Investigation of the prevalence of antibiotic resistant bacteria and their genes in wastewater

By

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Declaration

I declare that the dissertation herewith submitted for the Masters:

Microbiology

at the University of Zululand is my original work and has not been previously

submitted for a Degree at any other University. I further declare that all the

sources cited or quoted are acknowledged and indicated by means of

comprehensive list of references.

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I hereby approve the final submission of the following dissertation.

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Professor Mathews Simon Mthembu

This_____day_____2020, at the University of Zululand.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Antibiotics are therapeutic agents commonly used in the treatment of infections caused by microorganisms. Due to the increased demand of antibiotics, they have become environmental pollutant to both water and soil. This have caused adverse effects to human, animals, aquatic ecosystem and in the environment in general. These effects includes the development of antibiotic resistant microorganisms, which in turn makes the selection of appropriate antibiotics for particular infections difficult. Therefore, the presence and the prevalence of antibiotics in wastewater were investigated in this study and resistant microorganism and their resistant genes were identified. Gas chromatography with tandem mass spectrum was used to determine the presence of antibiotics in domestic wastewater. Resistance capabilities were detected using the disk diffusion method and resistance isolates were identified using the 16S rDNA sequencing. Polymerase chain reaction (PCR) with specific primers was used to detect resistant genes. Penicillin, ampicillin, meropenem and imipenem traces were determined in domestic wastewater and *Bacillus cereus* isolates were identified. CTX-M, TEM and SHV resistant genes were detected. These genes are commonly found in water and are implicated in numerous diseases such as gastrointestinal infections including diarrhoea, abdominal pains, fever, etc. It is essential to track the pattern of antibiotic resistance because this has a potential in controlling the spread of these genes, thus preventing infections caused resistant microorganisms.

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ABBREVATIONS

ARB:	Antibiotic resistant bacteria
ARG:	Antibiotic resistance bacteria
ESBL:	Extended spectrum beta-lactamases
WHO:	World Health Organisation
CDC:	Centre for disease control and prevention
PBP:	Penicillin binding protein
GT:	Glycosyltransferase
PT:	Transpeptidase
DNA:	Deoxyribose nucleic acid
RNA:	Ribonucleic Acid
mRNA:	Messenger ribonucleic acid
LPS:	Lipopolysaccharide
WWTP:	Wastewater treatment plant

REASEARCH OUTPUTS

CONFERENCES ATTENDED

Papers presented at regional conference

- Ndhlovu, TI and Mthembu, MS. 2018. Determining the presence of antibiotics in domestic wastewater effluents. *Faculty of Science and Agriculture Symposium.* University of Zululand, Science Centre, Richards Bay. South Africa.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Chemotherapeutic agents such antibiotics are widely used for the treatment and infection control for most common diseases. These agents use many mechanisms, including inhibiting nucleic acid, proteins, and cell wall synthesis as well as interference with the functioning of the cell membrane (Talaro, 2004; Shlaes, 2010). Antibiotics can also serve as metabolic antagonists or antimetabolites, where they use competitive inhibition to inhibit metabolites necessary for normal metabolism. This inhibition can results in improper cell growth thus resulting in cell death (Gonzales, 2019). Antimicrobial agents differ in their scope of activity (Madhab *et al.,* 2015). While some have a narrow spectrum, meaning they are effective against a limited type of microorganism, others have a broad spectrum or extended spectrum, meaning they are effective to a wider range of different microbes (Madhab *et al.,* 2015). These antibiotics have become environmental pollutants to both water and soil which causes adverse effects to humans, animals and aquatic ecosystem (Martinez, 2009). These compounds end up in wastewater through the disposal of unused antibiotics, effluents from pharmaceutical industries, domestic effluents (households), excretion to sewage treatment plants by humans and animals, and eventually end up in drinking water sources (Martin *et al*., 2012). Reports from the World Health Organisation noted that around 60% of the world's population practice improper sanitation since they do not have proper sanitation systems (WHO, 2017). This has led to the increase of contamination in water bodies (Statssa, 2017). Over the past several years, concentrations of antibiotics, beta-blockers, lipid regulators, steroids, hormones, anti-inflammatory drugs and cancer therapeutics have been detected in surface and drinking water, wastewater, and groundwater and are labelled as environmental

pollutants (Nikolaou, 2007;Chander *et al.,* 2014). The presence of these compounds has raised concerns to drinking water regulators, municipal as well as to the public at large (WHO, 2012).

Regarding the release of antibiotics to wastewater treatment plants, resistance to antibiotics is one of the increased major concerns worldwide (van Hoek *et al.,* 2011). Recent research has shown that infections caused by antibiotic resistant microorganisms have increased mortality compared to those caused by susceptible microorganisms (CDC, 2018). Munita and Arias (2016), reported that at least 23 000 people die each year as a result of infections caused by antibiotic resistant organisms, and it is estimated that these will cause around 300 million premature deaths worldwide by 2050. According to Chadha (2012) the continuous utilisation of antibiotics in livestock and agriculture also play a major role in the selection of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG's). The continuous detection of ARB in various environments has led to the prospect of chemotherapy to look a bit unpromising (Chattopadhyay *et al*., 2015). Zhang (2016) argued that these ARB have the ability to transfer their ARG's from one bacteria to another through horizontal gene transfer which is regarded as the main mechanism responsible for the spread of resistant genes. Therefore, it is important to investigate the prevalence of antibiotic resistant since it has been thought that individuals that take antibiotics to treat infections caused by extended-spectrum beta lactamases producing organisms (ESBLs) are at risk of treatment failure due to resistant genes (Bradford, 2001). Thus, the aim of this study was to investigate genes that causes resistant to commonly used beta lactam antibiotics such as penicillin, ampicillin, cefotaxime, imipenem and meropenem from rural and low income areas around Vulindlela area. These antibiotics were picked because they are the commonly used worldwide to eradicate infections

caused by gram-negative bacteria (Shaikh *et al*., 2015). This was achieved through determining the presence of antibiotic levels in wastewater, followed by isolating and identifying microorganisms with resistant genes. Then lastly, antibiotic resistant genes were detected to determine the common resistant genes that are most prevalent. The study is aiming to help control the spread of antibiotic resistant genes, thus increasing the effectiveness of the drugs against bacteria. It will also make local clinics to be aware of patients with prolonged illness which could be due to antibiotic resistant. The study also has a potential to create awareness to the public and municipalities to practise proper disposal of antibiotics and use approved water for their domestic purposes.

1.2 Hypothesis

Antibiotic residuals and other chemotherapeutic agents are present in wastewater even after treatment and they play a role in the development and spreading of antibiotic resistant bacteria and antibiotic resistance genes.

1.3 Research Question

Are there any antibiotic residues and antibiotic resistant bacteria found in wastewater treatment plants final effluents?

1.4 Aim and Objectives

1.4.1 Aim

The aim of this study was to investigate the presence of antibiotic resistant bacteria and their genes in wastewater in order to control their spread.

1.4.2 The objectives of the study were:

- To determine the presence of antibiotics in wastewater post treatment.
- To isolate and identify resistant bacteria.
- To detect antibiotic resistant genes as well as identify the most prevalent gene.

1.5 Literature Review

1.5.1 Introduction

One of the most important basic requirements to sustain human life is safe and clean supply of domestic water (Howard and Bartram, 2003). Life cannot be sustained beyond few days without water. Because of this, many waterborne diseases have been identified and continue to spread across the continent (Wang and Cao, 2014). Sobsey (2002) argued that about 1.73 million deaths per year may be attributed to poor water quality, supply and hygiene, thus leading to most diarrhoeal diseases and increased drug resistance. The importance of acceptable water quality for human health has been recognised for many years, and debate has been ongoing among organisations about the importance of safe and clean water (Supply *et al.,* 2000). Saywell, (1999) and Smith *et al.* (2000) noted that many diseases results from the consumption of water contaminated with toxic levels of chemicals such as antibiotics, fluoride, arsenic, nitrate and other contaminations from domestic practices. The contamination of water with these compounds has posed a challenge to human health as there is a possibility of getting infections caused by resistant microorganisms (Microbiology Society, 2018). Individuals infected by these resistant microorganisms can experience treatment failure and end up being hospitalized for longer periods, which is costly to both the healthcare facility and the patients (Pena *et al*., 2006).Therefore, it is important to trace the presence of antibiotics, resistant microorganisms and their genes in wastewater

since they are thought to play an important role in the developing and spreading of resistant genes among microbial species. Tracing resistant microorganisms is also important in the development of counter antibiotics to help eradicate resistant microorganisms by understanding their mechanisms of action in acquiring resistant genes.

1.5.2 The Presence of Antibiotics in Wastewater

Antibiotics are widely used in veterinary and human medical practices, agriculture and aquaculture products (WHO, 2012). This has led to the increased release of these compounds into the environment. Antibiotics end up in domestic water supply through human excretion, disposal of unused or expired drugs and also through veterinary drugs such as feed additives which end up in ground water, and eventually in drinking water tables (Ternes, 1998). Human use a variety of antibiotics daily to maintain a healthy life. However in developing countries such as South Africa, where some areas lack proper sanitation and proper disposal services, antibiotics becomes and other pharmaceutical compounds becomes environmental pollutants through excretion and disposal. About 22.1 % of South African population make use of improper sanitation facilities such as pit latrines (Statssa, 2017). Pit latrines lacks proper physical barriers in their infrastructures, this results in leaching of contaminants into groundwater (Beuke *et al.,* 2017). Pit latrines are also subjected to flooding, thus contributing largely in pollution of water sources (Todman *et al.,* 2015).

Some antibiotics end up onto soils through agricultural practices where they use pesticides, manure and when sludge generated from water treatment plants is used as fertilizer (Beausse, 2004; Martin *et al.,* 2012). The application of organic manure plays a role in introducing antibiotics such as tetracyclines and quinolones in the soil

(Hou *et al.,* 2015). These antibiotics can eventually end up in water sources through water run offs and also leaching into underground water tables (Sun *et al.,* 2017). The use of antibiotics in aquaculture to promote growth, prevent and treat infections also contributed largely in polluting the environment (Rico *et al.,* 2013; Nguyen *et al.,* 2015). Antibiotics belonging to beta lactam, macrolides, cyclines, quinolones, sulphonamides and other groups are allowed in most countries to be used in limited quantities in aquaculture industries (Binh *et al.,* 2018). The use of these antibiotics in this industry has resulted to the release of contaminated wastewater to the environment (Andrieu et al., 2015). Giang *et al* (2015) reported that 91.6 % of surface water that receive wastewater from aquaculture industry in Melta River, Vietnam contains 1 to 4 traces of antibiocs. Chander *et al* (2014) also noted that pharmaceutical industries play a huge role in the occurrence of antibiotics in the environment as they discharge larger amount of treated and untreated effluents in open areas and streams. Antibiotics are mainly distributed to the environment through aquatic medium followed by food chain dispersal (Nikolaou *et al.,* 2007; Chander *et al.,* 2016).

1.5.3 Mechanisms of Action by Antibiotics

Antibiotics are increasingly used worldwide to treat bacterial infections or hospital acquired infections. Shaikh *et al* (2015) reported that beta-lactam antimicrobial agents are the most commonly used antibiotics to eradicate infections caused by Gram-negative and Gram-positive microorganisms. There are five mechanisms of action which antibiotics use to accomplish their functions.

- They may interfere with the synthesis of the cell wall, where they prevent enzymes that are important for the formation of the peptidoglycan layer (Beta-lactam) (Benton *et al.,* 2007).

- Antibiotics may also inhibit protein synthesis (Tetracyclines interfere by binding on to the 30S subunit of ribosome), resulting in weak interaction of the ribosome-tRNA (Leach *et al.,* 2007).
- They also interfere with nucleic acid synthesis where they disrupt RNA polymerase.
- They inhibit metabolic pathways by blocking key steps of folate synthesis which is important in the synthesis of nucleotides.
- Some antibiotics also act by disorganizing the cell membrane by increasing membrane permeability, which then results to the leakage of bacterial content (Gram-positive) (Straus and Hancock, 2006).

1.5.3.1 Antibiotics that Interfere with Cell Wall Synthesis

According to Park and Strominger (1957) and Chander *et al* (2016) beta lactam drugs such as penicillin are the most used classes of antibiotics worldwide. They are known to interfere with the assembly of bacterial cell wall. The cell wall is made up of polysaccharide structure that protects and surrounds cytoplasmic membrane from osmotic rupture (Romaniuk and Cegelski, 2015). Bacterial cell wall polysaccharide structure is built with peptidoglycan polymers that consist of glycan chains and peptides that are cross linked to form a matrix structure. Ruiz (2016) reported that beta lactams interferes with peptidoglycan biogenesis by inactivating the penicillin-binding protein (PBP). Sauvage *et al* (2008) argues that bacteria encodes a variety of PBPs that plays a vital role in peptidoglycan assemble. These PBPs are grouped into class A PBPs (aPBPs) and class B PBPs (bPBPs). Class A has two activities: the glycosyltransferase (GT) activity which polymerize the glycan strand, and the transpeptidase (TP) activity for crosslinking glycan strands (Uehara and Berhardt,

2011). Class B only possesses the TP activity. Beta lactams target and modify the TP active site of the synthetic PBPs which results to the loss of cell integrity followed by cell lysis (Benton *et al.,* 2007). Cho *et al* (2014) demonstrated that antibiotics such as mecillinam do not only inhibit the TP activity of the PBPs but also stimulates a deleterious futile cycle of the cell wall synthesis and degradation resulting in a lethal malfunctioning of the cell machineries.

1.5.3.2 Interfering with Nucleic Acid Synthesis

Transcription is the first step of gene expression where DNA is transcribed to mRNA by RNA polymerase. Transcription of mRNA may be targeted by various antibiotic compounds. For example, rifampicin binds to the beta subunit of RNA polymerase, thus blocking the tunnel of the nascent chain of mRNA (Nguyen, 2018). Fidaxomicin binds to the sigma (70) subunit and the beta subunit of DNA dependent RNA polymerase, and disturbing the incorporation of the DNA into the polymerase (Leach *et al.,* 2007). Quinolones causes the breakage of DNA double strand by interfering with topoisomerase type II and IV during the replication cycle (Strohl, 1997).

1.5.3.3 Inhibition of Protein Synthesis

Translation, the last step in the synthesis of new proteins, is one of the usual targets by antibiotics. Casteels *et al* (1994) noted that tetracyclines, aminoglycoside and macrolides are the most commonly used translation inhibitors. Wong *et al* (2010) argued that these drugs inhibit protein synthesis via various mechanisms of action, including binding directly to the protein synthesis machinery, while others act by inhibiting specific factors required for translation. For example, aminoglycosides bind within the messenger RNA (mRNA) channel, thus inhibiting the correct codon-anticodon interaction that is necessary for the binding of N-Formylmethionine-transfer

RNA unit (fMet-tRNA) (Nguyen, 2018). Tetracylcines interfere by binding the 30S subunit of the ribosome, resulting in weak interaction of the ribosome-tRNA (Leach *et al.,* 2007). Macrolides act by inhibiting the elongation of polypeptide chains by binding to the 50S ribosomal subunit (Kannan *et al.,* 2014). Aminoglycosides are known to inhibit the initiation of protein synthesis as they also bind to the 30S subunit of ribosomes (Francis *et al.,* 2013).

1.5.3.4 Antibiotics Inhibiting Metabolic Pathways

Lobritz *et al* (2015) reported that there are some antibiotics that are known to block the key metabolic steps which result to disturbing the products of metabolic pathways. As an example, Green and Matthews, (2007) observed that some antibiotics block the steps of folate synthesis which is important for the synthesis of nucleotides. Tetrahydrofolate is one of the important cofactors of biochemical pathway for the production of nucleotides and amino acids (Wang *et al.,* 2011). The synthesis of tetrahydrofolate is a multistep process that requires multiple enzymes (Hitchings and Barchal, 1965; Wang *et al.,* 2011). Sulfonamides and trimethoprim are two classes of antibiotics that target this reaction cascade (Illarionova *et al*., 2002). Sulfonamides inhibit the condensation of p-aminobenzoic acid (pABA) with 7, 8 –dihydropterin-pyrophosphate to form 7, 8 –dihydropteroate by acting as a substrate analog of the enzyme dihydropteroate synthetase (Bock *et al*., 1974; Achari *et al*., 1997). Trimethoprim on the other hand target the reduction of dihydrofolate to tetrahydrofolate catalysed by dihydrofolate reductase (Gleckman *et al*., 1981; Capaso and Supuran, 2014).

1.5.3.5 Antibiotics Disorganising the Cell Membrane

Berglund *et al* (2015) reported that polymyxin B and E are peptide antibiotics that integrates into the membranes of the gram-negative bacteria and that these antibiotics target lipid A components which are the innermost components of the lipopolysaccharide (LPS), thereby increasing the permeability of the cell membranes, which leads to the leakage of bacterial content. Nisin is one of the known members of lantibiotics and is known for its ability to block lipid II, which is the precursor molecule for the biosynthesis of cell membrane (Mothia *et al.,* 2011). This results to the lysis of bacteria (Raetz and Whitfield, 2002). Daptomycin has bactericidal activity since it binds to the membrane in a calcium dependent manner, resulting in the efflux of potassium from bacterial cell, and eventually cell death occurs (Straus and Hancock, 2006).

1.5.4 Antibiotic Resistance and Resistant Genes

Most of the commonly used drugs such as antimicrobials have reduced effectiveness against common microorganisms, and these microbes are said to be antibiotic resistant (Shaikh *et al*., 2015). When microbes are showing resistance to more than one type of drug, they are called multidrug resistant microorganisms (Fisher and Mobashery, 2010). Bacteria may develop or acquire different genes that are responsible for their resistance and these genes continue to develop and cause major threats to the treatment of infectious diseases (Goossens, 2005). Thus, it is very important to understand the mechanisms that contribute to the development of resistant genes as well as understand the different pathways involved in the spreading of these resistant genes in order to cope with antibiotic resistance. Resistance genes are usually located on mobile genetic elements that functions as vectors, thus promoting rapid spread of these genes (Szczepanowski *et al.,* 2009). These mobile

genetic elements include transposons, plasmids and integrin (Seveno *et al.,* 2002). The first enzyme that was reported to show resistance was the AmpC beta lactamase of the *Escherichia coli* which destroyed penicillin (Abraham and Chain, 1940). Recently, the increase of resistance against beta lactam antibiotic is increasing at a rapid rate and has become a major common problem in the discovery of new antibiotics (van Hoek *et al.,* 2011). Bacteria adapt a common mechanism of resistance against beta lactams by expressing beta lactamases which are enzymes capable of destroying the beta lactam ring that is found in almost all beta lactams molecular structure (Paterson and Bonomo, 2005). Monobactams do not contain the beta lactam ring in their molecular structure but they still belong to beta lactams antibiotics (van Hoek *et al.,* 2011).

1.5.5 The South African Perspective of Antibiotic Resistance and Resistance Genes

South Africa is one of the developing country in the southern part of the African continent (Weforum, 2016). The Department of Water Affairs and Forestry (DWAF) reported that as the population size keeps on increasing rapidly, the demand for suitable water also increases (DWAF, 2004). Edokpayi *et al* (2015) stated that the increase in population puts pressure to wastewater treatment plants, as they are required to operate beyond their normal capacity, resulting in the release of large inadequately treated effluents.

There are 986 municipal WWTP's in South Africa (Diallo *et al.,* 2015), hence there is high level of pathogenic bacteria and organic compounds in rivers across the country and these cause health risks to people that use water directly from rivers and other surface water sources (Igwaran *et al.,* 2018). According to Igwaran *et al* (2018 isolates of diarrheagenic *E. coli* that were isolated in the Eastern Cape showed 100%

resistance to erythromycin and 48.6% to meropenem, and this concurred with the increase in the number of people with diarrhoea in the region. This demonstrates why diarrheagenic *E. coli* are regarded as principal causes of diarrhoea which has caused about 1 to 2.5 million deaths per year in developing countries including South Africa (Shabana *et al.,* 2013). In the Gauteng province, 44% enterococci isolates from five WWTP's that serve small town around Vaal catchment area contained tetracycline resistance gene tetM and 41% contained tetL which is another tetracycline resistance gene (Hamiwe *et al.,* 2018). In another study conducted from KwaZulu Natal, it was shown that 34% isolates of *E. coli* were resistance to ampicillin (Beukes and Schimidt, 2018). This demonstrates that antibiotic resistance bacteria and antibiotic resistance genes is a challenge that continue to have major impacts to public health as well as on the national economy. It has huge health burdens that need to be addressed (Canizalez-Roman *et al.,* 2016).

(i) Biochemical Aspect

Some bacteria have chemical processes within their cells that can largely contribute to antibiotic resistance (Michael-Kordatou *et al,* 2018). They have substances that can react with compounds found in antibiotics, thus inactivating the antibiotic, and thereby resulting in reduced effectiveness (Michael-Kordatou *et al.,* 2018). These processes include modification, group transfer and chemical inactivation (Braschi *et al.,* 2010). Target modification is the second known major resistance mechanism, where the target site is modified to inhibit antibiotic molecules from binding, thus the drug is unable to function (Wilson, 2014).

The most diverse family of resistant enzymes is the group of transferases (Blanchard, 2004). These transferases are mostly known for their ability to inactivate antibiotics

(such as aminoglycosides, chloramphenicol etc.) by chemical substitution where acetyl, adenyl or phosphoryl groups are inserted in the antibiotic molecule, thus changing the structure of the antibiotic and resulting in the loss of activity (Schwarz *et al*., 2004). Chemical substitution processes include O and N- acetylation, O-phosphorylation, O- glycosylation and thiol transfer (Matsuoka and Sasaki, 2004). These processes require co-substrates such as ATP, NAD^+ and acetyl-CoA in order to function properly and effectively, hence they are restricted to the cytoplasm (Dzidic *et al.,* 2008).

As bacteria continue to develop several strategies to circumvent the action of antibiotics and render them inactive, they deploy both specific and nonspecific mechanisms through antibiotic inactivation that bring about general innate immunity and enzyme based mechanisms that involves the alteration of the antibiotic structure to an inactive derivative that is incapable of destroying its target (D'Costa and Wright, 2017). These mechanisms include hydrolysis, as most commonly used antibiotics have chemical bonds such as esters and amides which are highly susceptible to hydrolysis.

Extended spectrum beta-lactamases (ESBLs) excrete these enzymes and they inactivate antibiotics by cleaving their chemical bonds (Bonnet, 2004). Some organisms may also act on antibiotics through inactivation via redox process where they exploited oxidation in order to reduce antibiotic activity. One example is the oxidation of tetracycline by TetX enzyme (Yang *et al*., 2004). A specific example of an organism is the *Streptomyces virginiae* which produces type A streptogram in virginiamycin M1. This organism protects itself from the antibiotic by reducing ketone group to alcohol (Shaikh *et al*., 2015).

(ii) Genetic Aspects

In most cases, antibiotic resistant is usually associated with numerous bacterial genetic changes. Bacteria can acquire foreign antibiotic resistant gene from the environment or neighbouring bacteria through mutation and horizontal gene transfer mechanism (Dzidic *et al.,* 2008). Mutation of the gene sequence of the target for some antibiotics results in the decreased effectiveness of the antibiotic. Resistant to antibiotics like fluoroquinolones and rifampin are usually caused by the mutation of the genes encoding the targets of these antibiotics, RNA polymerase beta subunit (RpoB) and DNA-topoisomerases (Ruiz, 2003; Shaikh *et al*., 2015). The difference in the expression of the antibiotic uptake or the one of the efflux system can be mutated (Ko¨ hler *et al*., 1997). For example, when the expression is reduced or there is an absence of the outer membrane porin D (OprD) in *Pseudomonas aeruginosa*, the permeability of the cell wall will be reduced, thereby resulting to the reduced effectiveness of carbapenems (Wolter *et al*., 2004). Another principal mechanism that is well known for the spread of antibiotic resistant gene is horizontal gene transfer (Liao *et al.,* 2019). Genes can be transferred horizontally through a variety of mechanisms which includes transformation, which is the uptake of short fragments of naked DNA by transformable bacteria. These fragments usually come from dead bacteria that release their genetic contents into the surrounding environment (von Wintersdorff *et al.,* 2016). Another mechanism is conjugation, which is the mechanism that involves the transfer of DNA material through sexual pilus and it requires cell to cell contact (Freitas *et al.,* 2017). The DNA transferred by this mechanism often involves plasmids which are circular DNA pieces that can replicate independently of the chromosome (Furuya and Lowy, 2006). Lastly, is the transduction mechanism which is the transfer of DNA from one bacterium to another through bacteriophages (Burmeister, 2015). Bacteriophages lack

the machinery to replicate their own genomes, thus these organisms infect bacteria in order to hijack its replication machinery (Alekshun and Levy, 2007).

1.5.6 Bacteria with Extended-Spectrum Beta-Lactamase (ESBL)

Beta lactamase enzymes that have ESBL activity against majority of beta lactams have evolved over the last decade (Bradford, 2001). An example of these is the CTX-M-I5 which was first identified in *Escherichia coli*, and now is also found in members of *Enterobacteraiceae* and has spread worldwide (Woodford *et al*., 2011). Extended-spectrum beta lactamases are a group of enzymes that have the ability to breakdown antibiotics that belong to penicillin and cephalosporin groups and make them ineffective. These enzymes are encoded by genes that can be easily exchanged between bacteria (Paterson and Bonomo, 2005). Cefotaxime-Munich is currently the most common genetic variant of ESBL. Beta lactamases are classified into Ambler molecular classification and the Bush-Jacoby-Medeiros functional classification (Ambler, 1980; Bush *et al*., 1995).

1.5.6.1 Ambler Molecular Classification

Ambler classified beta lactamases into four classes; named A to D based on their protein homology of enzymes. Class A, C and D are serine beta lactamases while class B enzymes are metallo-beta lactamases (Ambler, 1980).

- CTX-M Beta- Lactamases (class A)

These enzymes were named due to their ability to hydrolyse cefotaxime (Palzkill, 2018). They have been identified mainly in isolates of *E.coli, Salmonella enterica* serovar and other species of *Enterobacteriaceae* (Gazouli *et al*., 1998). These enzymes have a feature that makes them unique from other enzymes such as TEM or

SHV beta lactamases. The CTX-M can be better inhibited by tazobactam than by sulbactam and clavulanate (Bradford *et al*., 1998). CTX-M beta lactamases are found in the functional group two (Bush and Jacoby, 2010) and are thought to originate from chromosomal ESBL genes which are found in *Kluyvera spp*. which is an opportunistic pathogen of *Enterobacteriaceae* widely found in the environment (Bush and Jacoby, 2012).

- TEM Beta-Lactamases (class A)

The TEM originated in 1963 from the name of a patient Temoniera (CDC, 2018). TEM-1 is usually encountered in Gram-negative bacteria and is responsible for 90% of ampicillin resistance in *E.coli* (Cooksey *et al.,* 1990). This beta lactamase is also responsible for penicillin and ampicillin resistance of *Haemophilus influenza* and *Neisseria gonorrhoeae.*TEM-1 has substrate and inhibition profiles similar to those of SHV (Datta and Kontomichalou, 1965).

- SHV Beta-Lactamases (class A)

These enzymes are derived from *Klebsiella spp.* with the common one, SHV-1, found in *K. pneumonia* which is responsible for resistance to broad-spectrum penicillins such as ampicillin, tigecycline and piperacillin (Livermore, 1995). There are more than 60 SHV varieties known, with SHV-5 and SHV-12 being the most common (Paterson *et al*., 2003).

- OXA Beta-Lactamases (class D)

OXA beta lactamases have the ability to hydrolyse oxacillin (Shaikh *et al*., 2015). They are characterized by the rate of hydrolysis for oxacillin and cloxacillin greater than 50% than that of benzyl penicillin, and are poorly inhibited by antibiotics such as clavulanic

acid. OXA beta lactamases have been detected mainly in *P. aeruginosa* isolates (Weldhagen *et al*., 2003) but have also been found in other Gram-negative bacteria like *E. coli.*

1.5.6.2 Bush-Jacoby-Medeiros Functional Classification

This classification is based on functional characteristics of beta lactamases. Functional classification has three major groups where enzymes are grouped by their substrate and inhibitor profiles (Bush *et al*., 1995). This means enzymes are aligned based on their ability to hydrolyse specific classes of beta lactams and also on inactivation properties of beta lactamases inhibitors such as sulbactam, clavulanic acid and tazobactam (Bush *et al*., 1995).

- Group 1 cepholosporinases

This group of enzymes are encoded in many *Enterobacteriaceae* chromosomes and other organisms such as *Pseudomonas sp* (Jacoby, 2009). They are resistant to inhibition by clavulanic acid and more active on cephalosporins (Bauernfeind *et al*., 1999).

- Group 2 serine beta-lactamases

These lactamases are the largest group due to the increasing identification of ESBLs (Kernodle *et al*., 1989). These enzymes hydrolyses benzylpenicillin and many other penicillin derivatives but show poor hydrolysis of carbapenems and monobactams.

- Group 3 metallo-beta-lactamases

This of enzymes is unique to others structurally and functionally (Laraki *et al*., 1999). Structurally they require zinc ions on their active sites and functionally they have a unique ability to hydrolyse carbapenems (Marchiaro *et al*., 2008).

1.5.7 Significance of the Study

Antibiotic residue have been reported to be found in local main water streams, dams and ponds that are used by communities for drinking and other domestic uses (WHO, 2012). This is mainly through human and animal excretion, disposal of unused and expired antibiotics as well as residues that are not completely metabolised (Chander *et al.,* 2016). Wastewater treatment plants were designed to help eliminate this problem, but it has been shown that these plants do not completely remove these residues as they are found in the final effluents after treatment (Jang *et al.,* 2018). This has caused major concerns since it contributes largely to antibiotic resistance which has been responsible for a large number of deaths in the last decade (Shaikh *et al.,* 2015). Therefore, it is important to investigate the prevalence of antibiotic resistant since it has been thought that individuals that take antibiotics to treat infections are at risk of treatment failure due to resistant genes (Bradford, 2001). This limits treatment options and promotes prolonged hospitalization (Hamiwe *et al.,* 2018). Investigating the presence of these ARB and ARG's in water is important as it helps in assessing their potential health risks to the public (Hamiwe *et al.,* 2018). Studying and understanding antimicrobial susceptibility patterns is very important so that it can be easy to identify the shift in resistance patterns of pathogens, thus making it easy to develop control measures that will aid in stopping the spread of these antimicrobials and guide clinicians on antibiotic use (Friedman *et al.,* 2016).

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CHAPTER 2: DETERMINATION OF THE PRESENCE OF ANTIBIOTICS IN WASTEWATER

2.1 INTRODUCTION

The use of pharmaceuticals such as antibiotics is increasing yearly (Chander, 2016). This is due to the continuous improvement of healthcare systems and people trying to extend their life expectancy (Sim *et al.,* 2011). Kummerer (2004) argued that humans consume about 1ton per year of different pharmaceutical drugs with an average worldwide consumption of about 15 g per annum per person, while Derksen *et al.* (2004) noted that there are about 12 000 approved pharmaceuticals used by humans worldwide. Pharmaceutical industries produce about 350 various types of drugs, with approximately thousands of formulations from both large and small scale industries (Vijay, 2012). South African companies hold the number one position in Africa for its production of pharmaceuticals, with 276 companies licensed by the Health Department (Hassen, 2017). Due to this worldwide increase production, pharmaceutical industries produce large volumes of complex non-biodegradable toxic waste which end up in the environment (Chander, 2016). Frederic and Yves (2014) argued that healthcare facilities are also one of the main reservoirs that release antibiotics and bacteria into the environment through wastewater disposal. Wastewater from hospital containing different antibiotic residues enhance the development of antibiotic resistance because of the selective pressure that is placed on bacteria (Orias and Perrodin, 2013). Wastewater treatment plants are constructed to help eliminate antibiotic residues and pathogens in water (Igwaran *et al.,* 2018). However, wastewater treatment plants receives influents from different sources thus creating favourable conditions for the growth of antibiotic resistant bacteria and their genes (Zhang, 2016). Sludge that is also generated from treatment plants is often used as fertilizers for agricultural

purposes which lead to pharmaceutical (antibiotics) compound ending up into the soil or environment (Beausse, 2004).The acquired resistance genes carried by hospital contaminants can be easily transferred to other bacterial populations, and eventually reach water bodies that are used for agriculture or domestic purposes (Lien *et al.,* 2017). The release of these pharmaceutical compounds to the environment may not only lead to antibiotic resistance but also to other serious health implications to humans, animals and the environment at large (Houtman *et al.,* 2014). These include a decline in male fertility, defects in births, and testicular and breast cancer (Nikolaou *et al.,* 2007). The agricultural lands are impacted as their productivity may be lowered. Livestock death is also increasing due to resistance bacteria when they are being treated for disease such as shipping fever, calf scours and mastitis (WHO, 2013). Therefore, it is important to determine the presence of antibiotics in wastewater in order to create awareness and knowledge that will aid in controlling the spread of these antibiotic residues which could results in the development of antibiotic resistant. This may help in lowering and preventing the adverse effects of antibiotic contamination and antibiotic resistance in the community. Thus this study is aimed at identifying the presence of antibiotics and other pharmaceuticals in domestic wastewater.

2.2 Aims and Objectives

2.2.1 Aim

This chapter was aimed at identifying antibiotics and other pharmaceutical residues in domestic wastewater treatment plant in order to determine the extent of their removal by the plant.

2.2.2 Objective

- To determine antibiotic residues of the commonly used antibiotics from the influent and effluent loading of the wastewater treatment plant.

2.3 Materials and Methods

To determine the presence of the antibiotic residues, a 7890A GC system coupled with a 5975C VL MSD with Triple-Axis Detector (Agilent) was used since it is one of the advanced methods that is capable of determining the target compounds to the nanograms per litre level, and is commonly applied for the detection of antibiotics in wastewater and water in general (Fatta *et al*., 2007). This equipment aided in the detection of antibiotics present in the water samples. The selection of this method was dependent on the chemical and physical properties of the antibiotics.

2.3.1 Sample Collection

Samples were collected from Vulindlela Wastewater Treatment Plant. Two hundred millimetres of the influent and 200 millimetres of the final effluent was collected using Schott bottles daily for a period of five days and stored in a cooler box filled with ice and was transported to the laboratory for analysis.

2.3.2 Sample Analysis

Samples were filtered using Whatman filter paper (Meck) to ensure that substances such as papers, plastics and soil particles are not it the samples. For analysis of the presence of antibiotics gas chromatography with mass spectrometry (GC-MS) was used following manufactures manual. In this procedure, an Agilent 190915-433: 325 ºC: 30 m 250 µm 0.25 µm GC column was used and programmed as follows: The initial temperature was kept at 60 ºC for 4 min and then constantly increased by heating at

a rate of 4 ºC/min, all done at an injection temperature and pressure of 250 ºC and 29.127 kPa, respectively. A sample volume of 1.0 µl was manually injected using a 10.0 µl Agilent syringe and a 5975 Data analysis software 37 was used to analyse both the mass spectra and gas chromatograms. The helium (He) and hydrogen (H2) gases were used as a GC mobile phase and the MSD flame gas, respectively (both purchased from AFROX) (Kitson *et al.,* 1996).

2.3.3 Results Interpretation

The GC-MS detected molecules based on their molecular properties as some are hydrophilic while some are hydrophobic. Hydrophilic molecules completely dissolve (strong affinity) in water while hydrophobic molecules have less affinity to water making the detection with the GC-MS difficult. The mass charge ratio (m/z) was determined and compared with the database. The charge (z) was denoted as one (1), therefore, the detected m/z was regarded as the actual mass of the antibiotic (UCLA, 2016). The results were based on a five-day sampling period from the Vulindlela Wastewater Treatment Plant.

2.4 Results and Discussion

Antibiotic residues and their degradation end compounds are continuously found in the environment due to human and animal activities. This has raised concerns worldwide since it contributes to the development of antibiotic resistant bacteria as well as plays a role in spreading the resistance genes (Sebestyen *et al.,* 2018).

In this study the GC-MS produced a spectrum of mass charge ratio where it detected a variety of antibiotics and their residues based on their actual mass and their degradation end products. These antibiotics were identified by comparing the mass of

the detected molecule with the GC-MS database. Figure 1A presents the results of the day one spectrum of influent of the wastewater treatment plant. The spectrum indicated the presence of ampicillin, imipenem and meropenem based on their mass and the mass of their degradation end products mass when compared to the database (De Souza Barbaso *et al.,* 2019). Meropenem was identified by heptan-2-one which is one of its degradation end product (Mendez *et al.,* 2008). Cefotaxime and penicillin were not detected in the influent stage, meaning that the antibiotics were completely metabolised in the human system or they were not present in the influent (Vekey and Telekes, 2008).

ID : Bicyclo[2.2.1]heptan-2-one, 4,7,7-trimethyl-, semicarbazone

A

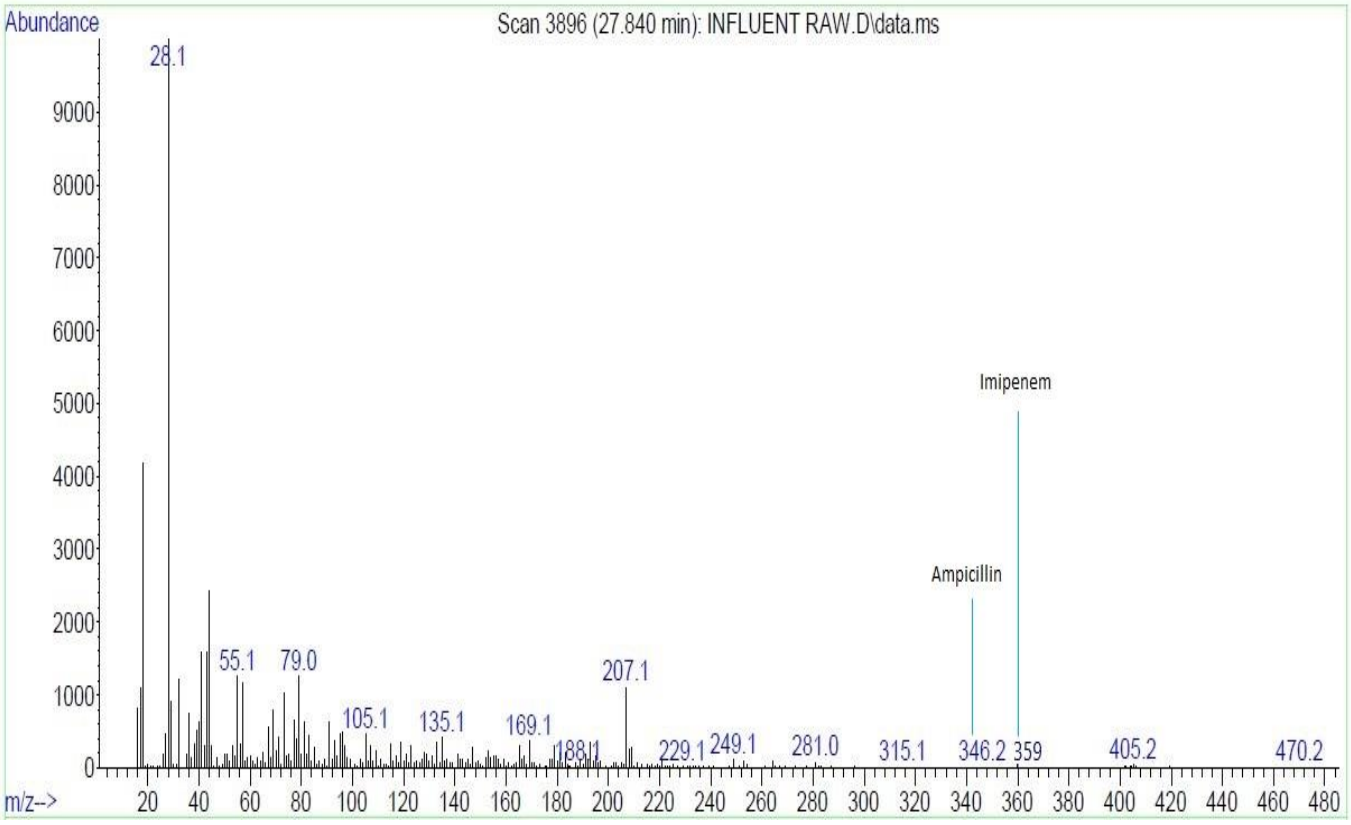

Figure 1A: Spectrum of the samples collected in day one showing antibiotic residues and other compounds from influent

Figure 1B presents the results of the day one spectrum of the final chlorinated effluent. This spectrum had ampicillin, penicillin, imipenem and meropenem. Penicillin was detected in this stage but not present in the influent stage. This may be due to the continuous flow of wastewater that is continuously introduced in the treatment plant or due to de-glucuronidation, where metabolites are transformed back to parent compound (Bouki *et al.,* 2013; Quach-Cu *et al.,* 2018). Other antibiotics residues identified by GC-MS were bicyclo[2,2,1]heptan-2-one, which is a known camphor for topical medication and a veterinary medicine, trimethly a residue of trimethylamine, semicarbazone such as nitrofurazone and thiosemicarbazones which are known to have antiviral and anticancer activities, acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethly-1,2,3,5,6,7,8,8a-octahydronaphthalen-2-ester.

B

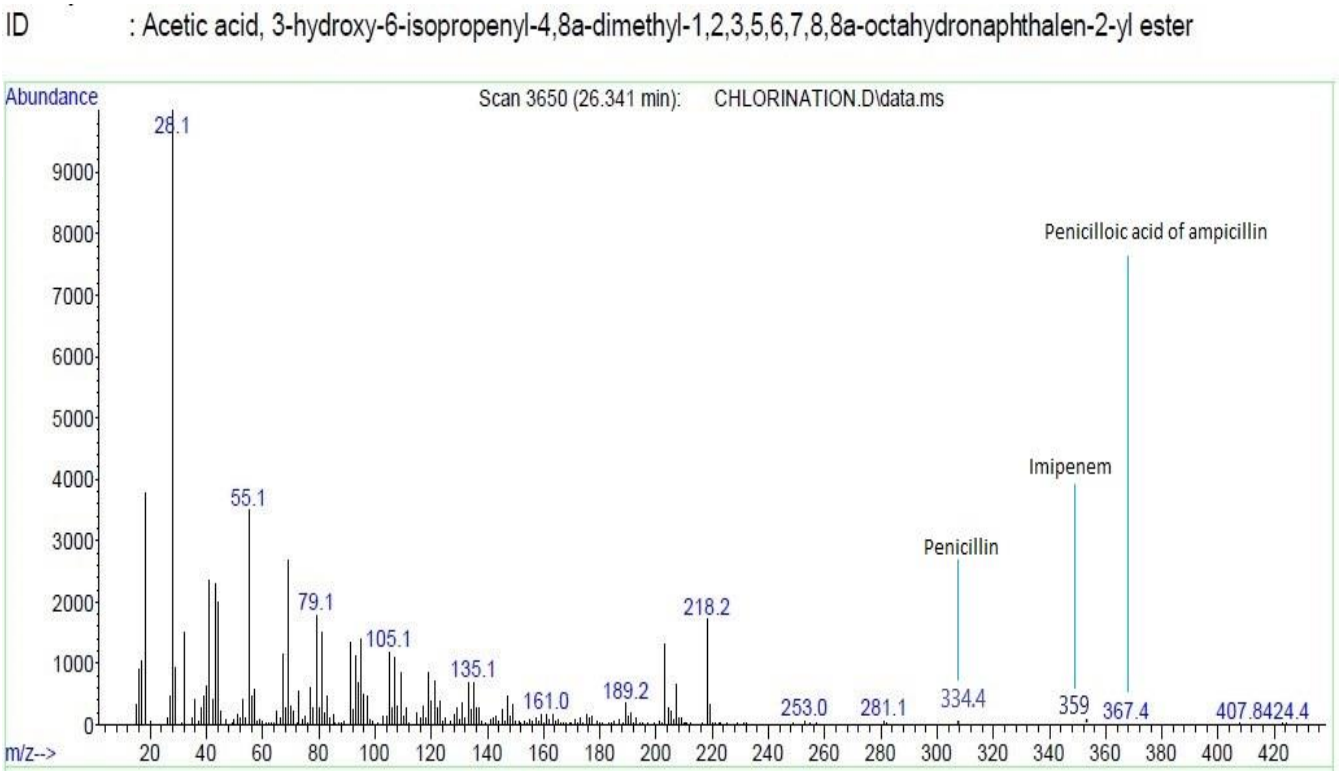

Figure 1B: Spectrum of the samples collected in day one showing antibiotic residues and other compounds from the final effluents after chlorination

Figure 2A presents a spectrum from day two of the influent. Ampicillin, penicillin, imipenem and meropenem were all detected in this influent based on their degradation end product mass and actual mass.

A

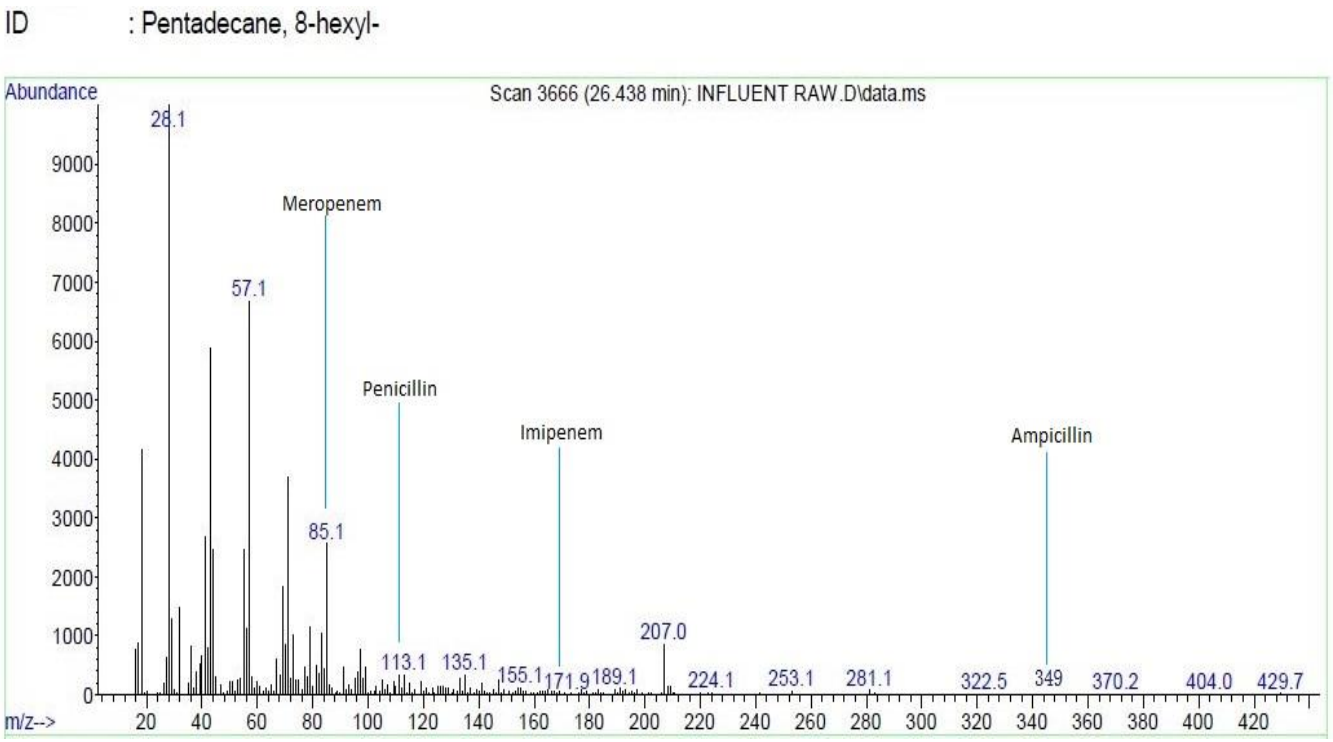

Figure 2A: Spectrums of the samples collected in day two showing antibiotic residues and other compounds from influent

Figure 2B presents the spectrum from day two of the chlorinated effluent. This effluent still contained ampicillin, penicillin, imipenem and meropenem residues even after chlorination (Li *et al.,* 2014). Cefotaxime was not detected in both influent and effluent. This means this antibiotic was completely removed or dissolved in the treatment processes. Other antibiotics residues identified by GC-MS in this effluent were pentadecane, 8-hexyl an alkane that plays a role as an animal metabolite, and propanamide a residue of D-Ala-6-LH-RH propylamide which is used to induce ovulation in amenorrheic patients.

B

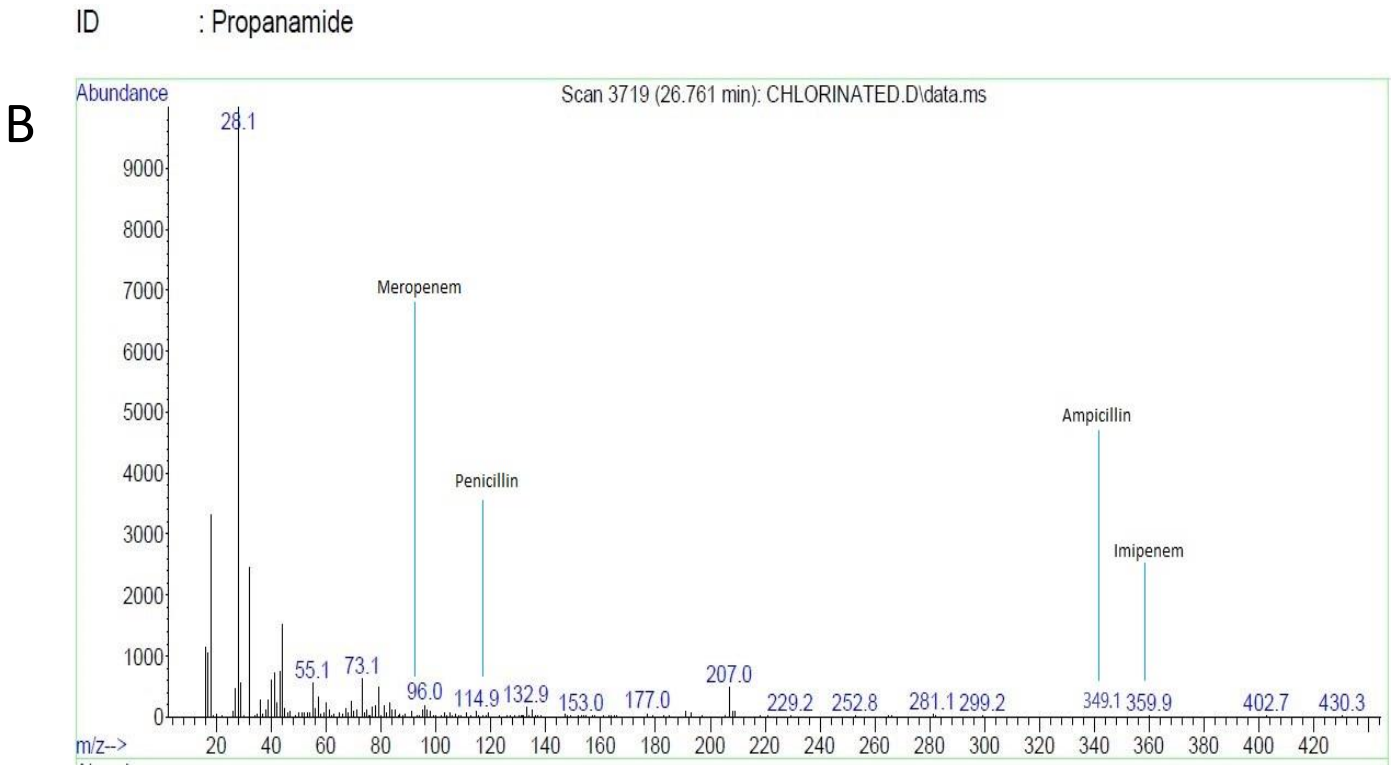

Figure 2B: Spectrums of samples collected in day two showing antibiotic residues and other compounds from final effluents after chlorination

Figure 3A presents a spectrum from day three of the influent of the treatment plant. The spectrum indicated the presence of ampicillin, Imipenem and meropenem by their degradation end products mass such as propyl-sodium, carboxylic acid and disodium salt. Penicillin and cefotaxime were not detected.

A

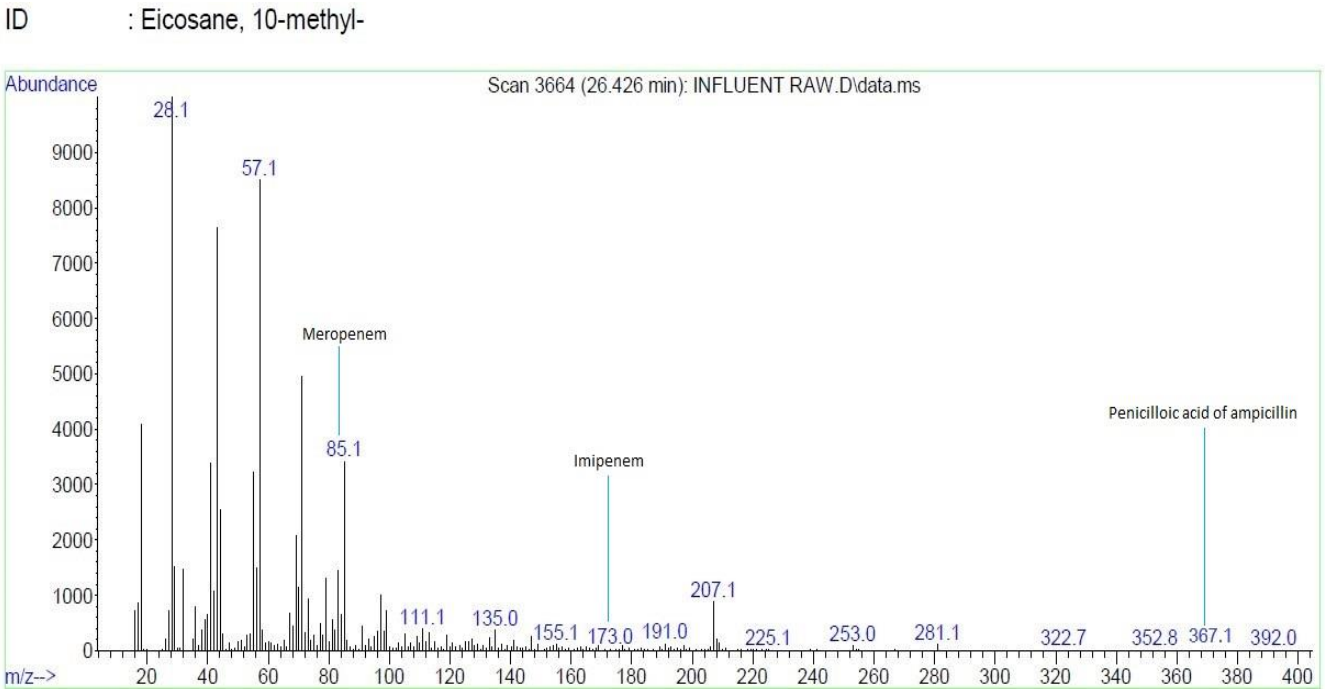

Figure 3A: Spectrums of samples collected in day three showing antibiotic residues and other compounds from influent

Figure 3B presents a spectrum of the final effluent after chlorination. In this effluent, penicillin, imipenem and meropenem were detected (De Souza Barbaso *et al.,* 2019). Ampicillin and cefotaxime were not detected as they were completely removed or not present in the sample. Other detected residues were eicosane, 10-methyl which is used in petrochemical industries, cosmetics and lubricants, and 3-propoxyamphetamine a psychelidic drug which works as a stimulant.

B

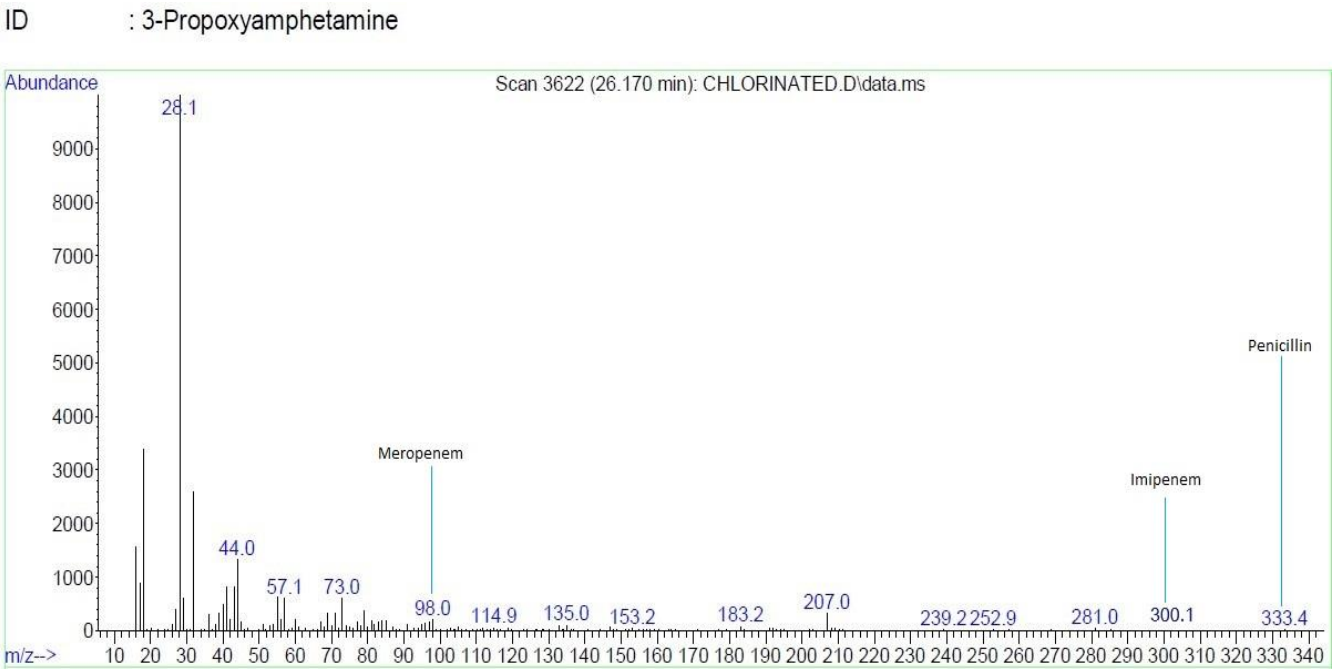

Figure 3B: Spectrums of samples collected in day three showing antibiotic residues and other compounds from final effluents after chlorination

Figure 4A presents the results of day four spectrum of the influent of the wastewater treatment plant. The findings shows the presence of ampicillin and meropenem, while penicillin, imipenem and cefotaxime were not detected. Other identified residue is propanamide.

A

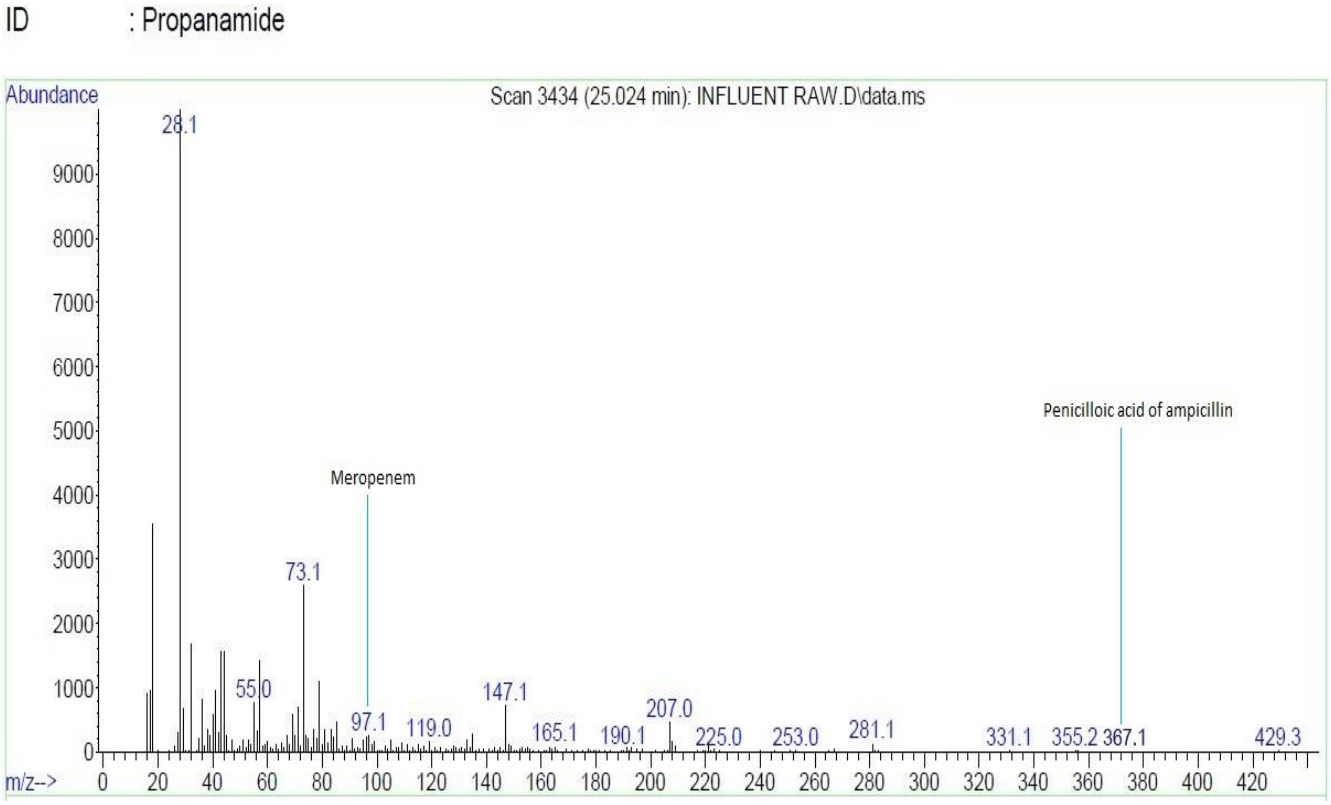

Figure 4A: Spectrums of samples collected in day four showing antibiotic residues and other compounds from influent

Figure 4B presents a spectrum of the final effluent after chlorination, where penicillin, imipenem and meropenem were detected in the sample by their end products (Mendez *et al.,* 2008). Ampicillin and cefotaxime were either completely removed or were not present at all, hence not detected.

B

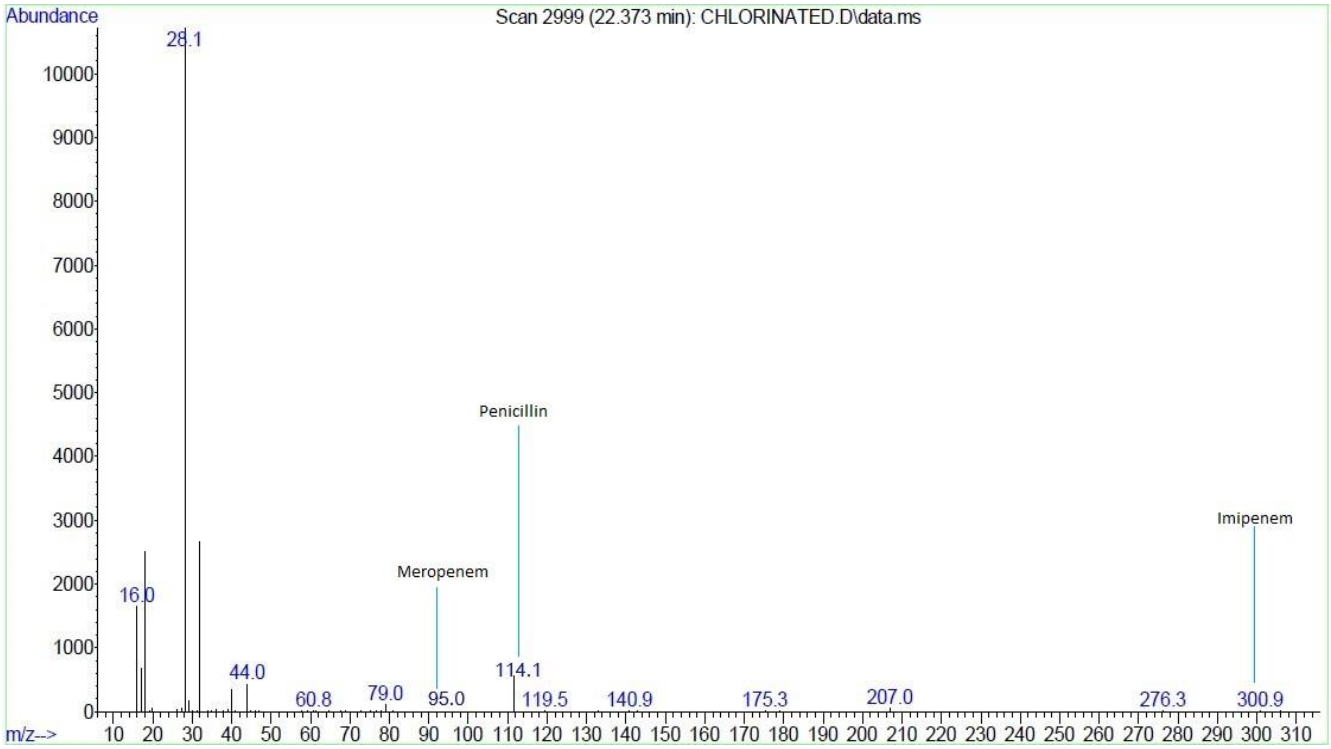

Figure 4B: Spectrums of samples collected in day four showing antibiotic residues and other compounds from final effluents after chlorination

Figure 5A presents a spectrum from day five of the wastewater treatment plant. This spectrum showed the presence of penicillin, imipenem and meropenem in the samples. Ampicillin and cefotaxime were also not detected in this influent.

A

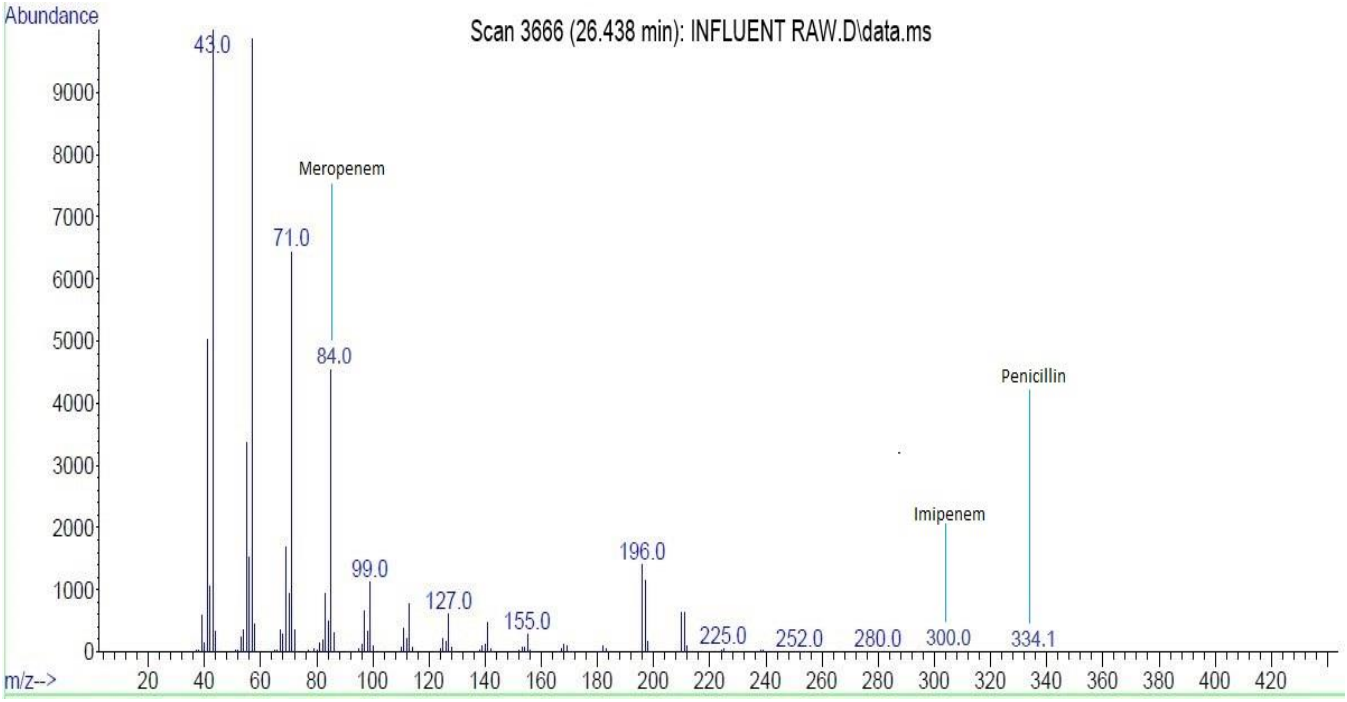

Figure 5A: Spectrums of samples collected in day five showing antibiotic residues and other compounds from initial influent

Figure 5B presents a spectrum of the final effluent after chlorination. This effluent showed the presence of penicillin, imipenem and meropenem based on their end product, while cefotaxime and ampicillin were not detected in this effluent (Li *et al.,* 2014).

B

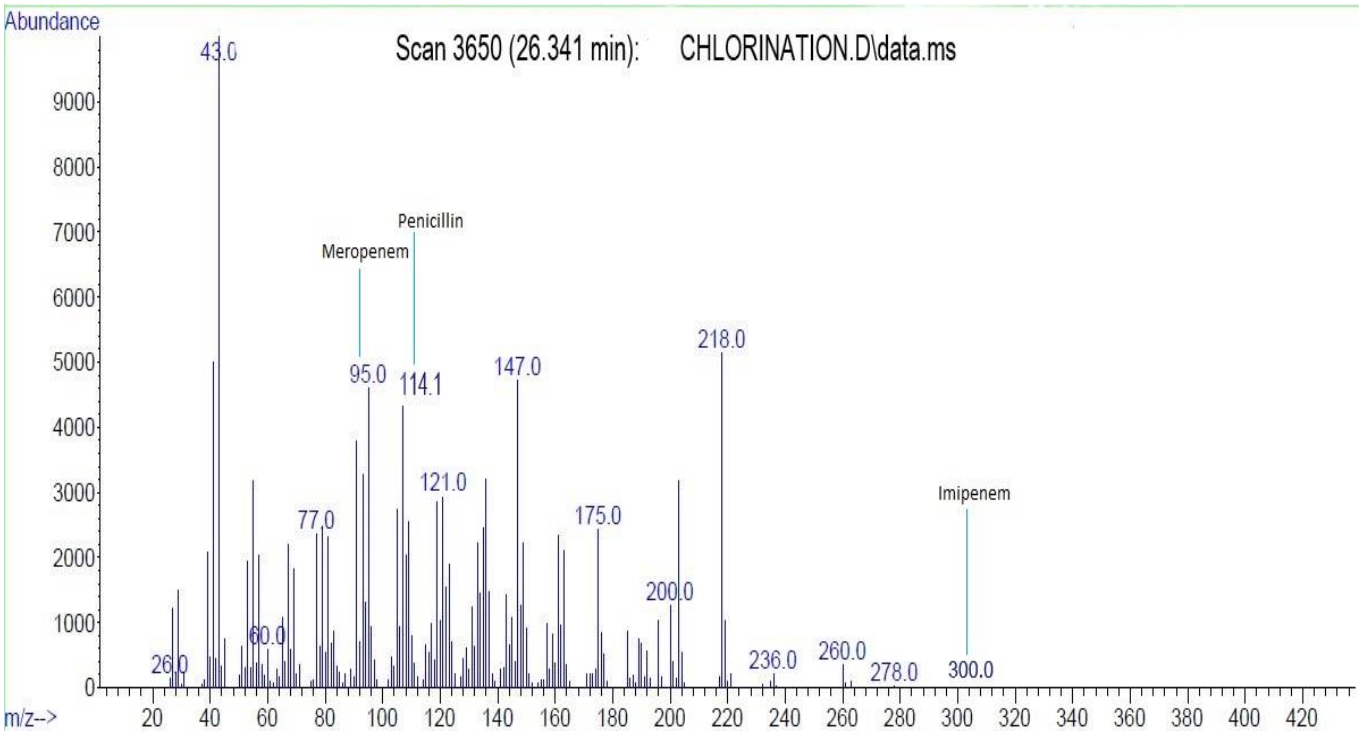

Figure 5B: Spectrums of samples collected in day five showing antibiotic residues and other compounds from final effluents after chlorination

The removal of the pharmaceutical compounds from wastewater during treatment differed depending on compound properties, various environmental conditions and different treatment processes (Diaz-Cruz and Barcelo, 2006). While some antibiotics were completely metabolized or stored in the body, resulting in no traces in the treatment plant (Chander *et al.,* 2016), some were volatile and were completely eliminated by the treatment plant processes (Igwaran *et al.,* 2018). Cefotaxime was not detected in all samples because this antibiotic and its residues are less stable in aqueous solution, thus was quickly degraded and removed during treatment (Berge *et al.,* 1983; Fabre *et al.,* 1984). Some antibiotics were not present in the initial influent stage but were found in the final effluent e.g. penicillin in day 1. This is because some antibiotic metabolites are transformed back to their parent compound under certain pressure conditions in the wastewater treatment procedure through a processes called

de-glucuronidation where there is an addition of glucuronic acid to the metabolites, resulting in the formation of the parent compound (Diaz-Cruz and Barcelo, 2006; Bouki *et al.,* 2013).

The presence of antibiotics in wastewater final effluents has raised many concerns worldwide. Their presence contribute to the poor drinking water quality which could lead to human and animal health implications and the environment is also affected as soil quality decreases (WHO, 2013). Wastewater treatment plants were designed to eliminate these concerns by removing these residues and release effluents that are not a healthy concern to humans, animals and the environment (Jia *et al.,* 2015). However, due to the changing of the environment, microorganisms and the increase in population size, some treatment plants are forced to operate above their normal capacity. This has led to the release of inadequately treated effluents that are in turn used by animals and humans for domestic purposes (WHO, 2017).

Kinge *et al.* (2010) determined the presence of ampicillin in Mmabatho Wastewater Treatment Plant in North West province, South Africa. It was reported that their presence affected the local community as there was a negative effect on antibiotic therapy within the community. Ramsamy *et al.* (2018) detected ampicillin, penicillin, imipenem and meropenem in a five-year surveillance in KwaZulu-Natal, South Africa which played a role in antibiotic resistant trends of ESKAPE pathogens which are implicated in life threatening nosocomial infections that have increased mortality rate and high healthcare costs. Iweriebor *et al*. (2015) further detected penicillin, cefotaxime and imipenem in domestic and hospital wastewater treatment plant in Eastern Cape, South Africa, which contributed in public health implications such as diagnosis dilemma, treatment failure especially to people whose immune system had been compromised. Qui *et al.* (2019) noted the presence of ampicillin and penicillin from

water samples in Huanpu River, China which caused difficulty in initiating treatments, while Hrenovic *et al*. (2016) detected imipenem and meropenem in municipal wastewater treatment plant final effluents in Croatia, and it was reported that these antibiotics contributed to resistance of *Acinetobacter baumannii* to disinfectants. A study conducted by Nikolaou *et al.* (2007) also reported the presence of penicillin in effluents along residential sites which caused major effects to the community of New Mexico as it was thought to contributed to penicillin resistant in the area. Some communities use water from the rivers receiving final effluents for irrigation of crops. This could lead to the indirect consumption of crops with antibiotic residues. Antibiotics are biological active, and this increases the possibilities of unintended effect on non-targeted receptors even at a lower concentrations (Jones *et al.,* 2005). Propoxyamphetamine (Figure 3B) was one of the pharmaceuticals detected in the final effluent which is one of the known psychedelic drugs used for hallucinations (Pubchem, 2017). This highlights some of the possible effects of the presence of these compounds in water. The spectrums showed that there are numerous antibiotic residues that survived the treatment from primary to tertiary stage (Table 1) and these are being released to the environment where some communities use this water with antibiotic traces for their domestic purposes.

Table 1: Summary of the antibiotic residues obtained in the influent and final effluent stages of wastewater treatment plant

	DAY 1		DAY 2		DAY 3		DAY 4		DAY 5	
ANTIBIOTICS	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
Ampicillin	+	+	+	+	+	_	+	_	_	_
Penicillin	_	+	+	+	_	+	_	+	+	+
Imipenem	+	+	+	+	+	+	+	+	+	+
Meropenem	+	+	+	+	+	+	+	+	+	+
Cefotaxime	_	+	_	_	_	_	_	_	_	_

+ denotes the presence of antibiotics

- denotes the absence of antibiotics

The presence of these antibiotic residues is a major worldwide concern as it has shown to be a threat to clinical cases by limiting treatment options for a variety of pathogens because of the development of resistance, thus contributing to treatment failure, longer hospitalization and high hospital costs (Kinge *et al.,* 2010).

2.5 Conclusion

In this chapter, antibiotics residues were detected at different stages of wastewater treatment plant from influent to the final effluent, which are then released to the surrounding environment. This work has shown that wastewater treatment plants may not be efficient enough when it comes to removing antibiotics and their residues from wastewater. Investigating the presence of antibiotic residues is important in alleviating

health implications to communities. Older adults, infants and people with chronic illness are easily affected since they have weak or compromised immune systems. These individuals can be affected even at a very low concentration (Ruhoy and Kaye, 2009). This highlights the importance of the continuous investigation of these residues in treated water. This study detected ampicillin, penicillin, imipenem and meropenem in Vulindlela Wastewater Treatment Plant. The presence of these antibiotics can be a health risk since they can contribute to antibiotic resistance which can in turn cause treatment failure and prolonged hospitalization. This study will create public awareness so that they can practice proper disposal of antibiotics and other chemicals. This will also make municipal and health officials to be aware of the risks posed by antibiotic residues in water, thus drawing attention to the need for developing more effective methods of treating domestic wastewater to target these compounds. The consumption of antibiotics differs from region to region therefore, different water sources should be monitored regularly for antibiotic residues.

2.6 Recommendations

- The liquid chromatography with tandem mass spectrum (LC/MS/MS) can be used as it is more sensitive and can analyse polar, non-volatile and acidic compounds than the GC/MS which is sensitive to volatile compounds.
- More commonly used antibiotics must also be investigated to widen the scope of antibiotic residues in wastewater after treatment.
- Sampling can also be extended to lower lying residential areas that are near the river that receive the effluent from the treatment plant in order to determine the total effects.

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CHAPTER 3: ISOLATION AND IDENTIFICATION OF ANTIBIOTIC RESISTANT BACTERIA AND THEIR GENES IN WASTEWATER

3.1 INTRODUCTION

The greatest medical achievement during the first century was the discovery of antibiotics by Alexander Fleming, where he discovered penicillin, a beta lactam antibiotic which showed high antimicrobial properties (Fleming, 1929). Since then, numerous antibiotics have been discovered and used to treat infections caused by microorganisms to humans and animals (Van Hoek *et al.,* 2011). These antibiotics are classified according to their mechanism of action with some inhibiting cell wall synthesis, protein synthesis etc. (Neu, 1992; Shaikh *et al.,* 2015). Microorganisms have continued to evolve and adapt to changing environmental conditions due to human activities, and this has led to the development of resistance to antibiotics (Van Hoek *et al.,* 2011). Antibiotic resistance is when microorganisms are able to withstand the effects of antibiotics which are designed to eradicate them and their infections (Ha *et al.,* 2019). When microorganisms are not susceptible to more than one antibiotic, they are regarded as multi-drug resistant (Magiorakos *et al.,* 2012). There are numerous human factors that contributes to antibiotic resistance. They include antibiotic misuse, where some individuals over use them and others use low dosages. Self-medication also plays a role in the development of antibiotic resistance, which is the use of antibiotics without professional prescription (Mason *et al.,* 2018). Self-medication give rise to misdiagnosis which could lead to the development of antibiotic resistance (Ha *et al.,* 2019). The concentration effect of antibiotics against a particular microorganism is very important since lower concentrations could lead to the survival of microorganism which could later results to the development of resistance (Coetzee and Bezuidenhout, 2015). World Health Organisation (WHO) (2015) reported that in

most developing countries, people misuse antibiotics by treating infections caused by viruses. The excessive use of antibiotics in agricultural practices can also increase antibiotic resistance in animals and plants, which can be easily transferred to humans through the consumption of livestock and crops (Founou *et al.,* 2016). World Health Organisation also reported that antibiotic resistance is spreading globally and new mechanisms of resistance are emerging. This affects the ability of treating the common infections, which results to prolonged illness, disability and even death (WHO, 2018). Microorganisms use different mechanisms to acquire resistance which include biochemical and genetic mechanisms (Bonnet, 2004). Biochemical mechanisms involve chemical processes and substances within microorganisms that contribute to the development of resistance. Biochemical mechanisms include antibiotic inactivation, inactivation by group transfer and by target modification (D'Costa and Wright, 2017). Research on bacterial pathogens has shown that there are numerous genetic changes in bacteria that lead to antibiotic resistance (Dzidic *et al.,* 2008). Therefore, bacteria can acquire resistance through various genetic mechanisms such as mutation and horizontal gene transfer (Burmeister, 2015).

Horizontal gene transfer is the principal mechanism that is well known in spreading the antibiotic resistance genes (Burmeister, 2015). SHV, cefotaxime-Munich (CTX-M) and TEM, which originated from a patient name Temoniera, are the most common genes encoding for beta lactamases enzymes that are able to break down antibiotics, thus rendering them ineffective, and these genes can be transferred between microorganisms (Bajpai *et al.,* 2017). Due to the continuous over use of beta lactam antibiotics, SHV, CTX-M and TEM genes are the most frequently occurring genes that plays a role in the development of resistance in a variety of microorganisms. Numerous studies in South Africa have reported an increase in resistance of some

microorganisms to commonly used antibiotics (Samie *et al.,* 2007; Kinge *et al.*, 2010; Iweriebor *et al.,* 2015). These studies shows that antibiotic resistance continues to prevail and spread across the country. As the antimicrobial market continue to be under threat due to the rising of antibiotic resistance bacteria, the occurrence of the antibiotic resistance and patterns need to be studied so that physicians, infection control practitioners and the public can rapidly identify bacterial resistance. This will aid in controlling and minimising the spread of antibiotic resistant bacteria and their genes as well as help in the selection of appropriate antibiotics for the treatment and the discovery of new resistant bacterial strains that can be further studied. Studying the occurrence of antimicrobial resistance can also help in developing other alternatives for fighting infections such as using plant extracts and antibiotics consortium (Aiyegoro and Okoh, 2009).

3.2 Aims and Objectives

3.2.1 Aim

The aim of this chapter was to detect the presence of antibiotic resistant bacteria and their genes (CTX-M, TEM, and SHV) in the influent and effluent stage after chlorination at Vulindlela Wastewater Treatment Plant.

3.2.2 Objectives

- To isolate and identify the resistant bacteria against commonly used antibiotics such as penicillin, ampicillin, cefotaxime, imipenem and meropenem.
- To detect the antibiotic resistant encoding genes and identification of the most prevalent gene.

3.3 Materials and Methods

Microorganism were isolated from the water samples and grown overnight on Mueller-Hinton agar. The susceptibility test for the identification of the resistant bacteria was conducted using a disk diffusion method. The Gram staining was used to identify the Gram negative bacteria because the antibiotics used commonly work against the infections caused by Gram negative bacteria. The DNA was extracted using the Quick-DNA micropep plus kit (Zymo Research) and the successful isolation was confirmed by running the agarose gel electrophoresis. The identification of the resistant isolates was conducted using 16S rRNA sequencing while the resistance genes were determined using the PCR amplification with specific primers (Table 2).

Table 2: The primers used in the detection of SHV, CTX-M and TEM resistant genes (Monstein *et al.,* 2007).

Primer name	Sequence (5' – 3')	Amplicon size (bp)
TEM (forward)	5'-TCGCCGCATACACTATTCTCAGAATG-3'	445
TEM (reverse)	5'-ACGCTCACCGGCTCCAGATTTAT-3'	445
CTX-M (forward)	5'-ATGTGCAGYACCAGTAARGTKATGGC-3'	593
CTX-M (reverse)	5'-TGGGTRAARTARGTSACCAGAAYCAGCG-3'	593
SHV (forward)	5'-ATGCGTTATATTCGCCTGTG-3'	747
SHV (reverse)	5'-TGCTTTGTTCGGGCCAA- 3'	747

3.3.1 Sample Collection

The samples were collected as previously explained in Chapter 2. Briefly, the water samples from the influent and effluent of the wastewater treatment plant were collected using the Schott bottles for five days and transported to the laboratory on ice-cold cooler box. In order to establish the occurrence of antibiotic bacteria and their genes in wastewater treatment plant, more than one sample needed to be collected since

wastewater enters the plant continuously. This changes wastewater composition hence the five-day interval was chosen.

3.3.2 Isolation and Purification of Isolates from Wastewater

The spread plates were prepared by spreading 0.1 ml of the water sample on the Mueller Hinton agar and incubated at 37ºC overnight. The colonies from the overnight culture were isolated and streaked onto the Mueller Hinton agar and incubated again at 37ºC overnight. To obtain the pure colonies, the isolated colonies were purified on agar plates using a four way streak method and incubated at 37ºC overnight.

3.3.3 Identification of Bacteria using Gram Staining

This technique helped in the identification of the Gram reaction of the bacteria since the antibiotics of choice are normally used to treat Gram negative bacteria. Thus, this study was targeting Gram negative bacteria. After the procedure, the microscopic examinations were conducted on the glass slide using the oil-immersion objective.

3.3.4 Antibiotic Susceptibility Test

The Kirby-Bauer disk diffusion method was used for the susceptibility testing of the bacteria. The susceptibility testing aided in determining the sensitivity of the microorganism to different antibiotics compounds. This technique is widely used worldwide to select the appropriate treatment against the particular microorganisms. The diameters of the zones of inhibition were measured to the nearest millimetre and were compared with the CLSI standard zones of inhibition.

3.3.5 DNA Extraction of Resistant Bacteria

Pure bacterial isolates were grown overnight in LB medium. After incubation, 600 µl were added to a 1.5 ml microcentrifuge. Following this, 100 µl of 7x lysis buffer was added and mixed by inverting, and then incubated for 1 to 2 minutes at room temperature (15 to 30ºC). Three hundred and fifty microliters of the cold neutralizing buffer was then added and thoroughly mixed and centrifuged for 4 minutes at 11000xg. The supernatant was transferred into the zymo-spin INN column with a collection tube, then centrifuged again for 15 seconds at 11000xg. The flow-through was discarded, then 200 µl of Endo-Wash buffer was added to the column and centrifuged for 30 seconds. Four hundred microliters of zyppy wash buffer was added to the column and centrifuged for 1 minute. The column was transferred into a clean 1.5 ml microcentrifuge tube and then 30 µl of zippy elution buffer was directly added to the column matrix and incubated at room temperature for 1 minute, and centrifuged for 30 seconds to elute the plasmid DNA (Zymoresearch, 2019). The success of the extraction of the DNA was confirmed by visualizing using the gel electrophoresis.

3.3.6 Gel Electrophoresis

For running the gel electrophoresis, 1% agarose gel was prepared by dissolving 1.5 g of agarose in 150 ml of 1x TAE buffer (4.84 g of Tris base, 2 ml of 0.5 M EDTA and 1.142 ml of glacial acetic acid) and boiled in a microwave for 5 minutes. After boiling, 15 µl of ethidium bromide was added. The gel was poured in a gel tray with 20 well-comb for making wells and cooled at a room temperature. After cooling, the DNA was loaded in the wells where, 5 µl was mixed with 1 µl DNA loading dye (Thermofisher). 3 µl of DNA ladder (100-1000 bp) (Thermofisher) was also loaded as a maker. The gel

was run at 100 volts for 30 minutes, and was then viewed using the Vilber Smart Imaging System (Inqaba biotec).

3.3.7 Polymerase Chain Reaction (PCR) Amplification of Resistance Gene

The direct PCR amplification was performed on the DNA extracted using group-specific PCR primers (Table 2). These primers were specific to the antibiotic resistance genes of interest. The PCR reactions were carried out following the order components in Table 3. The polymerase chain reaction was conducted under cycling parameters provided in Table 4 using the Eppendorf thermal cycler (Merck). The polymerase chain reaction products were visualized using the gel electrophoresis.

Table 3: The concentrations and volumes of polymerase chain reaction components used for the amplification of resistance genes in wastewater samples

Components	**Volume**
Forward primer	2 µl
Reverse primer	2 µl
DNTPs	2 µl
$MgCl_2$	1.5 µl
One Taq standard reaction buffer	5 µl
Template DNA	2 µl
One Taq DNA polymerase	1 µl
Nuclease-Free water	34,5 µl

Table 4: The polymerase chain reaction cycling parameters used in the amplification of the antibiotic resistant genes in wastewater samples

Process	**Temperature (^{0}C)**	**Time**	**Number of cycles**
Initial denaturation	50	2 minutes	1
Denaturation	95	10 minutes	1
Annealing	95	15 seconds	45
Extension	60	60 seconds	1

3.3.8 Identification of Resistant Bacteria by Sequencing the 16S rRNA

The genomic DNA of the resistant cultures were extracted using the Quick-DNA fungal/bacterial miniprep kit from Zymo Research. The 16S target region was amplified using OneTaq Quick-Load 2X Master Mix using the primers shown in Table 5.

Table 5: The primer sequences of the 16S rRNA used in the identification of the resistant bacterial isolates

Name of primer	**Target**	**Sequence (5' to 3')**
16S-27F	16S rRNA sequence	AGAGTTTGATCMTGGCTCAG
16S-1492R	16S rRNA sequence	CGGTTACCTTGTTACGACTT

The PCR products were run on a gel, and the gel was later extracted with Zymo-clean Gel DNA Recovery Kit. The extracted fragments were sequenced in the forward and reverse direction using a Nimagen, Brilliant Dye Terminator Cycle Sequencing Kit v3.1 and purified using ZR-96 DNA sequencing Clean-up Kit. The purified fragments were analysed using ABI 3500XL Generic Analyzer. The CLC Bio Main Workbench v7.3 was used to analyse the ab1 files generated by the ABI 3500XL Generic Analyzer, and the results were obtained using a BLAST method.

3.4 Results and Discussion

South Africa is one of the countries that are faced with poor water quality, and this has been implicated with numerous health effects. The Department of Water Affairs and Forestry (DWAF) identified the reuse of the daily discharged treated wastewater into the environment as one of the primary water source (DWAF, 2004). However, approximately 80% of wastewater especially in developing countries like South Africa are discharged without adequate treatment and this has led to the potential release of infectious water contaminants including bacteria and antibiotics, which could lead to

health risks to the public (Hamilton *et al.,* 2007; UN-Water, 2017). Wastewater treatment plants are a collection point for antibiotic resistant bacteria and resistance genes since influents from different sources end up in the treatment plant, thus creating an ideal environment for the development of resistant bacteria and the transfer of resistance genes (Pruden *et al.,* 2013).

3.4.1 Determining Antibiotic Resistant Bacteria using Susceptibility Testing

Pure colonies were successfully grown on Muller-Hinton agar. This study was targeting the Gram negative bacteria, therefore, the Gram staining reaction method was performed. The Gram reaction showed that the purified colonies were Gram negative *Bacillus* (rod shaped) since they retained the secondary stain (red/pink) (Figure 6). It is important to identify the Gram reaction of these microorganisms since the antibiotics tested were beta lactam antibiotics, and are commonly used to fight infections caused by Gram negative bacteria (Leboffe and Pierce, 2015; Shaikh *et al.,* 2015). Woodford *et al.* (2011) noted multiple resistance in Gram negative bacteria, and this played a role in the dissemination of antibiotic resistance.

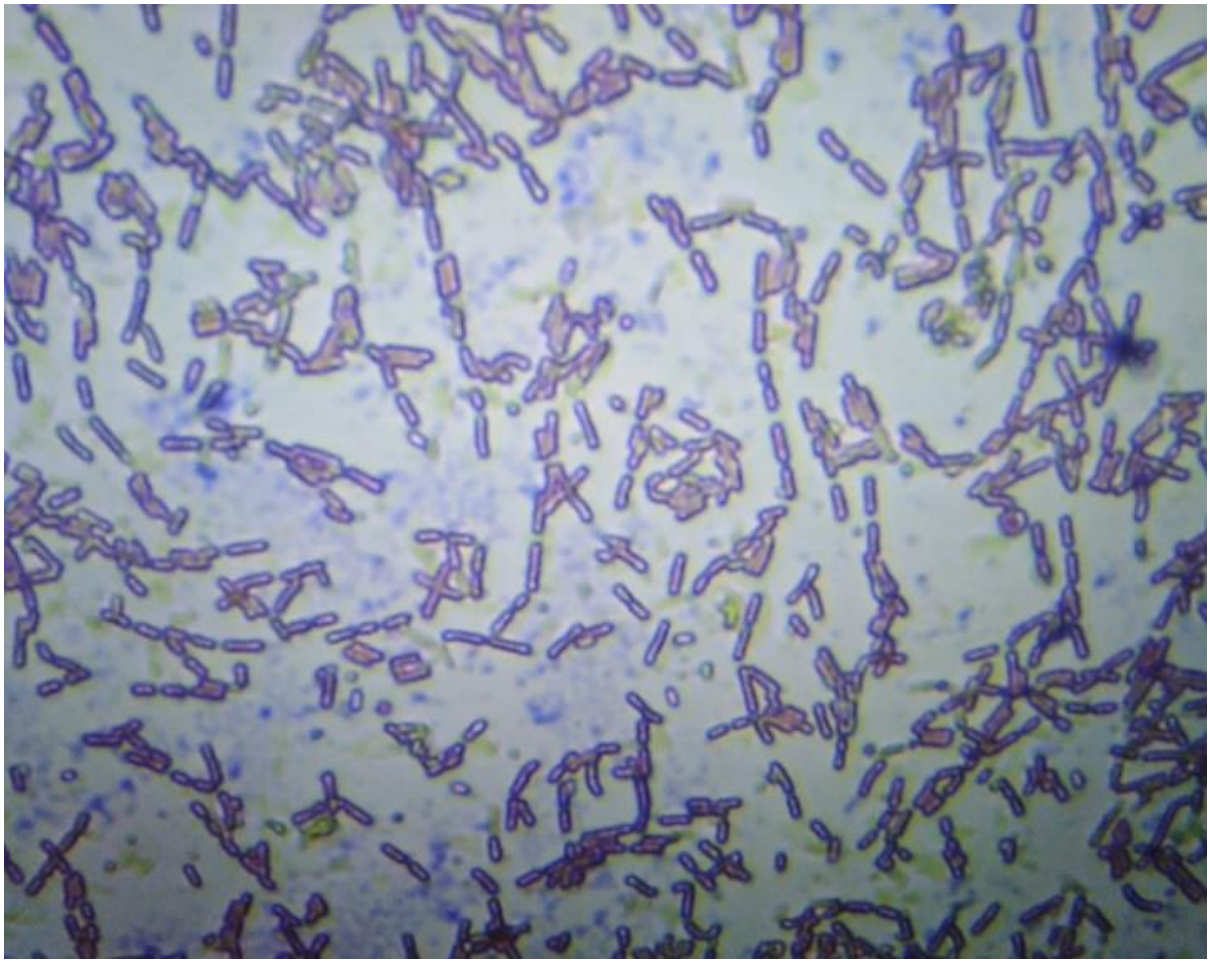

Figure 6: Gram staining of the Gram negative bacteria which are the bacteria that have retained the secondary dye and appear red/pink

The antibiotic resistance test showed different zones of inhibitions for different antibiotics used. These zones were compared to the standard zones of inhibition from the Clinical and Laboratory Standards Institute guidelines (2013). The isolates collected from the influent for five-day showed resistance to penicillin, cefotaxime, imipenem and meropenem while they had intermediate resistance to ampicillin (Table 6).

Table 6: Susceptibility testing of the five antibiotics against the influent isolates

Antibiotics	Influent susceptibility testing				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Penicillin	Resistant (14 mm)	Resistant (12 mm)	Resistant (11 mm)	Resistant (13 mm)	Resistant (15 mm)
Ampicillin	Intermediate (15 mm)	Intermediate (16 mm)	Intermediate (14 mm)	Intermediate (16 mm)	Intermediate (15 mm)
Cefotaxime	Resistant (20 mm)	Resistant (19 mm)	Resistant (21 mm)	Resistant (22 mm)	Resistant (18 mm)
Meropenem	Resistant (No zone)	Resistant (No zone)	Resistant (No zone)	Resistant (No zone)	Resistant (No zone)
Imipenem	Resistant (6 mm)	Resistant (4 mm)	Resistant (4 mm)	Resistant (3 mm)	Resistant (3 mm)

The Isolates from the five-day effluent after treatment showed the increased resistance against penicillin, meropenem and imipenem. This was caused by the continuous adaption of microorganisms against these particular antibiotics. The isolates were susceptible to ampicillin and had intermediate resistance to cefotaxime (Table 7).

Table 7: Susceptibility test of the five antibiotics against the effluent isolates

Antibiotics	Effluent susceptibility testing				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Penicillin	Resistant (No zone)	Resistant (No zone)	Resistant (No zone)	Resistant (No zone)	Resistant (No zone)
Ampicillin	Intermediate (16 mm)	Susceptible (20 mm)	Susceptible (19 mm)	Susceptible (18 mm)	Susceptible (22 mm)
Cefotaxime	Resistant (22 mm)	Resistant (19 mm)	Intermediate (23 mm)	Susceptible (26 mm)	Intermediate (25 mm)
Meropenem	Resistant (No zone)	Resistant (No zone)	Resistant (No zone)	Resistant (No zone)	Resistant (No zone)
Imipenem	Resistant (No zone)	Resistant (No zone)	Resistant (No zone)	Resistant (No zone)	Resistant (No zone)

Similar findings were reported by Lien *et al.* (2017) where they detected resistance to imipenem in wastewater influents. Their study showed that the resistance contributed in the development of multidrug resistance of *Escherichia spp* to ceftriaxone, amoxicillin and ceftazidime. In a study of tracking antibiotics in wastewater, Holzel *et al*. (2010) found the resistance of *Escherichia coli, Enterococcus faecium* and *Enterococcus faecalis* to ampicillin and intermediate resistance to imipenem from sludge. For five days, individual isolates tested from the influent in this study were resistant to cefotaxime. Isolates from day 1 and 2 of the effluent also showed resistance to cefotaxime, while day 3 and 5 isolates showed intermediate resistance. The isolates collected in day 4 were susceptible to cefotaxime. Luczkiewics *et al.* (2010) also detected the resistance of *Escherichia coli* and *Enterococcus faecalis* to cefotaxime in wastewater treatment that treats water for a population of about 570 000 people. The Resistance to cefotaxime has been implicated to diarrhea, fever and

abdominal pain (Manyi-Loh *et al.,* 2018). The Isolates from both the influent and effluent were all resistant to penicillin, imipenem and meropenem. This shows that microorganisms that are either resistant before being introduced to the treatment plant or those that developed resistance during treatment processes are not completely removed and are released to the environment where they can transfer resistance genes to other microbial communities, resulting in drastic effect to the environment, humans and animals Similar findings by Ramsamy *et al.* (2018) were reported where resistance of *Enterococcus faecium* to ampicillin, penicillin, imipenem and meropenem in KwaZulu-Natal was detected and it was implicated to life threatening nosocomial infections. Obi *et al.* (2004) also observed resistance of *Escherichia coli* to common antibiotics such as ampicillin, penicillin and tetracycline and this resistance caused diarrhoea in rural Venda communities in South Africa. Penicillin resistance is also implicated with allergic hypersensitivity reaction ranging from mild skin rash to life threatening anaphylaxis (Olatoye *et al.,* 2016). All these studies accord with this study which also detected resistance in penicillin, cefotaxime, meropenem and imipenem in wastewater influents and effluents and this can cause serious health problems to humans, animals and the environment. In this study, the tested isolates in the influent were resistance to penicillin and imipenem with zones of inhibitions of 11-15 mm for penicillin and 3-6 mm for imipenem, but in the effluent, the tested isolates were completely resistant to these antibiotics with no zones of inhibition observed. The changes in zones of inhibitions shows that if microorganisms are continuously exposed to antibiotics, they end up adapting and withstanding the effects of antibiotics. Samie *et al.* (2007) reported a 25% to 53% increase in antibiotic resistance of *Campylobacter jejuni* in Vhembe district, South Africa from 2002 to 2007, and this resistance was found to be an important agent of diarrhoea. A study by Kinge *et al.* (2010) also accord

with this study where the increase of resistance from 10% to 80% to ampicillin was observed in the wastewater treatment plant in the North West province, South Africa. This shows that microorganisms continue to develop more resistance during treatment process as they try to protect themselves from the pressure put upon them during the treatment processes (Rizzo *et al.,* 2013). Resistance of microorganisms does not only impact human health, but also possess a threat to animals and the environment. Caldwell *et al.* (2008) reported the decrease in reproductive success in fishes that are exposed to wastewater effluents that contains antibiotic residues and resistant bacteria. Communities from rural areas use effluents released to the river streams for irrigation of their crops. This impacts the soil quality and contributes to the resistance of the soil microorganisms which leads to decrease in productions and may indirectly affect humans who consume these crops especially elders and infants who have weak and compromised immune systems (Samie *et al.,* 2007; Chen *et al.,* 2011). Schwaiger *et al.* (2004) further reported toxic effect caused by diclofenac in fish. Diclofenac is a commonly used drug by humans to treat inflammatory diseases such as gout. It was reported that this drug was found in the liver, muscle tissues, gills and kidney of trout fish. This shows that prolonged exposure to antibiotics and other pharmaceuticals by the environment can lead to numerous health conditions in animals, humans and can also affect the environment (Touraud *et al.,* 2011).

3.4.2 Identification of Resistant Bacteria and Resistance Genes

The resistant bacterial isolates were characterised by sequencing the 16S rDNA, and BLAST results obtained corresponded with the sequences in the NCBI database which predicted that the resistant microorganism was *Bacillus cereus* for all the tested isolates. Most conducted researches on the resistant bacteria in wastewater treatment plants identified *Escherichia, Enterobacter, Staphylococcus, Pseudomonas,*

Enterococcus and *Campylobacter sp* to be the most prevalent (Samie *et al.*, 2007; Obi *et al.,* 2004; Ramsamy *et al.*, 2018). In contrast, this study identified a different microorganism that is usually not found to be resistant in wastewater treatment plants. The identified microorganism could lead serious health problems if it enters the food chain, as it could contribute to therapy failure when treating infections caused by *Bacillus cereus*. The transfer of resistant genes to other microorganisms can also occur, thus contributing to the spread of resistance to antibiotic which possess major threat to humans, animals and the environment. This also shows that wastewater treatment plants are not hundred percent efficient, pointing out the need of improving the treatment plant or developing other alternative treatments. These results shows that microorganisms continues to evolve and acquire resistance as they are subjected to various environmental conditions and also highlights the importance of the continuous investigation of resistance microorganisms in wastewater to identify new strains (Van Hoek *et al.,* 2011). The early identification of new resistance strains can aid in preventing the spread of their genes, thus avoiding future complications.

The extracted DNA of the resistant *Bacillus cereus* (Figure 7) was successfully amplified in a polymerase chain reaction in order to amplify genes that were encoding for the resistance of this microorganism. The specific primers used aided in amplification of the SHV, CTX-M and TEM genes.

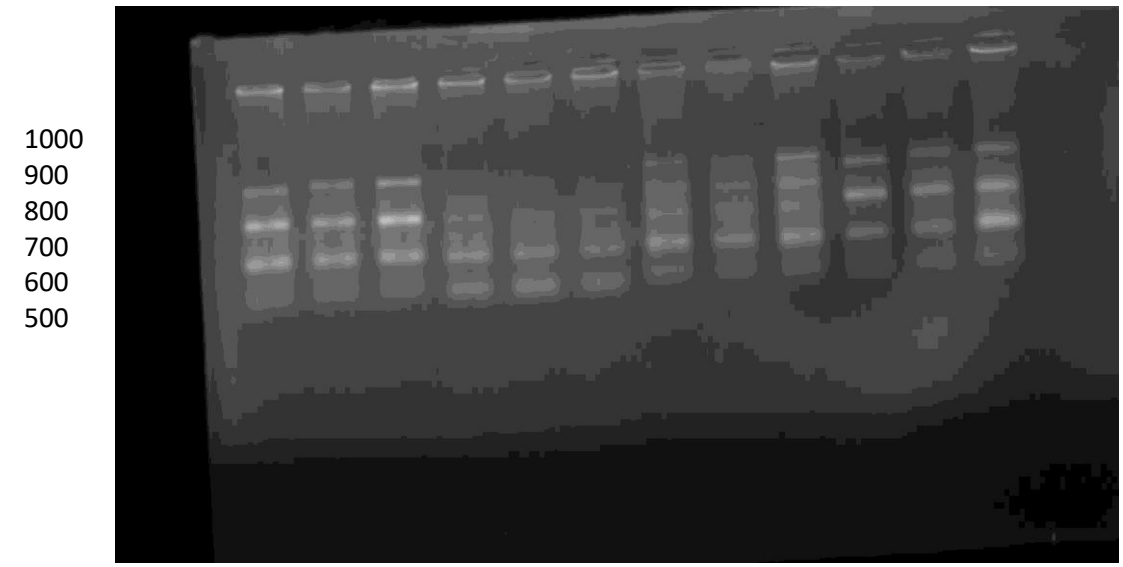

Figure 7: Gel electrophoresis image confirming the extracted DNA of the resistant microorganism

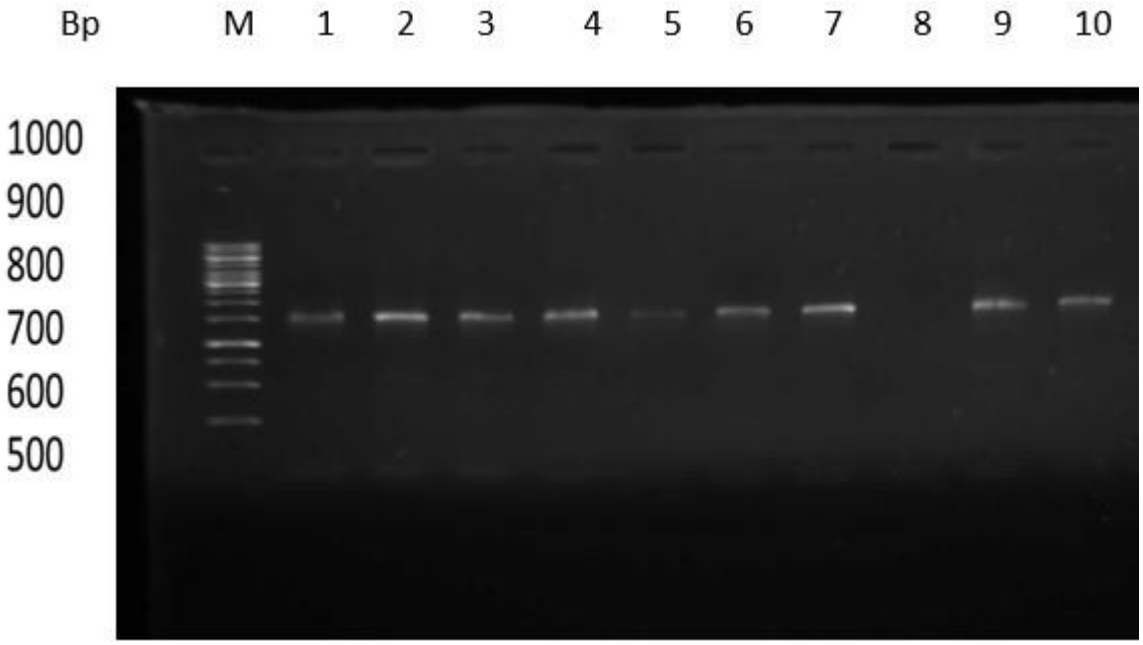

Figure 8: Gel electrophoresis image confirming the polymerase chain reaction amplification of the SHV gene. Lane 1 to 5 representing influents from day 1 to 5, while 6 to 10 represents effluents from day 1 to 5

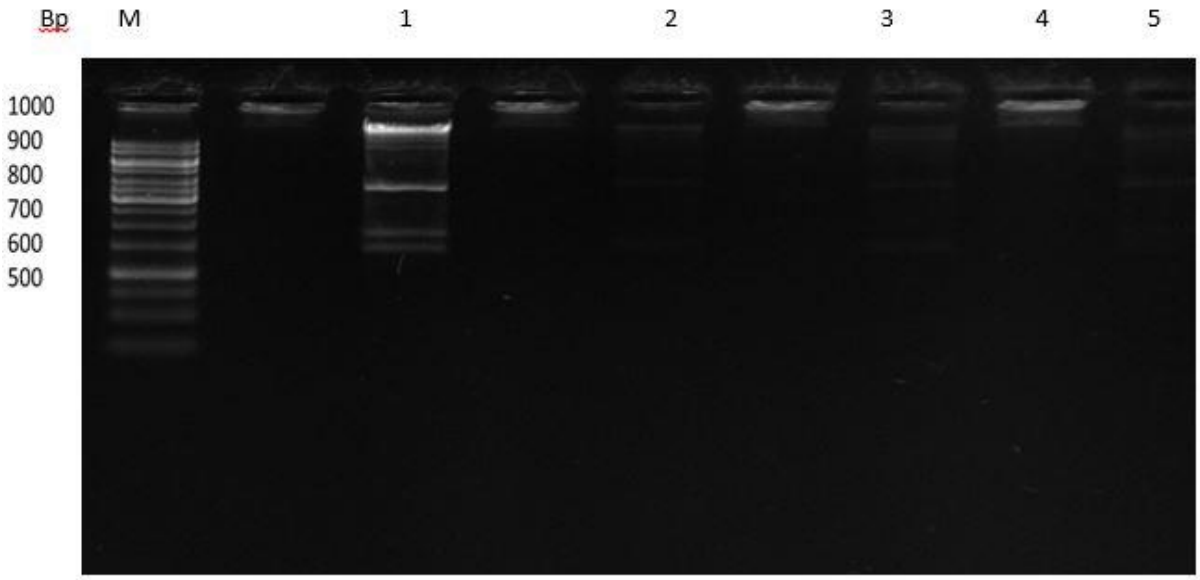

Figure 9: Gel electrophoresis image confirming the polymerase chain reaction amplification of the CTX-M gene. Lane 1 to 5 represent genes detected in the effluents of the wastewater treatment plant

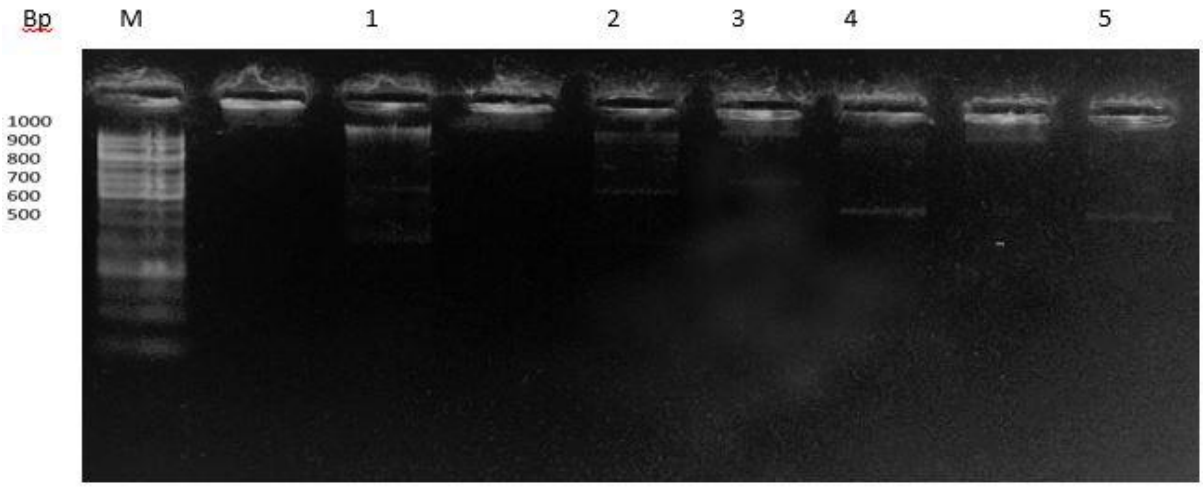

Figure 10: Gel electrophoresis image confirming the polymerase chain reaction amplification of the TEM gene. Lane 1 to 5 represent genes detected in the effluents of the wastewater treatment plant

The SHV gene was identified with amplicon of 747 bp (Figure 8) while CTX-M (Figure 9) and TEM (Figure 10) of amplicons, 593 bp and 445 bp respectively were also

identified (Monstein *et al.,* 2007). The SHV gene was detected in all influent isolates and only in four effluent isolates. The SHV gene was not detected in one isolate of the effluent, this could be due to the continuous feed of the influent to the treatment system. The detection of this gene shows that resistant microorganisms survives the treatment processes and can end up being released to the environment. The CTX-M and TEM genes were only detected in the effluent. This shows that during treatment processes susceptible microorganisms develops or acquire resistance genes in order to survive treatment processes. The detected genes are mostly found in beta lactamases and are known to be responsible for the resistance capabilities of beta lactams. Due to the continuous misuse of antibiotics the SHV, CTX-M and TEM genes are occurring frequently and these has led to the development of resistance in a variety of microorganisms. The genes detected in this study contributed to the multidrug resistance of *Bacillus cereus* to penicillin, cefotaxime, meropenem and imipenem. Multidrug resistance is a major threat to the treatment of infections as it contributes largely to treatment failure. Laffite *et al.* (2016) obtained similar findings where TEM, CTX-M and SHV genes were detected in wastewater effluents and urban rivers in the Democratic Republic of Congo. These genes were reported to be responsible for multidrug resistance of *Escherichia coli, Enterococcus sp.* and *Pseudomonas sp.* Veldsman *et al.* (2017) also detected SHV, TEM and CTX-M in 56 isolates in Pretoria Academic Hospital, South Africa, and these genes made it difficult to select antibiotics for the treatment of infections caused by *Klebsiella sp., Enterobacter sp.* and *E*scherichia *coli.* These studies accord with this study which also detected the SHV, CTX-M and TEM genes in wastewater samples. The presence of these genes may not only contribute to resistance of *Bacillus cereus* but can also be transferred to other microorganisms through horizontal gene transfer. Soge *et al.* (2009) further identified

TEM gene in drinking water which was implicated to a variety of nosocomial infections, arthritis and neonatal sepsis and the gene was also transferred to other microorganisms. In this work, the SHV gene was identified as the most prevalent gene as it was detected in all isolates tested followed by CTX-M and TEM genes. Lyimo *et al.* (2016) also detected the prevalence of the SHV gene in drinking water in Tanzania and this gene was reported to be responsible to a series of diarrhoea cases and urinary tract infections. However, numerous studies detected the CTX-M as the most prevalent resistant gene followed by the TEM gene then lastly the SHV gene (Laffite *et al.,* 2016; Veldsman *et al.,* 2017). This shows that microorganism's resistance varies depending on the environment, thus highlighting the importance of the continuous investigation of the resistance microorganisms and their genes in different environments and communities. Resistant genes from the wastewater treatment plants effluents can end up in the soil through surface and underground water run offs, thus contributing to the spread and development of resistant bacteria. Gao *et al.* (2015) found CTX-M gene in *Escherichia coli* isolates from the soil and pig farm samples. Study by Igbinosa and obuekwe. (2014); Adesoji *et al.* (2015) further reported the presence of TEM gene in the influent and effluent of treated water, which caused resistance of *Pseudomonas sp*. This resistance caused serious health problems which includes infections of the blood, urinary tract infections and pneumonia (Manyi-Loh *et al.,* 2018). This work showed that the treated wastewater effluents still contains microorganisms that could be a threat in humans, animals and the environment. Animals are affected when exposed to the effluents containing antibiotic residues and antibiotic resistant bacteria as reports have shown that their productivity decreases. Animals exposed to resistant bacteria can play a role in spreading resistance bacteria and their genes to humans through the food chain and the environment through excretion and death (Karkman *et*

al., 2018). Antibiotic resistance genes in wastewater treatment plants, drinking and surface water were also detected in Durban and Mafikeng, South Africa and this was reported to cause gastroenteritis, listeriosis and wound infections (Mulamattathil *et al.,* 2014; Olaniran *et al.,* 2015). Antibiotic resistance continues to prevail worldwide and has been regarded by the United Nations as one of the global health risks that requires urgent intervention (UN, 2016). This health risk largely effects low and middle income communities where resources are limited but it is more detrimental in rural areas where treated effluents, surface and underground water are used for domestic purposes, drinking and agriculture (Lien *et al.,* 2017). Results from this study highlights the importance of studying the emergence antibiotic resistance strains in order to be able to manage infections caused by resistant microorganisms and also be able stop the spread of resistance genes.

3.5 Conclusion

Antibiotic resistant bacteria and genes in wastewater treatment plant were detected in this chapter. These were SHV, CTX-M and TEM genes which contributed in the resistant of *Bacillus cereus* to penicillin, cefotaxime, meropenem and imipenem. This shows that treatment plants may not be efficient in removing the life threatening resistant microorganisms. The release of effluents that are not properly treated have adverse effects to humans especially those with compromised immune systems including elders and children aged three years and younger. This study highlighted the importance of investigating the presence of antibiotic resistance bacteria and their genes in order to raise awareness that could aid in avoiding complications that arises to the resistant microorganisms. The complications are human based only as the environment and animals are also impacted. This work also showed that microorganisms continue to acquire resistant genes as previously susceptible

microorganisms are now resistant to antibiotics. Therefore, it is of paramount importance to continue assessing the prevalence of this genes in order to avoid complications that might be unpleasant to humans, animals and environment.

3.6 Recommendations

- More research needs to be conducted in wastewater treatment plants from diverse communities to identify what triggers the development of resistance during treatment processes.
- Suitable methods for analysing antibiotic resistance gene transfer under real wastewater treatment plants conditions need to be developed.
- More studies on resistance microorganisms in animals needs to be conducted especially livestock that are consumed by humans.

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CHAPTER 4: EXECUTIVE DISCUSSION, CONCLUSION AND RECOMMENDATIONS

The use of antibiotics and other pharmaceutical compounds in treating infections caused by common microorganisms continue to rise worldwide. The population continues to increase, and this has led to the increase in the development and production of antibiotics, thus also increasing the inappropriate use of these compounds (Van Boeckel *et al.,* 2015). The misuse of antibiotics has led to the contamination of water streams and the soil with antibiotics and their residues. Wastewater treatment plants were designed to help eliminate water contamination by removing antibiotics and other pharmaceutical contaminants. However, research has indicated that these treatment plants do not completely remove these antibiotic residues since they still become available in the treated effluents (Jang *et al.,* 2018). The release of effluents with antibiotic traces has raised concerns to the public health because of their contribution to the development of antibiotic resistance (Shaikh *et al.,* 2015).

4.1 The Occurrence of Antibiotics in Wastewater and the Environment

Antibiotics are introduced into the environment through different anthropogenic activities (Venter *et al.,* 2017). This include the direct disposal of expired and unused antibiotics (Christou *et al.,* 2017). Most antibiotics are not completely metabolized in human and animal systems. A study by Zhang *et al*. (2014) found that 30 to 90% of most antibiotics are excreted from humans and animals through urine and fecal matter. Through these processes, antibiotics end in wastewater treatment plants and the environment. Antibiotics also end up on the soil through the use of pesticides, manure and when sludge from wastewater treatment plants is used as fertilizer (Martin *et al.,*

2012). These also end up in wastewater through surface and underground run offs. Hospitals, clinics and pharmaceutical industries are regarded as the main reservoirs that release antibiotic contaminants through disposal of large amounts of inadequately treated and untreated effluents to the environment (Chander *et al.,* 2014). In this study area, most of the communities do not have proper disposal facilities and sanitation resulting in dumping their waste on the surrounding environment, which end up in water sources which in turn they use it for irrigation and some domestic purposes.

4.2 Effects of Antibiotic Contaminated Effluents to Humans, animals and the Environment

The use of wastewater for irrigation plays a vital role in introducing antibiotics in the soil. The uptake of this antibiotic contaminated wastewater by vegetation may lead to the introduction of antibiotic into the human food chain which has been reported to cause negative health effects (Prosser and Sibley, 2015). One major health effect is the development of resistant microorganisms, where human associated susceptible pathogenic microorganisms becomes resistant by acquiring resistant genes from the environment or other resistant microorganisms (Berendonk *et al.,* 2015). The Center for Disease Control and Prevention reported that about 23 000 people die yearly as a result of infections caused by antibiotic resistant microorganisms (CDC, 2018). It is estimated that the number will increase to 300 million by 2050 (Munita and Arias (2016). Continuous exposure to antibiotic contaminants also affect various animal populations. Yu *et al.* (2011) observed the growth defects in nematodes following their exposure to antibiotics. Livestock death has also increased due to resistance bacteria when they are treated for diseases such shipping fever (WHO, 2013). Antibiotics that end up in soil through disposal, excretion and irrigation disturbs the soil biota, and this is associated with the decreased in soil quality (Becerra-Castro *et al.,* 2015). This work

has also concord with numerous researches by showing the occurrence of antibiotics in wastewater after treatment processes. This will aid in elevating the concerns around the release of antibiotics contaminated effluents, which has been shown to cause detrimental effects to human, animals and the environment. Most studies are conducted in urban or semi urban areas. However, this study was conducted in the rural environment, this will aid in extending the knowledge to the under privilege communities thus decreasing the number of deaths caused by resistant microorganisms.

4.3 Conclusion

In this work, the presence of antibiotics in wastewater before and after treatment was identified. This showed that wastewater treatment plants do not completely remove antibiotics and their residues, thus results to the presence of antibiotic residues and antibiotic resistant genes in the environment. One major concern implicated with the release of inadequately treated wastewater is the development of antibiotic resistant microorganisms. In this work, resistance to penicillin, meropenem, imipenem and cefotaxime were detected in the influent and effluent of the Vulindlela Wastewater Treatment Plant. There are numerous concerns regarding the release of antibiotic contaminated effluent and the release of resistant microorganism to the environment. These effluents can enter the food chain and caused serious health effects to humans especially those with compromised or not fully developed immune system. This study also highlighted the importance of developing other alternative measures to treat influents containing antibiotic residues. The work will create public awareness since the community was not aware of the development and the emergence of resistance microorganisms, and now will start practicing proper and safe disposal of antibiotics. This work will also contribute to the body of knowledge since health practitioners from

surrounding clinics and hospitals will now be aware of the presence of resistant microorganisms and will start paying attention to patient with prolonged hospitalization which could be due to resistance microorganism. The microorganisms identified in this study are not commonly known to be resistance to antibiotics and are usually destroyed during wastewater treatment processes. This highlights the need for continuous investigation of resistance microorganisms since these organisms continue to acquire and develop resistance. These microorganisms need to be identified and studied continuously in order prevent the health effects to humans, animals and the environment. This also shows the significance of improving wastewater treatment plants and/or possible developing the alternatives to address the challenges of the wastewater contaminated with antibiotics and pharmaceuticals compounds.

4.4 Recommendations

- More antibiotics that are commonly used to treat common infections need to be studied so that the occurrence, transfer and spread of resistant genes can be halted.
- This type of research needs to be continuous because microorganisms continue to evolve and new mechanisms of resistance continue to arise.
- The use of more advanced methods such as LC/MS/MS to detect antibiotic residues can yield more results that can be further analyzed such as the abundance of the detected compounds.

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