public health safety, which aim at reducing pathogens and organic components such as antibiotics prior to discharge. However, these discharges still pose a major concern to the environment.

In some parts of Africa, it has been reported that large volumes of treated or untreated wastewater adversely affect water bodies such as rivers, streams and ponds in both urban and peri-urban areas, specifically people using these water bodies as sources of water for irrigation (Zhang & Li 2011; Smillie *et al.*, 2010; Keraita & Drechsel, 2004). High levels of pollution, particularly microbiological contamination with ARB and the resistance genes have been detected in irrigation water (Zhang & Li 2011; Smillie *et al.*, 2010; Keraita & Drechsel, 2004).

The World Health Organization (WHO), the European Commission and the United States Centre for Disease Control and prevention (CDC) have highlighted the importance of studies on emergence of resistance genes together with the need for control strategies to reduce the development and spread of antibiotic resistance (Oteo *et al.*, 2005).

This study focused primarily on determining the occurrence, abundance, diversity and potential spread of ARGs and ARB in WWTP and the environment receiving effluent wastewater, and evaluates the antibiotic resistance gene dynamics by considering Botswana's socio-economic factors.

1.2. Statement of the problem

Antibiotic resistance is a major public health problem that requires immediate interventions. Currently, the understanding of concerns related to its impact are hampered by insufficient data, as surveillance of antibiotic resistance is limited to a few countries resulting in incomplete information on the true extent of this problem (WHO, 2011). Botswana is one of the African countries that lack antibiotic resistance surveillance information and hence more research is warranted.

1.3. Objectives

The main objective was to determine occurrence, abundance and diversity of ARGs and ARB in wastewater treatment facilities (WWTFs). To achieve this objective, the following specific aims were set;

1. To determine abundance by quantifying bacterial species that are present in GWWTP, PWWTF and their downstream environment using culture based method.
2. To determine the occurrence (presence/absence) of ARB in GWWTP, PWWTF and their downstream environment using culture based method.
3. To determine diversity by detecting the presence of various antibiotics resistance genes in GWWTP, PWWTF and their downstream environment using molecular based method.

1.4. Research question

Due to the geographical, climatic, social differences and the differences in the treatment infrastructures of wastewater among countries, we do not know of the similarities or variations in terms of diversity and occurrence of ARB and ARGs in wastewater and contaminated environments in Botswana. Several studies on antibiotic resistance have been conducted globally particularly in Asia, Europe and North America. In Botswana factors such as poor infrastructure in facilities like WWTPs, poor rainfall and lack of knowledge on the prudent use and disposal of antibiotics may have major implications in the rapid development and spread of antibiotic resistance. This could have a major impact to public health if resistance develops rapidly and spread to pathogenic bacteria.

The hypothesis of the study was that high numbers of ARB and ARGs are released from the WWTPs through the effluent into the downstream environment where they can proliferate and potentially spread resistance to clinical pathogens.

1.5. Significance of study

The issue of increasing ARGs in the community is a global emergency that requires a range of interventions and multidisciplinary teams' approach from all countries. Antibiotic resistance commonly occurs in microorganisms that are easily transmitted in the community such as those causing diseases like pneumonia, diarrhoea, tuberculosis, sexual transmitted diseases and malaria. This has increased mortality, the cost of fighting such diseases and also threats on the world's push to effectively treat people with HIV/AIDS especially in low income countries (Nugent *et al.*, 2012). To the best of our knowledge, no study on the characterization of ARB and ARGs has been conducted in wastewater treatment facilities (WWTFs) in Botswana and their downstream environment. This study will provide insights on the occurrence, abundance and diversity of ARB and ARGs from a non-clinical environment setup.

The public of Botswana needs to be aware of the social, economic burden, and human health implications of antibiotic resistance. This will help the government to implement measures such as improvement of WWTPs infrastructure, enforcement of regulations on use of effluent wastewater for agricultural purposes and public health policy-makers to develop the national antimicrobial resistance policy to prevent the emergence and spread to global community. The study will also sensitize the community on the misuse/abuse of antibiotics that contribute to rapid antibiotic resistance development, as well as the safe and sustainable use of wastewater effluent for irrigation purposes, ultimately protecting the environment and public health.

CHAPTER 2 LITERATURE REVIEW

2.1. Wastewater treatment plants

The most commonly and widely used environmental waste pollution control is the wastewater treatment. This is generally to avoid human and industrial wastes to be disposed to the environment as they can be dangerous to human health or cause damage to the natural environments (Sehar *et al.*, 2013).

Wastewater treatment plants collect waste from homes, businesses, agricultural environments, hospitals and many other industries and therefore contain different contaminants. These are defined by physical (colour, odour and solids materials), chemical (pesticides, nitrogen and gases) and biological (bacteria, fungi, protozoa and viruses) characteristics (Von Sperling, 2007). Wastewater passes through different stages of treatment prior to discharge into the environment such as streams and other receiving water bodies. The treatment stages involve preliminary, primary, secondary and tertiary treatment.

According to Arceivala *et al.*, (2006), preliminary treatment involves the use of screens to remove large and rough solid materials such as rags, plastics that may clog pipes or damage the downstream equipment of the plant. This is to enhance further treatment of the water.

Primary treatment involves several mechanical processes to remove suspended solid waste, oils, grease and fats. Some of the processes include sedimentation to remove inorganic solids and conventional treatment processes which involves the oxidisation of complex organic compounds by microorganisms through the continuous stirring of the sewage (Nkegbe & Koorapetse, 2005). The compounds are changed into simple volatile acid (Nkegbe & Koorapetse, 2005).

The secondary treatment involves the use of biological processes to remove dissolved organic matter. Microbes are utilized to consume the organic matter as food, converting it to carbon dioxide, water, and energy (Arceivala *et al.*, 2006). Some of the biological processes involved include; the trickling filters (bed of stones through which sewage passes) and the activated sludge process (stimulation of growth of oxygen-using bacteria to consume organic materials that are polluting the water) (Arceivala *et al.*, 2006). Effluent

from secondary treatment can further be treated using disinfectants such as chlorine to kill pathogenic bacteria and minimize odour (Malik, 2014).

Tertiary treatment involves mainly three stages of treatment, physical (filtration), chemical (disinfection) and irradiation (UV light) to eliminate pathogens (Von Sperling, 2007). Its purpose is to remove the remaining inorganic compounds and nutrients, such as the nitrogen and phosphorus. Furthermore, it also removes bacteria, viruses and parasites, which are harmful to public health.

Effluent (treated wastewater) is used for various purposes, such as irrigation, livestock drinking and aquaculture (Carr *et al.*, 2004). The treated wastewater may also reach groundwater, and further downstream environment through rivers.

Solid waste/sludge from WWTPs is used as fertilizer applied in agricultural soils (Mtshali *et al.*, 2014).

2.2. Antibiotics in wastewater treatment plants

Antibiotics are substances that have a cidal or static effect to slow or inhibit the growth of bacteria to improve human, animal and plant health. They are derived or chemically produced by microorganisms such as *Streptomyces*, *Penicilliums*, and *Bacilli* (Kohanski *et al.*, 2010). The main classes of antibiotics are penicillins, cephalosporins, macrolides, fluoroquinolones, tetracyclines, aminoglycosides and carbapenems (Frank & Tacconelli, 2012) (**Table 2.1**).

Some of the critically important classes of antibiotics are the fluoroquinolones, macrolides, 3rd and 4th generation cephalosporins and the carbapenems (WHO, 2012). Carbapenems are the broadest spectrum of activity and the most effective against gram-positive and gram-negative bacteria. They are often considered as antibiotics of last resort to patients with infections from drug resistant bacteria (Paterson & Bonomo, 2005).

Many countries do not have regulations on the use of antibiotics, making them cheap and easily available to the public, hence promoting overuse. Antibiotics used for non-therapeutic purposes in agriculture such as growth supplements and pesticides constitute as abuse and can lead to rapid development and emergence of resistance which can be passed on to pathogens (Spellberg *et al.*, 2014).

The overuse of antibiotics in food industries, medical and veterinary practices has also resulted in the increase of antibiotic residues being detected in different environments (Santos *et al.*, 2013). These antibiotics used in human and animals are frequently excreted in urine, faecal material and end up in WWTPs. This has led to various classes of antibiotics being widely detected in WWTPs (Zhang & Li, 2011). Many WWTPs are not efficient or not prioritized for removal of antibiotic resistance determinants; antibiotics, ARGs and ARB prior to release of effluent to downstream environment (Zhou *et al.*, 2013; Frigon *et al.*, 2013; Viau & Peccia, 2009).

Table 2.1. Classes of antibiotics and their antimicrobial properties

Class	Examples	Biological source	Spectrum effective against	Mode of action
Beta-lactams (penicillins and cephalosporins)	Penicillin Cephalosporin	<i>Penicillium notatum</i> <i>Cephalosporium</i> species	Gram-positive bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Semi synthetic beta-lactams	Ampicillin,	Synthetic penicillin	Gram-positive and Gram-negative bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Carbapenems	Meropenem	<i>Streptomyces cavuligerus</i>	Gram-positive and Gram-negative bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Aminoglycosides	Streptomycin	<i>Streptomyces griseus</i>	Gram-positive and Gram-negative bacteria	Inhibits translation (protein synthesis)
Macrolides	Erythromycin	<i>Streptomyces erythreus</i>	Gram-positive bacteria, Gram-negative bacteria not enteric, <i>Neisseria</i> , <i>Legionella</i> , <i>Mycoplasma</i>	Inhibit translation (protein synthesis)
Tetracyclines	Tetracycline	<i>Streptomyces</i> species	Gram-positive and Gram-negative bacteria, Rickettsias	Inhibit translation (protein synthesis)
Growth factor analogs	Sulfamethoxazole , Trimethoprim	Synthetic	Gram-positive and Gram-negative bacteria	Inhibits folic acid metabolism (anti-folate)

(Moore, 2013; Frank & Tacconelli., 2012)

2.3. ARB in wastewater treatment plants

Antibiotic resistance is the ability of bacteria to resist the effects of an antibiotic. It occurs when bacteria changes in a way that reduces the efficiency of antibiotics, the bacteria survive and continue to multiply causing more harm (WHO, 2016). According to Moore, (2017), different antibiotics have different genetic mechanisms by which they destruct, inhibit the multiplication and growth of bacteria. These mechanisms include inhibition of cell wall synthesis, disruption of cell-membrane function, inhibition of protein synthesis and inhibition of nucleic acid synthesis (**Table 2.1**).

However, bacteria have developed mechanisms that help them resist antibiotics. These can either be through chemical modification or degradation of the antibiotic so that it loses its activity and physical removal of the antibiotic from the cell through efflux pump or alteration of the bacteria's target site so that the antibiotic doesn't recognise it, hence loss of effectiveness (**Figure 2.2**).

Antibiotic resistance in bacteria develop through inheritance of trait that renders it naturally resistant or may be by means of gene mutation or acquired from another bacteria through horizontal gene transfer (Todar, 2010) (**Figure 2.1**).

The transmission route used by many bacterial pathogens of clinical importance, including resistant bacteria is usually from non-clinical environment. Pathogens affecting humans come from or transit through the environment to infect human host (Faucher *et al.*, 2015). Examples of the pathogens include ARB from environments such as WWTPs and downstream water sources (Middleton *et al.*, 2013; Korzeniewska *et al.*, 2013).

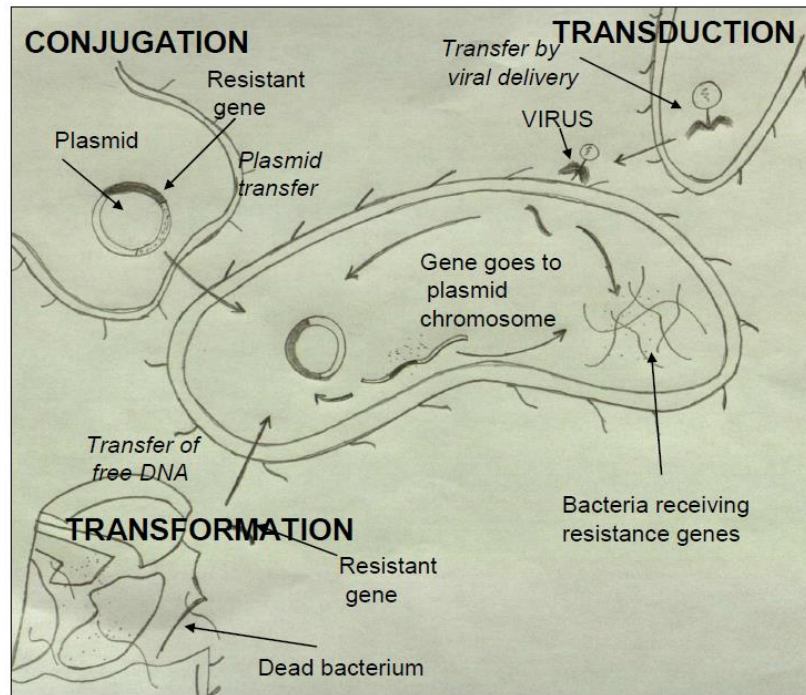
Although disinfection processes are generally effective in removal of pathogens, several studies have used different methods such as culture based method to clearly demonstrate that they do not always remove ARB (Negreanu *et al.*, 2012; Akiyama *et al.*, 2010). For example, tetracycline and sulphonamide resistant bacteria were found in final effluent of five WWTPs in Michigan, (Munir *et al.*, 2011).

Wastewater contains many pathogenic bacterial species including Enterobacteriaceae (*Klebsiella pneumonia*, *Escherichia coli* and *Pseudomonas aeruginosa*), Mycobacteriaceae (*Mycobacterium tuberculosis*) (Ye & Zhang., 2013) and Vibrionaceae (*Vibrio fluvialis*) (Ramamurthy *et al.*, 2014). These organisms are fast growing bacteria that have the

potential to adapt and also important vectors of antibiotic resistance genes (McGowan, 2006).

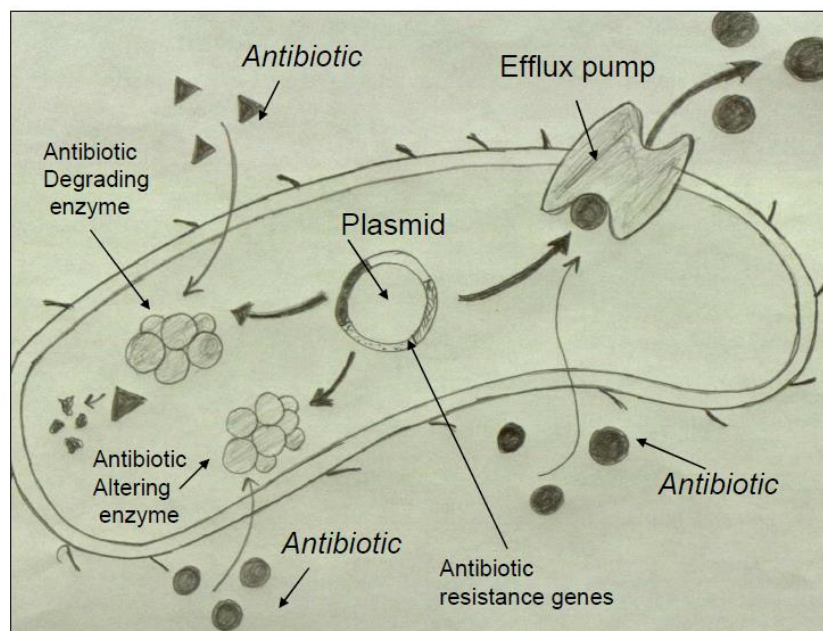
Many bacterial species resistant to different antibiotics have been detected in wastewater influent, effluent and downstream environment. In one of the studies, a number of Enterobacteriaceae were found in the Arga River, which receives effluent from WWTP in Spain, the species were resistant against 95% of the antibiotics tested (Goni-Urriza *et al.*, 2000). Another Enterobacter, vancomycin resistant-*Enterococcus* was found in municipal wastewater in Germany (Schwartz *et al.*, 2003). The same bacteria resistant to tetracycline were also found in municipal wastewater in Germany in a study by (D'costa *et al.*, 2006; Ferreira da Silva *et al.*, 2006).

Bacteria can also be resistant to more than one unrelated antibiotic, known as multidrug resistant. Some of the most important types of multiple drug that are becoming increasingly widespread in the community include Extended-spectrum beta-lactamases (ESBLs)- *Escherichia coli*, *Neisseria gonorrhoea*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (CDC, 2013). Methicillin-resistant *Staphylococcus aureus* (MRSA) is among the first and most important multiple drug resistant bacteria on the rise in many countries including sub-Saharan Africa (CDDEP, 2015). MRSA are strains of *Staphylococcus aureus* that are resistant to methicillin and related beta-lactam antibiotics like penicillin and cephalosporin. Carbapenem resistant Enterobacteriaceae (CRE) has also emerged as a major challenge in hospitals, CRE are a group of bacteria that have developed resistance against all available antibiotics, including carbapenems (Perez *et al.*, 2013).



Reproduced from: Nagulapally, (2007)

Figure 2.1. Mechanisms of horizontal gene transfer (HGT) in bacteria. The three types of mechanisms involved in HGT: Transformation (uptake of cell free DNA), Conjugation (bacteria mating), and Transduction (gene transfer by viruses).



Reproduced from: Nagulapally (2007)

Figure 2.2. Mechanisms of antibiotic resistance. The four mechanisms involved in antibiotic resistance are: drug inactivation, drug modification, and drug efflux and target alteration.

2.4. ARGs in wastewater treatment plants

Wastewater treatment plants are considered reservoirs of antibiotic resistance as they serve as a collection point for all types of chemical and biological components from a wide range of sources. Hence, there are conducive environment for bacterial genetic mutations resulting in the evolution and proliferation of ARGs. This is because of different mixtures of bacteria, abundant nutrients and presence of antibiotic residues. ARGs together with the mobile genetic elements such as plasmids, transposons and integrons are transferred from one bacterium to another, multiplying the number of resistant bacteria in wastewater. Studies use culture independent method (molecular) to detect antibiotic resistant genes in different environments. The most occurring ARGs in wastewater as indicated by several studies are the resistance genes against tetracycline and sulfonamides. For example, tetracycline resistance genes were found to be universal in all the 15 sampled sewage treatment plants in a study by Zhang and Li., (2011) in Hong Kong, China. In another study, *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetM*, *tetO*, *tetS*, and *tetQ* were found in wastewater from two WWTPs in Wisconsin, United States of America (Auerbach *et al.*, 2007).

The resistance gene; *sul1* was found in the upstream and downstream of one of the WWTP in United States of America, Pruden *et al.*, (2013). The gene was also detected in WWTPs by Berglund *et al.*, (2015) and Gao *et al.*, (2012). One of the reasons for the frequent occurrence of these genes may be because their phenotypes are the most used antibiotics in hospitals especially for treatment of methicillin-resistant *Staphylococcus aureus* infection which is one of the important pathogens in many countries. Many other studies also found the resistance genes against tetracycline and sulphonamide in wastewater in different environments, Gao *et al.*, (2012) found high levels of tetracycline (*tetO* and *tetW*) and sulphonamide (*sul1*) resistance genes in the final effluent of a WWTP in Michigan, United States of America.

Other important resistance genes against antibiotics such as macrolides, trimethoprim and penicillin which are clinically used in hospitals and veterinary clinics have also been found in WWTPs (Rahube *et al.*, 2014; Araujo *et al.*, 2010; Volkmann *et al.*, 2004). One of the previous reports found ARGs; *ampC* and *vanA* that confer resistance to ampicillin and vancomycin respectively, in five different WWTPs. The study further found the methicillin-resistance gene *mecA*, in samples taken from clinical wastewater (Volkmann *et al.*, 2004). Macrolides resistance genes *mph*, *mrx* and *mphR* were found in plasmids

(pTOR_01, pTOR_02, pEFC36a & pRWC72a) detected from wastewater in Canada (Rahube *et al.*, 2014). Another macrolides resistance genes isolated from wastewater were *ermA* and *ermB* in Portugal (Araújo *et al.*, 2010), *ermC*, *ermF*, *ermT* and *ermX* in agricultural wastewater from a study by Chen *et al.*, (2007). Trimethoprim resistance genes; *dhfr* and *dhfrA14* were found in WWTPs in Poland from a study by Ziemińska-Buczyńska *et al.*, (2015). Other genes; *dhfrA12* and *dhfrA1* were also found in WWTPs connected to the slaughterhouse (Moura *et al.*, 2007). Plasmid-mediated quinolone resistance (PMQR) genes such as *oqxA*, *oqxB*, *aac(6)-Ib* and *qnrS* were found in wastewater from feedlots and chemical plants in a study by Xiong *et al.*, (2014). *dfrA1* and *dfrA12*, which are trimethoprim resistance genes were found in WWTPs connected to a slaughterhouse (Moura *et al.*, 2007).

2.5. Water quality indicators in treated wastewater

Certain Bacteria are used in monitoring the quality of water from different sources. The bacteria belonging mainly to the Enterobacteriaceae family are frequently used as water quality indicators. Their detection and quantity usually indicate faecal contamination and may correlate to the presence of bacterial pathogen. They include the faecal Coliforms, *Campylobacter jejuni*, *Salmonella* species, *Pseudomonas aeruginosa* and *Escherichia coli* (Krauss *et al.*, 2011). Other bacterial indicators are gram positive *Streptococcus* and *Enterococcus* (Krauss *et al.*, 2011). There are many concerns regarding the transmission of water-borne pathogens particularly in environments where the effluent wastewater is discharged (e.g. downstream rivers) or used (e.g. for irrigation in agricultural fields). Several studies have reported high count of total coliforms and faecal coliforms in the fields where treated wastewater was used for irrigation (Al-Jaboobi *et al.*, 2013; Negreanu *et al.*, 2012; Malkawi & Mohammad, 2003).

Bacterial abundance was found to have a higher magnitude in treated wastewater than in fresh water in a study by Negreanu *et al.*, (2012) in Israel. Malkawi & Mohammad, (2003) found a high count (1.2×10^2 CFU/mL to 4.2×10^2 CFU/mL) of the faecal coliforms in irrigation water from WWTPs in Yemen, while an increase of 1.3×10^3 CFU/mL was observed in the concentration of faecal coliform in soil irrigated with wastewater compared to those irrigated with fresh water in United States of America (Ryua *et al.*, 2011). High count of faecal coliforms in water may indicate high contamination with pathogenic bacteria that can ultimately be transferred to humans through food, air and soil, thereby causing outbreaks of diseases such as typhoid fever, gastroenteritis and dysentery (Domingo *et al.*, 2012).

Considering all these studies, it becomes very critical also to monitor antibiotic resistance in bacterial populations.

CHAPTER 3 METHODOLOGY

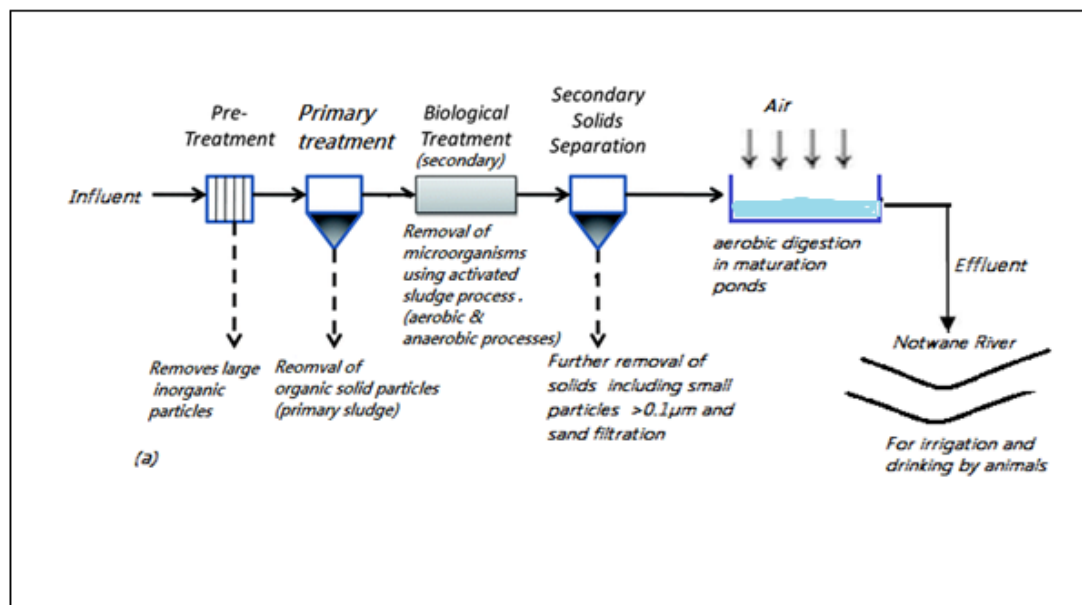
3.1. Description of study area

The study areas were 1) Gaborone; Gaborone wastewater treatment plant (GWWTP), downstream Notwane River receiving final effluent from GWWTP, 2) Palapye; Palapye wastewater treatment facility (PWWTF) and downstream man-made pond receiving the final effluent from PWWTF. Unless specified as influent, effluent or downstream, GWWTP and PWWTF acronyms will be used generally to differentiate the study areas in Gaborone and Palapye respectively. The two chosen areas are different in terms of population and infrastructure. Gaborone being the capital city, with a population of about 208,411 and Palapye being rural-urban town with a population of 30,883. The wastewater treatment facilities employ different treatment schemes or processes and different uses of the final effluent.

The GWWTP is situated along Notwane River, about 10km North East of Gaborone, Botswana. The treatment plant has a capacity to receive 40,000m³ waste per day, it receives water from households; sinks, washing machines, bathtubs and toilets combined with industrial wastewater from industries such as breweries, hospitals and agriculture in Gaborone and surrounding areas. It is the biggest in the country consisting of the primary treatment stage where large solid inorganic materials are filtered. The treatment plant also consists of secondary treatment which aims to degrade the biodegradable content of the sewage using activated sludge processes (**Figure 3.1**). The solid material (sludge) is then separated from liquid (effluent). GWWTP do not have a tertiary treatment; instead the effluent water is discharged into a series of maturation ponds for further biological degradation (Phuntsho *et al.*, 2009). The final effluent from maturation ponds is discharged into the Notwane River, which is the main source of water used by farmers to irrigate their crops (Nkegbe *et al.*, 2005). The water used for irrigation from this river is mostly effluent wastewater since there is normally no inflow from upstream especially during dry seasons.

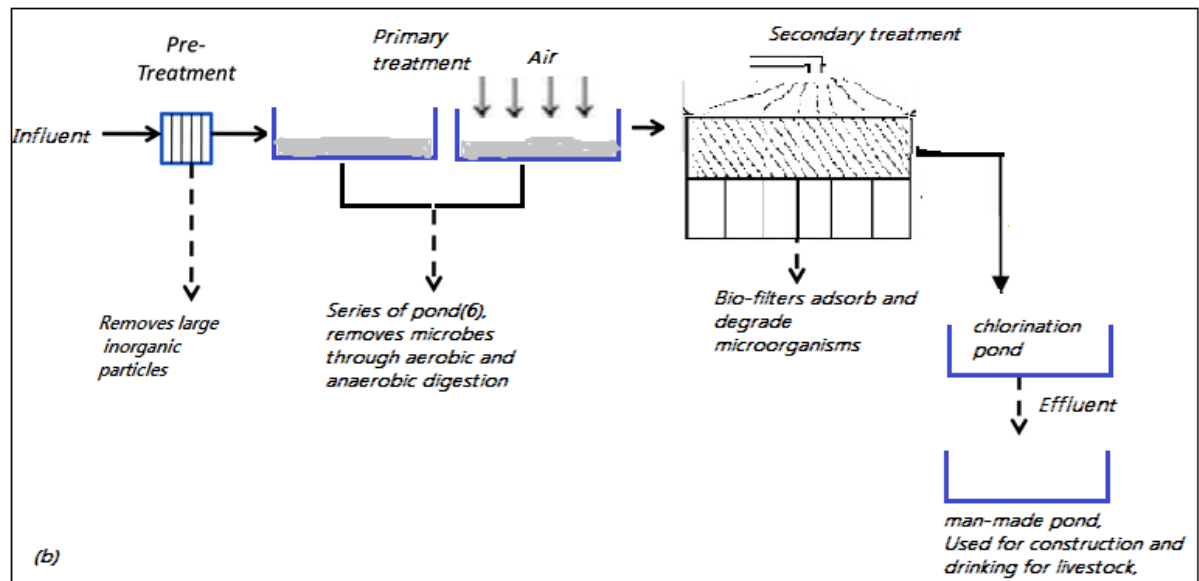
Palapye wastewater treatment facility (PWWTF) is situated just outside Palapye town in close proximity to land used generally for agriculture. Palapye is one of the fastest growing peri-urban areas with increasing population resulting from introduction of the new university (BIUST), expansion in coal mining (Morupule B power station), shopping complexes and industries (steel and glass production). PWWTF has a smaller receiving

capacity of about 14000 m³ per day. PWWTF treats its wastewater using a system known as Pond Enhanced Treatment and Operation (PETRO) (**Figure 3.2**). This is described by the use of anaerobic digestion by microorganisms in a series of ponds existing as primary treatment stage before the secondary treatment where aerobic degradation is used through the use of bio-filters (Shipin *et al.*, 1998). Bio filters are a pollution control technique in which medium grains like sand covered with biofilms are used to biologically remove nutrients, organic carbon and other pollutants in wastewater (Rocher *et al.*, 2012). PWWTF does not have a tertiary treatment stage, the final effluent is disinfected by the use of chlorine before being discharged to the downstream environment which is a man-made pond. The water is used by the public for different uses such as construction, watering gardens, and livestock also drink from it.



(Modified from Westerhoff *et al.*, 2011)

Figure 3.1. The wastewater treatment process in GWWTP and the downstream environment.



(Modified from Westerhoff *et al.*, 2011)

Figure 3.2. The wastewater treatment process in PWWTF and the downstream environment.

3.2. Sample collection

The sampling sources from Palapye were from S1 (22°32.399'S; 27°10.391'E) influent wastewater, S2 (22°32.323'S; 27°10.437'E), effluent and S3 (22°32.303'S; 27°10.541'E), downstream of PWWTF, a man-made pond. Distance between S2 and S3 is 0.18km (**Figure 3.3**). In Gaborone samples were from S1 (24°36.444'S; 25°57.811' E) influent wastewater (before treatment), S2 (24°36.575'S; 25°57.949' E) effluent (after treatment), and Dws1 (24°35.348'S; 25°58.959'E) and Dws2 (24°33.808'S; 25°58.533'E) downstream of GWWTP (along the Notwane river). The distance between S2 and Dws1 is 3.15km and the distance between Dws1 and Dws2 is 5.16km (**Figure 3.4**).

Samples were collected all year round to cover all the four seasons; spring (September), summer (January), autumn (March) and winter (June) and were collected once per month. Three samples per site were taken using grab sampling method, in the mornings between 8am and 10am. Samples were collected as previously described by Yuan *et al.* (2015). Briefly 750ml water samples collected in 250ml portions from three different points of the sites in sterile polystyrene bottles. Samples were placed in a cooler with ice-packs and transported to the laboratory and analysed within 12hrs.



Figure 3.3. Google view of PWWTF and downstream showing the sampling sites. Distance between S2 and S3 is 0.18km.

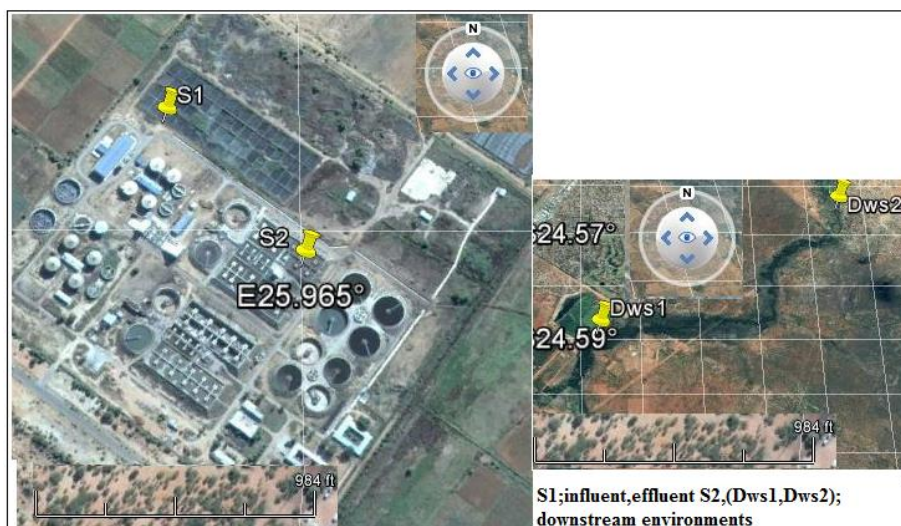


Figure 3.4. Google view of GWWTP and downstream showing the sampling sites.

(a) S1: Influent and S2: Effluent. (b) Downstream environment comprise of 2 sampling locations (Dws1, Dws2) and the distance between S2 and Dws1 is 3.15km and the distance between Dws1 and Dws2 is 5.16km.

3.3. Bacterial isolation and quantification

Fresh samples were analysed exactly as described by Yuan & colleagues (2015). Briefly 0.1ml aliquot of water samples from each of the ten-fold serial dilutions were spread plated on different selective agar media targeting different bacterial species that are of public health concern (**Table 3.3**). The plates were incubated at 37°C for 24hrs. Only plates with 30 to 300 colonies were considered quantifiable and were counted using a colony counter (BioCote, Stuart, UK).

Isolates growing in respective selective media were randomly picked and confirmed by gram stain procedure.

An average of eight bacterial colonies in different media were then randomly picked and further sub-cultured to obtain a pure culture. The pure culture isolates were grown in nutrient broth and subsequently stored in nutrient broth containing 50% glycerol (ratio, 1:1) at -80°C for further antibiotic resistance characterization.

3.3.1. Statistical analysis

An average counts from triplicate plates were used to obtain the mean and standard deviation. The mean and standard deviations were imported into Graph Pad Prism software (7th edition) and the difference between wastewater treatments plants and sample sources were determined by t-Test with P values less than 0.05 considered being significant.

Table 3.3. Different selective media that were used in the study with their targeted bacterial species and the pathogenicity.

Media	Targeted bacterial species	Pathogenicity
Harlequin pseudomonas agar base(LabM laboratories)	<i>Pseudomonas</i> species <i>Pseudomonas aeruginosa</i>	Eye, ear and skin infections
Harlequin salmonella™ ABC agar base(LabM laboratories)	<i>Salmonella</i> species <i>Salmonella typhimurium</i>	Enteric fever, typhoid fever, food poisoning and gastroenteritis
Lab 112 campylobacter selective media (LabM laboratories)	<i>Campylobacter</i> species <i>Campylobacter jejuni</i> <i>Campylobacter coli</i>	Inflammatory and Bloody diarrhea or dysentery.
Mannitol salt agar (Biolab laboratories)	<i>Staphylococcus</i> species <i>Staphylococcus aureus</i> <i>Staphylococcus epidermidis</i>	Skin infections, pneumonia and Bacteremia
Brucella agar (Conda laboratories)	<i>Brucella</i> species <i>Brucella abortus</i> , <i>Brucella melitensis</i>	Brucellosis-a zoonotic disease that may cause anemia, leucopenia and contagious abortion.
Chromo cult agar (Merk laboratories)	<i>Enterobacter aerogenes</i> <i>Escherichia coli</i>	Diarrhea, skin and soft tissue infections
Listeria selective media (sigma-Aldrich)	<i>Listeria</i> species <i>Listeria monocytogenes</i>	Listeriosis-A food borne disease that affect newborn babies and may cause sepsis and meningitis.

3.4. Antibiotic resistance characterization

Resistance analysis was done as previously described by Yuan *et al.*, (2015). Frozen isolates were thawed at room temperature and each bacterial isolate was aseptically sub-cultured into a number of nutrient agar plates containing different classes of clinically relevant antibiotics using a sterile tooth pick. Isolates were also sub-cultured on a nutrient agar without antibiotic as a control.

The selected antibiotics concentrations used were defined as the Minimum Inhibition Concentration (MIC) of bacteria listed in Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS, 2015) (**Table 3.4**). These are universally accepted concentrations used in hospitals for treatment of bacterial infections. Cyclohexamide (75µg/ml) was also supplemented to the plates for fungal growth control.

Table 3.4. Different classes of antibiotics and their minimum inhibitory concentration (MIC).

Class	Antibiotic	Concentration (µg/ml)
Penicillin	Penicillin(PEN)	16
	Ampicillin(AMP)	32
Quinolones	Ciprofloxacin(CIP)	4
Tetracycline	Tetracycline(TET)	16
Aminoglycosides	Streptomycin(STR)	64
Macrolides	Erythromycin(ERY)	8
Cephalosporin	Cephalosporin(CSP)	32
Carbapenem	Meropenem(MER)	4
Sulfonamides	Sulfamethoxazole(SMX)	512
	Trimethoprim(TMP)	64

The plates were then incubated for 24hrs at 37°C and resistance of isolates were recorded on the basis of growth in the presence of antibiotic. Isolates resistant to more than two antibiotics were considered as multidrug resistant bacteria.

3.4.1. Statistical analysis

Percentage values and graphs of isolated bacteria including resistant bacteria were calculated and designed using Microsoft Excel 2010 edition. The difference between

treatments plants and sample sources were determined by t-Test with P values less than 0.05 considered being significant.

3.5. Molecular characterization of antibiotic resistance

Different multiple resistant bacterial species from the different sources in PWWTF and GWWTP were selected and analysed for identification of ARGs using PCR method.

3.5.1. DNA extraction

DNA from bacterial isolates (volume of 1ml) was extracted as previously described by Mirmohammadsadeghi *et al.*, (2013), with few modifications. Briefly, the cell pellet were suspended in 600µl of cell lysate solution (500 mM Tris-HCl, 200 mM EDTA, 460 mM NaOH; pH 8 and 15% SDS pH 6.6) and incubated at 80°C for 5 minutes. Then 3µl of RNase was added and incubated at 37°C for 30 minutes. 200µl of potassium acetate was added followed by 600µl of Isopropanol and the mixture centrifuged to pellet DNA. The DNA was then concentrated using ethanol, hydrated in 100µl TE buffer and incubated overnight a room temperature.

The yield of extracted DNA was measured using a nano drop spectrophotometer (Lasec, Jenway Genova nano) at an absorbance of 260nm. The DNA quality was checked by gel electrophoresis (0.8% agarose gel (3µl DNA plus 2µl loading dye)) and then stored at -20°C until PCR amplification.

3.5.2. ARGs analysis

Antibiotic resistance genes were identified using qualitative PCR with primers specific for each gene (Table 3.5.1).

All PCR assays were performed in a 25µl volume reaction consisting of 12.5µl Emerald Amp[®] GT PCR Master Mix (TAKARA BIO INC), 1.5 µl each primer, 7.5µl nuclease free water and 2µl DNA template. Amplification was carried out in a PCR machine (ProFlex PCR system) using a temperature program consisting of initial denaturation of 95°C for 5 minutes, followed by 35 cycles of 98°C for 10 seconds, 1 minute at the respective annealing temperature of various primers, 72°C for 1 minute with a final extension at 72°C for 1 minute. The annealing temperatures were calculated using a T_m calculator (Thermo Fisher Scientific Inc) (**Table 3.5.1**). PCR products were analysed by gel electrophoresis using 1% (w/v) agarose stained in 4 µl /g ethidium bromide for 90 minutes in 1× TAE

buffer and viewed under UV light (Gel doc-IT® imager UVP, Cambridge, UK). The sizes of the PCR products were confirmed against Quick-Load 1 Kb DNA ladder (BiLabs inc, England).

The PCR products were also stored for further confirmation by sub-cloning and DNA sequencing.

Table 3.5.1. Primers and their annealing temperature used in the study

Gene	Resistance phenotype	DNA sequence 5'-3'	Annealing temperature	Product (bp)	Reference
<i>mphA</i>	Macrolides	F-GCCGATACCTCCCAACTGTA R-AGGCGATTCTTGAGCATTGC	52.8°C	403	Wang <i>et al.</i> , 2017
<i>sul1</i>	Sulfamethoxazole	F-GTGACGGTGTTCGGCATTCT R-TCCGAGAAGG TGATTGCGCT	54.7°C	921	Lanz <i>et al.</i> , 2003
<i>int1</i>	Class1 Integrase	F-GCATCCTCGGTTTTCTGG R-GGTGTGGCGGGCTTCGTG	50.4°C	457	Yousefi <i>et al.</i> , 2010
<i>dfr</i>	Trimethoprim	F-CCCAACCGAAAGTATG CGGTCTG R-GTATCTACTTGAT CGAT CAGG	45.6°C	171	Sunde, 2005
<i>strA</i>	Streptomycin	F-CCTGGTGATAACGGCAATTC R-CCAATCGCAGATAGAAGGC	50.0°C	548	Gebreyes <i>et al.</i> , 2002 Madsen <i>et al.</i> , 2000
<i>strB</i>	Streptomycin	F-ATCGTCAAGGGATTGAAACC R-GGATCGTAGAACATATTGGC	46.9°C	500	Gebreyes <i>et al.</i> , 2002 Madsen <i>et al.</i> , 2000
<i>tetA</i>	Tetracycline	F-CATATAATCATCACCAATGGCA R-GGCGGTCTTCTTCATCATGC	46.2°C	500	Boerlin <i>et al.</i> , 2005
<i>tetB</i>	Tetracycline	F-CATTAATAGGCGCATCGCTG R-TGAAGGTCATCGATAGCAGG	50.5°C	930	Memon <i>et al.</i> , 2016

<i>ermA</i>	Erythromycin	F-GTTCAAGAACAATCAATACAGAG R-GGATCAGGAAAAGGACATTTTAC	45.2°C	421	Lina <i>et al.</i> , 1999
<i>ermB</i>	Erythromycin	F-AAAACTTACCCGCCATACCA R-TTTGGCGTGTTTCATTGCTT	49.7°C	190	Knapp <i>et al.</i> , 2010 Zhai <i>et al.</i> , 2016
<i>ermC</i>	Erythromycin	F-GAAATCGGCTCAGGAAAAGG R-TAGCAAACCCGTATTCCACG	50.7°C	291	Graham <i>et al.</i> , 2011 Zhai <i>et al.</i> , 2016
<i>qnrA</i>	Quinolones	F-ATTTCTCACGCCAGGATTTG R-GATCGGCAAAGGTTAGGTCA	49.4°C	516	Wang <i>et al.</i> , 2008
<i>catB3</i>	Chloramphenicol	F-CTCTGAGGCAATGGTCATGC R-GCCGCTTTGATCTTCTCCAG	52.9°C		This study
<i>catA1</i>	Chloramphenicol	F-GGTGAGCTGGTGATATGGGA R-AGGGATTGGCTGAGACGAAA	52.8°C		This study

3.6. Metagenomics analysis

Microbial diversity, identification and quantification of bacterial population in the downstream environment of PWWTF was analysed by 16S rRNA sequencing on the Ion PGM™ System with Ion Reporter™ Software at the University of Pretoria.

3.6.1. Sample preparation

Sample-S3, (PWWTF downstream samples) were pooled together to a total volume of 50ml and centrifuged (SL 16R centrifuge, Thermo scientific, Germany), to release a pellet which was used for DNA extraction. Power soil DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA) was used following the manufacturer instructions and the extracted DNA was stored at -20°C until transportation to the University of Pretoria for metagenomic sequencing.

3.6.2. DNA sequencing and analysis

The procedure is illustrated by the **Figure 3.6.2**. In summary, the two primer pools (V2-4-8, V3-6-7-9) were simultaneously used to amplify 7 of the hyper variable regions of the 16S rDNA gene in bacteria. After generating the amplicons, the Ion Plus™ Fragment Library Kit was used to ligate barcoded adapters and synthesize libraries. Barcoded libraries from the sample were pooled and templated on the OneTouch2™ system followed by 400bp sequencing on the Ion PGM. Automated analysis, annotation and taxonomic assignment occurred via the Ion Reporter Software pipeline. Classification of reads was through alignment to either the curated MicroSEQ ID or curated Green genes databases (Salipante *et al.*, 2013).

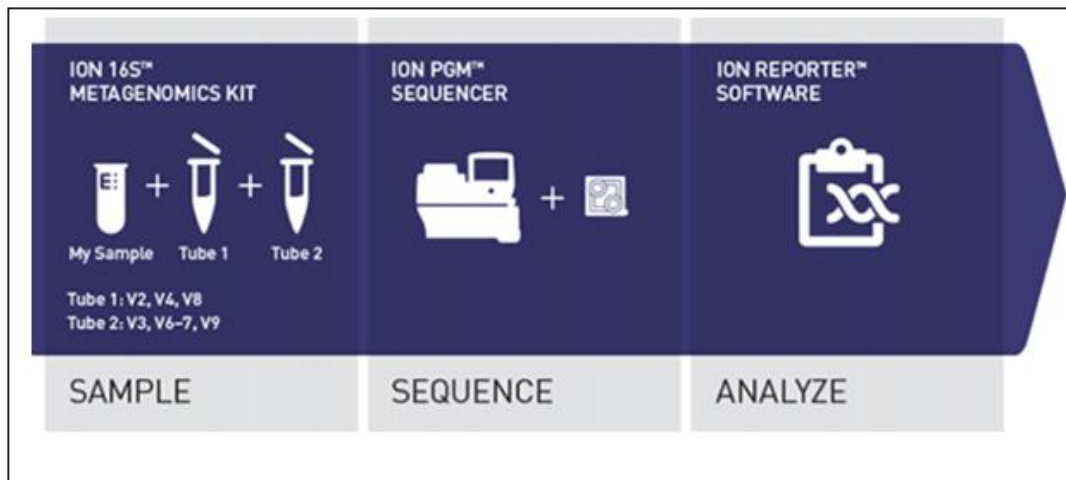


Figure 3.6.2. Ion 16S Metagenomics workflow (Salipante *et al.*, 2013).

CHAPTER 4 RESULTS

4.1. Bacterial quantification

The abundance of bacterial species was determined by quantifying different bacterial species in the influent, effluent and downstream water samples. Potentially pathogenic bacteria were found present at various quantities in almost all the sample sources of both treatment facilities (Figure 4.1).

Salmonella species from GWWTP were significantly higher in influent (2.82×10^5 CFU/mL) than effluent (9.53×10^2 CFU/mL), the *Salmonella* species numbers slightly decreased further in the downstream environment; environment 1 (1.56×10^2 CFU/mL) and environment 2 (1.57×10^2 CFU/mL). The downstream environment did not have any significant difference between them. *Listeria* species were also significantly higher in influent (1.40×10^8 CFU/mL), than effluent (5.6×10^4 CFU/mL). The *Listeria* species numbers significantly increased further in the downstream environment, environment 1 (4.27×10^6 CFU/mL) and environment 2 (3.70×10^5 CFU/mL). The other species, *Campylobacter* were also found significantly higher in GWWTP influent (2.16×10^6 CFU/mL) than effluent (9.30×10^3 CFU/mL), the bacterial numbers slightly decreased in the downstream environment, environment 1 (1.85×10^3 CFU/mL) and environment 2 (3.7×10^2 CFU/mL). For *Brucella* species, the numbers were also significantly higher in influent (1.95×10^6 CFU/mL) compared to effluent (2.04×10^4 CFU/mL), the numbers significantly increased further in the downstream environment, environment 1 (2.50×10^8 CFU/mL) and a slight decrease in environment 2 (4.69×10^7 CFU/mL) occurred.

Staphylococcus species were also quantified from GWWTP, and the numbers were relatively lower in the influent (1.14×10^4 CFU/mL), followed by a slight increase in the effluent (2.78×10^4 CFU/mL). The numbers for *Staphylococcus* species significantly increased further in downstream 1 (2.78×10^6 CFU/mL) followed by a decrease of 2 orders of magnitude in downstream environment 2 (3.7×10^4 CFU/mL). The numbers for *Escherichia coli* were significantly higher in GWWTP influent (8.80×10^6 CFU/mL) than effluent (1.03×10^1 CFU/mL) and downstream environment 1 (1.00×10^1 CFU/mL). There was no significant difference between effluent and downstream environment 1. *Escherichia coli* were below quantifiable limits in downstream environment 2. *Enterobacter aerogenes* were also significantly higher in GWWTP influent (8.30×10^6 CFU/mL) than effluent (4.1×10^4 CFU/mL).

Effluent from GWWTP had a significantly higher *Enterobacter aerogenes* numbers compared to the downstream environment, environment 1 (8.23×10^2 CFU/mL) and environment 2 (4.60×10^2 CFU/mL). The quantity of the *Enterobacter aerogenes* from environment 1 was relatively the same as in environment 2. Lastly in GWWTP, *Pseudomonas* species were also detected at significantly higher quantities in influent (3.93×10^6 CFU/mL) than effluent (1.01×10^3 CFU/mL). The numbers increased by one and two orders of magnitude in the downstream environment 1 (6.62×10^3 CFU/mL) and environment 2 (2.22×10^4 CFU/mL) respectively. There were no significant differences of species quantity between the downstream environment 1 and environment 2.

All the targeted bacterial species were also detected and quantified in PWWTF (**Figure 4.1**), *Salmonella* species were slightly higher in influent (7.06×10^3 CFU/mL) compared to the effluent (2.67×10^3 CFU/mL), there was an increase of at least one order of magnitude in the downstream environment (2.08×10^4 CFU/mL). The *Listeria* species were also significantly higher in PWWTF influent (1.75×10^5 CFU/mL), than effluent (4.2×10^3 CFU/mL). The species slightly increased in the downstream environment (4.10×10^4 CFU/mL). The other species, *Campylobacter* were also found to be higher in PWWTF influent (7.16×10^4 CFU/mL) than effluent (6.8×10^3 CFU/mL). The *Campylobacter* species significantly increased in the downstream environment (3.73×10^5 CFU/mL). *Pseudomonas* species were found to be significantly higher in PWWTF influent (2.82×10^{11} CFU/mL) than effluent (2.74×10^3 CFU/mL). Interestingly, there was also a significant increase of at least eight orders of magnitude in downstream environment (2.69×10^{11} CFU/mL).

The *Brucella* species were lower in PWWTF influent (1.17×10^5 CFU/mL), with a slight increase in the effluent (2.09×10^6 CFU/mL) and the downstream environment (4.23×10^6 CFU/mL). There was no significant difference in quantity between *Brucella* species from effluent and downstream environment. The numbers for *Staphylococcus* species were relatively equal between influent (5.53×10^2 CFU/mL), effluent (2.13×10^2 CFU/mL) and the downstreams environment (5.16×10^2 CFU/mL). *Escherichia coli* were higher in the influent (7.53×10^5 CFU/mL) but below limit of quantification in the effluent. However, the numbers recorded increased in the downstream environment (1.03×10^4 CFU/mL). *Enterobacter aerogenes* were higher in PWWTF influent (2.83×10^4 CFU/mL) than in effluent (3.53×10^3 CFU/mL). There was no significant difference between *Enterobacter aerogenes*

from effluent and the downstream environment (2.27×10^4 CFU/mL). Numbers of *Enterobacter aerogenes* from influent were also relatively equal to those from downstream environment as there was no significant difference.

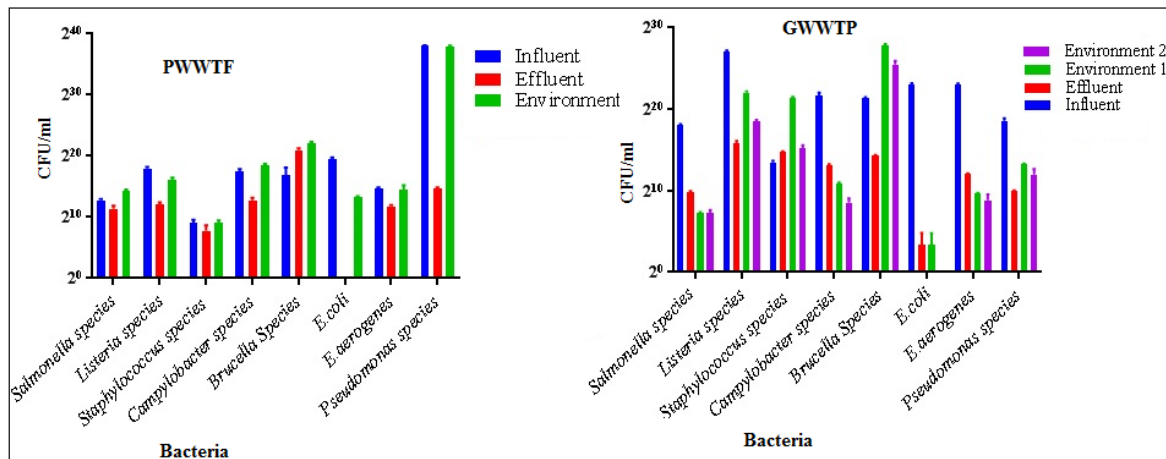


Figure 4.1. Quantification of bacterial species in GWWTP, PWWTF and downstream environment. (Environment 1 and 2 are sites Dsw1 and Dsw2 respectively which are 5.16km apart)

4.2. Bacterial isolation

A total of 972 bacterial isolates from influent, effluent and downstream environment of GWWTP and PWWTF were collected. From the total isolates, 565 (58%) were from GWWTP influent, effluent and downstream while 407 (42%) were from PWWTF influent, effluent and downstream. In the Gaborone study area, 195 (35%) of the isolates were from influent, 216 (38%) were from effluent, and 154 (27%) from downstream environment. In Palapye, 165 (41%) of isolates were from the influent, 102 (25%) from effluent, and 140 (34%) from downstream environment (**Figure 4.2**).

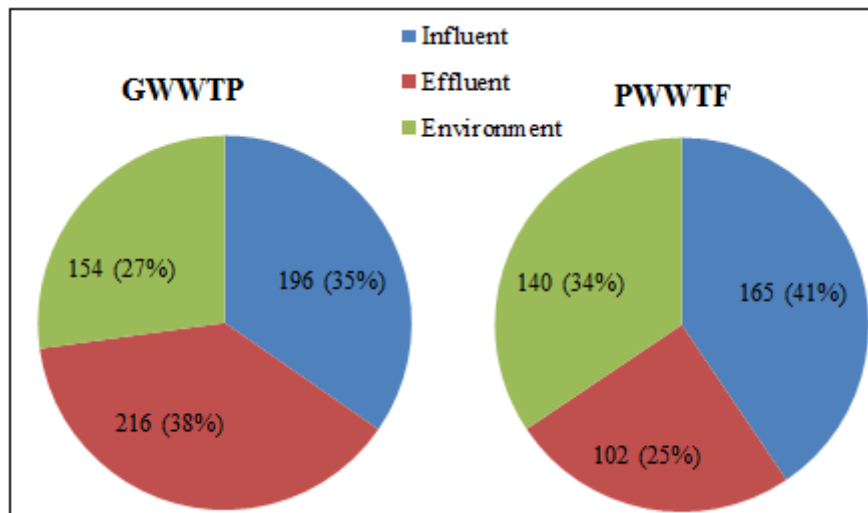


Figure 4.2. Number of bacterial isolates collected from the influent, effluent and downstream environment of GWWTP and PWWTF.

4.3. Antibiotic resistant bacteria

All the 972 isolates were then tested for antibiotic resistance against 10 antibiotics. 525(93%) of GWWTP were resistant to one or more antibiotics while 322 (79%) of PWWTF were also resistant to one or more antibiotics (**Figure 4.3.1**).

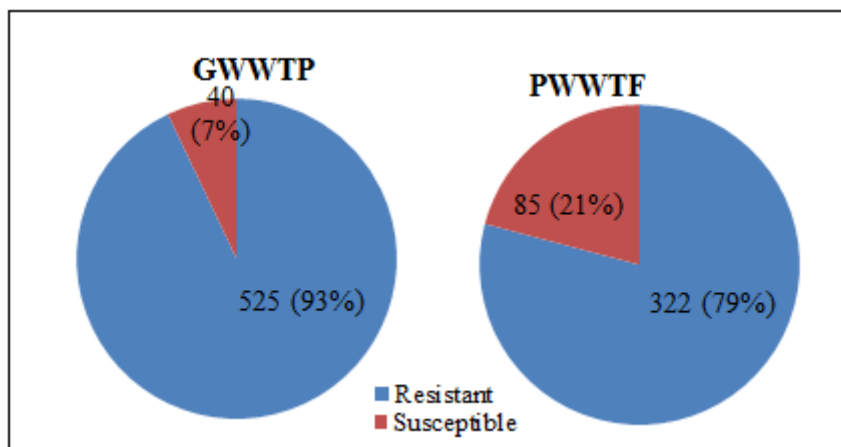


Figure 4.3.1. Percentage occurrence Of ARB in GWWTP and PWWTF study areas.

Considering only antibiotic resistant isolates, the percentage occurrence of ARB in PWWTF were, 127 (39%) from influent, 82 (25%) in the effluent and 113 (35%) in the downstream environment. The percentage occurrence was higher in the PWWTF influent and the downstream environment compared to effluent. In GWWTP, the effluent 204 (39%) and influent 190 (36%) had relatively equal percentage of ARB than downstream environment 131 (25%) (**Figure 4.3.2**).

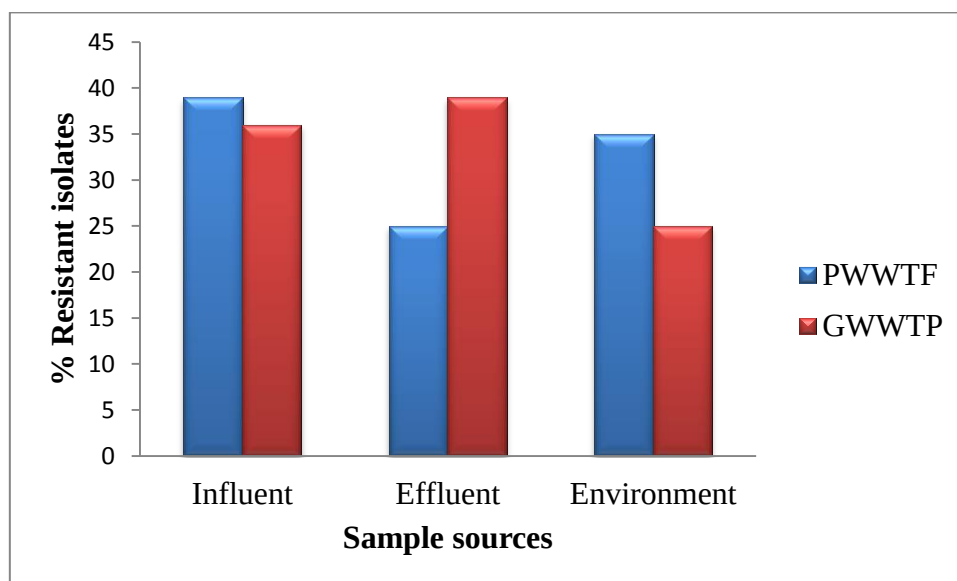


Figure 4.3.2. Percentage occurrence of ARB isolated from different sources (influent, effluent and downstream environment) in GWWTP and PWWTF study areas.

Resistant bacteria occurring at the treatment facilities were further characterized based on the different antibiotics they were resistant to. The percentage occurrence of antibiotic resistance for each individual antibiotic was expressed as percentage relative to total number of isolates tested for different sample sources (In PWWTF, influent-127, effluent-82, downstream environment-113 and from GWWTP, influent-190, effluent-204, downstream environment-131).

For comparative purposes, bacteria that had a resistance equal or greater than 50% for each antibiotic were considered highly frequent while those lower than 50% were considered as lower frequencies (**Figure 4.3.3**).

Antibiotic resistance in bacteria from GWWTP influent occurred at higher frequencies against erythromycin (94%), cephalosporin (83%), trimethoprim (77%), ampicillin (67%), sulfamethoxazole (66%), penicillin (63%) and tetracycline (58%). Lower frequencies

occurred against streptomycin (20%), ciprofloxacin (19%) and meropenem (4%). The GWWTP effluent bacteria were frequently resistant against cephalosporin (83%), penicillin (76%), erythromycin (74%), ampicillin (66%), tetracycline (66%) sulfamethoxazole (64%) and trimethoprim (53%). Lower frequencies were observed against streptomycin (17%), ciprofloxacin (12%) and meropenem (3%). In the downstream environment, ARB frequently occurred against erythromycin (85%), ampicillin (82%), trimethoprim (81%), cephalosporin (81%), penicillin (77%), sulfamethoxazole (69%) and tetracycline (54%). Lower frequencies were observed against streptomycin (12%), ciprofloxacin (10%) and meropenem (8%).

Resistant bacteria from PWWTF influent were frequently resistant against erythromycin (92%), penicillin (85%), trimethoprim (85%), cephalosporin (83%), ampicillin (75%), sulfamethoxazole (76%) and tetracycline (62%). Lower resistances were recorded against ciprofloxacin (39%), streptomycin (11%) and meropenem (2%). Bacteria occurring in the PWWTF effluent were frequently resistant against erythromycin (90%), cephalosporin (89%), penicillin (85%), ampicillin (73%), sulfamethoxazole (73%), trimethoprim (74%) and tetracycline (53%). Lower frequencies occurred against ciprofloxacin (24%), streptomycin (9%) and meropenem (9%). The downstream environment bacteria were frequently resistant against erythromycin (85%), penicillin (74%) and cephalosporin (58%). Lower frequencies were observed against trimethoprim (43%), ampicillin (41%) sulfamethoxazole (37%) tetracycline (21%), meropenem (11%), streptomycin (9%) and ciprofloxacin (3%).

Comparing the influent and effluent in GWWTP, relatively similar percentages of ARB were observed against cephalosporin (83-83%), meropenem (4-3%), ampicillin (67-66%) and sulfamethoxazole (66-64%). However, notable differences were observed in bacteria resistant against, streptomycin (20-17%), ciprofloxacin (19-12%) tetracycline (58-66%), penicillin (63-76%), trimethoprim (77-53%) and erythromycin (94-74%) between GWWTP influent and effluent. The percentage occurrence between GWWTP effluent and downstream environment were relatively similar on bacteria resistant against penicillin (76-77%) cephalosporin (83-81%) and ciprofloxacin (12-10%). Meropenem resistant bacteria had the least percentage occurrence at all sampling sources, influent (4%), effluent (3%) and downstream environment (8%).

In Palapye, the observed percentage occurrence of ARB between influent and effluent varied as follows; tetracycline (62-53%), trimethoprim (85-74%), cephalosporin (83-89%),

ciprofloxacin (39-24%), sulfamethoxazole (76-73%), ampicillin (75-72%), and meropenem (2-9%).

There was slight or no difference on percentage occurrence of ARB between PWWTF influent and effluent for penicillin (85-85%), erythromycin (92-90%) and streptomycin (8-8%). A notable decrease in percentage of ARB occurred between PWWTF effluent and downstream for bacteria resistant against tetracycline (53-21%), penicillin (85-74%), trimethoprim (74-43%), ampicillin (73-41%), ciprofloxacin (24-3%), sulfamethoxazole (73-37%) and erythromycin (90-85%) (**Figure 4.3.3**).

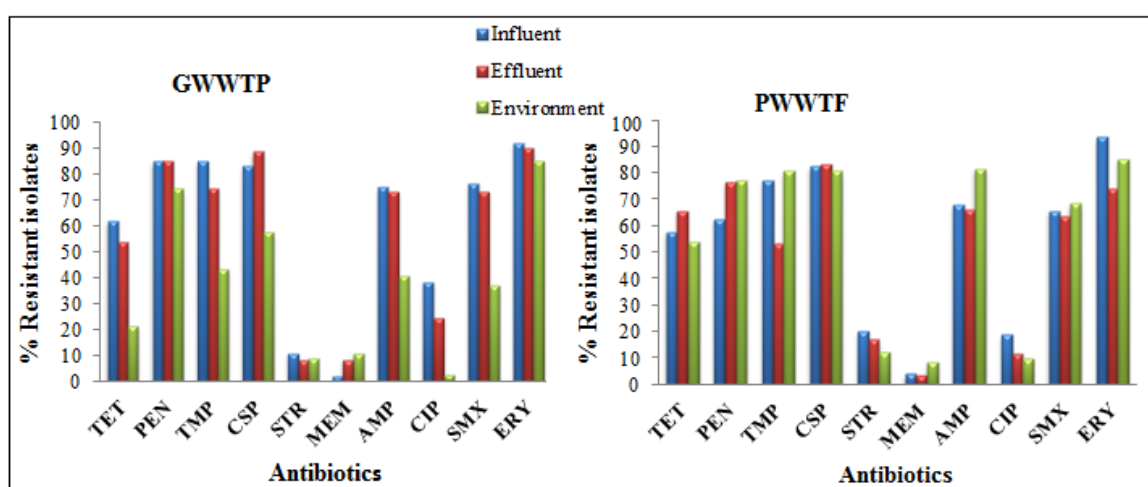


Figure 4.3.3. Bacterial isolates resistant against different antibiotics from influent, effluent and downstream environment of PWWTF and GWWTP.

TET; tetracycline, PEN; penicillin, TMP; trimethoprim, CSP; cephalosporin, STR; streptomycin, MEM; meropenem, AMP; ampicillin, CIP; ciprofloxacin, SMX; sulfamethoxazole, ERY; erythromycin.

The percentage occurrence of resistance against various antibiotics was also determined relative to individual bacterial species. The total number of resistant bacterial species were 847. Out of the total isolates, (525) were from GWWTP while (322) were from PWWTF. Isolates from GWWTP consisted of antibiotic resistant species; *Staphylococcus* species (20.9%), *Salmonella* (5.9%), *Campylobacter* (10.4%), *Listeria* (9.1%), *Brucella* (7.2%), *Escherichia coli* (13.1%) *Enterobacter aerogens* (9.3%) and *Pseudomonas* (23.8%). PWWTF had *Staphylococcus* species (14%), *Salmonella* (9.3%), *Campylobacter* (13.3%), *Listeria*

15.5%), *Brucella* (12.7%), *Escherichia coli* (13.7%) *Enterobacter aerogens* (12.7%) and *Pseudomonas* (8.7%).

The percentage occurrence of antibiotic resistant individual species was expressed as percentage relative to total number of isolates tested per sample source. For comparative purposes, bacterial species that had a resistance equal or greater than 50% were considered highly resistant while those less than 50%, were considered lower.

The *Staphylococcus* species from GWWTP influent were highly resistant against erythromycin (100%), cephalosporin (83%), trimethoprim (75%) and tetracycline (55%). Lower resistances occurred against sulfamethoxazole (43%), ampicillin (40%), penicillin (30%), streptomycin (8%) and meropenem (3%). Ciprofloxacin resistant *Staphylococcus* was not detected. The GWWTP effluent species were highly resistant against cephalosporin (91%), erythromycin (71%) and penicillin (53%). Lower resistances were observed against trimethoprim(17%), tetracycline (49%), sulfamethoxazole (42%), ampicillin (40%), streptomycin (8%), meropenem (4%) and ciprofloxacin (2%). In the downstream, higher resistances occurred against penicillin (64%), trimethoprim (64%), cephalosporin (64%), ampicillin (64%), erythromycin (64%) and sulfamethoxazole (55%). Lower resistances were observed against tetracycline (45%), streptomycin (9%) and meropenem (9%). Ciprofloxacin resistant *Staphylococcus* species were not detected (**Figure 4.3.4**).

A high resistance was observed against penicillin (85%), trimethoprim (85%), erythromycin (76%), cephalosporin (69%), ampicillin (54%) and sulfamethoxazole (54%) in *Pseudomonas* species from GWWTP influent. Lower resistances occurred against tetracycline(38%), ciprofloxacin (15%) and streptomycin (7%). Meropenem resistant *Pseudomonas* species was not detected in GWWTP influent. *Pseudomonas* species from GWWTP effluent were also highly resistant against erythromycin (100%), cephalosporin (100%), ampicillin (80%), penicillin (80%), sulfamethoxazole (80%) and tetracycline (60%). Lower resistances were observed against trimethoprim (40%), ciprofloxacin (20%) and streptomycin (10%), while meropenem resistant *Pseudomonas* species were not detected in the GWWTP effluent. Highly resistant *Pseudomonas* species occurred against erythromycin (100%), cephalosporin (86%), trimethoprim (79%), ampicillin (71%) and penicillin (64%) in the downstream environment, and lower resistances were observed against sulfamethoxazole(43%)

tetracycline (21%), ciprofloxacin (7%), streptomycin (14%) and meropenem (14%) (**Figure 4.3.4**).

Salmonella species from GWWTP influent showed high resistances against erythromycin (100%), tetracycline (67%), trimethoprim (67%) and ampicillin (67%). Lower resistances were observed against penicillin (33%), cephalosporin (33%) and sulfamethoxazole (33%). The *Salmonella* species in the influent did not express resistance against streptomycin, meropenem and ciprofloxacin. In the effluent high resistances were detected against erythromycin (80%), ampicillin (80%), cephalosporin (70%), tetracycline (60%), penicillin (60%), trimethoprim (60%) and sulfamethoxazole (60%) while lower resistance occurred against streptomycin (10%). Meropenem resistant *Salmonella* species were not detected in the GWWTP effluent. In the downstream environment, high resistances occurred against erythromycin (85%), cephalosporin (77%), penicillin (77%), ampicillin (77%), sulfamethoxazole (69%), trimethoprim (69%) and tetracycline (54%). Lower resistances were observed against streptomycin (0.6%). Meropenem and ciprofloxacin resistant *Salmonella* species were not detected downstream environment (**Figure 4.3.5**).

The *Campylobacter* species in the GWWTP influent were highly resistant against erythromycin (93%), trimethoprim (93%), penicillin (71%), cephalosporin (71%), ampicillin (64%) and tetracycline (50%). Lower resistances occurred against sulfamethoxazole (43%) and streptomycin (29%). Meropenem and ciprofloxacin resistant *Campylobacter* species were not detected in GWWTP influent. Higher resistances were also observed from the GWWTP effluent on *Campylobacter* bacteria against tetracycline (95%), penicillin (95%), sulfamethoxazole (95%), ampicillin (86%), trimethoprim (81%), cephalosporin (81%) and erythromycin (71%). The *Campylobacter* species showed lower resistances against ciprofloxacin (14%), streptomycin (10%) and meropenem (5%). The downstream environment *Campylobacter* species were highly resistant against trimethoprim (87%), ampicillin (69%), sulfamethoxazole (56%) and erythromycin (56%). Lower resistances occurred against penicillin (44%), cephalosporin (38%), tetracycline (31%), ciprofloxacin (19%), streptomycin (13%) and meropenem (6%) (**Figure 4.3.5**).

Listeria species from GWWTP influent showed higher resistances against penicillin (100%), erythromycin (100%), cephalosporin (89%) and trimethoprim (67%). Lower resistances were observed against tetracycline (44%), ampicillin (33%) and sulfamethoxazole (22%).

Streptomycin, meropenem and ciprofloxacin resistant *Listeria* species were not detected in GWWTP influent. The GWWTP effluent *Listeria* species showed higher resistances against penicillin (88%), cephalosporin (75%), trimethoprim (50%) and erythromycin (63%). Lower resistances were observed against sulfamethoxazole (44%) and tetracycline (38%). Streptomycin, meropenem and ciprofloxacin resistant *Listeria* species were not detected in GWWTP effluent. The *Listeria* species occurring in the downstream environment had higher resistances against erythromycin (94%), penicillin (81%), cephalosporin (81%), ampicillin (81%), trimethoprim (69%) and sulfamethoxazole (69%). Lower resistances occurred against tetracycline (44%), streptomycin (6%), and meropenem (6%). Ciprofloxacin resistant *Listeria* species were not detected in downstream (**Figure 4.3.6**).

Brucella species from GWWTP showed higher resistances against erythromycin (100%), penicillin (100%), trimethoprim (92%), ampicillin (92%), tetracycline (77%), sulfamethoxazole (77%) and cephalosporin (69%). Lower resistances occurred against ciprofloxacin (38%), streptomycin (8%) and meropenem (8%). In the GWWTP effluent, *Brucella* species had a higher resistance against ampicillin (100%), erythromycin (92%), tetracycline (85%), cephalosporin (85%), sulfamethoxazole (85%) and penicillin (77%). Lower resistances were observed against streptomycin (15%) and meropenem (8%). Ciprofloxacin resistant *Brucella* species were not detected (**Figure 4.3.6**).

Enterobacter aerogenes occurring in GWWTP influent were frequently resistant against erythromycin (94%), trimethoprim (88%), ampicillin (82%), penicillin (82%), sulfamethoxazole (82%) and cephalosporin (59%). Lower resistances were observed against tetracycline (47%), ciprofloxacin (11%) and streptomycin (6%). Meropenem resistant *Enterobacter aerogenes* was not detected in the GWWTP influent. The GWWTP effluent *Enterobacter aerogenes* had a higher resistance against penicillin (89%), cephalosporin (79%), erythromycin (74%), tetracycline (68%), ampicillin (63%), trimethoprim (58%), sulfamethoxazole (53%). Lower resistances occurred against streptomycin (21%) and ciprofloxacin (10%). Meropenem resistant *Enterobacter aerogenes* bacteria was not detected. The *Enterobacter aerogenes* occurring in the downstreams had a higher resistance against erythromycin (95%), penicillin (79%), ampicillin (79%), cephalosporin (79%), sulfamethoxazole (74%) and trimethoprim (74%). Lower resistances occurred against

antibiotics tetracycline (47%) and ciprofloxacin (10%). Streptomycin and meropenem resistant *Enterobacter aerogenes* were not detected in the downstream (**Figure 4.3.7**).

Lastly from GWWTP, *Escherichia coli* from influent showed higher resistances against erythromycin (81%), penicillin (81%), trimethoprim (62%) sulfamethoxazole (57%) and tetracycline (54%), lower resistances were observed against ampicillin (42%), ciprofloxacin (23%) and streptomycin (23%). The GWWTP effluent bacteria showed higher resistances against penicillin (71%), cephalosporin (64%), sulfamethoxazole (64%), tetracycline (57%) ampicillin (54%) and erythromycin (54%). Lower resistances occurred against trimethoprim (46%), streptomycin (14%) and ciprofloxacin (7%). Meropenem resistant *Escherichia coli* was not detected in GWWTP effluent. *Escherichia coli* from downstream environment showed a higher resistance against penicillin (75%), cephalosporin (75%), ampicillin (65%), tetracycline (60%), trimethoprim (60%), erythromycin (60%) and sulfamethoxazole (55%). Lower resistances occurred against streptomycin (25%) and ciprofloxacin (15%). Meropenem resistant *Escherichia coli* bacteria was not detected in downstream (**Figure 4.3.7**).

Considering PWWTF, the influent *Staphylococcus* species were highly resistant against erythromycin (100%), cephalosporin (94%), penicillin (75%), ampicillin (75%), trimethoprim (69%), sulfamethoxazole (63%) and tetracycline (50%). *Staphylococcus* resistance against ciprofloxacin (31%) and streptomycin (25%) were lower. Meropenem resistant *Staphylococcus* species were not detected in PWWTF. The PWWTF effluent species were highly resistant against erythromycin (100%), cephalosporin (89%), ampicillin (89%), trimethoprim (78%), sulfamethoxazole (67%) and tetracycline (56%). Lower resistances occurred against penicillin (44%), ciprofloxacin (44%), streptomycin (6%) and meropenem (6%). The downstream *Staphylococcus* species showed high resistances against erythromycin (100%), sulfamethoxazole (83%), penicillin (83%), trimethoprim (67%) and ampicillin (50%). Lower resistances occurred against cephalosporin (33%), streptomycin (33%), meropenem (26%) and ciprofloxacin (16%). Tetracycline resistant *Staphylococcus* species were not detected (**Figure 4.3.4**).

Pseudomonas species from PWWTF influent showed higher resistances against trimethoprim (100%), cephalosporin (88%), penicillin (82%), ampicillin (82%), erythromycin (82%) and sulfamethoxazole (70%). Lower resistances occurred against tetracycline (47%), streptomycin (12%), meropenem (12%) while ciprofloxacin resistant *Pseudomonas* was not

detected. In the PWWTF effluent the *Pseudomonas* species were highly resistant against cephalosporin (100%), trimethoprim (100%), erythromycin (100%), penicillin (100%), ampicillin (92%), sulfamethoxazole (83%) and tetracycline (58%). Lower resistances were observed against ciprofloxacin (33%) and meropenem (33%). Streptomycin resistant *Pseudomonas* species were not detected in PWWTF effluent. The downstream *Pseudomonas* species had higher resistances against erythromycin (78%), penicillin (84%), cephalosporin (68%) and ampicillin (58%). The *Pseudomonas* species showed lower resistances against trimethoprim (37%) sulfamethoxazole (32%), meropenem (16%), tetracycline (11%) and streptomycin (5%). Ciprofloxacin resistant *Pseudomonas* species were not detected(**Figure 4.3.4**).

The PWWTF influent *Salmonella* species were highly resistant against penicillin(100%), trimethoprim (90%), ampicillin (90%), sulfamethoxazole (90%), erythromycin (80%), ciprofloxacin (80%), cephalosporin (80%) and tetracycline (80%). *Salmonella* species resistance against streptomycin and meropenem were not detected in PWWTF influent. The PWWTF effluent *Salmonella* species showed higher resistance against penicillin (100%) only. In the downstreams, higher resistances occurred against erythromycin (100%), trimethoprim (80%), penicillin (60%) and cephalosporin (60%). Lower resistances occurred against ampicillin (40%) and sulfamethoxazole (20%). Tetracycline, streptomycin, meropenem and ciprofloxacin resistant *Salmonella* species were not detected (**Figure 4.3.5**).

High resistance of *Campylobacter* species from PWWTF influent occurred against trimethoprim (93%), sulfamethoxazole (86%), tetracycline (79%), penicillin (71%), cephalosporin (64%), ampicillin (57%) and ciprofloxacin (57%). Lower resistances occurred against streptomycin (7%). Meropenem resistant *Campylobacter* species were not detected in PWWTF influent. *Campylobacter* species from PWWTF effluent showed higher resistances against penicillin (88%), cephalosporin (88%), trimethoprim (75%), sulfamethoxazole (63%) and erythromycin (63%). Lower resistances occurred against ampicillin (38%) and tetracycline (38%). Streptomycin, meropenem and ciprofloxacin resistant *Campylobacter* species were not detected in PWWTF effluent. The downstream *Campylobacter* species resistance were highly detected against penicillin (100%), cephalosporin (75%), erythromycin (75%) and ampicillin (50%). Lower resistances occurred against trimethoprim (38%), sulfamethoxazole (25%) and streptomycin (13%). Tetracycline, meropenem and

ciprofloxacin resistant *Campylobacter* species were not detected in downstream (**Figure 4.3.5**).

Listeria species from PWWTF influent showed a higher resistances against erythromycin (100%), penicillin (93%), trimethoprim (80%), cephalosporin (80%), ampicillin (73%), sulfamethoxazole (67%), tetracycline (53%) and ciprofloxacin (53%). Lower resistance occurred against streptomycin (6%). Meropenem resistant *Listeria* species were not detected in PWWTF influent. In the PWWTF effluent, high resistances occurred against erythromycin (90%), trimethoprim (64%), cephalosporin (64%), penicillin (72%), ampicillin (55%) and sulfamethoxazole (55%). Lower resistance was observed against tetracycline(45%). Streptomycin, meropenem and ciprofloxacin resistant *Listeria* species were not detected in PWWTF effluent. The downstream species had higher resistances against erythromycin (81%), penicillin (63%) and cephalosporin (56%). Lower resistances occurred against trimethoprim (38%), ampicillin (13%), tetracycline (6%) and meropenem (6%). Streptomycin and ciprofloxacin resistant *Listeria* species were not detected in downstream (**Figure 4.3.6**).

Influent *Brucella* species from PWWTF showed higher resistances against erythromycin(92%), cephalosporin (92%), penicillin (92%), trimethoprim (85%), ampicillin (77%) and tetracycline (62%). Lower resistances of the species were observed against ciprofloxacin (15%), streptomycin (8%), and meropenem (8%). In the PWWTF effluent species, high resistances occurred against erythromycin (100%), sulfamethoxazole (100%), cephalosporin(100%), penicillin(93%), trimethoprim (86%), ampicillin (71%) and tetracycline (57%). Lower resistances were observed against ciprofloxacin(36%), streptomycin (14%) and meropenem (7%). In the downstreams, *Brucella* species showed higher resistances against penicillin (82%), erythromycin (82%) and cephalosporin (64%). Lower resistances were observed against trimethoprim (45%), sulfamethoxazole (45%), streptomycin (36%), ampicillin (27%), tetracycline (18%), meropenem (9%) and ciprofloxacin (9%) (**Figure 4.3.6**).

Escherichia coli from PWWTF influent showed a higher resistance only against erythromycin (50%). Lower resistances occurred against ampicillin (45%), cephalosporin (42%), streptomycin (4%), penicillin (33%), trimethoprim (42%), sulfamethoxazole(33%), ciprofloxacin (25%) and tetracycline (38%). Meropenem resistant *Escherichia coli* was not detected in PWWTF influent. In the PWWTF effluent, *Escherichia coli* showed a higher resistance against penicillin (85%). Lower resistances occurred against trimethoprim (14%),

ampicillin (14%) and erythromycin (14%). Cephalosporin, streptomycin, sulfamethoxazole, ciprofloxacin, tetracycline and meropenem resistant *Escherichia coli* were not detected in PWWTF effluent. The downstreams *Escherichia coli* had higher resistances against penicillin (100%), erythromycin (83%) and cephalosporin (83%). Lower resistances occurred against trimethoprim (33%) and sulfamethoxazole (16%). Tetracycline, streptomycin, ciprofloxacin and meropenem resistant *Escherichia coli* was not detected in downstream (**Figure 4.3.7**).

Enterobacter aerogenes from PWWTF influent showed higher resistances against cephalosporin (75%), ampicillin (75%), erythromycin (69%), penicillin (63%), trimethoprim (56%), tetracycline (56%) and sulfamethoxazole (50%). The *Enterobacter aerogenes* had lower resistance against ciprofloxacin (13%). Streptomycin and meropenem resistant *Enterobacter aerogenes* was not detected in PWWTF influent. In PWWTF effluent *Enterobacter aerogenes* was only resistant against penicillin (20%). *Enterobacter aerogenes* from downstreams had higher resistances against penicillin (67%), erythromycin (67%) and cephalosporin (50%). Lower resistances occurred against sulfamethoxazole (33%), tetracycline (16%), ampicillin (16%), trimethoprim (16%) and ciprofloxacin (8%). Streptomycin and meropenem resistant *Enterobacter aerogenes* bacteria were not detected in downstream (**Figure 4.3.7**).

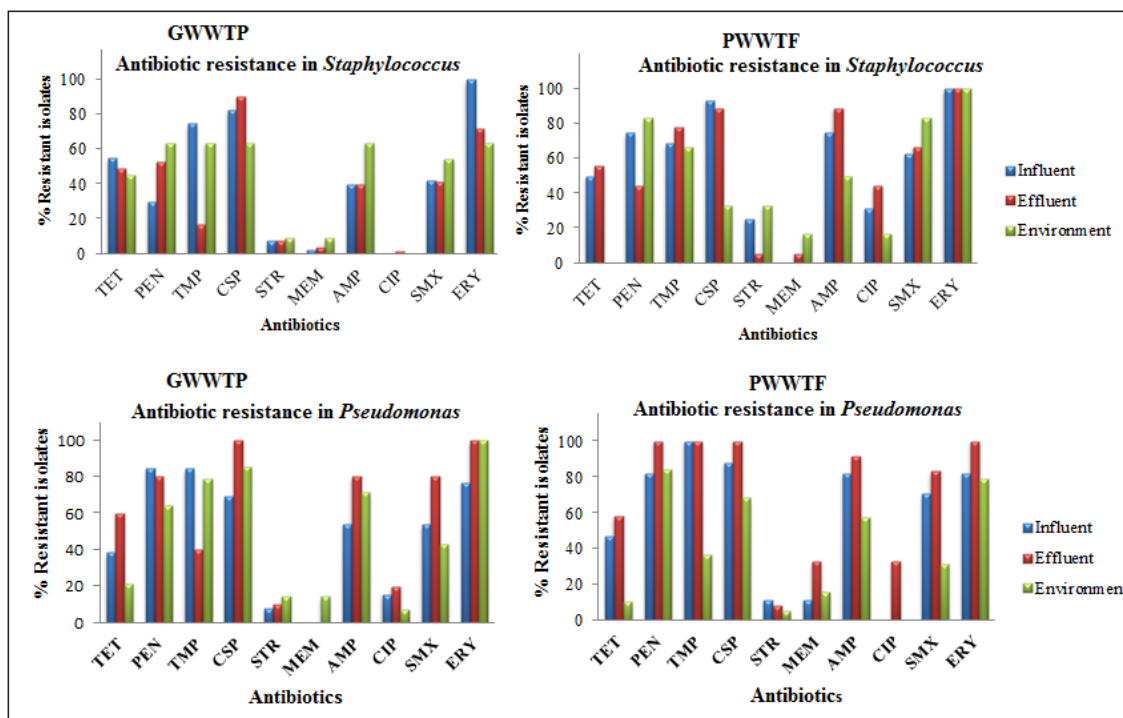


Figure 4.3.4. Antibiotic resistance in *Staphylococcus* and *Pseudomonas* species from PWWTF and GWWTP.

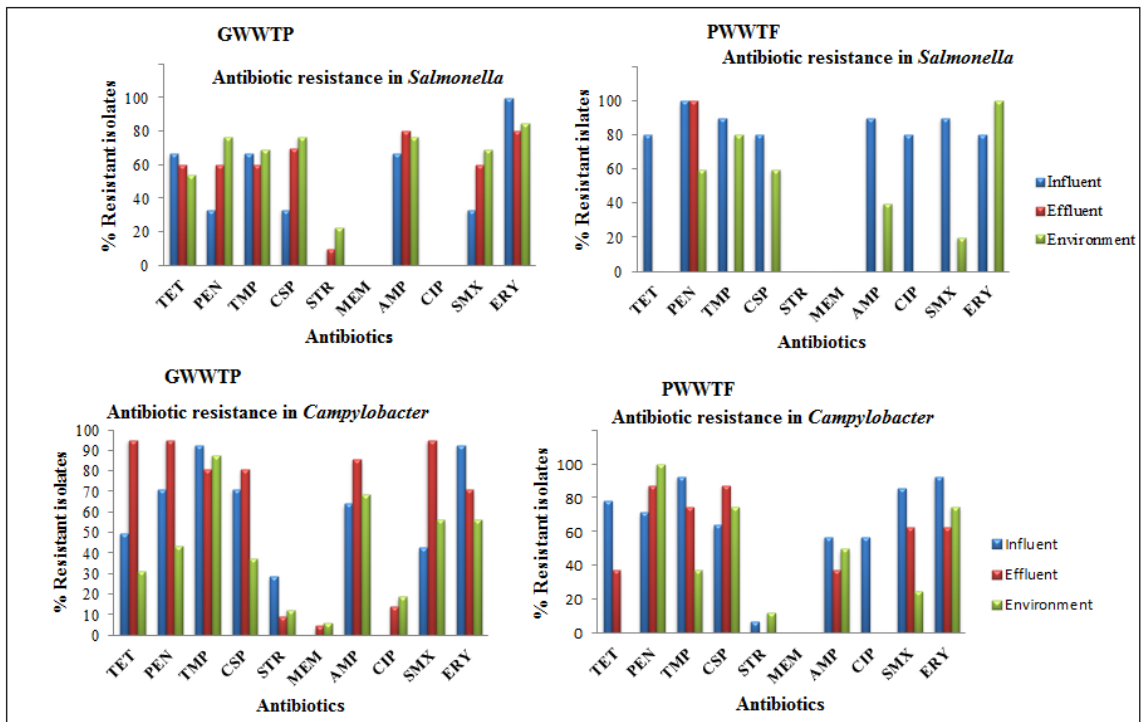


Figure 4.3.5. Antibiotic resistance in *Salmonella* and *Campylobacter* species from GWWTP and PWWTF.

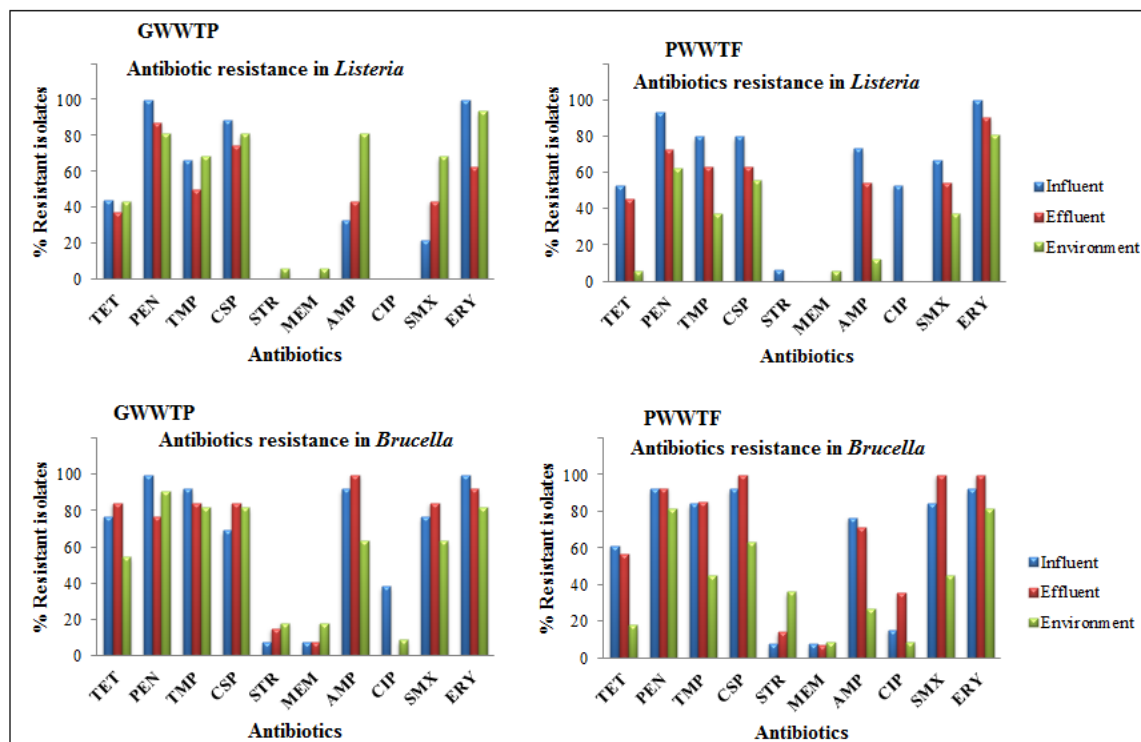


Figure 4.3.6.: Antibiotic resistance in *Listeria* and *Brucella* species from GWWTP and PWWTF.

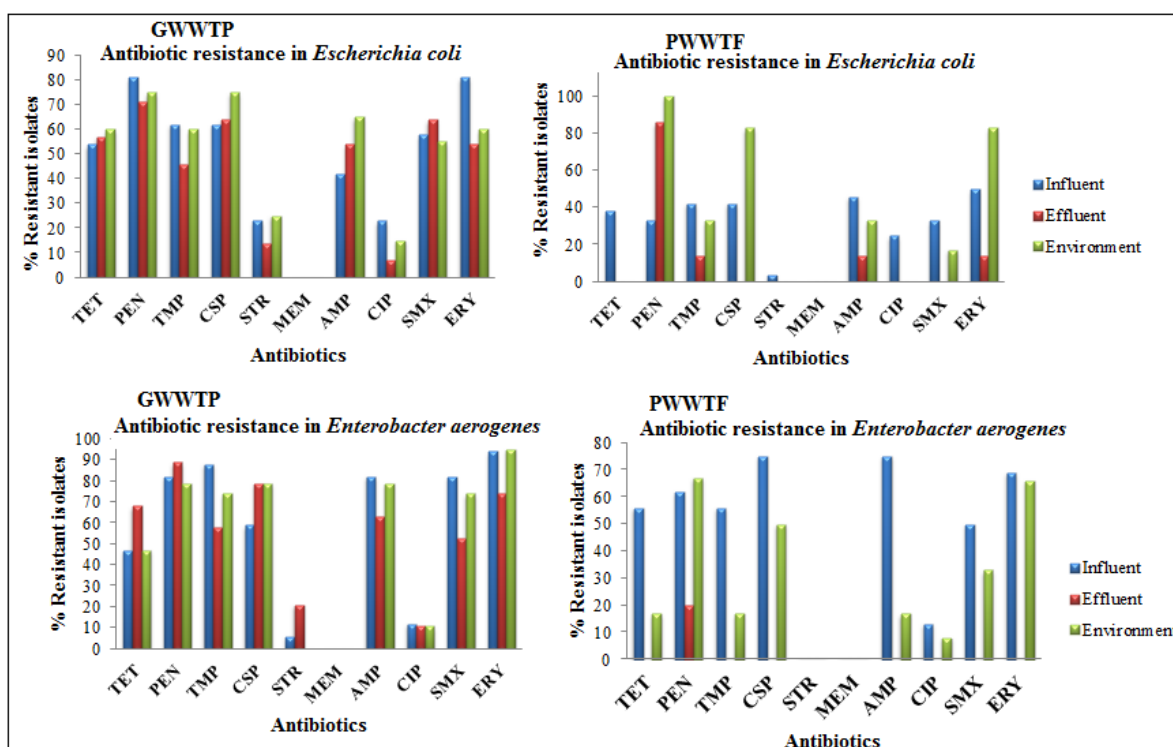


Figure 4.3.7. Antibiotic resistance in *Escherichia coli* and *Enterobacter aerogenes* from PWWTF and GWWTP

4.3.1. Multidrug resistance

The study also determined the occurrence of multidrug resistance (MDR) with isolates that were resistant to more than two antibiotics being considered as multidrug resistant species. Out of all (847) resistant isolates, 690 (81.4%) were MDR, 421 (61%) were from GWWTP while 269(39%) were from PWWTF. The percentage occurrence of MDR bacteria was expressed as percentage relative to total number of multiple resistant isolates in each sample sources (**Figure 4.3.8**).

Multidrug resistant bacteria in GWWTP influent mostly occurred against six antibiotics (20%), followed by five antibiotics (19%), seven antibiotics (15%), three antibiotics (15%), four antibiotics (10%) and nine antibiotics (4%). Effluent multidrug resistant isolates also occurred, mostly against six antibiotics (24%), three antibiotics (22%), five antibiotics (15%), eight antibiotics (15%), four antibiotics (13%), nine antibiotics (8%) and lastly seven antibiotics (2%). Multidrug resistant bacteria was also observed in the downstream environment, mostly against seven antibiotics (31%) followed by five antibiotics (20%), six

antibiotics (18%), eight antibiotics (11%), three antibiotics (9%), four antibiotics (8%) and nine antibiotics (2%) (**Figure 4.3.8**).

Multidrug bacteria also occurred in PWWTF influent, mostly against eight antibiotics (41%) followed by seven antibiotics (18%), four antibiotics (13%), six antibiotics (9%), three antibiotics (7%), five antibiotics (6%) and lastly nine antibiotics (4%). The effluent-multidrug bacteria mostly occurred against seven antibiotics (34%), six antibiotics (19%), eight antibiotics (13%), five antibiotics (11%), three antibiotics (10%), four antibiotics (9%) and less were resistant against nine antibiotics (3%). The multidrug bacteria was also observed in the downstreams, mostly against three antibiotics (28%) followed by five antibiotics (23%), four antibiotics (20%), six antibiotics (13%), seven antibiotics (13%) and lastly eight antibiotics (4%). Bacteria that were resistant against nine antibiotics were not detected (**Figure 4.3.8**).

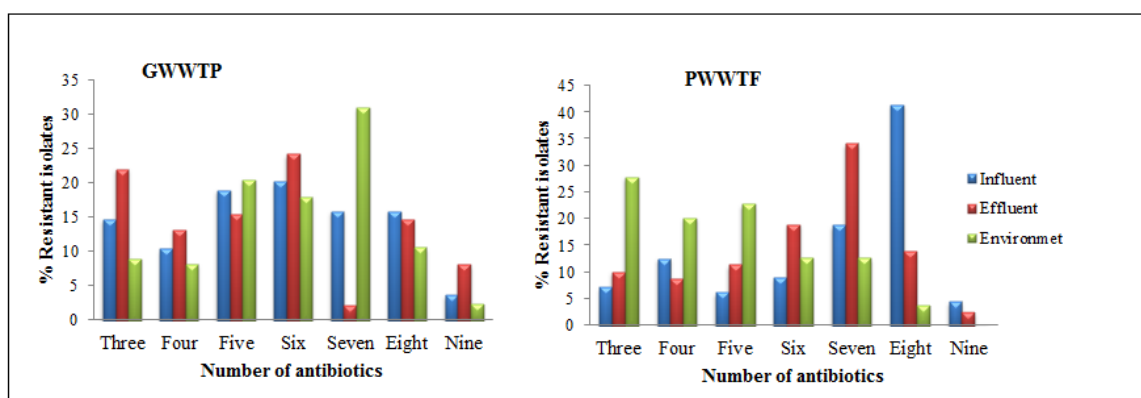


Figure 4.3.8. Multidrug resistant isolates in GWWTP and PWWTF.

4.5. Antibiotic resistance genes

Diversity of ARGs was determined from DNA isolated in selected multiple resistant isolates from Gaborone and Palapye study areas (**Table 4.5.1**). A total of 48 MDR isolates were selected for PCR analysis targeting fourteen ARGs described previously, 24 (50%) were from GWWTP and 24 (50%) were from PWWTF. ARGs that showed positive amplification to the DNA samples tested were *tetA*, *tetB*, *mphA* and *dfr*, *sul1*, *int1* and *strB* (**Figure 4.5.2**). Seven targeted ARGs (*strA*, *ermA*, *ermB*, *ermC*, *catA1*, *catB3* and *qnrA*) were not detected in all 48 selected MDR bacteria species.

Table 4.5.1. 48 multiple resistant bacterial isolates that were selected for detection of ARGs.

	GWWTP			P WWTF		
	Influent	Effluent	Environment	Influent	Effluent	Environment
<i>Staphylococcus</i>	Stp33	Stp22	Stp40	Stp44	Stp25	Stp27
species	(9*)	(8*)	(8*)	(9*)	(8*)	(8*)
<i>Pseudomonas</i>	Pse39	Pse21	Pse38	Pse36	Pse46	Pse1
species	(9*)	(9*)	(8*)	(9*)	(7*)	(7*)
<i>Salmonella</i>	Sal24	Sal26	Sal32	Sal47	Sal6	Sal35
species	(9*)	(6*)	(7*)	(8*)	(9*)	(7*)
<i>Campylobacter</i>	Cam9	Cam31	Cam23	Cam42	Cam5	Cam13
species	(9*)	(7*)	(7*)	(9*)	(7*)	(7*)
<i>Listeria species</i>	Lis17	Lis7	Lis45	Lis48	Lis10	Lis20
	(9*)	(6*)	(7*)	(9*)	(7*)	(7*)
<i>Brucella</i>	Bru14	Bru37	Bru16	Bru11	Bru18	Bru2
species	(9*)	(9*)	(9*)	(9*)	(9*)	(8*)
<i>E.coli</i>	E.co3	E.co29	E.co8	E.co30	E.co28	E.co12
	(9*)	(9*)	(9*)	(9*)	(8*)	(8*)
<i>E.aerogenes</i>	E.ae15	E.ae4	E.ae19	E.ae34	E.ae41	E.ae43
	(9*)	(9*)	(7*)	(9*)	(7*)	(7*)

(*); number of antibiotics the isolate is resistant to.

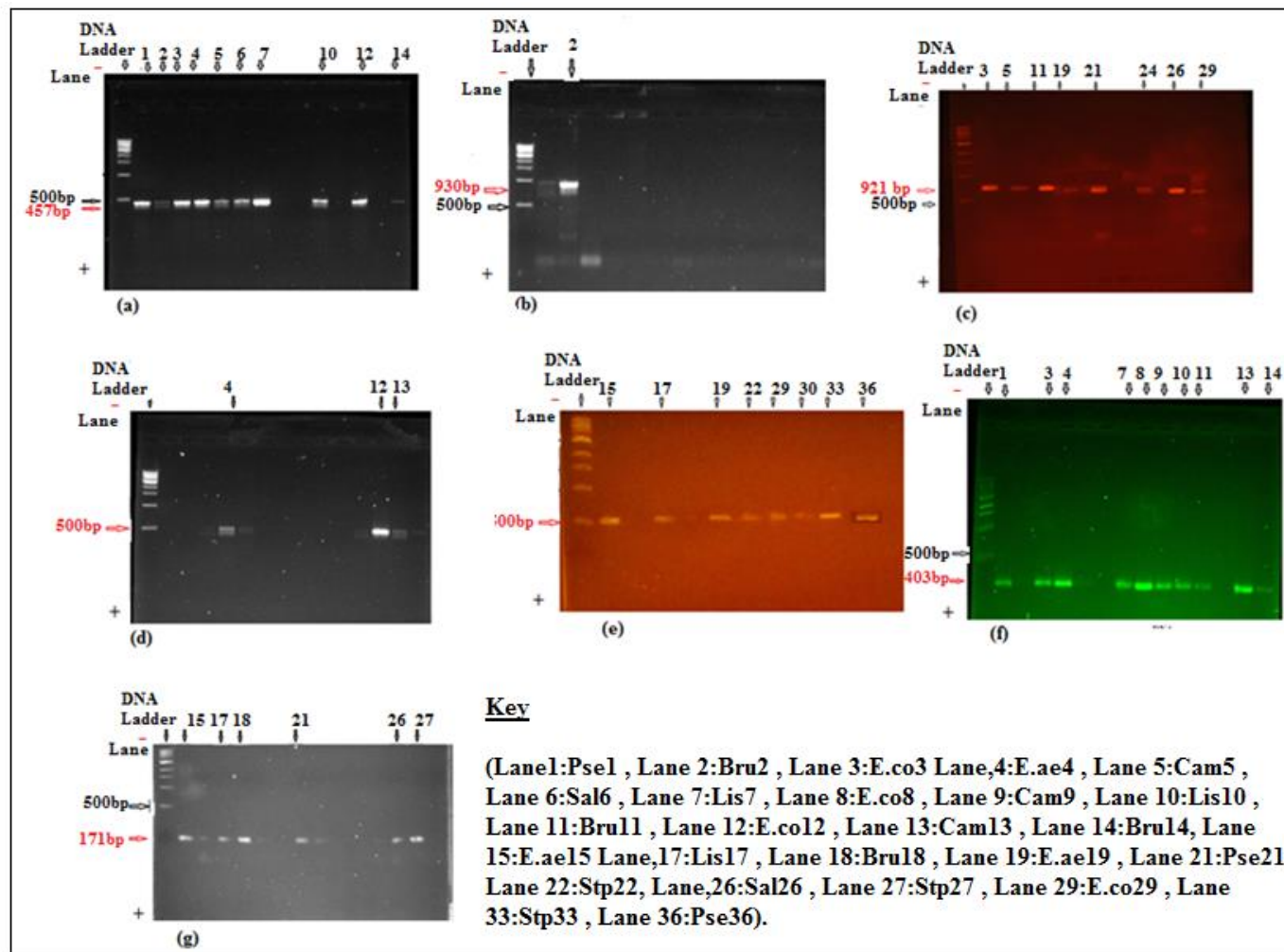


Figure 4.5.1. Summary of positive amplification of PCR target genes; a (*int1*), b (*tetB*), c (*sul1*), d (*strB*), e (*tetA*), f (*mphA*) and g (*dfr*) in selected multiple resistant bacterial isolates.

The frequency of detection of ARGs was expressed as percentage relative to total number of selected isolates in each sample source. For comparative purposes, genes that were detected at 50% or more were considered most frequent than those below 50 % (**Figure 4.5.2**).

ARGs that frequently occurred in GWWTP influent were; *tetA* (75%), *mphA* (62.5%) and *dfr* (50%). Lower frequencies were detected for *sul1* (25%) and *int1* (25%). *strB* and *tetB* genes were not expressed in the influent. In GWWTP effluent, *tetA* (62.5%) and *mphA* (50%) were frequently detected. *sul1* (37.5%), *dfr* (37.5%), *int1* (25%) and *strB* (12.5%) genes occurred at lower frequencies while *tetB* was not detected in the effluent. The downstream environment showed lower frequencies of all resistance genes targeted; *dfr* (37.5%), *mphA* (37.5%), *tetA* (25%), *sul1* (12.5%), *int1* (12.5%) and *strB* (12.5%). *tetB* was not detected.

ARGs were also detected in PWWTF influent, all the targeted genes occurred at lower frequencies in the bacterial species; *tetA* (37.5%), *sul1* (37.5%), *dfr* (25%), *mphA* (12.5%) and *strB* (12.5%). *tetB* and *int1* genes were not expressed in the influent. In PWWTF effluent, frequently detected genes were *dfr* (62.5%), *sul1* (37.5%), *int1* (37.5%), *tetA* (25%) and *mphA* (12.5%) all occurred at lower frequencies while *tetB* and *strB* genes were not detected. In PWWTF downstream environment, frequently detected genes were *dfr* (62.5%), *int1* (37.5%), *tetA* (37.5%), *mphA* (25%), *strB* (25%) and *tetB* (12.5%) were detected at lower frequencies while *sul1* gene was not detected (**Figure 4.5.2**).

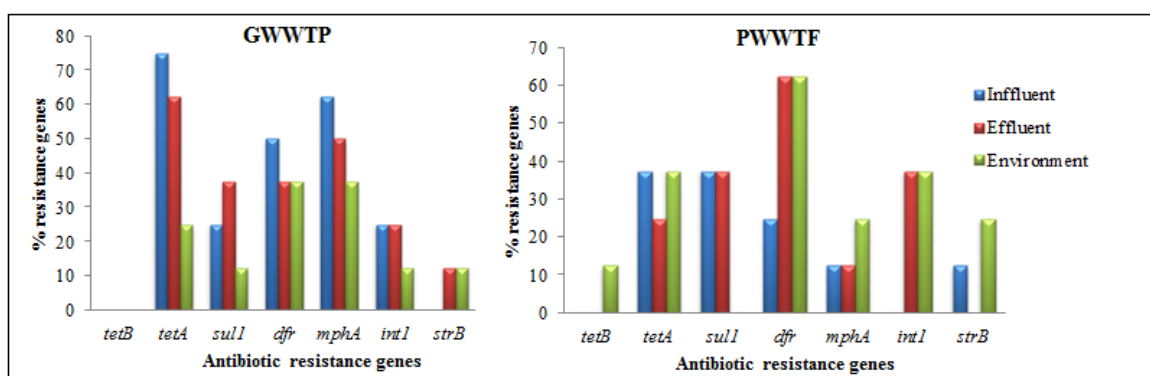


Figure 4.5.2. Diversity of ARGs in GWWTP and PWWTF.

Relative diversity of ARGs carried by individual bacterial species was determined.

Beginning with GWWTP, *Campylobacter* species from influent were found to carry ARGs *tetA*, *dfr* and *mphA* genes. *mphA* gene was the only one which was further detected in *Campylobacter* species from the effluent. There was no targeted gene detected from *Campylobacter* species in the downstream of GWWTP. In *Listeria* species, *tetA*, *dfr* and *mphA* genes occurred in influent. *Int1* and *mphA* were also observed from *Listeria* species in GWWTP effluent. The downstream species carried only *dfr* and *mphA* targeted genes.

The *Brucella* species from GWWTP influent carried *int1* and *mphA* genes. *tetA* occurred in the *Brucella* species in the effluent and the downstream species showed presence of *int1*, *strB* and *mphA*. Resistance genes that were observed in *Escherichia coli* from GWWTP influent were *tetA*, *int1*, *dfr*, *sul1* and *mphA*, effluent species had *tetA*, *sul1* and *mphA*. *tetA* and *mphA* further occurred in *Escherichia coli* from downstream. *tetA*, *dfr* and *mphA* were observed in *Enterobacter aerogenes* bacteria from influent. *tetA*, *int1*, *strB*, *dfr* and *mphA* were detected in species from GWWTP effluent. In the downstream, only *dfr* gene was detected. *tetA* was the only gene that was detected in *Staphylococcus* species from GWWTP influent and effluent. There was no other targeted gene that was detected in *Staphylococcus* species from the downstream environment. *tetA* and *sul1* occurred in *Pseudomonas* species from influent, effluent (together with *dfr*) and downstream environment (**Table 4.5.2**).

The diversity of ARGs carried by individual bacterial species was also analyzed in PWWTF. *tetA* and *strB* were observed in *Pseudomonas* species from PWWTF influent, effluent *Pseudomonas* species had *dfr* and *sul1* genes. The downstream *Pseudomonas* species showed presence of *tetA*, *int1*, *dfr* and *mphA*. In *Salmonella* species, *sul1* was observed in influent while effluent had *int1* and *dfr*. No ARG gene targeted was detected in *Salmonella* species from the downstream environment of PWWTF.

Campylobacter species from PWWTF influent were found to carry only *dfr* gene, in the effluent *tetA*, *int1*, *dfr* and *sul1* were detected in *Campylobacter* species. *tetA*, *strB* and *mphA* were observed in downstream environment. No gene target was observed in *Listeria* species from PWWTF influent and downstream while *tetA*, *dfr*, *mphA*, *int1* were detected in *Listeria* species from the effluent. *tetA*, *dfr*, *sul1* and *mphA* were observed in *Brucella* species from PWWTF influent. *dfr* was the only gene that further occurred in effluent. In the downstream environment of PWWTF, *tetB*, *int1* and *dfr* were detected.

No ARG gene target occurred in *Enterobacter aerogenes* from PWWTF influent and effluent while *tetA* and *dfr* genes were observed in the downstreams. In *Escherichia coli*, *tetA* was the only gene observed in PWWTF influent, no ARG gene target occurred in effluent while *int1*, *strB* and *dfr* occurred in the downstream. *sul1* was the only gene observed in PWWTF influent and effluent of *Staphylococcus* species while *dfr* occurred in the downstream (Table 4.5.3).

Table 4.5.2. ARGs present in GWWTP.

GWWTP			
	Influent	Effluent	Environment
<i>Staphylococcus</i> species	<i>tetA</i>	<i>tetA</i>	*
<i>Pseudomonas</i> species	<i>tetA</i> , <i>Sul1</i>	<i>tetA,sul1,dfr</i>	<i>tetA,sul1</i>
<i>Salmonella</i> species	*	<i>dfr,sul1</i>	<i>dfr</i>
<i>Campylobacter</i> species	<i>tetA</i> , <i>dfr</i> , <i>mphA</i>	<i>mphA</i>	*
<i>Listeria</i> species	<i>tetA</i> , <i>dfr</i> , <i>mphA</i>	<i>int1,mphA</i>	<i>dfr</i> , <i>mphA</i>
<i>Brucella</i> species	<i>int1,mphA</i>	<i>tetA</i>	<i>int1,strB,mphA</i>
<i>E.coli</i>	<i>tetA,int1,dfr,,sul1,mphA</i>	<i>tetA,sul1,mphA</i>	<i>tetA</i> , <i>mphA</i>
<i>E.aerogenes</i>	<i>tetA</i> , <i>dfr</i> , <i>mphA</i>	<i>tetA,int1,strB,dfr,mphA</i>	<i>Dfr</i>

* means no gene was detected

Table 4.5.3. ARGs present in PWWTF.

	PWWTF		
	Influent	Effluent	Environment
<i>Staphylococcus</i> species	<i>sul1</i>	<i>sul1</i>	<i>dfr</i>
<i>Pseudomonas</i> species	<i>tetA</i> , <i>StrB</i>	<i>dfr</i> , <i>sul1</i>	<i>tetA</i> , <i>int1</i> , <i>dfr</i> , <i>mphA</i>
<i>Salmonella</i> species	<i>sul1</i>	<i>int1</i> , <i>dfr</i>	*
<i>Campylobacter</i> species	<i>Dfr</i>	<i>tetA</i> , <i>int1</i> , <i>dfr</i> , <i>sul1</i>	<i>tetA</i> , <i>strB</i> , <i>mphA</i>
<i>Listeria</i> species	*	<i>tetA</i> , <i>dfr</i> , <i>mphA</i> , <i>int1</i>	*
<i>Brucella</i> species	<i>tetA</i> , <i>dfr</i> , <i>sul1</i> , <i>mphA</i>	<i>dfr</i>	<i>tetB</i> , <i>int1</i> , <i>dfr</i>
<i>E.coli</i>	<i>tetA</i>	*	<i>int1</i> , <i>strB</i> , <i>dfr</i>
<i>E.aerogenes</i>	*	*	<i>tetA</i> , <i>dfr</i>

*means no gene was detected

4.6. Microbial diversity in PWWTF downstream environment

The metagenomics approach was used to further characterize bacterial populations in PWWTF downstream environment. The data analysis reveals 4 most dominant phyla present in PWWTF downstream environment, which comprise of phylum Cyanobacteria (37%), Proteobacteria (35%), Firmicutes (14%) and the Bacteroidetes (11%) (**Figure 4.6.1**).

The most abundant class of bacteria was the Nostrophycideae (28%), Gammaproteobacteria (15%), Bacilli (13%), alphaproteobacteria (11%), betaproteobacteria (9%), and flavobacteria (9%). Other classes of bacteria detected were synechococophycideae (5%), oscillatoriohyphyceae (3%) and clostridia (1%) (**Figure 4.6.2**).

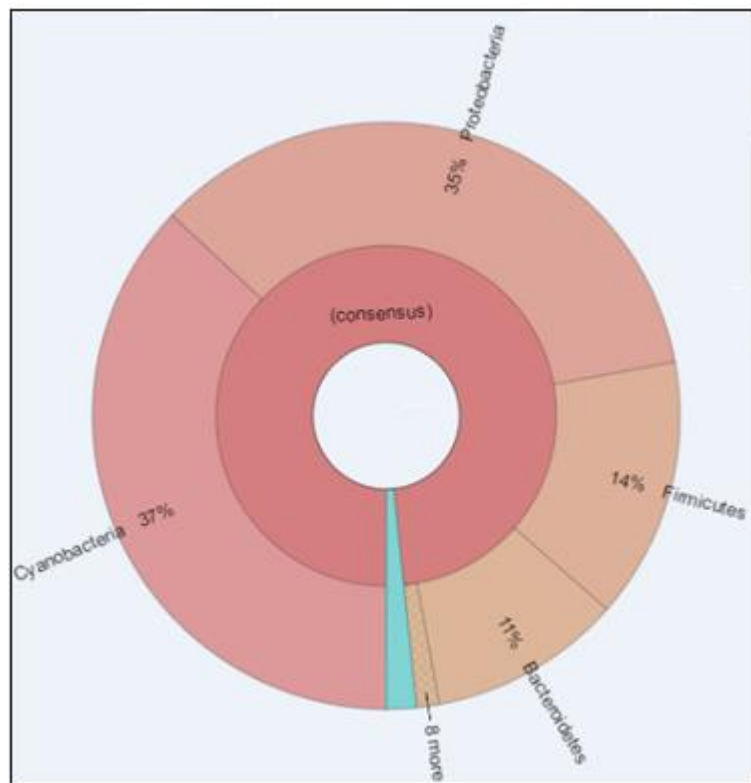


Figure 4.6.1. Visualization of bacterial diversity at the level of phylum.

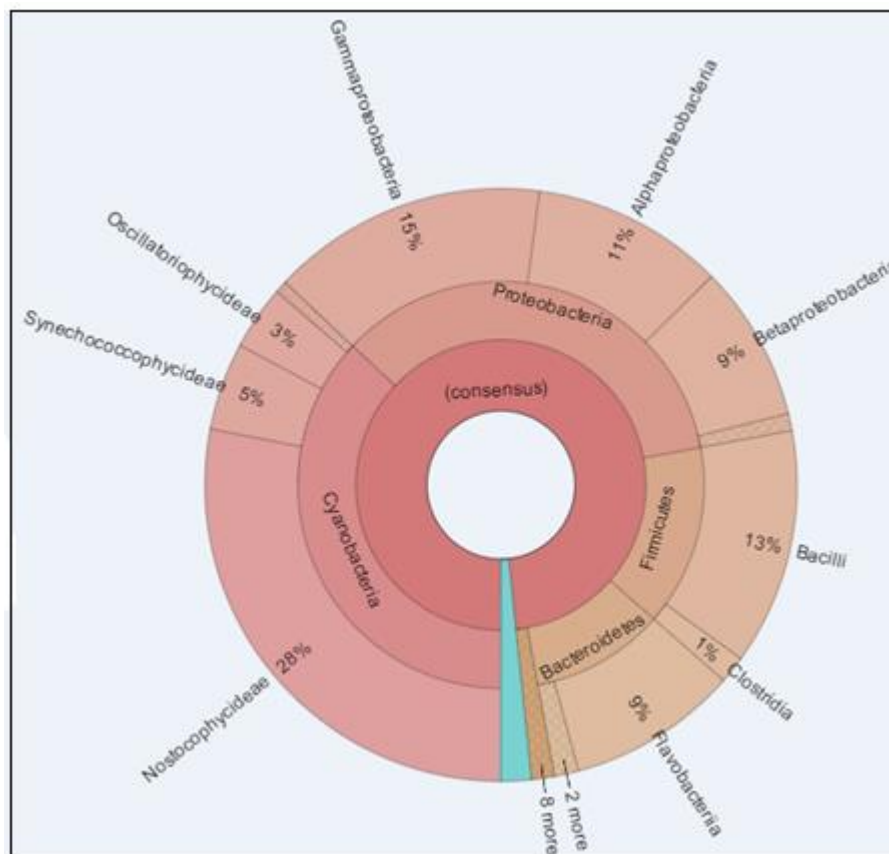


Figure 4.6.2. Visualization of bacterial diversity at the level of class.

At order level, the most abundant bacteria detected were the Nostocales (28%), Bacillales (10%), Flavobacteria (9%), Pseudomonates (7%), Xantomonadates (6%) and Sphinomonadates (6%). The other order detected were the Rhodocyclales (3%), Lactobacillales (3%) and Oscillatoriales (3%) (Figure 4.6.3).

The family of bacteria that was most abundant were the Nostocaceae (28%) followed by Planococcaceae (10%). Other families detected were the Flavobacteria (9%), Pseudomonadaceae (6%), Xanthomonadaceae (6%), and Sphingomonadaceae (6%), Phomidiaceae (3%), Rhodocyclaceae (3%), Comamonadaceae (2%) and Enterococcaceae (2%) (Figure 4.6.3).

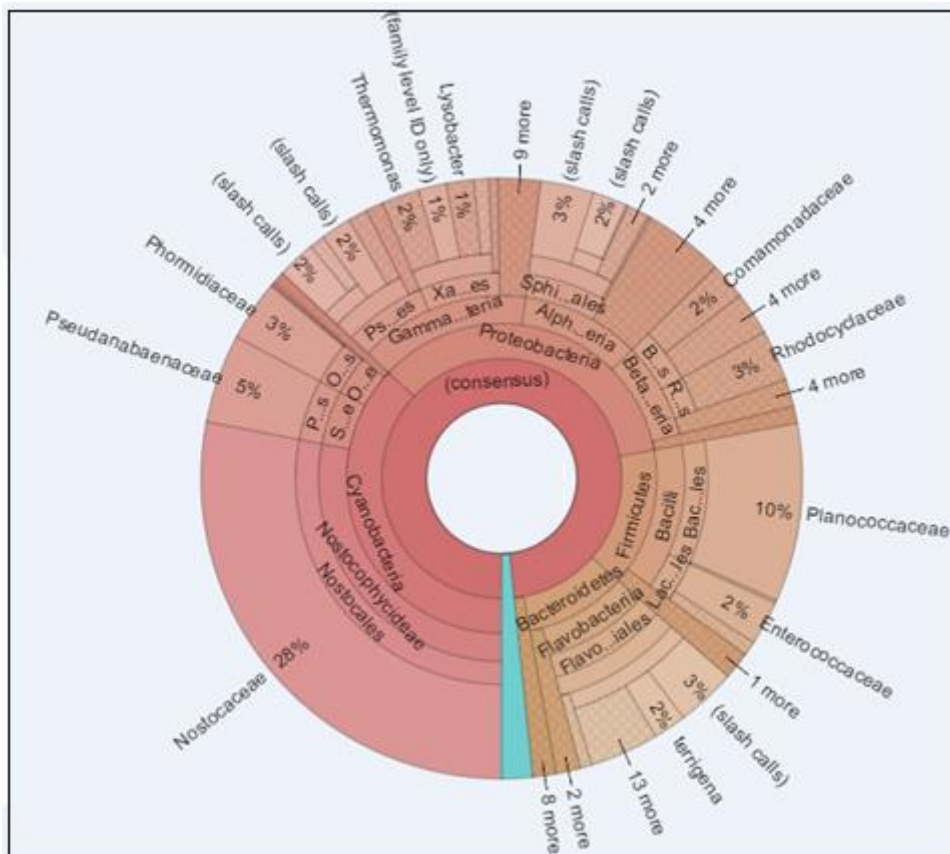


Figure 4.6.4. Visualization of bacterial diversity at genus level.

CHAPTER 5 DISCUSSION

WWTPs links different environments such as municipal sewage, industrial, hospital discharges and surface water, which often contains antibiotic residues, pathogenic and non-pathogenic bacterial populations from human and animals (Brown *et al.*, 2006; Watkinso *et al.*, 2009). This study examined the occurrence and abundance of potentially pathogenic and ARB, diversity of ARGs from PWWTF, GWWTP and their downstream environment.

The study has revealed high occurrences of clinically important bacteria (*Staphylococcus* species, *Escherichia coli*, *Brucella* species, *Campylobacter* species, *Listeria* species, *Salmonella* species, *Pseudomonas* species, and *Enterobacter aerogenes*) in all the sample sources from Gaborone and Palapye study sites except for *Escherichia coli* that were not quantifiable in the effluent samples of both treatment facilities. Most of the bacteria occurred at higher frequencies in influent samples, with slight decrease after treatment in the effluent samples. However, the results show a notable increase of many bacterial species in downstream environment.

These results suggest proliferation of bacteria due possible factors which may reflect conducive conditions that allows bacterial adaptation and multiplication in the downstream of the wastewater treatment facilities. The bacterial species detected in effluent are potentially pathogenic and may pose a serious concern to the human and livestock health, contributing to the spread of infectious diseases such as Brucellosis caused by *Brucella* species (Corbel, 2006), Campylobacteriosis caused by *Campylobacter* species and Listeriosis by *Listeria* species (Roberts, 2004). Although, some bacteria such as *Escherichia coli* were detected at lower frequencies in this study, other studies have detected higher numbers of these bacteria in WWTPs and the receiving environments. High abundance of *Pseudomonas* species and *Escherichia coli* were found in the sediments receiving effluent wastewater in a study by Devarajan *et al.*, (2016).

Other studies have found that WWTPs reduce the abundance of bacterial species in the effluent released to downstream environment (Lu & Lu, 2014; Drury *et al.*, 2013). However, the difference in final effluent of WWTPs may be influenced by the type of WWTP and hence the composition of the effluent, geography of the region, the size and flow rate of the downstreams (Atashgahi *et al.*, 2015).

This study looked at antibiotic resistance in the two treatment facilities and found out that higher amounts of ARB were present in the influent and many persisted in the treated wastewater with a slight percentage decrease. ARB further occurred in the receiving environments with a notable decrease from effluent for many bacterial species. Higher occurrences were observed in GWWTP than PWWTF. The results are not surprising, considering that Gaborone is the main Capital City with a higher population than Palapye. The city may generally be characterized by higher usage of antibiotics and release of various antimicrobial residues from hospitals and industries into the wastewater treatment plant

The bacterial species were resistant to clinically relevant classes of antibiotics (sulphonamides, tetracyclines, beta-lactams, macrolides, quinolones and aminoglycosides) in all the sample sources including effluent and downstream environment, with majority exhibiting multiple resistances to three or more antibiotics. Presence of multiple resistant bacteria in the effluent and downstream further pose a threat to the public health as they can lead to persistence, proliferation and further dissemination to human and animals. This can burden the healthcare system due acquisition of bacterial infections that cannot be treated because of multiple resistance.

The presence of ARB including multiple drug resistance in effluent wastewater and the downstream in the present study is supported by a study from Thomas & Nielsen, 2005, which stated that ARB may still persist in the final effluent after treatment. Several studies have also demonstrated the spread of ARB from wastewater treatment to the downstream environment (Figueira *et al.*, 2011; Kümmerer, 2009; Ferreira da Silva *et al.*, 2006). Multiple resistant *Staphylococcus aureus* (MRSA) was detected in Thibodaux sewage in USA (Boopathy *et al.*, 2017; Naquin *et al.*, 2014), and carry several resistance genes to beta-lactams antibiotics. The differences in efficiency of the treatment facilities may result from treatment processes used. Chlorination has been found as an ideal disinfectant for inactivating bacteria in WWTP, from a study by Iwane *et al.*, (2001) than activated sludge for wastewater treatment (Munir *et al.*, 2011).

This study revealed diversity of bacterial species carrying resistance genes to tetracycline (*tetA*, *tetB*), sulphonamides (*sul1*), trimethoprim (*dhfr*), macrolides (*mphA*), streptomycin (*strB*) and mobile gene cassettes (*intI*). The ARGs were also present in the bacterial species found in the effluent and downstream environment. The presence of ARGs in WWTPs discharges may also be a threat to the public as it indicates the release of the ARB

from treatment facilities to the downstream water sources. The ARGs can further be transferred to environmental bacteria and ultimately to pathogenic bacteria. The results are consistent with several studies that have also detected the presence and persistence of ARGs in WWTPs and downstream environment. For example in a study by Ziemińska-Buczyńska *et al.*, (2015) on communal WWTP in Zabrze (Poland), *dfp* and *sul1* genes were detected in most of the sample tested from influent, effluent and downstreams.

In another study, *sul1* together with *int1* were also detected in samples from WWTP (Sayah *et al.*, 2005). The other resistance genes *mphA* and *int1* were isolated from plasmids from WWTP in a study by Rahube *et al.*, (2014). *strB*, *tetA*, *tetB* together with other tetracycline resistance genes were also detected in WWTP samples and the downstream environment (Xu *et al.*, 2015; Schluter *et al.*, 2007).

Conclusively, GWWTP had more occurrence and diversity of ARB and ARGs than PWWTF. The differences in occurrence may be because of treatment processes used, the population size and the capacity of the treatment plants. These results confirm the study expectations and also supports a suggestion by Hong *et al.*, (2013), that most municipal wastewater treatment design are unable to completely remove ARB and their associated resistance genes.

Metagenomic analysis of downstream environment of PWWTF also revealed diversity and abundance of bacterial populations belonging to major phyla Cyanobacteria, Proteobacteria, Firmicutes and the Bacteroidetes among others. Proteobacteria is the most clinically important phylum of gram negative bacteria which encompasses several classes of bacteria; alpha, beta, and gamma. Many of the important clinical pathogens belong to the class gamma and these include; *Salmonella*, *Pseudomonas*, *Escherichia coli* and *Vibrio cholerae*. These bacteria cause important diseases such as diarrhea, typhoid fever and cystic fibrosis (Liang *et al.*, 2006). Another clinically important phylum that dominates in this study was the Firmicutes which comprise Clostridia and Bacilli classes of Gram positive bacteria such as *Clostridium leptum*, *Bacteroidetes* and *Escherichia coli* (Mariat *et al.*, 2009).

These findings are supported by a study from USA, which revealed Proteobacteria to be the most dominant phyla in WWTPs and their downstream environment (Drury *et al.*, 2013). It was also one of the dominant phyla in a river that receives final effluent from wastewater treatment in a study by Atashgahi *et al.*, (2015) in Belgium.

CHAPTER 6 CONCLUSION

The results obtained indicate that GWWTP and PWWTF have the potential of disseminating clinically important bacterial pathogens including those that carry resistance genes to multiple antibiotics to the receiving environments. This supports studies that consider WWTPs as reservoir of antibiotic resistance. The findings of the study support the hypothesis of the study that high numbers of ARB and ARGs are released from the WWTPs through the effluent into the downstream environment. The disseminated bacteria have the ability to proliferate and potentially spread resistance to clinical pathogens. This can lead to accumulation, persistence and transfer of resistance into the environmental bacteria including clinical pathogens. These antibiotic resistance determinants can spread further to humans through ingestion of effluent irrigated vegetables and livestock through drinking of the contaminated effluent.

This research is critical in the development of new water quality monitoring schemes and implementation of policies on the use of effluent wastewater for irrigation purposes.

Future studies should investigate the diversity of ARB and ARGs in the soils that receives the final effluent, like the irrigated fields and also the crops irrigated. In addition, studies that aim at monitoring ARB and ARGs by quantifying antibiotic resistance determinants are warranted.

Culture based and non-culture based methods were used in the research which have different limitations. Culture based method target viable bacteria while non-culture based target the specific sequences of DNA. In order to overcome the limitations associated with the methods, both approaches were used.

Although, the current methods used were capable of addressing the research objectives, improvement to the study can be done to also determine the effects of climatic conditions on occurrence, abundance and diversity of antibiotic resistance determinants in downstream environment.

6.1. Recommendations

The results will be shared with the government in order to consider the improvement of wastewater treatment infrastructure and use of more advanced treatment processes like ultra violet disinfection used in USA that may target other contaminants such as ARGs. In addition to culture-based and chemical methods, the use of molecular (culture independent) methods for monitoring the efficiency of WWTPs is highly recommended. Furthermore the government should revise its implementation policy of using effluent wastewater for irrigation purposes; further studies are needed before it can be implemented.

- More research on the environment dimension of antibiotic resistance is warranted as it links together clinical and non-clinical environments.
- Further work should include the identification of antibiotic residues in the raw wastewaters, the ones that persist after treatment and the concentrations correlation with the development of ARB.
- Performing conjugation analysis can help in evaluating the likelihood of resistance determinant transfer and the risk of it reaching pathogenic species.
- The public should avoid over-use of antibiotics, finish prescribed antibiotics and also practise proper disposal of antibiotics.

REFERENCES

- Akiyama, T., Asfahl, K.L., Savin, M.C. (2010). Broad-host-range plasmids in treated wastewater effluent and receiving streams. *Journal of Environmental Quality* 39:2211–2215
- Al-jaboobi, M., El-ariqi, S., El Housni, A., Zouahri, A., & Bouksaim, M. (2013). Assessment of the impact of wastewater use on soil properties.
- Aminov, R.I., 2009. The role of antibiotics and antibiotic resistance in nature. *Environmental Microbiology*. 11, 2970–2988.
- Amisu, K. O., Coker, A. O., On, S. L. W., & Isokpehi, R. D. (2003). *Arcobacter butzlii* strains from poultry abattoir effluent in Nigeria. *East African medical journal*, 80(4), 218-222.
- Auerbach, E. A., Seyfried, E. E., & McMahon, K. D. (2007). Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water research*, 41(5), 1143-1151.
- Arau ´jo, C., Torres, C., Silva, N., Carneiro, C., Goncalves, A., Radhouani, H. (2010). Vacomycin-resistant *Enterococcus* from Portuguese wastewater treatment plants. *Journal of Basic Microbiology*. 50: 605_9.
- Arceivala, S. J., & Asolekar, S. R. (2006). *Wastewater treatment for pollution control and reuse*. Tata McGraw-Hill Education.
- Atashgahi, S., Aydin, R., Dimitrov, M. R., Sipkema, D., Hamonts, K., Lahti, L., & Dejonghe, W. (2015). Impact of a wastewater treatment plant on microbial community composition and function in a hyporheic zone of a eutrophic river. *Scientific reports*, 5.
- Baquero, F., Martinez, J.-L. & Canto´n, R. (2008). Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology*. 19, 260–265.
- Berglund, B., Fick, J., & Lindgren, P. E. (2015). Urban wastewater effluent increases antibiotic resistance gene concentrations in a receiving northern European river. *Environmental toxicology and chemistry*, 34(1), 192-196.
- Boerlin, P., Travis, R., Gyles, C. L., Reid-Smith, R., Lim, N. J. H., Nicholson, V., & Archambault, M. (2005). Antimicrobial resistance and virulence genes of *Escherichia coli* isolates from swine in Ontario. *Applied and environmental microbiology*, 71(11), 6753-6761.

- Boopathy, R. (2017). Presence of Methicillin Resistant *Staphylococcus aureus* (MRSA) in sewage treatment plant. *Bioresource Technology*.
- Bouki, C., Venieri, D., & Diamadopoulos, E. (2013). Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: a review. *Ecotoxicology and Environmental safety*, 91, 1-9.
- Brown, K. D., Kulis, J., Thomson, B., Chapman, T. H., & Mawhinney, D. B. (2006). Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. *Science of the Total Environment*, 366(2), 772-783.
- Carr, R. M., Blumenthal, U. J., & Mara, D. D. (2004). Guidelines for the safe use of wastewater in agriculture: revisiting WHO guidelines. *Water Science and Technology*, 50(2), 31-38.
- CDC, (2013). Antibiotic resistance threats in the United States, <https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf>, Center for disease control and prevention
- CDDEP, (2015). Drug Resistance Index. Accessed on August 20, 2015. http://www.cddep.org/projects/resistance_map/drug_resistance_index_urinary_tract_and_skin_infections. Centre for Disease Dynamics, Economics & Policy
- Chen, J., Z. Yu, F.C. Michel, Jr., T. Wittum, and M. Morrison. 2007. Development and application of real-time PCR assays for quantification of *erm* genes conferring resistance to macrolides-lincosamides-streptogramin B in livestock manure and manure management systems. *Applied Environmental Microbiology*. 73:4407–4416. doi:10.1128/AEM.02799-06
- Corbel, M. J. (2006). *Brucellosis in humans and animals*. World Health Organization.
- D'costa, V. M., McGrann, K. M., Hughes, D. W., & Wright, G. D. (2006). Sampling the antibiotic resistome. *Science*, 311(5759), 374-377.
- Devarajan, N., Laffite, A., Mulaji, C. K., Otamonga, J. P., Mpiana, P. T., Mubedi, J. I., ... & Poté, J. (2016). Occurrence of antibiotic resistance genes and bacterial markers in a tropical river receiving hospital and urban wastewaters. *PloS one*, 11(2), e0149211.
- Domingo, J., & Ashbolt, N. (2012). Fecal pollution of water. Retrieved from <http://www.eoearth.org/view/article/152739>
- Ryua, H., Lub, J., Vogelc, J., Elka, M., Chávez-Ramírez, J., Ashbolt, N., & Santo Domingo, J. 2011. Development and Evaluation of a Quantitative PCR Assay

- Targeting Sandhill Crane (*Grus canadensis*) Fecal Pollution. *Applied and Environmental Microbiology*, 22(5), 3715-3726.
- Du, J., Geng, J., Ren, H., Ding, L., Xu, K., & Zhang, Y. (2015). Variation of antibiotic resistance genes in municipal wastewater treatment plant with A2O-MBR system. *Environmental Science and Pollution Research*, 22(5), 3715-3726.
- Drury, B., Rosi-Marshall, E., & Kelly, J. J. (2013). Wastewater treatment effluent reduces the abundance and diversity of benthic bacterial communities in urban and suburban rivers. *Applied and Environmental microbiology*, 79(6), 1897-1905.
- Faucher, S. P., & Charette, S. J. (Eds.). (2015). *Bacterial pathogens in the non-clinical environment*. Frontiers Media SA.
- Ferreira Da Silva, M. F., Tiago, I., Veríssimo, A., Boaventura, R. A., Nunes, O. C., & Manaia, C. M. (2005). Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant. *FEMS Microbiology Ecology*, 55(2), 322-329.
- Figueira, V., Vaz-Moreira, I., Silva, M., & Manaia, C. M. (2011). Diversity and antibiotic resistance of *Aeromonas spp.* in drinking and wastewater treatment plants. *Water Research*, 45(17), 5599-5611.
- Frank, U., & Tacconelli, E. (2012). *The Daschner Guide to In-Hospital Antibiotic Therapy: European Standards*. Springer Science & Business Media.
- Frigon, D., Biswal, B. K., Mazza, A., Masson, L., & Gehr, R. (2013). Biological and physicochemical wastewater treatment processes reduce the prevalence of virulent *Escherichia coli*. *Applied and Environmental microbiology*, 79(3), 835-844.
- Forsberg, K. J., Reyes, A., Wang, B., Selleck, E. M., Sommer, M. O., & Dantas, G. (2012). The shared antibiotic resistome of soil bacteria and human pathogens. *Science*, 337(6098), 1107-1111.
- Gao, P., Munir, M., & Xagorarakis, I. (2012). Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Science of the Total Environment*, 421, 173-183.
- Gebreyes, W. A., & Altier, C. (2002). Molecular characterization of multidrug-resistant *Salmonella enterica* subsp. *enterica* serovar Typhimurium isolates from swine. *Journal of Clinical Microbiology*, 40(8), 2813-2822.
- Goni-Urriza, M., Capdepuy, M., Arpin, C., Raymond, N., Caumette, P., & Quentin, C. (2000). Impact of an Urban Effluent on Antibiotic Resistance of Riverine

- Enterobacteriaceae and *Aeromonas* spp. *Applied and Environmental Microbiology*, 66(1), 125-132.
- Goossens, H. (2005). European status of resistance in nosocomial infections. *Chemotherapy*, 51(4), 177-181.
- Harwood, V. J., Levine, A. D., Scott, T. M., Chivukula, V., Lukasik, J., Farrah, S. R., & Rose, J. B. (2005). Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. *Applied and Environmental Microbiology*, 71(6), 3163-3170.
- Hong, P. Y., Al-Jassim, N., Ansari, M. I., & Mackie, R. I. (2013). Environmental and public health implications of water reuse: antibiotics, antibiotic resistant bacteria, and antibiotic resistance genes. *Antibiotics*, 2(3), 367-399.
- Iwane, T., Urase, T., & Yamamoto, K. (2001). Possible impact of treated wastewater discharge on incidence of antibiotic resistant bacteria in river water. *Water Science and Technology*, 43(2), 91-99.
- Jelic, A., Gros, M., Ginebreda, A., Cespedes-Sánchez, R., Ventura, F., Petrovic, M., & Barcelo, D. (2011). Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment. *Water research*, 45(3), 1165-1176.
- Keraita, B. N., & Drechsel, P. (2004). Agricultural use of untreated urban wastewater in Ghana. *Wastewater use in irrigated agriculture: Confronting the livelihood and environmental realities*, 101-112.
- Knapp, C. W., Dolfing, J., Ehlert, P. A., & Graham, D. W. (2009). Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environmental Science & Technology*, 44(2), 580-587.
- Kohanski, M. A., Dwyer, D. J., & Collins, J. J. (2010). How antibiotics kill bacteria: from targets to networks. *Nature Reviews Microbiology*, 8(6), 423-435.
- Korzeniewska, E., Korzeniewska, A., Harnisz, M. (2013). Antibiotic resistant *Escherichia coli* in hospital and municipal sewage and their emission to the environment. *Ecotoxicology and Environmental Safety*. 91 96–102
10.1016/j.ecoenv.2013.01.014
- Kummerer, K. (2009). Antibiotic in the aquatic environment—A review—Part II. *Chemosphere*, 75, 435–441.
- Krauss, S., Griebler, C., & Deutsche Akademie der Technikwissenschaften. (2011). *Pathogenic microorganisms and viruses in groundwater*. Acatech.

- Lanz, R., Kuhnert, P., & Boerlin, P. (2003). Antimicrobial resistance and resistance gene determinants in clinical *Escherichia coli* from different animal species in Switzerland. *Veterinary Microbiology*, 91(1), 73-84.
- LaPara, T., & Burch, T. U. C. K. E. R. (2011). Municipal wastewater as a reservoir of antibiotic resistance. *Antimicrobial Resistance in the Environment*, 241-250.
- Lu, X. M., & Lu, P. Z. (2014). Characterization of bacterial communities in sediments receiving various wastewater effluents with high-throughput sequencing analysis. *Microbial ecology*, 67(3), 612-623.
- Lina, G., Quaglia, A., Reverdy, M. E., Leclercq, R., Vandenesch, F., & Etienne, J. (1999). Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among staphylococci. *Antimicrobial Agents and Chemotherapy*, 43(5), 1062-1066.
- Liang, J. L., Dziuban, E. J., Craun, G. F., Hill, V., Moore, M. R., Gelting, R. J. & Roy, S. L. (2006). Surveillance for waterborne disease and outbreaks associated with drinking water and water not intended for drinking in United States, 2003--2004. *MMWR Surveill Summ*, 55(12), 31-65.
- Luo, Y., Yang, F., Mathieu, J., Mao, D., Wang, Q., & Alvarez, P. J. J. (2013). Proliferation of multidrug-resistant New Delhi metallo- β -lactamase genes in municipal wastewater treatment plants in northern China. *Environmental Science & Technology Letters*, 1(1), 26-30.
- Madsen, L., Aarestrup, F. M., & Olsen, J. E. (2000). Characterisation of streptomycin resistance determinants in Danish isolates of *Salmonella* Typhimurium. *Veterinary Microbiology*, 75(1), 73-82.
- Malik, M. (2014), Primary vs. Secondary treatment; Types of wastewater treatment plant. <http://archive.epi.yale.edu/case-study/primary-vs-secondary-types-wastewater-treatment>
- Malkawi, H. I., & Mohammad, M. J. (2003). Survival and accumulation of microorganisms in soils irrigated with secondary treated wastewater. *Journal of Basic Microbiology*, 43(1), 47-55.
- Mariat, D., Firmesse, O., Levenez, F., Guimarães, V. D., Sokol, H., Doré, J & Furet, J. P. (2009). The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiology*, 9(1), 123.
- McGowan, J. E. (2006). Resistance in non-fermenting gram-negative bacteria: multidrug resistance to the maximum. *The American Journal of Medicine*, 119(6), S29-S36.

- Memon, J., Kashif, J., Hussain, N., Yaqoob, M., Ali, A., Buriro, R., & Hongjie, F. (2016). Serotypes, Genotypes, Virulence Factors and Antimicrobial Resistance Genes of *Escherichia coli* Isolated in Bovine Clinical Mastitis from Eastern China. *Pakistan Veterinary Journal*, 36(4), 493-498.
- Middleton, J. H., & Salierno, J. D. (2013). Antibiotic resistance in triclosan tolerant fecal coliforms isolated from surface waters near wastewater treatment plant outflows (Morris County, NJ, USA). *Ecotoxicology and Environmental Safety*, 88, 79-88.
- Mirmohammadsadeghi, H., Abedi, D., Mohmoudpour, H. R., & Akbari, V. (2013). Comparison of five methods for extraction of genomic DNA from a marine Archaea, *Pyrococcus furiosus*.
- Moore, D. (2017). Antibiotic Classification & Mechanism. <http://www.orthobullets.com/basic-science/9059/antibiotic-classification-and-mechanism>.
- Moore, D. (2013). Antibiotic classification and mechanism. *Orthobullets. com*.
- Moura, A., Henriques, I., Ribeiro, R., & Correia, A. (2007). Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. *Journal of Antimicrobial Chemotherapy*, 60(6), 1243-1250.
- Mtshali, J. S., Tiruneh, A. T., & Fadiran, A. O. (2014). Characterization of sewage sludge generated from wastewater treatment plants in Swaziland in relation to agricultural uses. *Resources and Environment*, 4(4), 190-199.
- Munir, M., Wong, K., & Xagorarakis, I. (2011). Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Research*, 45(2), 681-693.
- Nagulapally, S. R. (2007). Antibiotic resistance patterns in municipal wastewater bacteria (Doctoral dissertation, Kansas State University).
- Naquin, A., Clement, J., Sauce, M., Grabert, R., Sherpa, M., & Boopathy, R. (2014). Presence of antibiotic resistant *Staphylococcus aureus* in sewage treatment plant. *Journal of Water Sustainability*, 4(4), 227-236.
- Nkegbe, E. & Koorapetse, I., 2005. Heavy Metal Compositions in Gaborone Industrial Effluent. *Journal of Applied Sciences*, 5(8), pp. 1418-1419.
- Negreanu, Y., Pasternak, Z., Jurkevitch, E., & Cytryn, E. (2012). Impact of treated wastewater irrigation on antibiotic resistance in agricultural soils. *Environmental Science & Technology*, 46(9), 4800-4808.

- Nugent, N. R., Lally, M. A., Brown, L., Knopik, V. S., & McGeary, J. E. (2012). OPRM1 and diagnosis-related posttraumatic stress disorder in binge-drinking patients living with HIV. *AIDS and Behavior*, 16(8), 2171-2180.
- Oteo, J., Lázaro, E., de Abajo, F. J., Baquero, F., Campos, J., & Spanish EARSS Group. (2005). Antimicrobial-resistant invasive *Escherichia coli*, Spain. *Emergence Infectious Diseases*, 11(4), 546-53.
- Paterson, D. L., & Bonomo, R. A. (2005). Extended-spectrum β -lactamases: a clinical update. *Clinical Microbiology Reviews*, 18(4), 657-686.
- Phuntsho, S., Shon, H. K., Vigneswaran, S., & Kandasamy, J. (2009). Wastewater stabilization ponds (wsp) for wastewater treatment. *Wastewater Treatment Technologies-Volume II*, 44.
- Perez, F., & Van Duin, D. (2013). Carbapenem-resistant Enterobacteriaceae: a menace to our most vulnerable patients. *Cleveland Clinic Journal of Medicine*, 80(4), 225.
- Pruden, A., Larsson, D. J., Amézquita, A., Collignon, P., Brandt, K. K., Graham, D. W., & Topp, E. (2013). Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environmental Health Perspectives (Online)*, 121(8), 878.
- Rahube, T. O., Viana, L. S., Koraimann, G., & Yost, C. K. (2014). Characterization and comparative analysis of antibiotic resistance plasmids isolated from a wastewater treatment plant. *Frontiers in Microbiology*, 5, 558.
- Rahube, T. O., & Yost, C. K. (2010). Antibiotic resistance plasmids in wastewater treatment plants and their possible dissemination into the environment. *African Journal of Biotechnology*, 9(54), 9183-9190.
- Ramamurthy T, Chowdhury G, Pazhani GP and Shinoda S(2014) *Vibrio fluvialis*: An Emerging Pathogen. *Frontiers in Microbiology*. 5:91. doi:10.3389/fmicb.2014.00091
- Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., & Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Science of the total Environment*, 447, 345-360.
- Roberts, M. T. (2004). Mandatory Recall Authority: A Sensible and Minimalist Approach to Improving Food Safety. *Food & Drug LJ*, 59, 563.
- Rocher, V., Paffoni, C., Gonçalves, A., Guérin, S., Azimi, S., Gasperi, J., & Pauss, A. (2012). Municipal wastewater treatment by biofiltration: comparisons of various

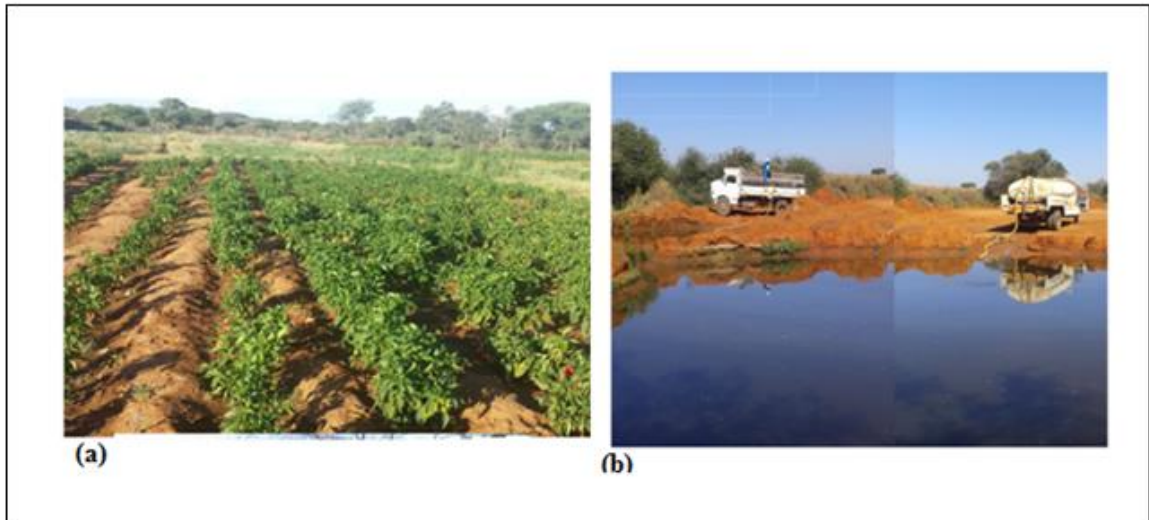
- treatment layouts. Part 1: assessment of carbon and nitrogen removal. *Water Science and Technology*, 65(9), 1705-1712.
- Rosal, R., Rodríguez, A., Perdigón-Melón, J. A., Petre, A., García-Calvo, E., Gómez, M. J., & Fernández-Alba, A. R. (2010). Occurrence of emerging pollutants in urban wastewater and their removal through biological treatment followed by ozonation. *Water Research*, 44(2), 578-588.
- Salipante, S. J., Sengupta, D. J., Rosenthal, C., Costa, G., Spangler, J., Sims, E. H., & McCoy, C. (2013). Rapid 16S rRNA next-generation sequencing of polymicrobial clinical samples for diagnosis of complex bacterial infections. *PloS One*, 8(5), e65226
- Santo Domingo, J., Schoen, M., Ashbolt, N., & Ryu, H. (2012). Using quantitative polymerase chain reaction for water microbial risk assessments. *Quantitative Real-time PCR in Applied Microbiology*. Caister Academic Press, Norfolk, UK, 121.
- Santos, L. H., Gros, M., Rodriguez-Mozaz, S., Delerue-Matos, C., Pena, A., Barceló, D., & Montenegro, M. C. B. (2013). Contribution of hospital effluents to the load of pharmaceuticals in urban wastewaters: identification of ecologically relevant pharmaceuticals. *Science of the Total Environment*, 461, 302-316.
- Sayah, R. S., Kaneene, J. B., Johnson, Y., & Miller, R. (2005). Patterns of antimicrobial resistance observed in *Escherichia coli* isolates obtained from domestic-and wild-animal fecal samples, human septage, and surface water. *Applied and environmental microbiology*, 71(3), 1394-1404.
- Schlüter, A., Szczepanowski, R., Pühler, A., & Top, E. M. (2007). Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. *FEMS Microbiology Reviews*, 31(4), 449-477.
- Schwartz, T., Kohnen, W., Jansen, B., & Obst, U. (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiology Ecology*, 43(3), 325-335.
- Shipin, O. V., Meiring, P. G., & Rose, P. D. (1998). PETRO [R] system: A low-tech approach to the removal of waste-water organic (incorporating effective removal of micro-algae by the trickling filter). *WATER SA-PRETORIA*, 24, 347-354.
- Sehar, S., Rabia, A., Naz, I., Ali, N., Ahmed, S. (2013). Reduction of Contaminants (Physical, Chemical, and Microbial) in Domestic wastewater through Hybrid

- Smillie, C., Garcillán-Barcia, M. P., Francia, M. V., Rocha, E. P., & de la Cruz, F. (2010). Mobility of plasmids. *Microbiology and Molecular Biology Reviews*, 74(3), 434-452.
- Spellberg, B., & Gilbert, D. N. (2014). The future of antibiotics and resistance: a tribute to a career of leadership by John Bartlett. *Clinical infectious diseases*, 59 (supplement 2), S71-S75.
- Sunde, M. (2005). Prevalence and characterization of class 1 and class 2 integrons in *Escherichia coli* isolated from meat and meat products of Norwegian origin. *Journal of Antimicrobial Chemotherapy*, 56(6), 1019-1024.
- Thermo Fisher Scientific Inc (2016)
<https://www.thermofisher.com/bw/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html>
- Thomas, C. M., & Nielsen, K. M. (2005). Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nature Reviews Microbiology*, 3(9), 711-721.
- Todar, K. (2010). Antimicrobial Agents used in the treatment of infectious disease. *Department of Bacteriology University of Wisconsin-Madison*.
- Todar K., 2009. Antimicrobial agents used in treatment of infectious disease. In: Todar K, ed. *Todar's Online Textbook of Bacteriology*. 2009 Available at <http://textbookofbacteriology.net/antimicrobial.html> Accessed 19 January 2010
- Viau, E., & Peccia, J. (2009). Evaluation of the enterococci indicator in biosolids using culture-based and quantitative PCR assays. *Water research*, 43(19), 4878-4887.
- Volkman, H., Schwartz, T., Bischoff, P., Kirchen, S., & Obst, U. (2004). Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). *Journal of Microbiological Methods*, 56(2), 277-286.
- Von Sperling, M. (2007). *Basic principles of wastewater treatment*. IWA publishing.
- Walsh, T. R., Weeks, J., Livermore, D. M., & Toleman, M. A. (2011). Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *The Lancet Infectious Diseases*, 11(5), 355-362.

- Wang, J., Li, Y., Xu, X., Liang, B., Wu, F., Yang, X., & Li, H. (2017). Antimicrobial Resistance of *Salmonella enterica* Serovar Typhimurium in Shanghai, China. *Frontiers in Microbiology*, 8.
- Watkinson, A. J., Murby, E. J., Kolpin, D. W., & Costanzo, S. D. (2009). The occurrence of antibiotics in an urban watershed: from wastewater to drinking water. *Science of the Total Environment*, 407(8), 2711-2723.
- West, B. M., Liggit, P., Clemans, D. L., & Francoeur, S. N. (2011). Antibiotic resistance, gene transfer, and water quality patterns observed in waterways near CAFO farms and wastewater treatment facilities. *Water, Air, & Soil Pollution*, 217(1-4), 473-489.
- Westerhoff, P., Song, G., Hristovski, K., & Kiser, M. A. (2011). Occurrence and removal of titanium at full scale wastewater treatment plants: implications for TiO₂ nanomaterials. *Journal of Environmental Monitoring*, 13(5), 1195-1203.
- WHO, (2016). *Antibiotic resistance fact sheet*. <http://www.who.int/mediacentre/factsheets/antibiotic-resistance/en/>
- WHO, (2012). *Critically important antimicrobials for human medicine*. World Health Organization.
- WHO, (2011). Message of the Regional Director on World Health Day, 7 April 2011, WHO African Region.
- Xiong, W., Sun, Y., Ding, X., Zhang, Y., & Zeng, Z. (2014). Antibiotic resistance genes occurrence and bacterial community composition in the Liuxi River. *Frontiers in Environmental Science*, 2, 61.
- Xu, J., Xu, Y., Wang, H., Guo, C., Qiu, H., He, Y., & Meng, W. (2015). Occurrence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river. *Chemosphere*, 119, 1379-1385.
- Ye, L., & Zhang, T. (2013). Bacterial communities in different sections of a municipal wastewater treatment plant revealed by 16S rDNA 454 pyrosequencing. *Applied Microbiology and Biotechnology*, 97(6), 2681-2690.
- Yousefi, S., Nahaei, M. R., Farajnia, S., Ghojzadeh, M., Akhi, M. T., Sharifi, Y., & Ghotaslou, R. (2010). Class 1 integron and imipenem resistance in clinical isolates of *Pseudomonas aeruginosa*: prevalence and antibiotic susceptibility. *Iranian Journal of Microbiology*, 2(3), 113-119.

- Yuan, Q. B., Guo, M. T., & Yang, J. (2015). Fate of antibiotic resistant bacteria and genes during wastewater chlorination: implication for antibiotic resistance control. *PloS One*, 10(3), e0119403.
- Zhai, W., Yang, F., Mao, D., & Luo, Y. (2016). Fate and removal of various antibiotic resistance genes in typical pharmaceutical wastewater treatment systems. *Environmental Science and Pollution Research*, 23(12), 12030-12038.
- Zhang, T., & Li, B. (2011). Occurrence, transformation, and fate of antibiotics in municipal wastewater treatment plants. *Critical reviews in Environmental Science and Technology*, 41(11), 951-998.
- Zhang, T., Zhang, M., Zhang, X., & Fang, H. H. (2009). Tetracycline resistance genes and tetracycline resistant lactose-fermenting Enterobacteriaceae in activated sludge of sewage treatment plants. *Environmental Science & Technology*, 43(10), 3455-3460.
- Zhang, X. X., & Zhang, T. (2011). Occurrence, abundance, and diversity of tetracycline resistance genes in 15 sewage treatment plants across China and other global locations. *Environmental Science & Technology*, 45(7), 2598-2604.
- Zhou, L. J., Ying, G. G., Liu, S., Zhao, J. L., Yang, B., Chen, Z. F., & Lai, H. J. (2013). Occurrence and fate of eleven classes of antibiotics in two typical wastewater treatment plants in South China. *Science of the Total Environment*, 452, 365-376.
- Ziembińska-Buczyńska, A., Felis, E., Folkert, J., Meresta, A., Stawicka, D., Gnida, A., & Surmacz-Górska, J. (2015). Detection of antibiotic resistance genes in wastewater treatment plant—molecular and classical approach. *Archives of Environmental Protection*, 41(4), 23-32.

APPENDIX A



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Figure A1-Different uses of final effluent from the treatment plants. (a)-GWWTP effluent is used for irrigation by local vegetable farmers along the Notwane River and (b)-made-made ponds where the public draw water for different uses such as construction .

APPENDIX B

Table B1-Quantification of bacterial isolates in GWWTP and PWWTF.

GWWTP												
	Influent			Effluent			Environment 1			Environment 2		
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
<i>Salmonella</i> species	2.83x10 ⁵	2.91x10 ⁵	2.74x10 ⁵	9.9x10 ²	9.71x10 ²	9x10 ²	1.64x10 ²	1.47x10 ²	1.59x10 ²	1.21x10 ²	1.5x10 ²	2.00x10 ²
<i>Listeria</i> species	1.39x10 ⁸	1.29x10 ⁸	1.54x10 ⁸	6.9x10 ⁴	5.7x10 ⁴	4.2x10 ⁴	4.8x10 ⁶	4.1x10 ⁶	3.9x10 ⁶	4.1x10 ⁵	3.9x10 ⁵	3.1x10 ⁵
<i>Staphylococcus</i> species	1.12x10 ⁴	1.3x10 ⁴	1.01x 10 ⁴	2.89x10 ⁴	2.76x10 ⁴	2.69x10 ⁴	2.98x10 ⁶	2.76x10 ⁶	2.61x10 ⁶	3.1x10 ⁴	5.1x10 ⁴	2.9x10 ⁴
<i>Campylobacter</i> species	2.46x10 ⁶	3.61x10 ⁶	4.1x10 ⁵	8.89x10 ³	1x10 ⁴	9x10 ³	2.024x10 ³	1.834x10 ³	1.690x10 ³	2.0x10 ²	4.1x10 ²	5.0x10 ²
<i>Brucella</i> Species	2.89x10 ⁶	2.71x10 ⁶	2.5x10 ⁶	2.11x10 ⁴	1.96x10 ⁴	2.07x10 ⁴	2.69x10 ⁸	2.49x10 ⁸	2.33x10 ⁸	6.17x10 ⁷	5.10x10 ⁷	2.79x10 ⁷
<i>E.coli</i>	9.5x10 ⁶	8.x10 ⁶	8.9x10 ⁶	TFC	31	TFC	TFC	30	TFC	TFC	TFC	TFC
<i>E.aerogenes</i>	9.3x10 ⁶	7.5x10 ⁶	8.1x10 ⁶	4.4x10 ³	3.8x10 ³	4.2x10 ³	8.60x10 ²	8.21x10 ²	7.90x10 ²	7.6X10 ²	4.2X10 ²	2.OX10 ²
<i>Pseudomonas</i> species	4.9x10 ⁵	3.8x10 ⁵	3.1x10 ⁵	1.060x10 ³	1.001x10 ³	9.90x10 ²	1.x10 ⁴	8.9x10 ³	9.7x10 ³	1.2X10 ³	4.6X10 ³	6.1X10 ³

PWWTF									
	Influent			Effluent		Environment			
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
Salmonella species	7.8x10 ³	6.1x10 ³	7.3x10 ³	3.7x10 ³	1.5x10 ³	2.8x10 ³	2.16x10 ⁴	1.78x10 ⁴	2.31x10 ⁴
Listeria species	2.99x10 ⁵	2.69x10 ⁵	2.01x10 ⁵	4.3x10 ³	2.9x10 ³	5.4x10 ³	9.1x10 ⁴	6.6x10 ⁴	4.8x10 ⁴
Staphylococcus species	3.70x10 ²	5.10x10 ²	7.80x10 ²	3.3x10 ²	0	3.1x10 ²	7.1x10 ²	3.1x10 ²	5.3x10 ²
Campylobacter species	1.19x10 ⁵	2.39x10 ⁵	1.79x10 ⁵	9.3x10 ³	6.2x10 ³	5x10 ³	3.1x10 ⁵	4.2x10 ⁵	3.9x10 ⁵
Brucella Species	2.22x10 ⁴	1.98x10 ⁴	3.1x10 ⁵	2.01x10 ⁶	1.76x10 ⁶	2.51x10 ⁶	4.4x10 ⁶	5.1x10 ⁶	3.2x10 ⁶
E.coli	8.6x10 ⁵	5.9x10 ⁵	8.1x10 ⁵	0	0	0	1.1x10 ⁴	9.8x10 ³	1.01x10 ⁴
E.aerogenes	2.99x10 ⁴	2.71x10 ⁴	2.81x10 ⁴	3.1x10 ³	4x10 ³	3.5x10 ³	3.3x10 ⁴	5.1x10 ³	3x10 ⁴
Pseudomonas species	2.99x10 ¹¹	2.8x10 ¹¹	2.68x10 ¹¹	2.54x10 ⁴	2.94x10 ⁴	2.75x10 ⁴	2.81x10 ¹¹	2.71x10 ¹¹	2.55x10 ¹¹

APPENDIX C

Table C1-Total isolated bacteria from the treatment facilities.

	GWWTP	PWWTP
Influent	196	165
Effluent	216	102
Environnement	154	140
Total	407	566

Table C2-Total ARB from the treatment facilities.

	PWWTF		GWWTP	
	Resistant	Susceptible	Resistant	Susceptible
Influent	127	38	191	5
Effluent	82	20	204	12
Environment	113	27	131	23
Total	322	85	526	40

Table C3- Total ARB according to antibiotics

	PWWTF		
	Influent	Effluent	Environment
TET	79	44	24
PEN	108	70	84
TMP	108	61	49
CSP	106	73	65
STR	14	7	10
MEM	3	7	12
AMP	95	60	46
CIP	49	20	3
SMX	97	60	42
ERY	117	74	96

	GWWTP		
	Influent	Effluent	Environment
TET	110	134	71
PEN	119	156	101
TMP	147	109	106
CSP	157	170	106
STR	38	35	16
MEM	8	7	11
AMP	129	135	107
CIP	36	24	13
SMX	125	130	90
ERY	178	151	112

Table C4- Multidrug resistant bacteria in the treatment facilities.

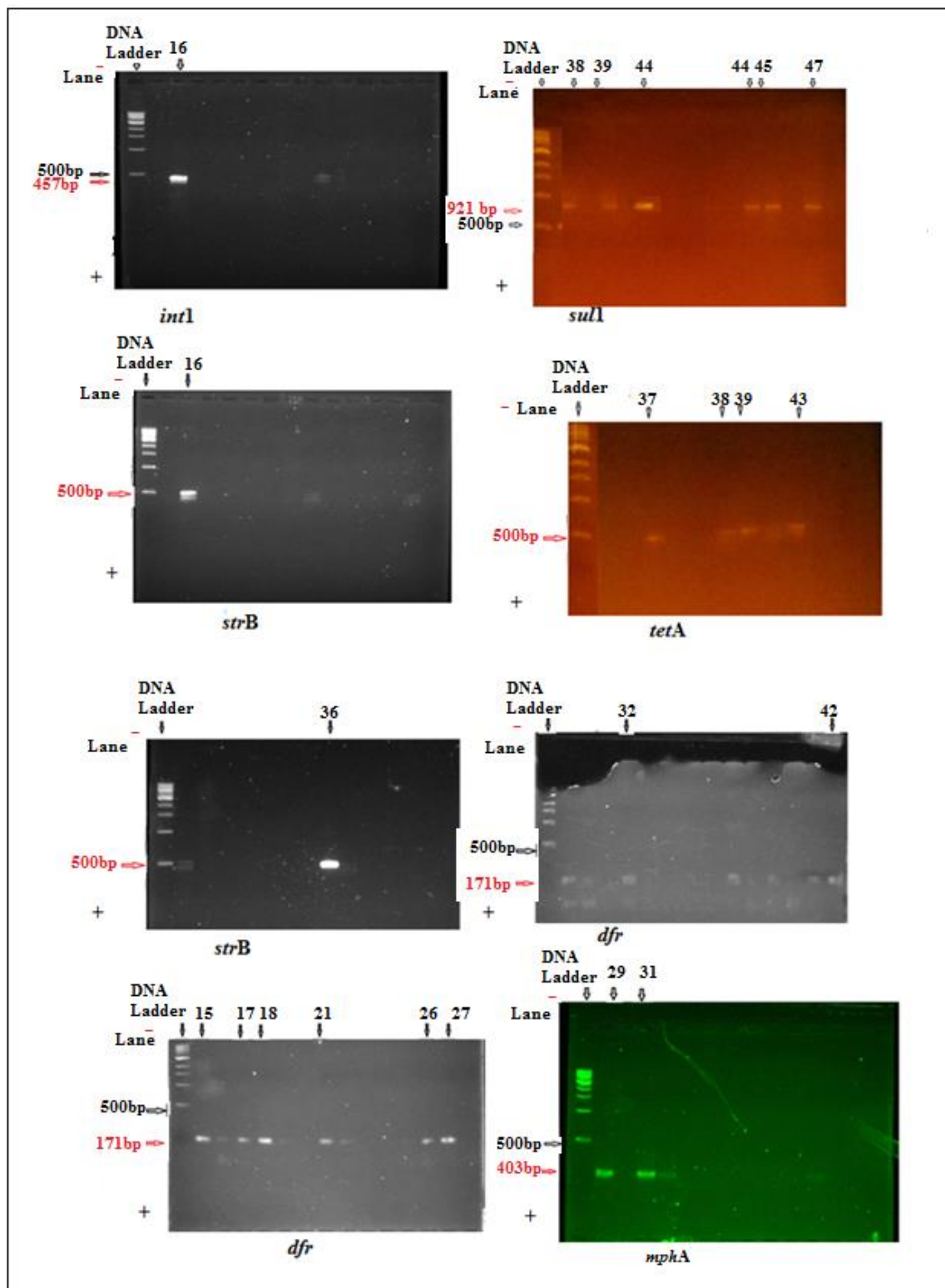
Number of antibiotics a bacteria is resistant to	GWWTP			PWWTF		
	Influent	Effluent	Environment	Influent	Effluent	Environment
Three	24	30	11	8	8	22
Four	17	18	10	14	7	16
Five	31	21	25	7	9	18
Six	33	33	22	10	15	10
Seven	26	3	38	21	27	10
Eight	26	20	13	46	11	3
Nine	6	11	3	5	2	0
TOTAL	163	136	122	111	79	79

APPENDIX D

Table D1-Presence of antibiotic resistance genes in the selected samples

	<i>tetA</i>	<i>tetB</i>	<i>int1</i>	<i>strB</i>	<i>dfr</i>	<i>sul1</i>	<i>mphA</i>
Pse1	+	-	+	-	+	-	+
Bru2	-	+	+	-	+	-	-
E.co3	+	-	+	-	+	+	+
E.ae4	+	-	+	+	+	-	+
Cam5	+	-	+	-	+	+	-
Sal6	-	-	+	-	+	-	-
Lis7	-	-	+	-	-	-	+
E.co8	+	-	-	-	-	-	+
Cam9	+	-	-	-	+	-	+
Lis10	+	-	+	-	+	-	+
Bru11	+	-	-	-	+	+	+
E.co12	-	-	+	+	+	-	-
Cam13	+	-	-	+	-	-	+
Bru14	-	-	+	-	-	-	+
E.ae15	+	-	-	-	+	-	+
Bru16	-	-	+	+	-	-	+
Lis17	+	-	-	-	+	-	+
Bru18	-	-	-	-	+	-	-
E.ae19	+	-	-	-	-	+	-
Lis20	-	-	-	-	-	-	-
Pse21	+	-	-	-	+	+	-
Stp22	+	-	-	-	-	-	-
Cam23	-	-	-	-	-	-	-
Sal24	-	-	-	-	-	-	-
Stp25	-	-	-	-	-	+	-
Sal26	-	-	-	-	+	+	-
Stp27	-	-	-	-	+	-	-
E.co28	-	-	-	-	-	-	-
E.co29	+	-	-	-	-	+	+
E.co30	+	-	-	-	-	-	-
Cam31	-	-	-	-	-	-	+
Sal32	-	-	-	-	+	-	-
Stp33	+	-	-	-	-	-	-
E.ae34	-	-	-	-	-	-	-
Sal35	-	-	-	-	-	-	-
Pse36	+	-	-	+	-	-	-
Bru37	+	-	-	-	-	-	-
Pse38	+	-	-	-	-	+	-
Pse39	+	-	-	-	-	+	-
Stp40	-	-	-	-	-	-	-
E.ae41	-	-	-	-	-	-	-
Cam42	-	-	-	-	+	-	-

E.ae43	+	-	-	-	+	-	-
Stp44	-	-	-	-	-	+	-
Lis45	-	-	-	-	+	+	-
Pse46	-	-	-	-	+	+	-
Sal47	-	-	-	-	-	+	-
Lis48	-	-	-	-	-	-	-
	-	-	-	-	-	-	-



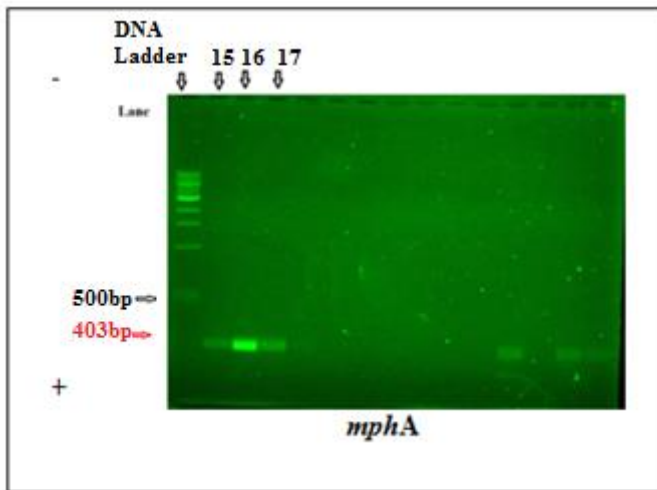


Figure D2: Positive amplification of PCR target genes in selected bacterial isolates.

Lane15: E.ae15, Lane 16: Bru16, Lane 17: Lis17, Lane 18: Bru18, Lane 21:Pse21, Lane 26: Sal26, Lane 27:Stp27, Lane 32:Sal32, Lane 36: Pse36, Lane 37:Bru37, Lane 38:Pse38, Lane 39: Pse39, Lane 42: Cam42, Lane 43: E.ae43, Lane 44:Stp44, Lane 45:Lis45, Lane 46:Pse46, Lane 47:Sal47.

APPENDIX E

Table E1-Antibiotic resistance in bacterial species from GWWTP

GWWTP				
		High resistance	Low resistance	Susceptible
<i>Staphylococcus</i> species	Influent	ERY,TRI,TET, CEP	SUL,AMP, PEN,STR	CIP
	Effluent	CEP,ERY,PEN	TRI,TET,SUL,AMP, STR,MER,CIP	-
	Downstreams	PEN,TRI,ERY, CEP,AMP,SUL	TET,CIP,STR	MER
<i>Pseudomonas</i> Species	Influent	PEN,TRI,ERY, CEP,AM,SUL	TET,CIPR,STR	MER
	Effluent	ERY,CEP,AMP, PEN,SUL,TET	TRI,CIP,STR	MER
	Downstreams	ERY,CEP,TI,A MP,PEN	SUL,TET,CIP,STR, MER	
<i>Salmonella</i> Species	Influent	ERY,TET,TRI, AMP	PEN,CEP,SUL	STR,MER, CIP
	Effluent	ERY,AMP,CEP, TET,PEN,TRI,S UL	STR	MER
	Downstream	ERY,RI,PEN,C EP,AMP,TET	STR	MER,CIP
<i>Campylobacter</i> Species	Influent	ERY,TRI,PEN, CEP,AMP,TET	SUL,STR	MER,CIP
	Effluent	TET,PEN,SUL, AMP,TRI,CEP, ERY	CIP,STR,MER	
	Downstreams	TRI,AMP,SUL, ERY	EN,CEP,TET,CIP,STR, MER	
<i>Listeria</i> Species	Influent	PEN,ERY,CEP, TRI	TET,AMP,SUL	STR,MER, CIP
	Effluent	PEN,ERY,CEP, TRI	TET,AMP,SUL	STR,MER, CIP
	Downstreams	ERY,PEN,CEP H,AMP,TRI,SU L	TET,STR,MER	CIP
<i>Brucella</i> Species	Influent	ERY,PEN,TRI, AMP,TET,SUL, CEP	CIP,STR,MER	
	Effluent	AMP,ERY,TET, CEP,SUL,PEN	STR,MER	CIP
	Downstreams			
<i>Enterobacter aerogenes</i>	Influent	ERY,TRI,AMP, PEN,SUL,CEP	TET,CIP,STR	MER
	Effluent	PEN,CEP,ERY,	STR,CIP	MER

		TET,AMP,TRI, SUL		
	Downstreams	ERY,PEN,AMP ,CEP,SUL,TRI	TET,CIP	STR,MER
<i>Escherichia coli</i>	Influent	ERY,PEN,TRI, SUL,TET	AMP,CIP,STR,AMP	MER
	Effluent	PEN,CEP,SUL, TET,AMP,ERY	STR,TRI,CIP	MER
	Downstreams	PEN,CEP,AMP, TET,TRI,ERY,S UL	STR,CIP	MER

Table E2-Antibiotic resistance in bacterial species from PWWTF

PWWTF				
		High resistance	Low resistance	Susceptible
<i>Staphylococcus</i> species	Influent	ERY,CEP,PEN,AMP,TRI,SUL,TET	CIP,STR	MER
	Effluent	ERY,CEP,AMP,TRI,SUL,TET	EN,CIP,STR,MER	
	Downstreams	ERY,SUL,PEN,TRI,AMP	CEP,STR,MER,CIP	TET
<i>Pseudomonas</i> species	Influent	TRI,CEP,PEN,AMP,ERY,SUL	TET,STR,MER	CIP
	Effluent	CEP,TRI,ERY,PEN,AMP,SUL,TET	CIP,MER	STR
	Downstreams	ERY,PEN,CEP,AMP,	TRI,SUL,MER,TET,STR	CIP
<i>Salmonella</i> species	Influent	PEN,TRI,AMP,SUL,ERY,CIP,CEP,TET		STR,MER
	Effluent	PEN		TRI,AMP,SUL,ERY,CIP,CE,TET,STR,MER
	Downstream	ERY,TRI,PEN,CEP	AMP,SUL	TET,STR,MER,CIP
<i>Campylobacter</i> species	Influent	TRI,SUL,TET,PEN,CEP,AMP,CIP	STR	MER
	Effluent	PEN,CEP,TRI,SUL,ERY	AMP,TET	STR,MER,CIP
	Downstreams	PEN,CEP,ERY,AMP	TRI,SUL,STR	TET,MER,CIP
<i>Listeria</i> species	Influent	ERY,PEN,TRI,CEP,AMP,SUL,TET,CIP	STR	MER
	Effluent	ERY,TRI,CEP,PEN,AMP,SUL	TET	STR,MER,CIP
	Downstreams	ERY,PEN,CEP	TRI,AMP,TET,MER	STR,CIP
<i>Brucella</i> species	Influent	ERY,CEP,PEN,TRI,AMP,TET	CIP,STR,MER	
	Effluent	PEN,ERY,CEP	TRI,SUL,STR,AMP,TET,MER,CIP	
	Downstreams	ERY	AMP,CEP,STR,	

			PEN,TRI,SUL, CIP,TET	
<i>Enterobacter aerogenes</i>	Influent	CEP,AMP,ERY, PEN,TRI,TET, SUL	CIP	STR,MER
	Effluent		PEN	CEP,AMP,ERY, TRI,TET,SUL, STR,MER,CIP
	Downstreams	PEN,ERY,CEP	SUL,TET,AMP, TRI,CIP	STR,MER
<i>Escherichia coli</i>	Influent	ERY	AMP,CEP,STR,P EN,TRI,SUL,CIP ,TET	MER
	Effluent	PEN	TRI,AMP,ERY	CEP,STR,SUL, CIP,TET,MER
	Downstreams	PEN,ERY,CEP	TRI,SUL	TET,STR,CIP, MER