FATE OF ANTIBIOTIC RESISTANT *ENTEROCOCCI* AND SELECTED TETRACYCLINE RESISTANCE GENES DURING WASTEWATER TREATMENT

BY

LEKITA SINGH

Submitted in fulfilment of the academic requirements for the degree of Master of Science (MSc) in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science at the University of KwaZulu-Natal (Westville Campus), Durban.

As the supervisor of the candidate, I approve this dissertation for submission

Signed: _____ Name: _____ Date: _____

PREFACE

The experimental work described in this dissertation was carried out in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science at the University of KwaZulu-Natal (Westville Campus), Durban, South Africa from March 2014 – May 2016, under the supervision of Prof. A.O. Olaniran.

These studies represent original work of the author and have not been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

DECLARATION 1– PLAGIARISM

I, Lekita Singh declare that:

1. The research reported in this dissertation except where otherwise indicated, is my original research.
2. This dissertation has not been submitted for any degree or examination at any other University.
3. This dissertation does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced or adapted from other persons.
4. This dissertation does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other sources have been quoted, then:

a. Their words have been re-written but the general information attributed to them has been referenced.

b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This dissertation does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the reference sections

Signed

……

Declaration Plagiarism 22/05/08 FHDR Approved

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

DECLARATION 2– PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this dissertation (include publications in preparation, submitted, in press and published and give details of the contributions of each authors to the experimental work and writing of each publication).

Publication 1

NOT APPLICABLE

Signed:

……

Declaration Plagiarism 22/05/08 FHDR Approved

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

"Most people say that it is the intellect which makes a great scientist. They are wrong: it is character" - Albert Einstein

The challenging, yet amazing journey of this Masters degree has expanded and enriched not only my intellectual treasure, but most importantly my character. The scientist I have become today are due to the great efforts of the many remarkable people that are special to me. To these people I am forever grateful.

- First and foremost, God for blessing my every academic and personal voyage.
- Prof. A.O. Olaniran for project supervision, support and guidance. Prof Olaniran contributed to a rewarding Masters experience by allowing me intellectual freedom in my research, mentoring me, supporting my attendance at various conferences and academic endeavors, engaging me in new ideas and being a constant source of cheer and encouragement.
- Prof. B. Pillay for support and guidance at conferences and various academic ventures
- The National Research Foundation and The Medical Research Council for their financial support.
- My parents, Mr and Mrs Singh, brother, sister, grandparents and Kaveshen Govindasamy, for their constant love, support and motivation. They have been my greatest pillar of strength and inspiration.
- All the staff and students at the Discipline of Microbiology, University of KwaZulu-Natal, Westville campus for their support, assistance and encouragement
- My friends and senior students of Lab 4 (Joash Govindsamy, Po-Cheng Tang, Ashmita Arjoon, Leanne Pillay, Ejovwokoghene Collins Odajare, Ndumiso Mkize, Noyise

Ntoshobeni and Ajit Kanwal) for assistance, encouragement and their endless bounds of cheer and motivation

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The problem of antibiotic resistance has been deemed as a 'serious threat' by the World Health Organization and continues to be a cause for concern, worldwide. Antibiotics have played a critical, yet remarkable role in the clinical management of bacterial diseases and have had an astounding effect on human mortality. However, the effectiveness and wide availability led to the misuse and overuse of antibiotics, giving rise to antibiotic resistant bacteria (ARB). *Enterococci* are a group of clinically significant bacteria that have gained much attention as a result of their antibiotic resistance. Wastewater treatment plants (WWTPs) have been implicated as the leading reservoir for ARB and antibiotic resistance genes (ARGs). The main objective of this study was to ascertain the role of WWTPs in Durban, South Africa as potential reservoirs for antibiotic resistant *Enterococci* (ARE) and their related ARGs. Using membrane filtration technique, *Enterococcus* selective agar and selected antibiotics, ARE were enumerated in samples (influent, activated sludge, before chlorination and final effluent) collected from two WWTPs, as well as from upstream and downstream of the receiving surface water. Two hundred *Enterococcus* isolates recovered from the treated effluent and receiving surface water were identified by biochemical and PCR-based methods, and their antibiotic resistance profiles determined. Molecular detection and quantification of selected tetracycline resistance genes was conducted by conventional PCR and droplet digital PCR (ddPCR), respectively, on the metagenomic DNA isolated from the water samples. The highest count of tetracycline resistant *Enterococci* was obtained at both WWTPs with 618000 CFU/100ml and 735000 CFU/100ml in the influent samples for WWTP1 and WWTP2, respectively. These values were significantly decreased by up to 99% in the final effluent samples of both WWTPs.

Antibiotic resistant *Enterococci* also decreased from upstream to downstream samples in the receiving water bodies of both WWTPs. Two hundred selected isolates were identified as *E. faecalis* at 34.5 %, followed by *E. faecium* and *E. hirea* at 26% and 25.5%, respectively. The antibiotic susceptibility testing revealed that 83% of the identified *Enterococci* isolates (n=200) were multidrug resistant. Multidrug resistance pose a serious threat to public health in effectively treating infectious diseases, often resulting in limited and extended treatment options. This problem is often associated with *Enterococci* gaining tetracycline resistance. Tetracycline is listed as an essential medicine by the World Health Organization and is used as first line therapy for many diseases. Five tetracycline resistance genes (*tet* K, *tet* L, *tet* M, *tet* O, *tet* S) were detected in the selected isolates by means of a multiplex PCR, with *tet* M being the most prevalent at 49%. Absolute quantification of selected tetracycline resistance genes from metagenomic DNA samples (influent and final effluent) using ddPCR showed that both WWTPs removed more than 82% of *tet* M and *tet* O genes during the treatment process. Findings from this study validates the high efficiency of both WWTPs in removing ARE and tetracycline resistance genes in wastewater influent during the treatment process. Despite this high efficiency during the treatment process, as well as the observed decrease from upstream to downstream of the receiving rivers, the presence of ARE and tetracycline resistance genes in the final treated effluent is a cause for concern. To prevent WWTPs from being a leading reservoir for ARB and ARGs, it is recommended that stringent treatment processes as well as tertiary treatment is followed in order to discharge final effluent that contains low levels of ARB and ARGs into the environment.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Antibiotics, a class of antimicrobial agents that are capable of killing or inhibiting the growth of bacteria, have been deemed a triumphant pharmaceutical agent in human and animal therapy (Bouki *et al*., 2013). They are generally used as treatment agents against infections in humans and animals and as growth promoters in animal farming (Jury *et al*., 2010). However, due to factors such as over-use, misuse, lack of education and bacterial evolution, antibiotic resistance has become a major problem in the clinical world. The World Health Organization (WHO) recently regarded the problem of antibiotic resistance as being "so serious that it threatens the achievements of modern medicine" (WHO, 2014b). Some localities have been described as reservoirs for antibiotic resistance as they provide environments that are conducive for the growth, spread and prevalence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs).

Major hotspots that are considered reservoirs of ARB and ARGs are hospitals and clinical settings, the gastrointestinal tract (GIT) of humans and animals, some food sources and wastewater treatment plants (WWTPs). Antibiotic resistant bacteria found in hospital settings are of particular concern as these microorganisms are usually multidrug resistant (Mulvey and Simor, 2009). The high prevalence of ARB in hospitals usually occurs as a result of high antibiotic selective pressure. Other reasons may involve the spread of resistant bacteria from patients that were infected with a resistant organism prior to admission to hospital. It may also be due to the easy route of contamination in surgical operations as well as the frequent spread of infections among patients as a result of inappropriate hygiene practices (Mulvey and Simor, 2009). The GIT of humans and animals are known to be a major reservoir of ARB, as the GIT

of most mammals inhabits a vast number of commensal bacteria (Silva *et al*., 2011). This symbiotic relationship between the bacteria and their host, allows one organism to benefit while the other is unaffected. Invasion of the host's intestine by bacteria causes the immune system of the host to screen pathogenic microorganisms from commensal microorganisms and eliminate the pathogenic microorganisms. Commensal microorganisms are commonly involved in host nutrition and health, promoting nutrient delivery, inhibiting pathogen colonization and sustaining intestinal immune system homeostasis (Silva *et al*., 2011). The GIT is considered to be a major reservoir of ARB as it provides an optimal environment for the spread of ARB and ARGs. This is as a result of the high bacterial populations present, thus allowing for horizontal gene transfer to easily and frequently take place (Huddleston, 2014). Factors that favour the spread of ARB and ARGs within the GIT are high cell density and antibiotic selective pressure. Many studies have shown that WWTPs are the leading reservoir for ARB and ARGs (Gallert *et al*., 2005; Kümmerer, 2009; Martínez, 2009). High levels of antibiotics enter WWTPs as a result of incomplete metabolism by humans or direct disposal of antibiotics (Nagulapally *et al*., 2009). Several studies have shown the presence of ARB and ARGs in wastewater, even in final treated effluent, despite conventional treatment processes followed by most WWTPs (Adams *et al*., 2002; Davies, 2012).

One clinically important organism that has gained much attention for its intrinsic and acquired antibiotic resistance are the *Enterococci* (Hollenbeck and Rice, 2012), which are a group of Gram positive, facultative anaerobic bacteria. They are cocci (spherical) in shape; occur as single cells, in pairs or as short chains (Ciftci *et al*., 2009). These organisms are non-spore forming, yet are tolerant to a wide range of environmental conditions such as, extreme temperature ranging from 10-45 °C, extreme pH ranging from 4.5-10.0 and high sodium chloride concentrations (Fisher and Phillips, 2009). The predominant species of *Enterococci*

cultured from humans, resulting in more than 90% of clinical infections, are *Enterococcus faecalis* and *Enterococcus faecium*. *Enterococcus avium*, *Enterococcus gallinarum*, *Enterococcus casseliflavus*, *Enterococcus durans*, *Enterococcus raffinosus* and *Enterococcus mundtii* are other enterococcal species that lead to human infections, including bacteraemia, urinary tract infections, peritonitis and surgical site infections (de Perio *et al*., 2006). In western countries, *Enterococci* have been found to be the second most common cause of nosocomial urinary tract infections and the third most common cause of nosocomial bacteraemia (Praharaj *et al*., 2013). Studies have shown that *Enterococci* are the third most commonly isolated nosocomial pathogen, accounting for 12% of all hospital infections (Hidron *et al*., 2008). With increasing antibiotic resistance, *Enterococci* are recognized as harmful nosocomial pathogens that can be difficult to treat (Fraser, 2016). A key reason associated with the longevity of *Enterococcus* in hospital settings, as well as the high incidence of enterococcal infections in humans, is due to their inherent ability to acquire antibiotic resistance (intrinsic resistance) to many commonly used antibiotics, as well as their ability to develop resistance to currently used antibiotics. They are able to acquire this resistance by means of mutation or via transfer of plasmids and transposons (Clevel, 1990). *Enterococci* are also commonly found in the intestinal microbiota of humans and animals and are widely distributed in food products (Klein, 2003).

This chapter provides an overview of the major hotspots for ARB, particularly antibiotic resistant *Enterococci* (ARE), and the associated ARGs. Wastewater treatment plants being indicated as hotspots of ARE and ARGs, and the prevalence of ARE and associated ARGs in treated effluent are highlighted. Finally, common treatment processes for efficient removal of ARB and ARGs, as well as the fate of ARE during wastewater treatment processes are discussed.

1.1. Major hotspots for antibiotic resistant *Enterococci* (ARE) and antibiotic resistance genes (ARGs)

1.1.1. Hospitals and clinical settings

Enterococci are commonly associated with nosocomial infections and are most common in intensive care units (Olawale *et al*., 2011). Hospital effluent consist of both medical and non-medical related waste, including those from the emergency, operating, research, pharmaceutical, kitchen and laundry activities (Majlesinasr, 1998). Hospital effluents consist of pathogens, medicine, antibiotics, chemicals, pharmaceutical runoff and organic matter (Radha, 2009). Such an environment provides organisms with optimal conditions to grow and thrive in. The resulting high bacterial load allows for easy transmission of genes between bacteria.

One common pathogen resident to hospital settings are *Enterococci*, which are commonly found in the gastrointestinal tract (GIT) of humans and animals and are the major cause of hospital-acquired infections (Shankar *et al*., 2002). These infections include bacteraemia, urinary tract infections, endocarditis and wound infections (Sava *et al.,* 2010). Treatment of infection caused by these bacteria are complex as they have intrinsic resistance and can easily acquire resistance to many antibiotics (Mohanty *et al.,* 2006). Resistance to high levels of aminoglycosides, beta lactams, glycopeptides and vancomycin pose a challenge to effective treatment of infections (Oberoi *et al.,* 2010). The selective pressure of antibiotics in a hospital setting may be the main reason for the selection of ARE.

Vancomycin resistant *Enterococci* (VRE) are common in hospitals and lead to many cases of colonization and infection of patients (Mutters *et al.,* 2013). Infections caused by VRE are problematic within a hospital, especially in patients that are immunosuppressed (Neely and Maley, 2000). Vancomycin resistance occurs by the modification of the bacterial cell-wall to

prevent binding of glycopeptides. Acquisition of clinically relevant, plasmid-associated genes, *vanA* or *vanB* encodes for vancomycin resistance in *Enterococci* (Leclercq and Courvalin, 1997). Nosocomial infections may be attributed to contamination of VRE or contact with contaminated medical equipment (Bonten *et al*., 1996).

The spread of VRE is difficult to control in hospital settings and can easily cause outbreaks. An example of one such outbreak occurred in 1997, through an electric ear-probe thermometer (Porwancher *et al*., 1997). In another incident, 34 patients, hospitalized in the hematology-oncology children's wards, were infected with VRE. Tests showed the origin of a single outbreak strain, on a video game console in one of the affected wards **(**Drews *et al*., 2008). The transfer of ARB between people and the surrounding environment within a hospital is largely due to the ability of the organism to thrive on a specific environmental surface. Antibiotic resistant *Enterococci* have the ability to survive on a number of surfaces, such as glass and countertops (Bonilla *et al*., 1996; Neely and Maley, 2000), bed rails and stethoscopes (Noskin *et al*., 1995; Neely and Maley, 2000). Neely and Maley (2000), reported that *Enterococci* as well as VRE, were able to survive on different types of fabrics. Additionally, they reported that the privacy drapes (made of polyester), which were touched by both staff and patients, housed *Enterococci* for days to months, proposing that it could be a reservoir for ARE (Neely and Maley, 2000).

1.1.2. **Gastrointestinal tract of mammals**

Various microbial communities are natural inhabitants of humans and animals. These microbial communities colonize various parts of the body, including the skin, oral cavity, upper respiratory tract, vagina and GIT (Lebreton *et al*., 2014). Unique physicochemical and histological characteristics featured by each body part, offers a selective environment for

various microorganisms to adapt and inhabit. The GIT contains the greatest number and largest diversity of microorganisms (Ley *et al*., 2006), with the human colon containing approximately 10^{12} different bacteria per gram of content (Sartor, 2008). The GIT is known to act as a reservoir for the transfer and spread of antibiotic resistance (Patel, 2003).

Bacteria belonging to the genus *Enterococci* are natural residents of the GIT of mammals and more than 90% of healthy humans are hosts to *Enterococci*. These bacteria are part of the commensal flora of humans and animals, and have been associated with hospital acquired infections, such as endocarditis, bacteremia and neonatal infections, since the 1970s (Gunasekera and Perera, 2007). Other infections caused by *Enterococci* are urinary tract infections, surgical wound infections, neonatal sepsis and meningitis (Fisher and Phillips, 2009). *Enterococci* are predominant in the small and large intestine of humans, yet constitute a mere 1% of the gut microflora (Eckburg *et al*., 2005; Hayashi *et al*., 2005). They are also found in human and animal feces, oral cavity and sometimes in the stomach (Smyth *et al*., 1987; Bik *et al.*, 2005). *Enterococci* form part of the normal intestinal flora of humans with values up to 10^8 CFU/g in stool (Noble, 1978; Huycke *et al.*, 1998), with *E. faecalis* and *E. faecium* as the common species found in human feces (Tannock and Cook, 2002). Layton *et al*. (2010) reported large quantities of *Enterococci* species in human feces with the most common also being *E. faecalis* and *E. faecium*. Along with human adults, colonization of the GIT of newborn babies also occurs either during or soon after birth (Mackie *et al*., 1999). *Enterococci* are commonly among the first microorganisms to colonize the gut of an infant as early as the first day of life (Orrhage and Nord, 1999; Fanaro *et al*., 2003). These bacteria are transferred from breast milk, vaginal and gastrointestinal flora, or the environment. The GIT of animals is one of the greatest reservoirs for *Enterococci* (Gilmore *et al*., 2013). The most common species isolated from pigs, cats and dogs were *E. faecalis* and *E. faecium* (Devriese

et al., 1992; Devriese *et al*., 1994). Other animals that have shown to be inhabited by *Enterococci* in their GIT include wild boars and fish (Almeida *et al*., 2011), wild rabbits (Silva *et al*., 2010), wild geese (Han *et al*., 2011) and cattle (Bekele and Ashenafi, 2010).

Antibiotic resistance occurs within bacterial populations via genetic mutation, expression of resistance genes or horizontal gene transfer. The GIT serves as a reservoir for ARB, as it encompasses factors that enable the emergence and transfer of resistance genes. These factors are attributable to the high cell density within the GIT as well as increased exposure to antibiotics (Huddleston, 2014). Bacterial biofilms found in the GIT provide suitable environments for horizontal gene transfer as they provide a high cell density for easy transfer of genes as well as provide physical protection to the cells against adverse conditions. The biofilms encompass many bacterial species with high antibiotic resistance in comparison to their free-living counterparts (Anwar *et al*., 1990; Mills *et al*., 2013). Exposure of microorganisms to antibiotics, within the GIT, will increase their resistance to the antibiotic. This was proven in a study comparing the metagenomes of various individuals, which showed that the ARGs that were more predominant in all the metagenomes were for those antibiotics that were used over a longer period of time in treatment as well as those used in livestock; as compared to those antibiotics that were more recently used for treatment and not at all in animals (Forslund *et al*., 2013). The ARGs most common in the human gut microbiome are the genes for antibiotics that are generally used in livestock (Forslund *et al*., 2013). This demonstrates that antibiotics used in animal husbandry results in an increased antibiotic resistance in humans via human consumption of the animal (Schjorring and Krogfelt, 2011). Antibiotic resistance genes may also be transferred from an animal source to bacteria within the human gut, when the animal is consumed by humans (Lester *et al.*, 2006). This was shown in an in vivo study where isolates of *E. faecium*, obtained from an animal, were able to transfer a vancomycin resistance gene (*vanA*) to another *E. faecium* isolate within the intestines of

certain humans (Lester *et al*., 2006). Microorganisms can therefore be exposed to antibiotics, and its subsequent effect is that it can enter the human GIT through consumption of undercooked meat. These bacteria readily colonize the large intestine of mammals and thus serve as a source of ARGs. A study conducted by Ghosh *et al*. (2013) detected resistance genes against 53 different antibiotics in the gut of 275 individuals. The study further showed that the resistance genes possibly occurred as mobile genetic elements, with 97% of the metagenomes containing resistance genes of tetracycline and 95% to bacitracin (Ghosh *et al*., 2013). In another study, an analysis of the metagenome of the gut from 162 people, showed the presence of 1093 ARGs (Sommer *et al*., 2009).

Enterococci are lactic acid bacteria and exist in the gut of mammals by converting carbohydrates into lactic acid (Carr *et al*., 2002). The GIT of humans and animals house many different types of lactic acid and non-lactic acid bacteria. In comparison to the environment (soil and aquatic environments), the GIT is a greater reservoir for ARGs (Sommer *et al*., 2009; Hu *et al*., 2013). *Enterococci* found in the GIT can result in infections through the fecal-oral route, by nosocomial infections or by contamination of food sources. The GIT is reservoir to a large diversity and density of ARB as well as ARGs that are primarily transferred through horizontal gene transfer.

1.1.3. **Food sources**

Enterococci have also been established in large numbers, in raw and preserved food, food of animal origin as well as vegetables (Giraffa, 2002). The presence of *Enterococci* in food is generally an indicator of low sanitary levels and food contamination; however, these sought after bacteria are also used as starter-cultures in the food industry as a result of their ability to produce lipase, protease and volatile compounds ensuring organoleptic features in some kinds of food (Giraffa, 2002; Furlaneto-Maia *et al*., 2014). They are also valuable for their probiotic

characteristics and bacteriocin production. Due to the ubiquitous nature of *Enterococcus* on raw meat and in dairy products, their ability to render an environment acidic and their thermotolerance, they have become valuable additives in fermented foods such as cheese and sausages (Giraffa, 2002). Their staggering ability to withstand pasteurization and high acid and salt concentrations, enable *Enterococcus* spp. to resist the technological processes involved in the manufacturing of certain food products. It is due to this characteristic that they are able to contaminate products as well as be used as starter-cultures in fermented foods (Foulquié-Moreno *et al*., 2006). *Enterococcus faecium* and *E. faecalis* are the two most common species isolated from food (Giraffa, 2002; Foulquié Moreno *et al*., 2006). In studies conducted by Hayes *et al*. (2003) and Gomes *et al*. (2008), *Enterococcus* spp. have been isolated from foods, such as raw and pasteurized milk, raw meat products, cheeses and vegetables, with contamination rates ranging from 52.5% to 99%. Countries such as Greece, Italy, Spain and Portugal often use *Enterococci* in the production of cheeses (Foulquie-Moreno *et al*., 2006). The bacteria assist in the ripening and aroma enhancement in the cheeses, caused by their proteolytic and esterolytic properties and the production of other compounds (Foulquie-Moreno *et al*., 2006).

A major problem associated with contamination of food products by the bacteria is spoilage. Due to the ability of *Enterococci* to withstand high temperatures and levels of salinity, studies have shown that salted, fermented meats as well as cooked, processed meats were readily contaminated by *Enterococci* (Teuber *et al*., 1996; Franz *et al*., 1999; Teuber *et al*., 1999). Both *E. faecalis* and *E. faecium* are common species of *Enterococci* involved in food spoilage. They have been shown to be able to withstand cooking at temperatures as high as 68 ºC for 30 min

(Gordon and Ahmad, 1991). *Enterococci* are therefore the cause of raw and processed food spoilage and are unavoidable even with present applied food technologies.

Despite the high prevalence of *Enterococci* in various food sources, the major problem is that these food sources may serve as a potential reservoir for ARE. The problem associated with utilizing *Enterococci* spp. in the production of certain foods, is that antibiotic resistance is increased as resistance genes in these bacteria can easily be transferred by means of mobile genetic elements (Hasman *et al*., 2005). The exceptional ability of *E. faecalis* to acquire and transfer ARGs, make them troublesome in their use for food production (Çitak *et al*., 2004). The high levels of antibiotic resistance in *Enterococci*, as well as the extensive findings of the bacteria in food products, are major contributors to food sources serving as hotspots for ARE. Antibiotic resistant *Enterococci* have been found in meat, dairy and processed foods (Chajęcka-Wierzchowska *et al*., 2012). Studies have shown *E. faecalis* and *E. faecium*, isolated from cheese, to be resistant to multiple antibiotics such as penicillin, tetracycline, chloramphenicol, erythromycin, gentamicin, lincomycin, rifampicin, fusidic acid and vancomycin (Teuber *et al*., 1999). A recent study conducted by Furlaneto-Maia *et al*. (2014**)** showed resistant phenotypes of *Enterococci* spp., isolated from soft cheese in South Brazil. These phenotypes were resistant to many classes of antibiotics that are commonly used in human treatment. This study identified the presence of a food-strain *Enterococci* containing multiple antibiotic resistance. All the isolates that were resistant to vancomycin were resistant to other clinically important antibiotics such as erythromycin, tetracycline, amikacin, norfloxacin, cephalothin and nalidixic acid, therefore leaving few therapeutic options (Furlaneto-Maia *et al*., 2014**)**. The spread of VRE in food samples was explained by Riboldi *et al*. (2009) to be a result of the widespread use of the antibiotics in agriculture as animal growth promoters. The increase of ARE in food sources

pose a danger to the society as there could also be a possible connection between the nosocomial *Enterococci* and those found in food (Donabedian *et al*., 2003).

Dairy products, that are not free of ARE before consumption, act as a reservoir for ARB creating a direct connection between animal indigenous microflora and the human GIT (Witte, 1998). There is also a connection between antibiotics used in human treatment and animal husbandary. This may lead to the emergence of resistant strains in various animal products (Van den Bogaard and Stobberingh, 2000). Foods contaminated with ARE can be directly transferred to humans upon consumption of the contaminated food. The ARE can survive the gastric passage and subsequently enter the environment via the human feces.

1.1.4. **Wastewater treatment plants**

Coliforms and *Enterococci* are common indicators of fecal contamination and antibiotic resistance in wastewater (Rizzo *et al*., 2014). Wastewater treatment plants have been reported to be major reservoirs of ARB and ARGs. Wastewater contains both ARB and antibiotic residues, which may increase antibiotic resistance in WWTPs under favourable conditions (Martínez, 2009). Since its discovery, antibiotics have been widely used in the clinical, veterinary, agricultural and farming settings. Antibiotics are commonly used to treat human and animal infections, it is used as fodder additives in the poultry industry and it is sometimes used in animal husbandary (Kummerer, 2009). Due to the vast use of antibiotics by humans and animals, most antibiotics and antibiotic residues enter WWTPs as a result of incomplete metabolism through human excretion. Improper disposal of unused antibiotics also increase the quantity of antibiotics in WWTPs. The high levels of antibiotics that enter the WWTP may provide a selective pressure for ARB, which may be a major contributing factor that makes

WWTPs such major reservoirs of ARB and ARGs. Raw wastewater, that predominantly consists of hospital effluent, most commonly contain ARE (Rizzo *et al.*, 2014).

The treatment process, followed by WWTPs, involves three main stages comprising of a physical, biological and chemical stage (Bouki *et al.*, 2013). As a result of the treatment processes, a considerable reduction in bacterial population and ARB occurs (Huang *et al*., 2012). Studies have reported that WWTPs may offer favourable conditions for the increase in ARB and therefore also in ARGs (Guardabassi and Dalsgaard, 2002; Huang *et al*., 2012). Variations in the design of WWTPs and operation procedures may have an influence on the presence and spread of ARB and ARGs (Guardabassi and Dalsgaard, 2002). It has been noted that WWTPs are capable of transferring 10^9 - 10^{12} CFU/ml (each day) of ARB from final effluent into the environment (Novo and Manaia, 2010). This large discharge of ARB into the environment highlights the major role WWTPs play in ARB dissemination. Various environmental conditions may also impact the proliferation of ARB and transfer of ARGs in WWTPs (Davies, 2012).

1.2. Overview of the general processes involved in a wastewater treatment plant

Wastewater treatment plants are equipped to purify and disinfect polluted effluent of domestic, clinical, agricultural and industrial settings. Three major stages involved in wastewater treatment processes that decontaminate waste effluent include primary, secondary and tertiary treatments. A combination of physical, chemical and biological systems are included in the various processes (Figure 1.1).

1.2.1. **Primary treatment process**

The initial treatment process is regarded as the pre-treatment process. This is a physical process that begins with screening, which allows for the removal of large objects and pollutants (Hendricks and Pool, 2012). This process is important in preventing the large debris from damaging or clogging pumps and tanks further down the treatment process (Abdel-Raouf *et al.*, 2012). Screening devices are generally wire mesh, perforated plates, grating or metal bars. Most large-scale plants utilize automated mechanical screens that vary in size (Tchobanoglous and Burton, 1991). The solids collected from the screening process are later disposed off in landfills or via incineration (Tchobanoglous and Burton, 1991). This step in the treatment process may also have a grit channel, which adjusts the velocity of influent to allow material like sand, stones, grit and other smaller objects to settle to the bottom. A procedure called 'flow equalization' is used to increase the effectiveness of secondary treatment processes and clarifiers by ensuring uniform flow conditions, pollutant levels and temperature. Flow equalization may be used prior to discharge into lakes and rivers or prior to advanced wastewater treatment processes. Equalization basins may also be used to briefly hold influent during plant maintenance and to dilute influent of high toxicity before it enters secondary treatment and inhibits the biological processes (EPA, 2004).

Once screening is completed, primary sedimentation takes place. This step is utilized to remove dissolved organic, inorganic and suspended material. Suspended solids are then removed by means of sedimentation, filtration, chemical coagulation or settling. In primary sedimentation tanks (clarifier), suspended solids slowly sink to the bottom and form a mass called 'primary sludge.' Primary sludge is then removed from the primary sedimentation tanks by utilizing mechanical equipment. Primary sludge may be removed continuously or periodically, depending on the individual treatment plants (EPA, 2004).

1.2.2. **Secondary treatment process**

The next stage in the treatment process is the secondary treatment. This stage utilizes biological treatment that allows for removal of most of the organic material. Secondary treatment is achieved by the attached growth process and the suspended growth process. In the attached growth process, microorganisms (bacteria, algae and fungi) proliferate on the surface of stone media (EPA, 2004). The wastewater is passed over the media, along with oxygen, to allow for increased microbial growth. This process involves the use of trickling filters, rotating biological contactors and biotowers (EPA, 2004). The media bed, commonly made of stones, rocks or plastic, enables microorganisms to attach and grow on it. The treatment is effective as the microorganisms utilize the oxygen and organic matter as food sources, thus removing the organic pollutants. Thereafter, the water is transferred into a secondary treatment tank to allow any biomass, which may have passed into the water from the media beds, to settle to the bottom of the tank. In the suspended growth process, removal of biodegradable organic matter and organic nitrogen-containing matter occurs by transforming ammonia nitrogen to nitrate. Here, microorganisms are suspended in aerated water and activated sludge (Hendricks and Pool, 2012). In this process, a rich aerobic environment is created to increase the rate of microbial breakdown of organic matter. The improved growth conditions allow for increased microbial growth. The excess biomass then settles to the bottom of a secondary treatment tank before the water is treated further. The biomass can be reused as activated sludge for the next round of treatment. Suspended growth processes is more advantageous than the attached growth processes as it uses smaller units and therefore requires less space, it also produces less odours and is free of flies when operated optimally. However, activated sludge processes are generally more expensive than attached growth processes due to higher energy needed to run the aeration systems (Vilanova *et al*., 2011).

1.2.3. **Tertiary treatment process**

The final stage in the treatment of wastewater is the tertiary treatment. This stage of treatment is to improve the final effluent quality before it is discharged into receiving rivers and lakes. The tertiary stage involves chemical treatment process, such as chemical precipitation, adsorption and disinfection (Tchobanoglous and Burton, 1991). In chemical precipitation, the flocculation of fine solids occur before sedimentation takes place in order to improve removal of suspended solids and phosphorus. Chemical coagulants that are commonly used in wastewater treatment include alum, ferric chloride, ferric sulfate, ferrous sulfate and lime (Tchobanoglous and Burton, 1991). Adsorption is a process that uses a solid object (usually activated carbon) in the normal biological treatment in order to remove any remaining dissolved organic matter (Tchobanoglous and Burton, 1991). Activated carbon exists in the granular and powdered form and is either filled in a fixed-bed column through which the wastewater runs, or it is added directly to the wastewater in a basin. Powdered activated carbon is then left to settle to the bottom of the basin and removed by addition of a polyelectrolyte coagulant or is filtered (Tchobanoglous and Burton, 1991). The disinfection process removes disease causing microorganisms from the wastewater. This process may include physical agents (heat and light), mechanical agents (screening, filtration and sedimentation), radiation (gamma rays) and chemical agents (chlorine, soap and detergents) (Qasim, 1999). Disinfectants act by damaging the cell wall of microorganisms, changing cell permeability and inhibiting enzyme activity. When using chlorine as the disinfectant, prior to discharge of the final effluent into receiving water bodies, a process of dechlorination may take place. The step of dechlorination is not practiced by all WWTP however, it is often a priority in food and beverage producing industries in order to prevent the undesirable chlorine taste. Dechlorination removes free chlorine present in effluent from the chlorinated wastewater. This is done to prevent

chlorine compounds from reacting with any organic compounds in the effluent and producing toxic products that may have adverse effects on the environment. This process is achieved by the use of activated carbon, or by the addition of reducing agents such as sulphur dioxide, sodium sulfite or sodium metabisulfite (Qasim, 1999).

The removal of ARE and ARGs in wastewater is of primary concern before release of treated effluent into the environment to prevent subsequent detriment to the human population at large. The primary and secondary treatments in the WWTP are inadequate in removing more than 90% of pathogens and ARB in raw wastewater (Sobsey *et al.,* 1998). Even secondary treated effluents sometimes contain high bacterial loads. Therefore, tertiary treatment (disinfection) is a fundamental barrier in removing pathogens and ARB in treated effluents. The disinfection process in wastewater treatment is essential to control pathogens and ARB in treated wastewater and receiving water bodies (Bouki *et al.,* 2013).

The most popular form of disinfection in WWTPs is chlorination. This is the preferred choice of disinfection due to it being a well-established technology, is economically practical, allows for simple application and is mass produced and easily available (Bouki *et al*., 2013). Also, residual chlorine, present in the final effluent, enables prolonged disinfection even after the initial chlorination step (EPA, 1999). Chlorine is able to remove unpleasant odours, dosage levels can easily be altered and is able to oxidize some organic and inorganic matter (EPA, 1999). As a disinfectant, chlorine is efficient in eliminating many bacteria; however it has lower efficiency against bacterial spores, viruses and protozoan cysts (Davies *et al.,* 2009; Maier *et al.,* 2009). The process of chlorination however, does not always completely remove bacteria and many bacteria, which are carried in the wastewater, develop resistance mechanisms and persist in the final treated effluents (Maier *et al.,* 2009). This then leads to the release of ARBs into the environment and may have detrimental impacts on public health.

Chlorination often results in the production of chlorinated compounds as possible toxic by-products which may even be carcinogenic to humans (Sobsey *et al.*, 1998). Also, treated effluents that have been chlorinated, often have to undergo dechlorination before being discharged into the environment (Sobsey *et al*., 1998). Other limitations associated with chlorination are that chlorine is a toxic, corrosive compound and therefore requires high safety regulations when handling, transporting and storing. The ability of chlorine to oxidize certain organic material may create more toxic compounds such as trihalomethane (EPA, 1999).

The process of chlorination occurs by chlorine (in the gaseous, liquid or solid phase) being added to the wastewater. This then triggers hydrolysis and ionization that result in the production of hypochlorous acid and hypochlorite ions. The hypochlorite ions bond with ammonia in the wastewater to form monochloramine (combined chlorine) and dichloramine (EPA, 1999). Optimal disinfection is achieved by allowing for proper mixing to enable the reaction between the free chlorine and the ammonia. This prevents extended periods of chlorine exposure and formation of chlorine by-products (EPA, 1999). Another factor that improves disinfection is contact time. Contact chambers should be constructed to have round edges to minimize dead flow areas. This would enable for substantial contact time between the bacteria and chlorine, thus utilizing a minimum chlorine concentration and shorter contact time (EPA, 1999).

Several factors affect the success of ARB and ARG removal. Cell structure, bacterial molecular characteristics and the design of the WWTP all have an effect on the efficiency of ARB and ARG removal (Batt *et al*., 2006). Independent uses of chemical or biological processes are not proficient in eliminating ARB during treatment, but rather require a combination of these processes (Garcia *et al.*, 1995; Adams *et al.*, 2002). A study conducted by Guardabassi *et al.* (2002) showed that tertiary treatment had a major reduction in the number of bacteria that were

multi-drug resistant. Earlier studies, however, have shown the opposite where tertiary treatment resulted in an increase in multi-drug resistant bacteria in the final effluent (Morozzi *et al*., 1988; Andersen, 1993). Contrary to these studies that show a reduction in the number of ARB after chlorination, Shi *et al.* (2013) reported that chlorination increased expression of ARGs in bacterial communities. Such findings were also reported in other studies, which showed that chlorination resulted in certain bacteria becoming more resistant to certain antibiotics by inducing a stress tolerance (Armstrong *et al*., 1982; Shrivastava *et al*., 2004). The increase in antibiotic resistance, following chlorination, can be attributed to an increased expression of bacterial efflux pumps that allow the bacterial cell to expel antibiotics within the cell **(**Xi *et al*., 2009).

Other studies have shown that chlorination induced the development of antibiotic resistance (Rutala *et al.,* 1997; Fraise, 2002). It was suggested that bacteria that were able to withstand the stress of disinfection, were more antibiotic resistant (Armstrong *et al*., 1982). However, it was later shown that a major aspect that affects the efficiency of removal in the tertiary step is chlorine dosage (Huang *et al*., 2011). This was verified in a study that showed that different concentrations of sodium hypochlorite (8.0 to 30.0 mg/liter), used in the disinfection stage, resulted in varying levels of reduction in *Enterococci* populations (Tree *et al.,* 2003). After 5 to 15 minutes of exposure to sodium hypochlorite, depending on the concentration, *Enterococci* decreased by nearly 5 orders (Tree *et al.*, 2003). The chlorine dosage depends on chlorine demand, discharge requirements and wastewater characteristics (EPA, 1999).

The many benefits associated with the use of chlorine as the disinfection process in wastewater treatment, has rendered this chemical a common choice in most WWTPs. However, the production of toxic by-products and hazardous characteristics it poses to the environment,

human and animal health has resulted in the use of other modes of disinfection processes such as UV radiation, advanced oxidation processes and microfiltration.

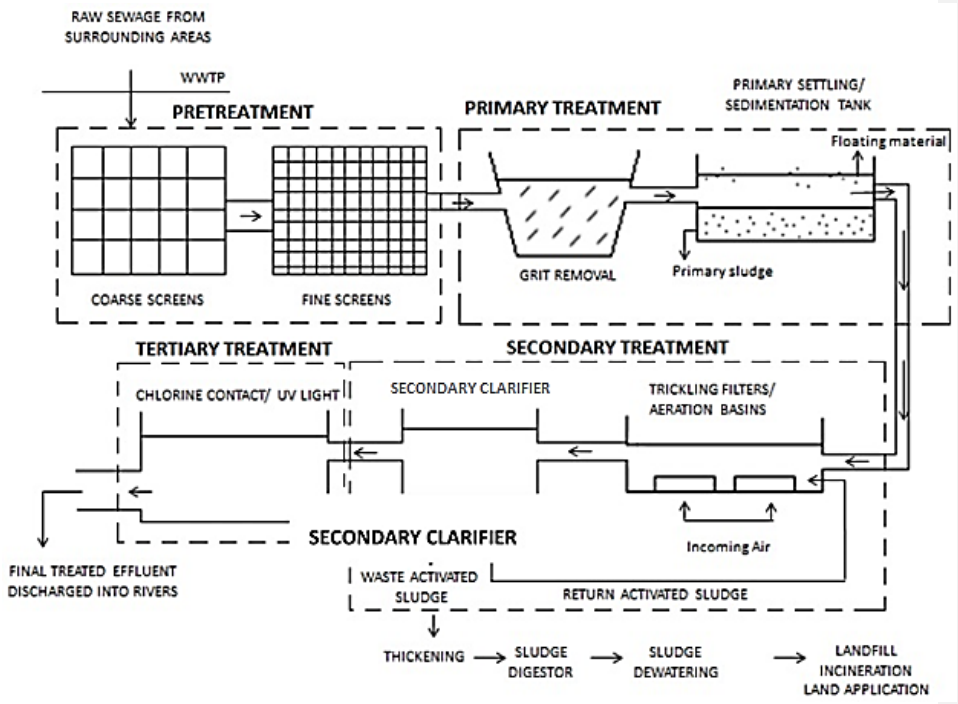


Figure 1.1: The general overview of treatment processes involved in a wastewater treatment plant (Naidoo and Olaniran, 2014)

The size of ARB populations and ARGs usually decrease during the various wastewater treatment processes (Wagner and Loy, 2002; Guardabassi *et al*., 2002; Huang *et al*., 2012). However, many other studies have shown the contrary; with WWTPs enhancing the proliferation of ARB (Guardabassi and Dalsgaard, 2002; Schwartz *et al*., 2003). Ultimately, the fate of ARB and ARGs, at every treatment stage within the WWTP, are dependent on

different treatment operations and plant designs (Batt *et al.*, 2006; Kim *et al*., 2006). Activated sludge, utilized during the secondary treatment stage, usually contain high levels of ARB (Ge *et al*., 2012). The tertiary stage is therefore important in reducing the high ARB load (Ge *et al*., 2012). Studies have revealed that the biological treatment stage may positively affect the spread of ARB and ARGs (Auerbach *et al*., 2007; Kim *et al*., 2007; Luczkiewicz *et al*., 2010). Duong *et al.*, (2008) reported that the biological treatment process reduced 99% of ARB. Several studies reported a decrease in ARB and ARGs, from the raw influent to the final effluent samples, achieved by the chlorination disinfection step (Shrivastava *et al.*, 2004; McKinney *et al.*, 2009; Templeton *et al.*, 2009). Chemical or biological processes alone are not effective in the removal of ARB, however a combination is more effective (Garcia *et al*., 1995; Adams *et al*., 2002).

1.3. Prevalence of ARB and ARGs in wastewater treatment plants

The main aim of WWTPs is to treat wastewater to a standard that is safe enough to discharge into the environment or reuse for other purposes. These facilities should be equipped to produce an end product that is free of physical, biological and chemically toxic material. It is conventional practice for WWTPs to reduce the total load of bacteria in the final effluent (Zhang and Farahbakhsh, 2007). The three stages involved in wastewater treatment (primary, secondary and tertiary) have a significant effect on the diversity and density of the bacterial populations present in the wastewater (Wagner and Loy, 2002). Studies have shown that the treatment process, generally, reduces the bacterial load present as well as the populations of ARB (Guardabassi *et al*., 2002; Huang *et al*., 2012). The treatment process however, lacks efficiency in total removal of ARB and ARGs (Munir *et al*., 2011). The presence, proliferation and persistence of ARB and their resistance genes in wastewater may be attributed to the fact

that WWTPs serve as reservoirs for antibiotic resistance (LaPara and Burch, 2012). Ferreira da Silva *et al.* (2006) found *E. hirae*, *E. faecium* and *E. faecalis* as the three prevalent species in raw wastewater in a WWTP in Portugal. Following treatment by the WWTP, the number of *E. hirae* decreased, the number of *E. faecalis* remained the same as in the raw wastewater, while the number of *E. faecium* increased. *Enterococcus faecalis* and *E. faecium* were resistant to ciprofloxacin, erythromycin and tetracycline. These resistant *Enterococci* were not completely removed during the treatment process, instead a noted increase in resistance to ciprofloxacin was noted in the final effluent (Ferreira da Silva *et al*., 2006). Possible reasons for WWTPs being able to support or increase antibiotic resistance could be as a result of horizontal gene transfer, improved survival of the bacterial population as well as elevated levels of antibiotics in treated effluent (Ferreira da Silva *et al.*, 2006; Li *et al*., 2009). The elevated use of antibiotics for various activities is a major factor in the increase of antibiotic resistance. A survey conducted between 2000 and 2010, revealed an increase of 36% in the global consumption of antibiotics (Van Boeckel *et al.*, 2014).

Due to WWTPs not being able to completely remove ARB and ARGs during the treatment process and as a result of the activated sludge containing high levels of antibiotics, an increased selective pressure is created that allows for proliferation of ARB. The presence of antibiotic residues, high microbial diversity and presence of gut-associated ARB provide a favourable environment for ARB proliferation and ARG dissemination in activated sludge (Rizzo *et al*., 2013). Activated sludge tanks, bioreactors and trickling filters accumulate bacteria, such as *Enterococci*, by providing conditions that encourage their proliferation to enhance the biological treatment process. A study conducted by Sahlström *et al*. (2009) discovered the presence of VRE in 77 sludge samples. This study revealed widespread presence of VRE in activated sludge obtained from WWTPs. It further revealed the risks associated with antibiotic

resistance being extended if these sludge samples were used as fertilizers in farms. Activated sludge from WWTPs may therefore facilitate the spread of ARE into the environment. This may thus act as a reservoir for ARE as it provides conditions suitable for their survival and gene transfer (Iversen *et al*., 2004; Vilanova *et al*., 2004). Following this phase in the treatment plant, ARE are able to escape the WWTP and infect humans and animals via water contamination or the food chain (Iversen *et al.*, 2004; Hayes *et al.*, 2003; Guardabassi and Dalsgaard, 2004). Studies have shown the presence of ARE in the environment caused by its release from an antibiotic resistant hotspot. One such study showed high prevalence of ARE in two recreational beaches in Brazil. This study showed that the beaches contained a high rate of ARE as a result of domestic wastewater being discharged onto the beaches (Oliveira and Pinata, 2008). In another study, VRE was detected in marine environments as a result of fecal waste (Whitman *et al.*, 2003). Resistance to vancomycin by *Enterococcus* is a predominant factor that has been reported in many studies. Resistance to vancomycin constitute 34% of the 55% of the total antimicrobial resistance among *Enterococci* spp. recovered from marine outfalls in Brazil (Carvalho *et al.*, 2014). A study in Sweden also showed the presence of VRE, with a value of 19% in treated wastewater and 36% in treated clinical wastewater (Iversen *et al*., 2002). These studies all highlight the fact that environments containing or contaminated by wastewater, serve as hotspots of ARE. These environments generally contain high loads of organic matter that assist in the transport, transfer and spread of ARGs or plasmids (Carvalho *et al*., 2014). Plasmids are common in *Enterococci* and are liable for much of the horizontal gene transfer. It was found that many resistance profiles were related to plasmids in *Enterococci* (McBride *et al*., 2007).

1.4. Factors influencing ARB and ARG concentration in treated wastewater effluent

Wastewater treatment plants have been reported as major reservoirs of ARB and ARGs in many recent studies (Gallert *et al*., 2005; Ferreira da Silva *et al.*, 2006; Kümmerer, 2009; Servais and Passerat, 2009). However, various contributing factors may influence ARB and ARG concentration in treated effluent. The use of physical, biological and chemical processes, are implemented in WWTPs to ensure that their objective of a safe, pathogen-free effluent is achieved (Hong *et al.*, 2013). Influent received from human, hospital, industrial and veterinary sources are known to harbor high levels of antibiotics and ARB (Wright, 2010; Grassi *et al*., 2013). The treatment processes involved in WWTPs are not sufficiently well designed to decrease ARB and ARGs, which may potentially encourage the spread of antibiotic resistance in the environment (Hong *et al.*, 2013; Lupo *et al*., 2012). Factors that commonly affect ARB and ARG concentration in WWTPs are discussed below:

1.4.1. **Source of influent**

An increased number of ARB in the raw influent has an increased effect on the number of ARB present in the final effluent. This primarily has to do with the type of influent that a plant receives (Novo and Manaia, 2010). Common types of influent are typically received from domestic, hospital, industrial, agricultural, pharmaceutical and veterinary sources (Figure 1.2) (Wright, 2010; Grassi *et al.*, 2013). Hospitals are one of the greatest platforms for antibiotic usage and therefore assist in the spread of antibiotic resistance (Stalder *et al.*, 2014). It was reported, in the United States, that more than 10 million kilograms of antibiotics were used, for non-therapeutic use, in poultry, cattle and swine production (UCS, 2001). This widespread use of antibiotics for human and animal purposes generally result in large amounts of the antibiotics being deposited into WWTPs. Antibiotics enter WWTPs via incomplete-metabolism through human excretion. Antibiotic residues are commonly found in the environment by means of

human and animal waste (Figure 1.2). These drugs enter the environment via feces and urine, unused antibiotics that are flushed down toilets, improper disposal of medical waste from clinical settings and septic tanks that leak into soil and ground water. Once in the environment, these residues may be degraded or enter rivers, sediment or soil from WWTPs. Antibiotics used in animal husbandary may be found in soil via animal waste.

Raw influent received from hospital settings generally contain greater loads of ARB (Baquero *et al*., 2008). One study assessed the role of hospital activities in the spread of antibiotic resistance by analyzing integrons and gene cassettes in hospital wastewater (Stalder *et al*., 2014). It was reported that hospital wastewater contained large quantities of integrons and that the gene cassette diversity and gene cassette arrays were very similar to that found in the sludge (Stalder *et al*., 2014). Various cases of ARGs were reported in hospital effluents, including *van*A genes (Schwartz *et al*., 2003), *mec*A genes encoding methicillin resistance in *Staphylococci* (Heuer *et al.*, 2002) and genes encoding gentamicin resistance in *Acinetobacter*, *Pseudomonas* and Enterobacteriaceae (Heuer *et al*., 2002). The high presence of antibiotics, ARB and ARGs are factors that contribute to hospitals being the greatest reservoirs of antibiotic resistance dissemination into WWTPs.

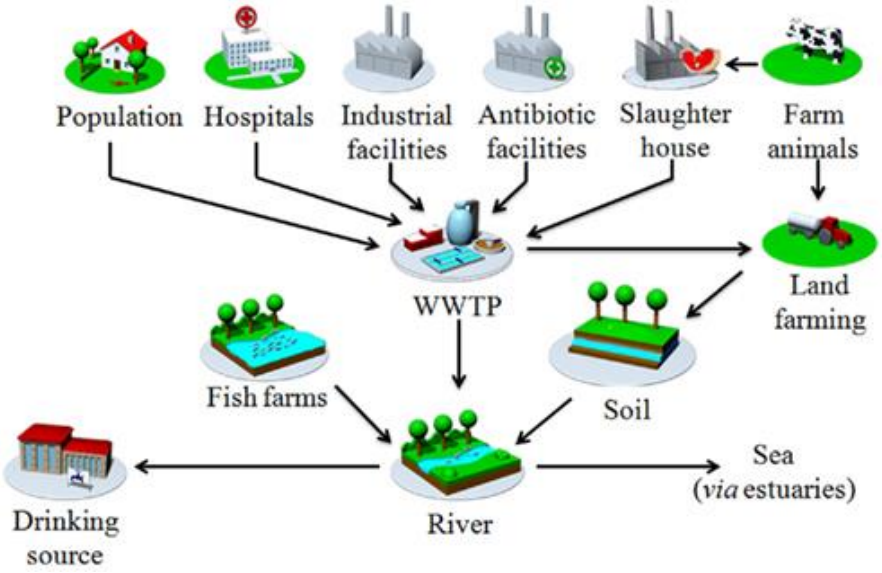


Figure 1.2: Common sources of influent received by wastewater treatment plants (Stalder *et al.*, 2012)

1.4.2. **Treatment methods in WWTPs**

The different forms of biological treatments and hydraulic retention time affect the removal efficiency of ARB and ARGs. Increased hydraulic retention time support increased transfer of ARGs (Tchobanoglous *et al.*, 2003). Hydraulic retention time refers to the ratio of the volume of waste to the flow rate. The hydraulic retention time can be increased by reducing the hydraulic loading rate. Kim *et al*. (2007) studied the prevalence of tetracycline resistant bacteria with regards to activated sludge organic loading rate and the bacterial growth rate. The study showed that tetracycline resistance increased by increasing the organic loading and growth rate (sludge retention time) (Kim *et al.*, 2007). Different treatment processes also affect the prevalence of ARB and ARGs in wastewater. Such processes include activated sludge, membrane biological reactor and rotatory biological contractors. Of the different processes, the

membrane biological reactor removed the greatest quantities of ARB and ARGs (Munir *et al*., 2011). Various environmental factors also play a role in affecting ARB dissemination in WWTPs. Extreme temperatures, light, salinity and low nutrient levels may affect bacteria by causing nonculturable to remain viable and transfer genes (Arana and Barcina, 2008). Therefore, adverse environments may not harm the bacteria, but merely induce a viable but nonculturable state. Also, environmental factors regarding predation may also have an effect. Predation by protozoa is able to reduce bacterial spread and therefore ARB and ARGs (Matz and Kjelleberg, 2005; Pernthaler, 2005).

1.5. Alternative tertiary treatment solutions for significant reduction in levels of ARB and ARGs in treated wastewater effluent

The final barrier for ARB and ARGs dissemination into the environment, from WWTPs, should be the disinfection stage of the treatment regime. The conventional tertiary process of chlorination has fallen short of being efficient in removing ARB and ARGs during the treatment process (Karumathil *et al.*, 2014**).** It is therefore necessary to find alternative disinfection processes that do not pose a risk to the environment, human and animal health. Such alternatives include ultraviolet (UV) radiation, advanced oxidation processes and microfiltration (Hijnen *et al*., 2005; Breazeal *et al*., 2013; Rizzo *et al.*, 2014).

1.5.1. Ultraviolet disinfection

Contrary to chemical disinfection, many WWTPs have adopted UV radiation as an alternative. The implementation of UV light for disinfection has become popular, with many treatment plants switching from chlorination as a disinfection process to UV technology (Das, 2001). UV radiation is a physical process that operates by exposing wastewater to a UV source (usually mercury arc lamps) before the final effluent can be released into receiving water bodies. This

non-ionizing radiation agent penetrates the bacterial cell wall, mutates its DNA (mainly by pyrimidine dimerization) and inhibits bacterial proliferation (Dodd, 2012; Biswal *et al.*, 2014). DNA mutation induces expression of the DNA repair system and in the case of pathogenicity and antibiotic resistance; it induces the integrase recombination genes (Courcelle *et al*., 2001; Quiroz *et al.,* 2011). With regards to pathogenicity, expression of the gene classes may result in deletion of some pathogenicity islands, thus causing the strain to be non-pathogenic rather than killing off the bacterial cell (Soto *et al.,* 2006; Almagro-Moreno *et al*., 2010; Frigon *et al*., 2013). Processes similar to this may also occur with ARGs as they are sometimes clustered in integrons containing integrase recombination genes (Biswal *et al*., 2014). Some bacteria, that may possibly be resistant to antibiotics, are able to become reactivated and survive the disinfection step by utilizing mechanisms of photoreactivation (Masschelein, 2002). This occurs by the pyrimidine dimers (formed during exposure to UV light), forming a complex with photoreactivating enzymes that assist in photolysis to repair the original monomer (Masschelein, 2002).

The physical and chemical quality of the wastewater that determines the success of UV radiation. An increased concentration of particulate components in the wastewater may result in decreased efficiency of the treatment step. The benefits of using this form of disinfection is that UV radiation is a faster process, cost effective, does not create toxic by-products and it does not result in prolonged residual content in the water (Das, 2001). Limitations of UV disinfection are that it is not always effective against retroviruses and rotaviruses, monitoring its efficiency are complex and there is no residual protection. Also, turbidity and the presence of total suspended solids can decrease its efficiency (Darby *et al.*, 1995).

UV disinfection is able to alter the DNA of ARB and potentially decrease ARGs (Dodd, 2012). Another study however, disproved this by showing that UV radiation used in WWTPs was able

to reduce ARB but not ARGs (McKinney and Pruden, 2012). An added advantage of UV disinfection would be its ability to thwart the progression of horizontal gene transfer to bacteria downstream of a receiving water body in order to prevent transfer of ARGs. McKinney and Pruden (2012) reported on the ability of UV disinfection to damage various resistance genes, intra- and extra-cellular, in methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE), *Escherichia coli* SMS-3-5, and *Pseudomonas aeruginosa*. The study revealed that MRSA and VRE (both Gram positive) exerted increased resistance to UV disinfection compared to *E. coli* and *P. aeruginosa* (both Gram negative). The increased resistance to UV radiation may be attributed to their reduced genome size, where a lower potential for pyrimidine dimmers are formed due to the small size. They also reported that damage to ARGs require increased UV doses compared to ARB. The study concluded that thymine dimers are not solely responsible for ARB destruction and UV disinfection may not be the best tool for ARG removal (McKinney and Pruden, 2012). UV radiation is also known to have an effect on the conjugative transfer of ARGs between bacteria. Low doses of UV had shown to possess little effect on the rate of conjugative transfer and merely decreased bacterial populations without affecting cellular structure (Guo *et al*., 2015). Cell permeability was barely affected, thus decreasing the ability of bacterial pilus to be inserted into other bacteria, reducing the chance of ARG transfer (Guo *et al*., 2015).

1.5.2. **Advanced oxidation processes**

A successful form of disinfection of wastewater is the advanced oxidation processes (AOP) (Zapata *et al*., 2010). Heterogeneous photocatalysis with TiO_2 (titanium dioxide) is among the AOPs, that has gained much attention as a disinfection process (Dunlop *et al*., 2011; Robertson *et al*., 2012). This form of disinfection does not produce toxic by-products, it is cost effective as it can utilize solar power to operate and it is effective against most bacteria, viruses and

protozoa (McCullagh *et al*., 2007; Malato *et al*., 2009). Additionally, TiO_2 is popular as a semiconductor as it is widely available and stable. UV-titanium dioxide photocatalysis is a form of AOP that has shown to be highly effective against most microorganisms (McCullagh *et al*., 2007). This functions by TiO_2 particles being exposed to near UV radiation that then produce charge carriers. These carriers move to the surface of semiconductors, react with hydroxyl groups and dissolved oxygen and produce reactive oxygen species such as peroxides and superoxide radicals (McCullagh *et al*., 2007). Microbial inactivation, resulting from photocatalytic processes, is due to the interaction of reactive oxygen species with components of the bacterial cell surface (McCullagh *et al.*, 2007). Photogenerated holes move to the titanium particle-solution interface and produces hydroxyl radicals by oxidising hydroxyl ions or water. Additional reactive oxygen species are produced from the reduction of dissolved oxygen such as hydrogen peroxide, hydroperoxyl radical and superoxide radical anion (McCullagh *et al.*, 2007). A recent study reported that cell wall modification, induced by radicals, was primarily responsible for the increased biocidal effect of TiO_2-based nanomaterials (Kubacka *et al*., 2014).

The spread of ARGs in WWTPs during the AOP may occur when stressed bacterial cells replicate and transfer their genes in order to survive and maintain their populations. One study has determined that high antibiotic use lead to the enhancement of multidrug resistance via radical induced mutation (Kohanski *et al*., 2010). Another study showed that excessive proliferation and genetic transfer had occurred by a bacterial population that was under great environmental stress and virtually inactive (McMahon *et al*., 2007). The survival mechanism supposedly occurs to enable stressed organisms preserve their species by rapidly replicating and transferring genetic material (McMahon *et al*., 2007). The increase in the transfer of ARGs occur if the treatment process is not followed fully, preventing complete inactivation of

microorganisms before release into the environment. Ensuring appropriate treatment time will prevent the spread and release of ARGs into rivers and lakes (Dunlopa *et al*., 2015).

1.5.3. **Microfiltration**

Membrane technologies, which include microfiltration, ultrafiltration, nanofiltration, and reverse osmosis is another type of disinfection utilized by WWTPs. This technology is a physical process that filters out microorganisms. Reactive chemicals are not required, no toxic by-products are formed in this disinfection process and it is efficient against bacteria, viruses, algae and protozoans. Limitations to this technology is the high cost, its concentrated backwash can result in microbial contamination and handling risks associated with cleaning of the membranes. The most popular membrane technology employed by WWTPs is microfiltration. This disinfection process operates by passing the wastewater through membrane fibres, which are cylinders that contain numerous microscopic pores. The membrane pore sizes of microfilters range from 0.1-10 μm in diameter (Jacangelo and Buckley, 1996). Liu *et al.* (2014) reported that nanofiltration had the ability to adequately remove antibiotics from treated effluent, however required an advanced oxidation processes to further remove residual antibiotics. Microfiltration and ultrafiltration function by being connected to bioreactors. However, due to the small size of antibiotics, the microfilter and ultrafilter are unable to remove these minute particles. Therefore, nanofiltration is used as it allows for better removal of antibiotics (Clara *et al.,* 2005). The organic fouling that commonly occurs on the membrane, form an added barrier in the removal of antibiotics (Hong *et al.,* 2013). This type of filtration causes an increased retention time for antibiotics, therefore causing an increased reaction time for degradation and hydrolysis of the antibiotics via biological processes (Hong *et al.,* 2013).

Membrane pore size is known to affect the success potential in the removal of ARGs. Analysis conducted by Breazeal *et al.* (2013), showed that as pore sizes decrease, the ARGs removal increases. The presence of colloids, found in wastewater, influences the removal of ARGs by attaching the ARGs to its surface. The study showed that the colloidal material significantly assisted in removal of *bla*TEM and *van*A at a pore size of 10 kDa (Breazeal *et al*., 2013). The study concluded that membrane treatment shows great success in removing ARGs in treated effluents with membrane sizes of 10 kDa and smaller (Breazeal *et al*., 2013).

1.6. Scope of the present study

Water is fundamental for the existence of all life forms (Russo *et al.*, 2014). It is a basic human right for every individual to have access to safe, affordable and sufficient water. However, due to the growing population, increasing demands and recent droughts, countries like South Africa lack access to clean and fresh water (Eyewitness News, 2015; Biowatch, 2016). In order to meet the growing demand for fresh water, appropriately treated wastewater is reused. The final treated effluent is usually discharged into receiving water bodies to be reused by municipal authorities for further treatment and distributed to households and industries (Hong *et al.*, 2013). It is therefore imperative that WWTPs follow strict treatment practices in order to prevent a negative impact that sewage contamination may have on the environment during discharge into receiving rivers and lakes (Jhansi and Mishra, 2013). The treatment of many bacterial infections and the increased developments in agricultural practices are owed to the use of antibiotics (Bouki *et al.*, 2013). However, due to the misuse and overuse of antibiotics, the world is rapidly heading to a post-antibiotic era, as a result of antibiotic resistance. Wastewater treatment plants have been implicated as hot spots for antibiotic resistant *Enterococci* (ARE) and antibiotic resistance genes, which results in the discharge of poor quality effluent into the receiving water bodies (Martínez, 2009; Mema, 2010). *Enterococci* are

problematic as they have a high tendency to acquire and spread antibiotic resistance. The presence of ARE therefore needs to be monitored and controlled. This is highly important to ensure prevention of severe enterococcal infections and increase in its multidrug resistance. This study therefore aimed at assessing the efficiency of two wastewater treatment plants, in Durban, in removing antibiotic resistant *Enterococci* during the treatment process and to establish their influence on receiving water bodies. The antibiotic resistance patterns, detection of specific tetracycline resistance genes and genetic finger-printing profile of *Enterococci* sp., recovered from the final effluent and receiving river samples, was evaluated. Furthermore, the efficiency of the WWTPs for the removal of selected tetracycline resistance genes was determined.

1.7. Hypotheses

It is hypothesized that wastewater treatment plants in Durban, South Africa, are reservoirs for antibiotic resistant *Enterococci* and tetracycline resistance genes. It is further hypothesized that the final effluent, discharged into receiving water bodies, negatively impact the environment by increasing the prevalence of antibiotic resistant *Enterococci.*

1.8. Objectives

1.8.1. To determine the efficiency of the wastewater treatment plants in removing antibiotic resistant *Enterococci* during the treatment process and to identify predominant *Enterococci* species.

1.8.2. To establish the antibiotic resistance profile of selected *Enterococci* isolates.

1.8.3. To establish the genetic diversity of the *Enterococci* isolates recovered from treated effluent and receiving surface water.

1.8.4. To determine the efficiency of the treatment process for the removal of tetracycline resistance genes from the influent.

1.9. Aims

1.9.1. To determine the incidence of antibiotic resistant *Enterococci* (ARE) in 4 pre-determined sampling sites of two wastewater treatment plants in Durban as well as upstream and downstream of their receiving water bodies.

1.9.2. To determine the percentage removal of ARE during the treatment process.

1.9.3. To identify selected *Enterococci* using standard biochemical tests as well as PCR-amplification using genus and species specific primers.

1.9.4. To determine the antibiotic resistance/susceptibility profile of selected *Enterococci* using the Kirby-Bauer disc diffusion method.

1.9.5. To quantify various tetracycline resistance genes in the influent and final effluent using droplet digital PCR assays.

1.9.6. To conduct genetic fingerprinting of *Enterococci* using the Random Amplified Polymorphic DNA (RAPD) technique.

1.10. Experimental Design

In order to achieve the stated objectives, the present study was divided into four main chapters as described below.

Chapter One

Introduction and Literature Review

This chapter summarized the major hotspots for ARE and ARGs, with particular focus on WWTPs. Common treatment processes for ARB and ARG removal as well as the fate of ARE during wastewater treatment processes were also discussed.

Chapter Two

This chapter focuses on the monthly enumeration of the total and antibiotic resistant *Enterococci* at four sampling points within the two wastewater treatment plants (raw influent, activated sludge, before chlorination and final effluent) as well as upstream and downstream of the receiving water bodies. The prevalence and removal of ARE during the treatment process and the subsequent effect on the environment was also discussed. Additionally, the identification to the genus and species level, of selected ARE, was determined.

Chapter Three

This chapter investigates the antibiotic resistance profile as well as presence of multidrug resistant *Enterococci* obtained from the final effluent and upstream and downstream of the

receiving water bodies. The detection of selected tetracycline resistance genes from selected isolates is also examined in this chapter. Furthermore, selected tetracycline resistance genes were quantified from metagenomic DNA, of the influent and final effluent samples, using the droplet digital PCR technique in order to establish the efficiency of the WWTPs in removing these genes.

Chapter Four

This chapter provides a summary of the main findings reported within the different chapters of the study. It also highlights possible limitations and probable future studies in line with the current study.

CHAPTER TWO

PREVALENCE AND FATE OF ANTIBIOTIC RESISTANT *ENTEROCOCCI* IN WASTEWATER EFFLUENTS AND RECEIVING RIVERS

2.1. Introduction

Water is a vital commodity for the existence of all life forms and acts as the core of sustainable and socio-economic development, and is fundamental in the control of diseases, improvement of global health and increase in the standard of living (Russo *et al.*, 2014). It serves as a key component in the production and manufacturing of industries and provides an assortment of uses and services to human beings. This indispensable resource is crucial for survival and population advances (WHO/UNICEF, 2013). However, as a result of water scarcity, many regions including Africa are water stressed areas (Jimenez, 2008). Due to the growing population, increasing demands and recent droughts, countries such as South Africa are facing major difficulties in meeting mandates for clean, fresh water. Recently, national government has declared three South African provinces (North West, Limpopo and KwaZulu-Natal) as disaster zones in terms of water crisis (Eyewitness News, 2015). Globally, 750 million people lack access to safe drinking water with almost 2 million children below the age of 5, dying annually from water related diseases (Eliasson, 2015). It is a basic human right for every individual to have access to safe, affordable and sufficient water that is free from microorganisms, chemical contaminants and radiological hazards (WHO, 2015a; UN, 2015). According to the South African constitution pertaining to environmental rights; Section 24 (a) states that: “Every human has the right to an environment that is not harmful to human health or well-being.” It also states in Section 24 (b) that: “Everyone has the right to have the

environment protected". The right to protect our water resources is one of great responsibility as this resource must be safeguarded in order to preserve its value (Hendricks and Pool, 2012**)**. Furthermore, it is a responsibility to ensure that proper means are put into place in order to distribute clean, safe drinking water.

In order to meet the growing demand of fresh water, a good solution would be to reuse water. The reuse of appropriately treated wastewater, defined as 'reclaimed water', is an alternative that many countries such as South Africa are undertaking. Guidelines according to the U.S. Environmental Protection Agency (EPA, 1999) stipulated that wastewater treatment plants (WWTPs) should perform secondary and/or tertiary treatment processes to obtain reclaimed water that is suitable for reuse, with a reduction in the organic and inorganic load according to results obtained from biochemical oxygen demand (BOD) and chemical oxygen demand (COD) tests amongst others (Asano *et al.*, 2007; Hong *et al.*, 2013).

Wastewater treatment plants operate by conducting primary (physical treatment), secondary (biological treatment) and optionally tertiary (chemical) treatment processes. Conventionally, WWTPs proceed by removing large particles and suspended solids from the raw influent. This is followed by biological treatment utilizing activated sludge, which is then transferred to a secondary clarifier for further removal of suspended particles and organic matter. The final process may include post-treatment operations such as disinfection (EPA, 2004). The final treated effluent is usually then discharged into receiving water bodies to be reused by municipal authorities for further treatment and distribution to households and industries (Hong *et al.*, 2013). It is therefore imperative that WWTPs follow strict and appropriate treatment practices in order to prevent a negative impact that sewage contamination may have on the environment during discharge into receiving rivers and lakes (Jhansi and Mishra, 2013). Developed

countries follow proper wastewater treatment protocols, which are lacking in many developing countries where access to safe drinking water and adequate sanitation are rare. This is disturbing as many communities of these countries, rely on treated effluent-treating river water for domestic and agricultural purposes (Mpenyana-Monyatsi *et al.,* 2012). Despite the stringent processes WWTPs should follow in order to effectively remove the load of enteric microorganisms; machinery malfunction and personnel short-comings, may result in WWTPs being regarded as reservoirs for these enteric microorganisms. Wastewater treatment plants have therefore been implicated as hot spots for antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs), resulting in the discharge of poor quality effluent into the receiving water bodies (Martínez, 2009; Mema, 2010).

Current guidelines regulating the quality of reclaimed water and treated wastewater that is discharged into receiving bodies do not include contaminating substances such as antibiotics, ARB and ARGs. Meanwhile, the concentration of residual antibiotics in wastewater is related to the prevalence of ARB and ARGs (Zhang *et al.,* 2009b). Many countries, including South Africa, have conducted studies pertaining to the prevalence and removal of bacteria in raw influent and treated final effluent (Ternes, 1998; Olańczuk-Neyman *et al.,* 2001; Jhansi and Mishra, 2013; Samie and Ntekele, 2014; Naidoo and Olaniran, 2014; Osuolale and Okoh, 2015). These studies have shown that although WWTPs are able to remove a large amount of these bacteria during the treatment process, there is not always complete eradication in the final treated effluent. Due to variable mixtures of bacteria, abundant nutrients and antimicrobial agents, municipal wastewater is considered favourable for both the survival and the transfer of bacterial resistance. Extensive use of antibiotics for medical and animal husbandry purposes allows for their frequent entry into WWTPs, allowing for an increased selective pressure to be exerted on bacteria (Klare *et al.,* 1995). This promotes the presence and proliferation of ARB

populations and thus increases the presence and transfer of ARGs between bacteria. However, it has generally been noted that the treatment allows a large reduction in bacterial prevalence and total number of ARB (Guardabassi *et al*., 2002; Huang *et al*., 2012).

Enterococci are Gram-positive, non-spore-forming microorganisms that belong to the lactic acid bacteria group. Despite *Enterococci* generally being non-pathogenic, they can become opportunistic pathogens if the commensal relationship with the host is disturbed (Fernandes and Dhanashree, 2013). *Enterococci* are among the predominant bacterial flora in the mammalian gastrointestinal tract. These bacteria have however, resulted in the second leading cause of nosocomial urinary tract infections and the third leading cause of nosocomial bacteraemia and are the cause of a variety of diseases including surgical wounds and bloodstream infections and endocarditis (Spencer, 1996; Cheng *et al.,* 2012). A highly problematic attribute of *Enterococci* is its high tendency to acquire and spread antibiotic resistance. These bacteria have gained resistance to a wide range of antibiotics including chloramphenicol, erythromycin, tetracycline, fluoroquinolones and vancomycin (Medeiros *et al.,* 2014). Multidrug resistance disrupts treatment of enterococcal infections and the therapeutic spectrum of these cases are limited (Oberoi and Aggarwal, 2010). Previous studies have shown the presence of antibiotic resistant *Enterococci* (ARE) in health care units, wastewater effluents and in food (Sherperd and Gilmore, 2002; Rizzo *et al*., 2013; Varela *et al*., 2013). The presence of ARE therefore needs to be monitored and controlled. This is highly important to ensure prevention of severe enterococcal infections and increase in its multidrug resistance. The primary aim of this chapter was to investigate the efficiency of the WWTPs in removing ARE during the treatment process, to establish their influence on receiving water bodies and to identify important species.

2.2. Materials and Methods

2.2.1. Sampling site and sample collection

Two wastewater treatment plants (WWTPs), differing in the types of influent they receive, were sampled within Durban, South Africa. WWTP1 received a Greendrop score of 86% for the year 2011 by the eThekwini Municipality and is one of the five largest WWTPs in Durban with an overall inflow of approximately 45 000 m^3 per day (DWA, 2011). It has a carrying volume of approximately 70 Ml/day. Majority of the influent received by the plant originates from domestic wastewater. Small quantities of wastewater received by the plant originate from industrial practices. During the tertiary treatment, the water is treated with chlorine. The influent received from WWTP2 are derived from domestic and hospital waste. This WWTP attained a Greendrop score of 99.4% for year 2011 also by the eThekwini Municipality and has a treatment capacity of 25 Ml/day (DWA, 2011). The technology used by both plants involves activated sludge and mechanical aeration as well as anaerobic digestion and belt press dewatering. Following tertiary treatment, the treated effluent of both WWTPs are released into major rivers in the Durban area. These rivers are widely used by many local inhabitants for domestic, agricultural and recreational activities. The receiving river of treated effluent from WWTP1 (RR1) has a catchment size of 441km^2, while the river length is 225 km from source to mouth. This river as well as its catchment area has various uses and includes conserved natural areas to highly urbanized and industrial areas (RHP, 2011). The second receiving river (RR2) of treated effluent from WWTP2 is 28 km long and empties into the Indian Ocean north of Durban. A lagoon is positioned at the river mouth, which is surrounded by a local conservancy. This area includes a 26 hectares Lagoon Nature Reserve (Kibirige *et al.,* 2006). Samples were collected monthly for a period of 4 months (June to September 2014) from four pre-determined sampling sites within the plants i.e. raw influent, activated sludge, secondary

effluent (prior to disinfection) and final effluent (after chlorination). Upstream and downstream of the respective receiving rivers were also sampled at approximately 500 m from the discharge point of the WWTPs. All samples were collected approximately at 20-30 cm below the water surface in 5L containers that were disinfected with 70% (v/v) alcohol and rinsed with water at the respective sampling points prior to collection. Samples were maintained on ice and transported to the Department of Microbiology at the University of KwaZulu-Natal (Westville Campus) and stored at 4 ºC until processed. The collected water samples were processed within 24 h of collection (Gao *et al*., 2012).

2.2.2. **Determination of pH and temperature**

The physical parameters, measured for all samples were temperature and pH. Temperature was measured on-site, immediately after collecting samples, using a standard mercury thermometer. The pH readings were conducted using a laboratory pH probe (Beckman 320 pH meter).

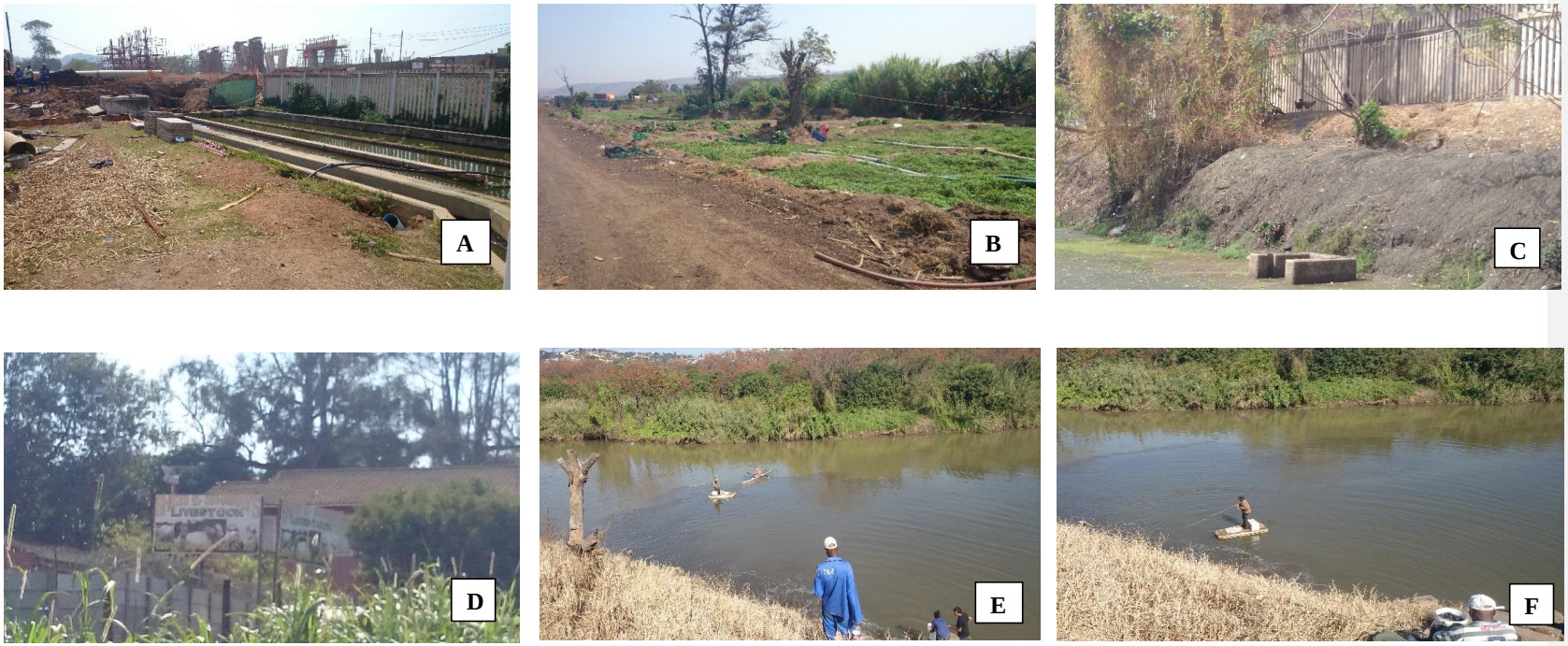


Figure 2. 1: Depictions of noteworthy observations of surrounding sampling points during the sampling period. **A**- Major construction and pedestrian traffic observed at 'after chlorination' point of WWTP2. **B**- Vegetable farm along sampling point of RR2. **C**- Free-running poultry at river bank of RR1. **D**- Small scale livestock farm alongside river bank of RR2. **E**- Local inhabitants and pedestrians engaging in recreational activity (canoeing), fishing and washing at river bank of RR1. **F**- Fisherman and local inhabitant engaging in domestic activity at river bank of RR1.

2.2.3. **Enumeration of antibiotic resistant *Enterococci***

2.2.3.1 **Membrane filtration and plating**

Bacteriological analyses were performed using the membrane filtration method (Myers and Sylvester, 1997). The sample containers were shaken, prior to filtration, in order to evenly distribute the bacteria within the samples. Appropriate volume of serially diluted water samples were filtered through 0.45 µm pore size cellulose nitrate filters (Millipore, South Africa). Filters were placed on *Enterococcus* Selective Agar (Sigma-Aldrich, US), with and without antibiotics, and incubated at 37 °C for 48 h (Luczkiewicz *et al.*, 2010). Presumptive *Enterococci* population was enumerated and expressed as colony forming units per hundred millilitres (CFU/100ml). The following concentrations of the various antibiotics were used as described by the Clinical and Laboratory Standards Institute (CLSI, 2007) for *Enterococci*: ampicillin (16 µg/ml), erythromycin (8 µg/ml), tetracycline (16 µg/ml), ciprofloxacin (4 µg/ml) and vancomycin (32 µg/ml). The chosen antibiotics are currently used in medical treatment of bacterial infections and have been commonly researched in recent studies (Luczkiewicz *et al.*, 2010; Czekalski *et al.*, 2012; Marti *et al.*, 2013). Tests were conducted in duplicate.

2.2.3.2 **Data analysis**

The percentage prevalence of antibiotic resistant *Enterococci* (ARE) was calculated using the formula: $\frac{A}{B} \times 100$ (Eq. 1),

Where A is the number of *Enterococci* isolates resistant to an antibiotic and B is the total number of *Enterococci* present in the wastewater sample (Tao *et al.*, 2010).

The efficiency of ARE removal by the WWTPs was calculated using the formula:

$$\frac{A-B}{A} \times 100 \text{ (Eq.2)},$$

Where A is the number of ARE present in the influent wastewater sample and B is the number of ARE present in the final treated effluent wastewater sample (Tao *et al.*, 2010).

2.2.4. **Identification of presumptive *Enterococci* species**

Two hundred of the *Enterococci* isolates recovered from the final effluent and receiving river water were purified on nutrient agar. Presumptive identification of the isolates at genus level were conducted by means of biochemical analysis, while confirmation at the genus and species level was conducted by means of Polymerase Chain Reaction (PCR) analysis.

2.2.4.1 **Biochemical analysis**

Characterization of presumptive *Enterococci* included a catalase test, growth on Bile Esculin Agar (BEA) and aesculin degradation as well as growth at 45 °C in Tryptone Soy Broth and growth in the presence of 6.5% NaCl (Ferreira de Silva *et al.*, 2006). All tests were conducted in duplicate.

2.2.4.2 **Molecular identification via PCR assays**

Presumptive *Enterococci* were subjected to multiplex PCR for further identification to the genus and species level using primers listed in Table 2.1 (Deasy *et al*., 2000; Jackson *et al.*, 2004). DNA was isolated using the boiling method (Tao *et al.*, 2010) and used as template for PCR. PCR amplification was conducted using a T100™ Thermal Cycler (Bio-Rad, USA). The PCR reactions contained a final volume of 22.5 µl, comprising of 2 groups each containing 20 µl mastermix. The first group selected for *E. faecalis* and *E. faecium*. The second group selected for *E. hirea*. The base mastermix was made up of 3 mM $MgCl_2$, 0.2 mM dNTP's, 1 × PCR buffer, 3.5 U *Taq*, 1.25 µl (16 mM) of each genus specific primer, 1.25 µl (16 mM) of each species specific primer with exception of 2.5 µl (16 mM) of *E. faecalis* primer. The following PCR protocol was used for both groups: initial denaturation at 95°C for 4 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, elongation at 72°C for 1 min and

final extension at 72°C for 7 min. The resulting PCR products were validated by conducting a 2% agarose gel electrophoresis at 60 V for 90 min in 1% TAE buffer, stained with ethidium bromide (0.5 μg/ml) and visualized using G: BOX imaging system (Syngene).

Table 2.1: Genus and species specific primers used in PCR assays for *Enterococcus* identification (Deasy *et al*., 2000; Jackson *et al*., 2004)

Target	Primer name	Sequence 5' → 3'	Product size (bp)	PCR group	Annealing temperature (°C)
Enterococci	E1 E2	TCA ACC GGG GAG GGT ATT ACT AGC GAT TCC GG	733		55
E. faecalis	FL1 FL2	ACTTATGTGACTAACTTAACC TAATGGTGAATCTTGGTTTGG	360	1	55
E. faecium	FM1 FM2	GAAAAAACAATAGAAGAATTAT TGCTTTTTTGAATTCTTCTTTA	215	1	55
E. hirae	HI1 HI2	CTTTCTGATATGGATGCTGTC TAAATTCTTCCTTAAATGTTG	187	2	55

2.3. Results

2.3.1. pH and temperature profiles of the water samples

The temperature and pH levels obtained for the various samples during the sampling period are shown in Table 2.2. The temperature at both WWTPs during the sampling duration ranged between 22-25 ºC, 20-26 ºC, 18-25 ºC, and 17-25 ºC for influent, activated sludge, before chlorination and after chlorination samples, respectively. The temperature obtained for both receiving river samples were similar to the wastewater samples, with temperatures ranging between 15-23 ºC for upstream river samples and 17-24 ºC for downstream river samples.

Varying pH levels were noted for all samples during the sampling period with the minimum pH value of 6.43 and maximum of 7.65. Sludge samples however showed a slightly more acidic pH (4.94) in June 2014 for WWTP2 and in August for WWTP1, with pH values of 4.94 and 4.92 obtained respectively (Table 2.2).

Table 2.2: pH and temperature profiles of the wastewater samples from June to September 2014

Month	Sampling Point	WWTP1		WWTP2	
		Temperature (°C)	pH	Temperature (°C)	pH
June 2014	Influent	22	7.51	23	6.73
	AS	23	6.43	23	4.94
	BC	18	7.33	21	6.51
	AC	17	7.25	19	6.43
	US	16	7.51	16	7.10
	DS	17	7.17	18	6.79
July 2014	Influent	22	7.28	22	7.23
	AS	22	6.00	20	6.89
	BC	18	7.10	20	7.24
	AC	17	7.15	18	7.25
	US	17	7.15	15	7.59
	DS	17	7.38	17	7.62
August 2014	Influent	25	7.13	23	7.41
	AS	23	7.03	23	5.25
	BC	21	7.36	22	6.83
	AC	21	7.37	22	7.19
	US	21	7.27	20	7.65
	DS	21	7.32	18	7.50
September 2014	Influent	24	7.12	24	6.98
	AS	24	4.92	26	5.34
	BC	23	7.07	25	6.89
	AC	22	7.16	25	7.09
	US	23	7.33	20	7.17
	DS	24	7.62	24	7.35

AS= Activated sludge; BC= Before Chlorination; AC= After Chlorination; US= Upstream River; DS= Downstream River

2.3.2. **Profiles of antibiotic resistant *Enterococci***

The average CFU/100ml of *Enterococci* obtained on *Enterococcus* Selective Agar with or without antibiotic used in the estimation of the prevalence of antibiotic resistant *Enterococci* (ARE) in WWTP1 and WWTP2, are presented in Table 2.3. The highest average ARE counts (in CFU/100ml) obtained for WWTP1 was for the influent and activated sludge samples and ranged from 42700 - 618000 CFU/100ml and 90500 - 871000 CFU/100ml, respectively (Table 2.3). A decrease in average CFU/100ml can be noted from samples before chlorination to the after chlorination samples. These values ranged from 700 - 8700 CFU/100ml (before chlorination) to 0 - 80 CFU/100ml (after chlorination). Low values were obtained upstream and downstream of the receiving river with values ranging from 0 - 200 CFU/100ml and 0 - 100 CFU/100ml respectively. A decrease in average CFU/100ml values can be observed from the upstream to the downstream river samples. Tetracycline resistant *Enterococci* were noted as being the highest in comparison to the population of *Enterococci* resistant to the other four antibiotics tested, with ampicillin resistant *Enterococci* being the lowest. Similar results can be noted in the average CFU/100ml values obtained in WWTP2 as depicted in Table 2.3, showing that the influent and activated sludge samples also had the highest ARE counts. The average CFU/100ml values for the influent ranged from 38000 - 735000 CFU/100ml, while that for activated sludge was greater and ranged from 235000 - 2370000 CFU/100ml. A decrease in ARE from the before chlorination to the after chlorination can also be seen in WWTP2. As for WWTP1, low ARE values for WWTP2 were also obtained in the upstream and downstream river samples with values ranging from 10 to 700 CFU/100ml and 0 to 30 CFU/100ml, respectively (Table 2.3).

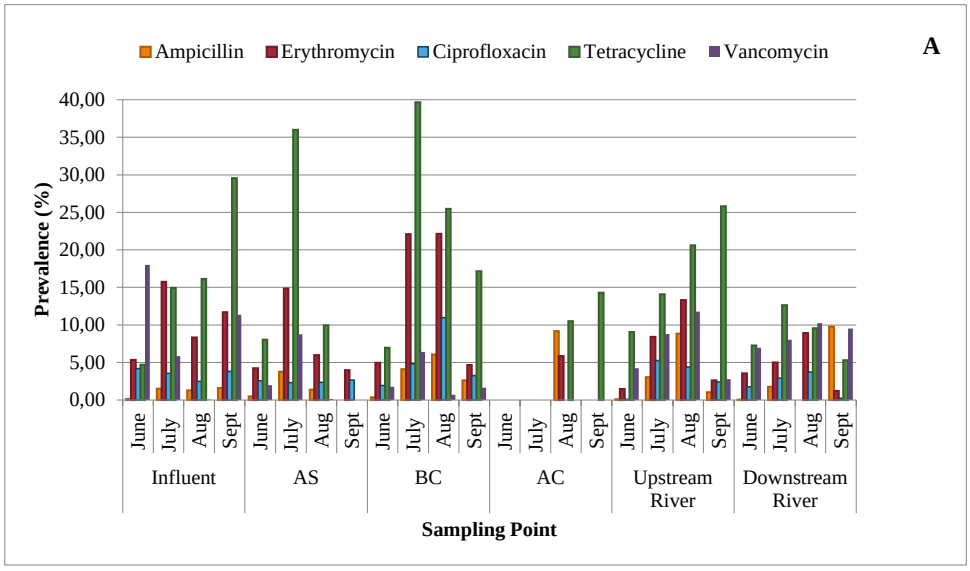
Table 2.3: Average antibiotic resistant *Enterococci* (CFU/ 100ml) at various sampling points during the sampling period in both WWTPs

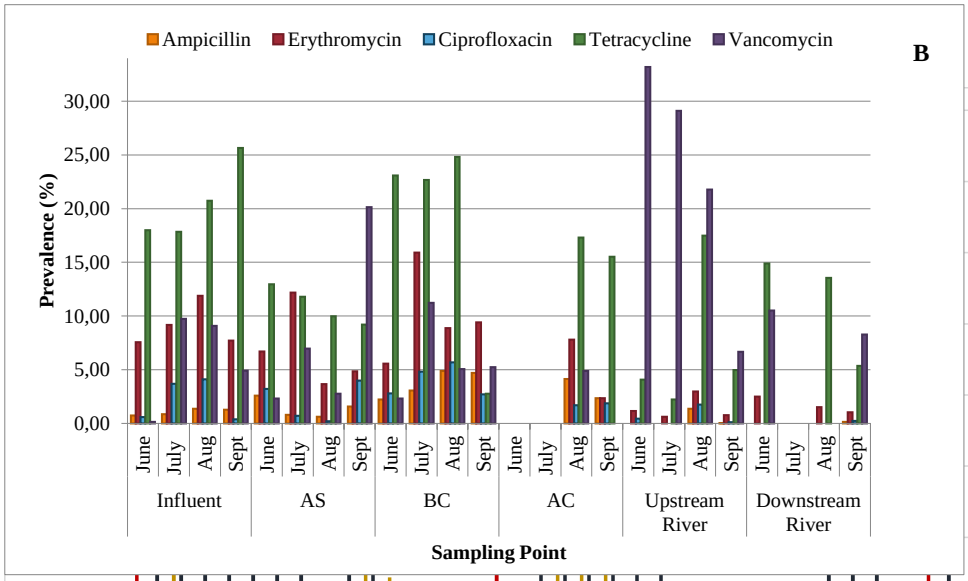
	Antibiotic	Influent	Sludge	Before Chlorination	After Chlorination	Upstream River	Downstream River
				Average (CFU/100ml) ± SD (Range)			
WWTP1	Control	3790000 ± 3.6 (3200000-4000000)	4316000 ± 28 (75000-7200000)	35400 ± 0.3 (3500-66000)	700 ± 0.0 (0-2900)	1100 ± 0.0 (510-1800)	1100 ± 0.0 (1020-1600)
	Ampicillin	42700 ± 0.2 (6000-63000)	90500 ± 1.1 (0-272000)	1600 ± 0 (13-4350)	70 ± 0.0 (0-300)	30 ± 0.0 (1-55)	0
	Erythromycin	393000 ± 1.8 (213000-630000)	394000 ± 4.3 (3000-1070000)	5600 ± 0.1 (173-14650)	40 ± 0.0 (0-200)	100 ± 0.0 (13-152)	52 ± 0.0 (57-91)
	Ciprofloxacin	135000 ± 0.4 (80000-168000)	104000 ± 0.7 (2000-166000)	2500 ± 0 (68-7250)	0	0	0
	Tetracycline	618000 ± 3.8 (189000-1170000)	871000 ± 10.8 (0-2590000)	8700 ± 0.1 (243-16800)	80 ± 0.0 (0-300)	200 ± 0.0 (78-254)	100 ± 0.0 (98-130)
	Vancomycin	351000 ± 2.8 (1900-720000)	186000 ± 2.8 (0-630000)	700 ± 0 (62-1550)	0	100 ± 0.0 (0-159)	100 ± 0.0 (83-112)
WWTP2	Control	3500000 ± 5.1 (2930000-4250000)	31370000 ± 247.3 (3250000-59800000)	2000 ± 0.0 (1400-2700)	100 ± 0.0 (0-400)	3200 ± 0.0 (3000-3600)	400 ± 0.0 (0-10200)
	Ampicillin	38000 ± 0.1 (21800-54000)	235000 ± 1.9 (26000-450000)	50 ± 0.0 (30-80)	0	10 ± 0.0 (0-45)	0
	Erythromycin	318000 ± 0.7 (222000-418000)	1058000 ± 8.4 (257000-2190000)	100 ± 0.0 (100-400)	0	45 ± 0.0 (19-97)	0
	Ciprofloxacin	75000 ± 0.6 (17000-145000)	353000 ± 4.9 (23000-1140000)	80 ± 0.0 (40-100)	0	20 ± 0.0 (0-60)	0
	Tetracycline	735000 ± 2.3 (527000-1090000)	2370000 ± 24.1 (383000-5960000)	400 ± 0.0 (350-600)	20 ± 0.0 (0-70)	200 ± 0.0 (70-600)	30 ± 0.0 (0-55)
	Vancomycin	214000 ± 1.4 (4500-324000)	1930000 ± 24.5 (89000-5760000)	100 ± 0.0 (30-300)	0	700 ± 0.0 (700-1300)	0

2.3.2.1 **Prevalence of antibiotic resistant *Enterococci***

High prevalence of ARE was obtained at both WWTPs, with values reaching a maximum of 40% (Figure 2.2 A and B). Variable trends, of the ARE prevalence, were observed over the sampling period for both WWTPs. The influent and activated sludge samples contained the greatest prevalence of ARE with lower values observed in the before and after chlorination samples (Figure 2.2 A and B). Despite the final effluent showing a reduced ARE prevalence (after chlorination), higher levels of ARE were detected in the receiving river samples (RR1 and RR2) throughout the sampling period. Generally, a lower ARE prevalence from upstream to downstream of both river samples can be seen in Figure 2.2 (A and B). This shows that the final effluent from the WWTPs reduced the ARE prevalence in the respective receiving rivers. Generally, high prevalence of tetracycline resistant *Enterococci* were observed throughout the 4 month sampling period across all sampling points. The highest tetracycline resistant *Enterococci* values can be seen in the influent sample for WWTP1 and WWTP2 for September 2014 (29.55% and 25.65%, respectively), it can also be seen in the activated sludge samples of WWTP1 in July 2014 (35.97%) as well as for the before chlorination sample in WWTP1 for July 2014 (39.67%). This high prevalence was closely followed by high erythromycin resistant *Enterococci*, with prevalence being the highest in the before chlorination samples in WWTP1 for July and August 2014 (22.11% and 22.12%, respectively) and in WWTP2 for July 2014 (15.92%) as well as for the influent samples of WWTP1 in July 2014 (15.75%). High prevalence of vancomycin resistant *Enterococci* can be noted across all months in the upstream and downstream samples of both RR1 and RR2. This high resistance can be seen in RR2, in the upstream river samples with prevalence values of 33.2%, 29.11%, 21.78% and 6.68% obtained in June, July, August and September, respectively. These values were the highest ARE values obtained for the whole sampling period in the upstream river samples of RR2.

Vancomycin resistant *Enterococci* were also high in the downstream river samples of RR2 in June 2014 (10.50%) and September 2014 (8.28%). High prevalence was also observed in downstream river samples of RR1 from June to Sept 2014 with values of 6.97%, 8.01%, 10.25% and 9.52%, respectively. With the increased ARE prevalence observed for the after chlorination point of WWTP2 in August 2014, the prevalence of vancomycin resistance was also notably high at 4.88%. Resistance to vancomycin was also observed to be the highest for influent of WWTP1 in June 2014 (18%) and activated sludge for September 2014 of WWTP2 (20.14%). Vancomycin resistant *Enterococci* also showed to be second and third highest in most samples from influent, activated sludge and before chlorination. The ARE prevalence in the after chlorination samples were low to nothing in June and July 2014 for WWTP1, but higher in August and September 2014. A notable decrease in ARE prevalence can be seen before chlorination to after chlorination. Similar results of ARE prevalence were obtained for WWTP2.


A
Ampicillin
Erythromycin
Ciprofloxacin
Tetracycline
Vancomycin
Prevalence (%)
40,00
35,00
30,00
25,00
20,00
15,00
10,00
5,00
0,00
June
July
Aug
Sept
Influent
AS
BC
AC
Upstream River
Downstream River
Sampling Point


B
Ampicillin
Erythromycin
Ciprofloxacin
Tetracycline
Vancomycin
Prevalence (%)
30,00
25,00
20,00
15,00
10,00
5,00
0,00
June
July
Aug
Sept
Influent
AS
BC
AC
Upstream River
Downstream River
Sampling Point

Ampicillin
Erythromycin
Ciprofloxacin
Tetracycline
Vancomycin
10,00
5,00
0,00
June
July
Aug
Sept
1
2
Influent
Sludge
BC
AC
Upstream River
Downstream River
Sampling Point

Figure 2.2: Prevalence of antibiotic resistant *Enterococci* at (A) WWTP1 and (B) WWTP2

AS= Activated sludge; BC= Before Chlorination; AC= After Chlorination

2.3.2.2 **Removal of antibiotic resistant *Enterococci***

Figure 2.3 represents the average ARE removal percentage for both WWTP1 and WWTP2. The percentage removal for both WWTP1 and WWTP2 were all above 99%. Up to 100% removal of ciprofloxacin resistant *Enterococci* was obtained for WWTP1, with the other ARE having a removal value of 99.83% to 99.99%. A 100% removal of erythromycin, tetracycline and vancomycin resistant *Enterococci* was also obtained in WWTP2, with the remaining ARE having a value of 99.99% removal.

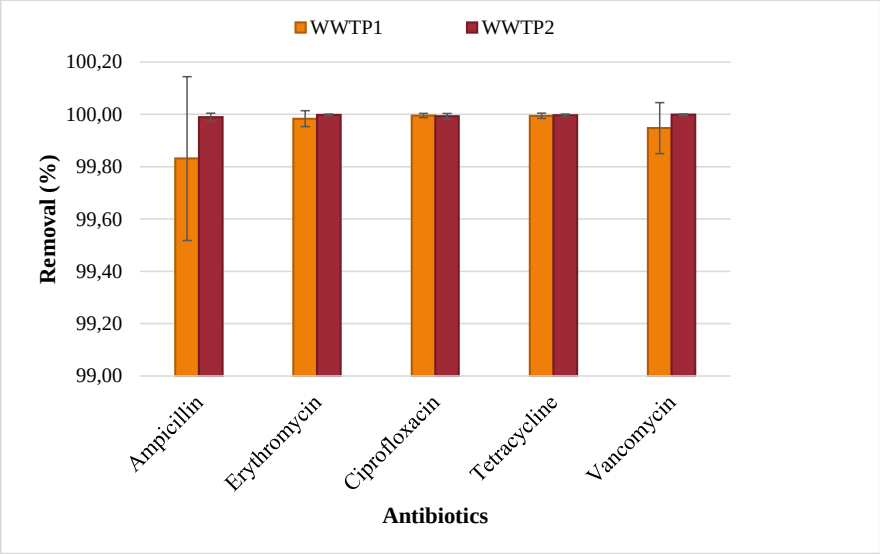


Figure 2.3: Removal of antibiotic resistant *Enterococci* after the treatment process at both WWTPs

2.3.3. **Species distribution of the *Enterococci* isolates**

Following a negative catalase test, isolates were tested for their ability to grow on bile easulin agar, producing a blackening halo, while also being able to degrade easculin. These isolates also tested positive for their ability to grow at elevated temperatures (45 ºC) as well as at high salt concentrations (6.5% NaCl). Isolates that passed these biochemical tests were then confirmed as *Enterococci* at the genus and species level utilizing multiplex PCR amplification. Figure 2.4 shows the PCR products of the genus and species on a 1.5 % agarose gel. The *Enterococci* genus amplicon product was 733 bp, while *E. faecalis* was 360 bp, *E. faecium* was 215 bp and *E. hirea* was 187 bp. Lane 1 represents a 100 bp ladder, Lane 2, 3 and 4 shows the positive controls of *E. faecalis, E. faecium* and *E. hirae*, respectively. While lane 6 to 13 shows representative isolates, with lane 14 containing a negative control. Table 2.4 highlights that *E. faecalis* was the most abundant species at 34.5% of the total isolates identified, followed by *E. feacium* and *E. hirea* at 26% and 25.5%, respectively. The remaining 14% confirmed as *Enterococci* isolates, but were not identified as being *E. faecalis, E. faecium* or *E. hirea*.

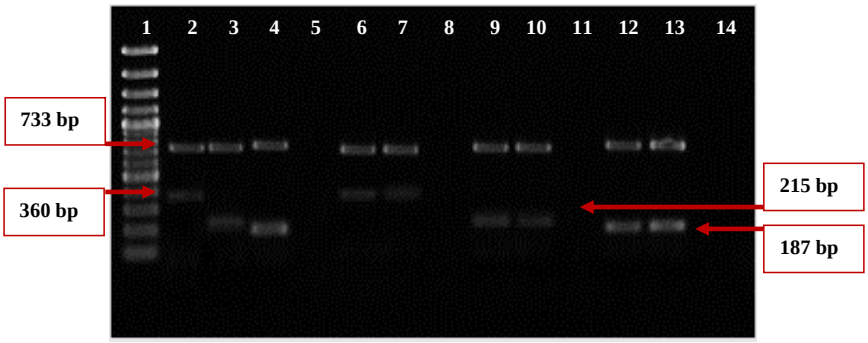


Figure 2.4: PCR detection of genus and species specific genes of *Enterococci*. Genus-specific bands are indicated at 733 bp and species-specific bands are indicated at 360, 215 and 187 bp. Lane 1: 100 bp ladder; Lane 2- 4: positive controls of *E. faecalis, E. faecium* and *E. hirae;* Lane 6-7: *E. faecalis*; Lane 9-10: *E. faecium*; Lane 12-13: *E. hirae*; Lane 14: negative control

Table 2.4: Distribution of *Enterococci* species identified in this study

Species	Total	Total (%)
E. faecalis	69/200	34.5
E. faecium	52/200	26.0
E. hirea	51/200	25.5
Enterococcus spp.	28/200	14.0

2.4. Discussion

Following the era of the antibiotic discovery, humans have become dependent on these miracle drugs for the control of infectious disease and in animal husbandry. In a study conducted in 2002, it was shown that the approximate global quantity of antibiotics consumed was between 100 million to 200 million kilograms (Wise, 2002). Large quantities of these antibiotics enter WWTPs from improper disposal, land run-off or from human faeces as a result of the antibiotics not being fully metabolized in the body (Hong *et al.,* 2013). Conventionally, wastewater in WWTPs is initially screened for large objects and suspended particles followed by biological treatment using activated sludge. Subsequently the water is then transferred into secondary clarifiers for further removal of suspended particles and organic matter. This step is sometimes followed by a tertiary step (disinfection) before being discharged into receiving water bodies or reused for agricultural purposes (Hong *et al.,* 2013). With many countries opting for reuse of wastewater as reclaimed water, it is necessary that wastewater is treated in an appropriate and efficient manner. One contaminant that often goes by unnoticed are ARB. The present study analysed the efficiency of two WWTPs that discharge their final treated effluent into two major rivers in Durban which are commonly used and further processed by local municipalities as a supply of drinking water to houses and industries and are also used by local inhabitants directly as a water source. The prevalence of *Enterococci* and antibiotic resistant *Enterococci* (ARE) was investigated in this study with particular focus on *Enterococci* resistant to ampicillin, erythromycin, tetracycline, ciprofloxacin and vancomycin. These antibiotics were selected as they are currently used in the treatment of many enterococcal infections (Gavaldà *et al.,* 2007; Kristich *et al.,* 2014). Urinary tract and soft-tissue infections are commonly treated with ampicillin or vancomycin in severe cases

(Cunha, 2006; Baddour *et al.,* 2015). Ciprofloxacin are used with much success for urinary tract infections and erythromycin has shown to be effective in supressing symptoms in endocarditis. Also, intravenous ampicillin may be used as a cure for enterococcal meningitis, while vancomycin, aminoglycosides, ciprofloxacin and erythromycin in the treatment of this disease (Dhanalakshmi *et al.,* 2015).

Enterococci are commonly found in the intestine of most mammals. They can also be found in many aquatic environments, as they are able to survive and propagate in adverse environments of extreme pH, temperature and sodium chloride concentrations (Feuerpfeil *et al.,* 1999). This genera of bacteria are nosocomial pathogens that cause a variety of diseases including urinary tract infections, endocarditis and bacteremia (Spencer, 1996). Nosocomial infections are problematic as they lead to functional disability and emotional stress and may reduce the quality of life (WHO, 2002). Such infections are one of the leading causes of death (Ponce-de-Leon, 1991). High hospitalization expenses resulting from increased length of stay is another major problem as well as loss of work (Kirkland *et al.*, 1999). Extensive antimicrobial agents are used to treat patients. By means of selection and exchange of genetic resistance, multidrug resistant bacteria are on the rise. Resistances to antibiotics used to treat bacterial infections, particularity enterococcal infections, are a major problem as many of these antibiotics are obsolete and can no longer be used in effective treatment. Many hotspots for these resistant bacteria have been identified, with WWTPs being of particular concern as the final effluents are discharged into receiving water bodies and thus impacts the environment. The final effluent from WWTPs have to be of a high quality as WWTPs have to comply with the National Water Act (1998) of South Africa for discharge of the final effluent into a surface water source or the ocean directly (DWAF, 2004). It is therefore imperative that WWTPs are efficient in their treatment process, ensuring that water of

high quality is released back into the environment and prevents the spread of antibiotic resistance. In addition to the selective pressure of high antibiotic concentrations and its constituents being present in wastewater, large microbial populations and the high nutrient levels from faecal matter, may contribute to the final effluent being a carrier of ARB and thus a contaminating factor of ARB to water bodies (Salyers *et al.*, 2004).

In the present study, temperature and pH measurements were conducted at the four pre-determined sampling points within the WWTPs and upstream and downstream of their receiving rivers. According to the Government Gazette of South Africa (1984), the standards of wastewater or the final effluent arising in the catchment area draining water to any river, state that temperature levels should not exceed 25 ºC and pH levels should be between 5.5 and 7.5. *Enterococci*, however, are able to grow at temperatures ranging from 5 to 50 ºC and also have the ability to grow in a range of pH levels from 4.6 to 9.9 (Fisher and Phillips, 2009; Van den Berghe *et al.*,2006). These bacteria are able to withstand such varying temperatures and pH levels due to their specialized physical characteristics. *Enterococcus faecalis* has the capability of tolerating extreme pH levels as a result of its durable and impermeable membrane (Fisher and Phillips, 2009; Nakajo *et al.*, 2005). The measured temperature values obtained for all the water samples during the sampling periods fall within the standards stipulated in the Government Gazette (1984), indicating that the treated effluent do not pose any danger to the homeostatic balance of the receiving rivers (Jaji *et al*., 2007).

The pH levels obtained for most of the samples during the sampling period, ranging from 6.43 to 7.65 were also in accordance with the Government Gazette (1984). Most of the pH values, also complied with the World Health Organization standard of 7.0 to 8.5 (WHO, 1989). The values obtained were also similar to values obtained by Igbinosa and Okoh (2009). The pH is an important

water quality factor as it establishes if the water can be used for other purposes. It is also important that pH levels for the final effluent are within the stipulated guidelines when being released into the environment as aquatic and plant life may not survive under extreme alkaline or acidic pH values. The pH protection limits for fisheries and aquatic life, recommended by the EU, range from 6.0 to 9.0 (Chapman, 1996).

Enterococci are hardy organisms and have the ability to withstand harsh environmental parameters (extreme temperature, pH and salinity levels) and are therefore able to persist in the environment (Leclercq *et al.,* 2013). Generally a decrease in the population number of *Enterococci* are observed when *Enterococci* are released from the gastrointestinal tract of warm-blooded animals into new environments such as water systems that poses many stress factors (Muruleedhara *et al.,* 2012). Environmental factors such as sunlight have been known to have an adverse effect on bacterial populations (Downes and Blunt, 1877). Sunlight is known to damage microbial DNA or lead to microbial inactivation by means of direct absorption of ultraviolet light or by means of indirect formation of endogenous and exogenous reactive oxygen species (Muruleedhara *et al.,* 2012). Due to this stress factor, it is would be expected that bacteria found in open-air wastewater tanks, exposed to large amounts of sunlight, would not be found in large numbers as a result of poor survival and propagation.

In the present study, ARE prevalence as high as 40% was obtained for both WWTPs. The prevalence of ARE was highest in the influent and activated sludge samples possibly as a result of high microbial load and optimal growth conditions for the bacteria. Generally biological reactors such as activated sludge tanks are rich in bacteria and favour their proliferation (Jury *et al.,* 2010). A reduction in ARE prevalence in treated effluent, following the disinfection step, showed the

effectiveness of the chlorination used by these WWTPs in removing the ARE. The ARE population in the upstream river samples of both RR1 and RR2 are higher than that in the downstream samples, possibly due to the positive effect of the final effluent discharge from both plants on the receiving rivers. The observed higher prevalence of ARE in the upstream river samples of RR2 than in RR1 may be due to a medium scale vegetable farm operating along the sampling point as well as a small scale livestock farm, as can be seen in Figure 2. 1 (B and D). This is because manure and antibiotic runoff from these farms may have lead to the increase in ARE prevalence. Up to 90% of an antibiotic dose can be passed in an animal's urine and up to 75% in their faeces (Sarmah *et al.,* 2006) and these antibiotics can seep into groundwater. Animal waste and manure which is used as fertilizer can also be a contributing factor to the spread of antibiotic resistance genes (ARGs) and contamination of soil and groundwater. Use of antibiotics for agricultural purposes has shown to be linked to ARB prevalence in surface water in the United States and Mexico (Sarmah *et al.,* 2006). A contributing factor to the higher ARE prevalence in the upstream river samples of RR1 as compared to the downstream river samples, may be due to the increased anthropogenic activity present at this sampling point. As can be seen in Figure 2. 1 (C, E and F), free-running poultry were present along the river bank as well as local inhabitants, fisherman and pedestrians engaging in recreational and domestic activities.

The lowest resistance observed against ampicillin for all samples, across all months, in this study is unusual as ampicillin are first line antibiotics and are most commonly used in the treatment of bacterial infections. However, the high level of susceptibility towards this antibiotic was in accordance with a previous study that revealed high susceptibility of environmental *Enterococci* towards beta-lactams (Ferreira da Silva *et al.,* 2006). These observations were consistent with many other studies testing *Enterococci* in animal and food products and in clinical isolates (Peters

et al., 2003; Fluit *et al.,* 2000). In the present study, the higher level of resistance towards tetracycline obtained in WWTP2 than in WWTP1 may be attributed to the hospital influent received by WWTP2. This is in accordance with previous studies reporting higher tetracycline resistance in clinical isolates (Fluit *et al.*, 2000; Mondino *et al.,* 2003). From the results obtained, resistance to erythromycin was second highest. Enterococcal resistance to macrolides, such as erythromycin, is alarming due to the increasing prevalence of resistance phenotypes (Ferreira da Silva *et al.,* 2006).

The nosocomial infections caused by *Enterococci*, such as urinary tract and surgical wound infections, bacteremia and endocarditis are often treated by the glycopeptide antibiotics, specifically vancomycin (Spencer, 1996). Resistance to this antibiotic is problematic in the therapy of these infections (Schwartz *et al.,* 2003). In a study conducted in Sweden, 60% of raw wastewater contained vancomycin resistant *Enterococci* (VRE) with 19% of final effluent containing VRE and low levels found in surface water (Iversen *et al.,* 2002). In the present study a high VRE prevalence can be noted across all months in the upstream and downstream river samples of RR1 and RR2. These values were the highest ARE values obtained for the whole sampling period in the upstream river samples of RR2. Increased ARE prevalence was observed for the after chlorination point of WWTP2 in August 2014, where vancomycin resistance was also notably high. Vancomycin resistant *Enterococci* also showed to be second and third highest in most samples from influent, activated sludge and before chlorination. High resistance to vancomycin, frequently noted in WWTP2 is not surprising as this WWTP received a portion of its waste from hospital settings. It has been shown that hospital waste contains VRE (Novais *et al.,* 2005). The concentration of this antibiotic in hospital waste is presumably higher than in domestic or industrial waste, which mainly is received in WWTP1 (Jury *et al.,* 2010).

Many classes of antibiotics have been reported in wastewater since the late 1990s including sulfonamides, trimethoprim, β-lactams, fluoroquinolones, macrolides and tetracyclines (Ghosh *et al.,* 2016; Sinthuchai *et al.,* 2016). It is therefore not surprising that bacteria resistant to these antibiotics are still prevalent, as shown in the present study. The observed high reduction in ARE (up to 99%) observed for both WWTPs corroborates a previous study conducted by Ferreira da Silva *et al.* (2006). They studied a similar WWTP to WWTP1 in which the plant received mostly domestic sewage and some industrial effluents and followed the activated sludge process. Both WWTPs have been awarded high green drop statuses for the year 2011. This was as a result of stringent treatment processes followed by the plants as well as maintenance upgrades. This is evident from the high removal percentages of ARE in both WWTPs. The high removal rate of ARE in the WWTPs may have been because of the recent upgrades at WWTP1 and the stringent treatment processes followed by both WWTPs that allowed them to achieve such high Greendrop scores in 2011. The decline in ARE from influent to final effluent samples are in accordance with other studies that showed such a decline following treatment (Garcia *et al.,* 2007).

A bile-esculin positive test is one in which the organism is able to turn the agar from a pale yellow to a dark brown to black colour following at least 48 h incubation (Chuard and Reller, 1998). This test is commonly used for presumptive identification of group D *Streptococci* and *Enterococci* as not many bacteria can hydrolyze esculin in the presence of bile. The ability of these organisms to withstand environments of high salinity and temperature levels were also used as an important biochemical test. Following PCR amplification *E. faecalis* was the most abundant species identified at 34.5% of the total isolates, followed by *E. faecium* and *E. hirea* at 26% and 25.5%, respectively. The remaining 14% were confirmed to belong to the genus *Enterococci*. The prevalence of *E. faecium* and *E. hirea* were each shown to be at less than 35% in wastewater

effluent, with *E. faecalis* at less than 20%. The prevalence of specific enterococcal species are dependent on season and climatic variability (Byappanahalli *et al.,* 2012). *Enterococcus faecalis* is commonly related to causing urinary tract, neonatal and central nervous system infections, endocarditis and bacteremia (Sievert *et al.,* 2013; Mikalsen *et al.,* 2015). *Enterococcus faecalis* and *E. faecium* are predominantly found in human and animal faeces and sewage (Manero *et al*. 2002; Abamecha *et al.,* 2015). This accounts for the high prevalence of these species found in the study. A study conducted by Bonjoch *et al.* (2011) showed that certain *Enterococci* species were most prevalent, in aquatic environments, during the winter. One such species is E. *hirea*, which could be why such high numbers of *E. hirea* was identified in this study as the study was conducted mainly during winter (Leclercq *et al.,* 2013). *Enterococcus faecalis, E. faecium, E. durans* and *E. hirae* are used as sewage contamination and water quality indicators as well as indicators of the presence of disease causing pathogens that are found in the gastrointestinal tract of most mammals (Kaltenthaler and Pinfold, 1995; Byappanahalli *et al.,* 2012).

2.5. Conclusion

This chapter investigated the efficiency of two WWTPs (within Durban, South Africa), in removing ARE during the treatment process, by evaluating the prevalence and removal of ARE at stipulated sites within the WWTPs. The influence of final effluent on the environment was also studied by analysing the ARE prevalence upstream and downstream of the receiving water bodies. Additionally, the identity of predominant *Enterococci* species was determined by conducting standard biochemical tests and PCR assays.

Previous studies, conducted in South Africa, have shown WWTPs to be reservoirs for ARE (Olaniran *et al.*, 2012; Iweriebor *et al.*, 2015). However, the results of this study show that WWTPs cannot be generalized as being such reservoirs. The temperature and pH levels of the water samples were satisfactory and within the specified standards of the Government Gazette. Both WWTPs were efficient in removing ARE during the treatment process by more than 99% (with no final effluent sample exceeding 80 CFU/100ml of ARE). This therefore shows that final effluent discharged from the WWTPs do not contribute to an increase in ARE in the environment. This is evident by the decrease in ARE prevalence from upstream to downstream of the receiving water bodies as a result of the dilution effect of the final effluent on the upstream water. The three species identified, are commonly found in faecal matter and aquatic environments. They are important indicators of water quality and disease causing pathogens. *Enterococcus faecalis*, was the predominant species identified, and is known to cause many human infections. It is therefore imperative that frequent monitoring of WWTP processes be conducted in order to maintain adequate final effluent and environmental water quality as well as to avert the spread of ARE.

CHAPTER THREE

ANTIBIOTIC RESISTANCE PROFILES OF *ENTEROCOCCI* SP. RECOVERED FROM TREATED EFFLUENT AND RECEIVING SURFACE WATER, AND FATE OF TETRACYCLINE RESISTANCE GENES DURING THE WASTEWATER TREATMENT PROCESS

3.1. Introduction

The introduction of antibiotics in the 1940's have saved countless lives' and revolutionized the medical, agricultural, social and economic world. This miracle drug has been used in the treatment and eradication against many deadly bacterial infections and has also allowed for great advances in agricultural practices (Bouki *et al.,* 2013). Antibiotics have evolved in their role as a mere treatment option against infections to an infection preventative and as a protective measure in patients suffering from cancer and immuno-deficiencies (Gelband *et al.,* 2015). The invaluable applications of antibiotics has resulted in its widespread use and increased consumption (Aminov, 2009). A major problem associated with their over-use and misuse is the development of antibiotic resistance which continues to be a catastrophic crisis worldwide, since the ineffectiveness of antibiotics is highly problematic in controlling infectious diseases, thus increasing the death rate. This major problem was recently acknowledged and highlighted in a report by the World Health Organization (2014b) in which it was stated that antibiotic resistance is no longer a prediction for the future but is a current problem worldwide and that antibiotic resistance affects the effective treatment of common infections in the community and hospitals. It was further reported that without immediate action, the world is headed to a post-antibiotic era. The problem of antibiotic resistance is further compounded by the rapid spread of antibiotic resistance genes (ARGs)

amongst bacteria (Wang and Schaffner, 2011). The spread of ARGs have often been found in environments such as wastewater, surface water, drinking water, soil and sediment (Brooks *et al.*, 2007; Munir *et al.*, 2011).

Wastewater and natural aquatic environments commonly inhabit ARB, ARGs and multidrug resistant bacteria. Anthropogenic and agricultural practices are major contributors to the spread of ARGs in the environment (Pei *et al.*, 2006). Antibiotic resistance may be intrinsic or acquired in bacteria. Extra-chromosomal elements that carry genes conferring antibiotic resistance have an enhanced ability to transfer ARGs via conjugation that lead to multidrug resistant bacterial infections in humans (Clewell, 2014). The increased spread of resistance in the environment is often due to the transfer of ARGs via horizontal gene transfer (HGT) by transduction, transformation or conjugation (Burmeister, 2015). Transferable ARGs are found on plasmids, integrons and transposons. These vectors transport the genes between bacteria of the same species and between bacteria of other genera or species. Bacterial procurement of ARGs from resistant donors can convert a previously susceptible bacteria to be antibiotic resistant (Burmeister, 2015).

Large quantities of ARGs have been reported in agricultural settings as well as in wastewater treatment plants (WWTPs) and their treated effluents (Schwartz *et al.*, 2003; da Costa *et al*., 2006; Pruden *et al.*, 2006; Iweriebor *et al.*, 2015; Yuan *et al.*, 2015; Karkman *et al.*, 2016). Wastewater received from clinical settings are major reservoirs of antibiotics, ARB and ARGs that may then be transferred into the environment. Not all WWTPs are designed to completely or efficiently remove ARB and ARGs. The combination of gut-associated resistant bacteria, antibiotic residues, high nutrient levels and the large microbial consortium found in the activated sludge shows that the secondary treatment process in a WWTP provides a favourable setting for the transfer of ARGs

(Michael *et al.,* 2013). This elevated transfer of mobile genetic elements between bacteria in activated sludge have been shown in many studies (Zhang *et al.,* 2009a; Colomer-Lluch *et al.,* 2011). The fate of ARGs in the environment rests on the ability of the bacterial host to survive and propagate in the environment following discharge from the WWTP (Proia *et al.,* 2015).

One bacterial group of particular importance with regards to antibiotic resistance are the *Enterococci.* The commensal nature of *Enterococci* make them important microorganisms in the intestine of most mammals. These organisms are however, the cause of many nosocomial infections (Gunasekera and Perera, 2007). Research has shown that *Enterococci* is the second most frequent cause of hospital acquired infections globally (Karki *et al.*, 2015). *Enterococci* are among the leading causes of nosocomial infections of the urinary tract, surgical wounds, bloodstream and endocarditis, amongst others (Hidron *et al.,* 2008). This group of bacteria could possibly obtain resistance to most clinically useful antibiotics (Hollenbeck and Rice, 2012). Due to *Enterococci* being infamously known for their ease in acquiring antibiotic resistance, they play an important role in the spread of resistance at the intra-and inter- specific levels. Antibiotic resistant *Enterococci* (ARE) may flourish in various environments such as the gastrointestinal tract of most mammals, food sources, clinical settings and WWTPs. From these respective hotspots, ARE can easily spread to humans directly via drinking water sources or indirectly via consumption of infected meat and vegetables (Leclercq, 2009; Marshall and Levy, 2011; Getachew *et al.,* 2013). The perpetual nature of these ubiquitous organisms, in the environment, are worrisome as they can outlast other organisms, such as fecal coliforms, and thus have an increased likelihood of re-infecting humans and animals (Kuhn *et al.,* 2000). Due to the vast acquisition of resistance to penicillin, vancomycin and high level aminoglycoside, the treatment of enterococcal infections

pose a difficult challenge (Mohanty *et al.,* 2006). Multidrug resistance hinders treatment of enterococcal infections thus limiting therapeutic options (Oberoi and Aggarwal, 2010).

Resistance to tetracyclines is frequently found in bacteria isolated from environmental samples (Billington *et al.,* 2002). A study conducted by Esiobu *et al.* (2002) found that almost 89% of bacteria they isolated from soil and water samples were resistant to tetracycline. Tetracyclines are broad-spectrum antibiotics and are active against many Gram negative and Gram positive bacteria. They are also used in the prevention of malaria and as livestock growth promoters (Chee-Sandford *et al.,* 2009). The widespread use of this antibiotics have lead to the high prevalence of tetracycline resistant bacteria. It was reported that approximately 5 million kilograms of tetracycline was used in the U.S per year just in agricultural settings (FDA, 2014). Wastewater treatment plants have also shown to contain high levels and diversity of tetracycline resistance genes and can therefore easily be transferred into the environment through discharge of the treated effluent into receiving water bodies (Pruden *et al.,* 2006; Auerbach *et al.,* 2007). *Enterococcus faecalis* are able to transfer tetracycline resistance genes by 10- to 100- fold greater than it can most other ARGs (Torres *et al.,* 1991).

Special techniques and tests are used to quantify the concentrations and prevalence of ARGs in various samples. One such technique is Real-Time PCR (RT-PCR), which is most traditionally used for absolute or relative quantification of deoxyribonucleic acid (DNA) copies in a sample. This technique utilizes a thermocycler, enzyme polymerase, specific oligonucleotides, DNA, intercalating dye and $MgCl_2$. The amplified products is detected and quantified by measuring the fluorescence intensity during the PCR cycles, which is a proportional value to the concentration of the DNA product (Ahrberg *et al.,* 2016). Absolute quantification, using RT-PCR, is not always

a favourable technique, as it is very labour-intensive, consistent standards and reference controls are needed in every run (Wong and Medrano, 2005). Droplet digital PCR (ddPCR), a novel method, of directly quantifying DNA copies in different samples, to ensure more accurate quantification results of ARGs in the environment, has been developed (Jones *et al.,* 2014). Droplet digital PCR allows for determination of absolute quantification of target DNA without the need for a standard curve of the reference. DNA is split into approximately 20 000 droplets allowing for amplification to occur in each droplet and uses fluorescence probes (Hindson *et al.,* 2011). This technique allows for quantification of very low DNA concentrations in a highly accurate and precise manner (Norton *et al.,* 2013). Droplet digital PCR is now the preferred method to qPCR (Miotke *et al.,* 2014).

The aims of this chapter was to assess the antibiotic resistance patterns of *Enterococci* sp. recovered from the final effluent and receiving river samples, to detect the presence of specific tetracycline resistance genes in the isolates and determine the genetic finger-printing profile of the isolates. Furthermore, the efficiency of the WWTPs for the removal of selected tetracycline resistance genes was determined.

3.2. Materials and Methods

3.2.1. Antibiotic susceptibility testing

The antibiotic susceptibility pattern of 200 *Enterococci* sp. recovered from the final treated wastewater effluent of the two WWTPs and the receiving rivers in Durban, South Africa, were conducted using the Kirby-Bauer disc-diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI, 2007). The antibiotics used for this test represent antibiotics

from different classes. The bacterial inoculum used for the antibiotic susceptibility test was prepared by growing the *Enterococci* isolates in nutrient broth for 24 h and then standardized to obtain a turbidity of 0.08 to 0.10 at 625nm (CLSI**,** 2006). The antimicrobial discs shown in Table 3.1, were impregnated on Meuller-Hinton agar, 25 mm apart once the plates were swabbed with the standardized inoculum and allowed to air dry. Plates were then incubated at 37 °C for 48 h. Isolates were categorized as susceptible (S), intermediate (I) or resistant (R) to each antibiotic based on the zone diameter analysis (CLSI, 2007). The inhibition zone diameters were measured to the nearest millimeter and recorded. Tests were conducted in two replicates. MAR index was calculated using the following formula (Blasco *et al.,* 2008):

$$\text{MAR} = \frac{a}{b} \text{ (Eq. 3),}$$

Where a is the number of antibiotics to which the isolate was resistant to; b is the total number of antibiotics against which individual isolate was tested.

Table 3.1: Concentrations of antibiotic discs used in Kirby Bauer Disk Diffusion test

Antibiotic Class	Antibiotic	Antibiotic abbreviation	Concentration (µg)
β-lactams	Ampicillin	AMP	10
Glycopeptides	Vancomycin	VAN	30
Aminoglycosides	Gentamicin	GEN	10
Aminoglycosides	Gentamicin	GEN	120
Macrolides	Erythromycin	ERY	15
Sulfonamides	Trimethoprim-sulfamethoxazole	SXT	1.25/23.75
Quinolones	Ciprofloxacin	CIP	5
Tetracycline	Tetracycline	TET	30
Phenicol	Chloramphenicol	CHL	30
Ansamycin	Rifampicin	RIF	5

3.2.2. **Detection of tetracycline resistance genes**

The presence of tetracycline resistance genes were determined in selected *Enterococci* isolates via multiplex PCR using a T100™ Thermal Cycler (Bio-Rad, USA). The five targeted tetracycline resistance genes were *tet* K, *tet* L, *tet* M, *tet* O and *tet* S. The primers listed in table 3.2 were used for PCR amplification of the target genes. The 50 µl reaction mixes was made up of 1 × PCR buffer, 3 mM $MgCl_2$, 300 µM dNTP's, 2.5 U *Taq*, 0.5 µg (2µl) DNA, specific primers for *tet* K (0.25 µM), *tet* L (0.2 µM), *tet* M (0.1 µM), *tet* O (0.15 µM) and *tet* S (0.1 µM) (Adapted from Ng *et al.*, 2001). The following PCR protocol was used: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, elongation at 72°C for 1.5 min and final extension at 72°C for 7 min. The resulting PCR products were validated by conducting a 1.5% agarose gel electrophoresis run at 90 V for 90 min. Thereafter, products were visualized using the G: BOX imaging system (Syngene) after staining in ethidium bromide (0.5 µg/ml).

Table 3.2: Primer sequence and product size of various tetracycline resistance genes (Ng *et al.,* 2001)

Primer name	Sequence 5' → 3'	Product size (bp)
tet(K)F	TCG ATA GGA ACA GCA GTA	169
tet(K)R	CAG CAG ATC CTA CTC CTT	
tet(L) F	TCG TTA GCG TGC TGT CAT TC	267
tet(L)R	GTA TCC CAC CAA TGT AGC CG	
tet(M)F	GTG GAC AAA GGT ACA ACG AG	406
tet(M)R	CGG TAA AGT TCG TCA CAC AC	
tet(O)F	AAC TTA GGC ATT CTG GCT CAC	515
tet(O)R	TCC CAC TGT TCC ATA TCG TCA	
tet(S) F	CAT AGA CAA GCC GTT GAC C	667
tet(S)R	ATG TTT TTG GAA CGC CAG AG	

3.2.3. Genetic fingerprinting

Randomly amplified polymorphic DNA (RAPD)-PCR was conducted for strain typing of the *Enterococci* isolates. DNA was isolated from the isolates using the boiling method (Tao *et al.,* 2010) and used as template in the PCR assay. PCR amplification was conducted using a T100™ Thermal Cycler (Bio-Rad, USA). Each 25 µl reaction consisted of 2.5 µl (10×) PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.8 µM primer M13R2 (5´-GGAAACAGCTATGACCATGA-3´), 1 U *Taq* and 2 µl DNA. PCR conditions included initial denaturation at 94 ºC for 5 min, 40 cycles of denaturation at 94 ºC for 30 s, annealing at 38 ºC for 1 min and elongation at 72 ºC for 1.5 min, a final elongation was conducted at 72 ºC for 5 min

(Martın *et al.*, 2009). PCR amplified products were subjected to gel electrophoresis in a 1.5% agarose gels which was run at 100 V for 120 min. Thereafter, products were visualized using the G: BOX imaging system (Syngene) after staining in ethidium bromide (0.5 µg/ml). DNA ladders of 1 kb was used as the molecular weight marker and normalization gel standards for RAPD profiles. Conversion, normalization and further analysis of RAPD patterns were done using the Bionumerics 6 software (Applied Maths). The dendrogram was drawn using the clustering (UPGMA) and tree building feature in the same software.

3.2.4. Absolute quantification of selected tetracycline resistance genes

Influent and final treated wastewater effluent of the two WWTPs was used for this section of the study. Samples from the first and third month of the sampling period (June and August 2014) were used in this phase of the study. DNA isolation was conducted within 48 h of sample collection.

3.2.4.1. DNA Isolation

Five hundred millilitres of water sample, was vigorously agitated to evenly distribute bacterial populations, and pre-filtered to remove larger debris and particles prior to DNA isolation. Genomic DNA was isolated using the PowerWater DNA Isolation Kit (MO BIO, Laboratories, Inc). DNA concentrations were determined spectrophotometrically using a NanoDrop spectrometer. Isolated DNA was then stored at -80 ºC until further use.

3.2.4.2. Droplet digital PCR and quantification assay

The tetracycline resistance genes quantified in the wastewater samples were the *tet* L, *tet* M and *tet* O using primers shown in Table 3.2. Droplet digital PCR assays were performed using the Bio-Rad QX200 Droplet Digital PCR System. This system partitioned samples into 20,000 droplets (QX200 Droplet Generator, Bio-Rad). PCR amplification was carried out within each droplet

using the C1000 Touch™ thermal cycler (Bio-Rad, USA). The PCR amplification reaction mixture contained 1 × QX200 ddPCR EvaGreen Supermix (Bio-Rad), 100 nM forward and reverse primer, 0.5 ng DNA, and RNase-/DNase-free water. The following PCR conditions were followed: initial denaturation for 5 min at 95 ºC, 40 cycles of denaturation for 30 s at 95 ºC, annealing for 1 min at 60 ºC, signal stabilization for 5 min at 4 ºC and 5 min at 90 ºC. A ramp rate of 2 ºC /sec was used. Following PCR, droplets were streamed in single file on a QX200 Droplet Reader (Bio-Rad), which counted the fluorescent positive and negative droplets in order to calculate target DNA concentration. The QuantaSoft™ software was used to measure the number of positive and negative droplets for each sample. The software then fit the fraction of positive droplets to a Poisson algorithm in order to determine the starting concentration of the target DNA molecule in units of copies/µl input. The assay experiments were conducted in two replicates. The removal efficiency values were calculated using the formula:

$$\frac{A-B}{A} \times 100 \text{ (Eq. 4)},$$

Where A is the concentration (copies/µl) in the influent wastewater sample and B is the concentration (copies/µl) in the final treated effluent wastewater sample.

3.3. Results

3.3.1. Antibiotic susceptibility profile of the *Enterococci* isolates

The antibiotic resistance profiles of 200 *Enterococci* isolates obtained from before and after chlorination points, and upstream and downstream of the receiving rivers are shown in Table 3.3. More than 13 % and 6 % of the isolates showed resistance to all ten antibiotics tested in WWTP1 and WWTP2, respectively. *Enterococci* isolated from WWTP1 showed greater overall antibiotic resistance than those from WWTP2. In WWTP1, the isolates showed high resistance to trimethoprim-sulfamethoxazole (84%), gentamicin (81%) and tetracycline (80%). Similarly, isolates from WWTP2 showed high levels of resistance to gentamicin (74%), tetracycline (77%) and trimethoprim-sulfamethoxazole (69%). These high levels of resistance was followed by 69% and 63% of the isolates being resistant to rifampicin in WWTP1 and WWTP2, respectively. Resistance to ciprofloxacin was found in more than 31% of the isolates, while more than 45% of the isolates were resistant to erythromycin. Resistance to the remaining antibiotics were found to be less than 30% each. Fewer isolates showed resistance to high-level gentamicin (120 µg), vancomycin and chloramphenicol. A large number of the isolates (83 %) displayed multidrug resistance.

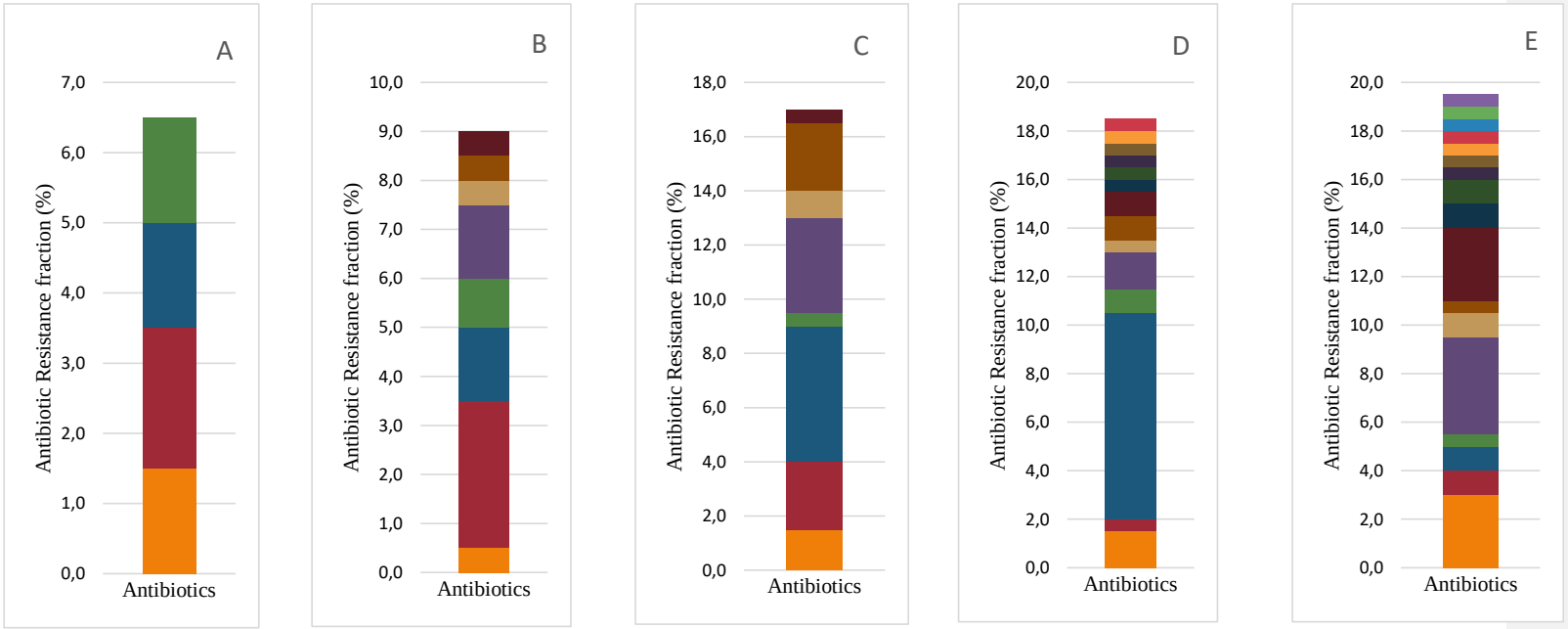
The antibiotic resistance patterns of the *Enterococci* isolates, evident in Figure 3.1, show nine different resistance patterns. Resistance to only one antibiotic was seen in 6.5 % of the isolates (Figure 3.1 A), while 9% were resistant to two antibiotics (Figure 3.1 B). A higher percentage of isolates showed resistance to three (17%), four (19%), five (19.5%) and six (14.5%) antibiotics (Figure 3.1 C-F). A lower proportion of isolates showed resistance to seven (9.5%) and eight

(2.5%) antibiotics, with only 0.5% being resistant to all nine antibiotics, as seen in Figure 3.1 (G-I). Four *Enterococci* isolates were susceptible to all the antibiotics tested.

Table 3.3: Antibiotic resistance profile of *Enterococci* species isolated from both WWTPs and their receiving rivers (n=200)

	No. of Isolates (%)										
	WWTP1					WWTP2					
Antibiotic	**BC (n=31)**	**AC (n=16)**	**US (n=26)**	**DS (n=29)**	**Total (n=102)**	**BC (n=31)**	**AC (n=18)**	**US (n=25)**	**DS (n=24)**	**Total (n=98)**	**Overall Total (n=200)**
VAN	5 (16)	0 (0)	6 (23)	4 (14)	15 (15)	0 (0)	1 (6)	3 (12)	5 (21)	9 (9)	24 (12)
GEN (10)	25 (81)	12 (75)	17 (65)	29 (100)	83 (81)	19 (61)	17 (94)	16 (24)	21 (88)	73 (74)	156 (78)
***GEN (120)**	11 (35)	0 (0)	1 (4)	4 (14)	16 (16)	2 (6)	3 (17)	0 (0)	1 (4)	6 (6)	22 (11)
ERY	19 (61)	4 (25)	11 (42)	14 (48)	48 (47)	12 (39)	11 (61)	14 (56)	7 (29)	44 (45)	92 (46)
SXT	25 (81)	14 (88)	21 (81)	26 (90)	86 (84)	21 (68)	14 (78)	14 (56)	19 (79)	68 (69)	154 (77)
CIP	16 (52)	6 (38)	12 (46)	12 (41)	46 (45)	7 (23)	11 (61)	5 (20)	7 (29)	30 (31)	76 (38)
TET	22 (71)	15 (94)	22 (85)	23 (79)	82 (80)	24 (77)	13 (72)	21 (84)	17 (71)	75 (77)	157 (79)
CHL	4 (13)	0 (0)	3 (12)	6 (21)	13 (13)	5 (16)	1 (6)	3 (12)	0 (0)	9 (9)	22 (11)
RIF	21 (68)	11 (69)	18 (69)	20 (69)	70 (69)	22 (71)	13 (72)	12 (48)	15 (63)	62 (63)	132 (66)
AMP	6 (19)	4 (25)	6 (23)	9 (31)	25 (25)	5 (16)	8 (44)	1 (4)	3 (13)	17 (17)	42 (21)

VAN: Vancomycin; GEN: Gentamicin; ERY: Erythromycin; SXT: Trimethoprim-Sulfamethoxazole; CIP: Ciprofloxacin; TET: Tetracycline; CHL: Chloramphenicol; RIF: Rifampicin; AMP: Ampicillin; * High level gentamicin resistance; BC: Before Chlorination; AC: After Chlorination; US: Upstream River; DS: Downstream River



A: One Antibiotic Pattern

RIF TET VAN SXT

B: Two Antibiotic Patterns

RIF,SXT GEN,SXT
GEN, RIF GEN,TET
ERY,TET TET,RIF
CIP,TET SXT,CIP

C: Three Antibiotic Patterns

GEN,ERY,SXT GEN,TET,RIF
ERY,SXT,TET GEN,SXT,RIF
TET,CHL,AMP GEN,SXT,TET
SXT,TET,RIF GEN,ERY,TET

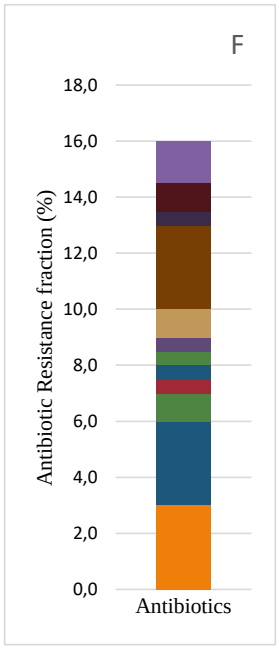
D: Four Antibiotic Patterns

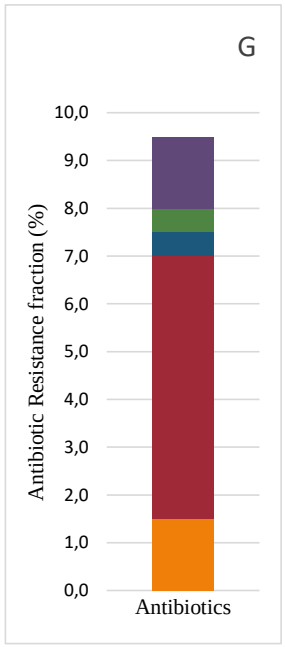
VAN,GEN,ERY,TET GEN,ERY,CIP,TET
SXT,TET,RIF,AMP VAN,GEN,SXT,TET
ERY,CIP,TET,RIF CIP,TET,RIF,AMP
GEN,SXT,CIP,RIF GEN,SXT,RIF,AMP
GEN,ERY,SXT,CIP GEN,ERY,SXT,TET
ERY,SXT,TET,RIF GEN,SXT,TET,RIF

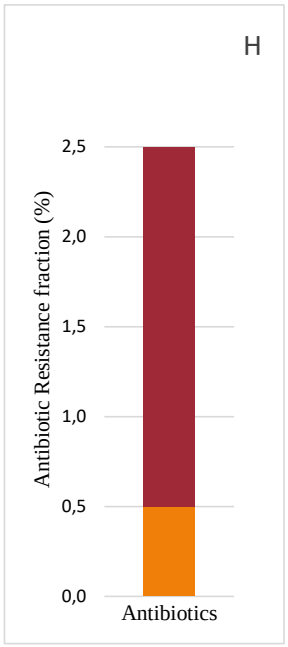
E: Five Antibiotic Patterns

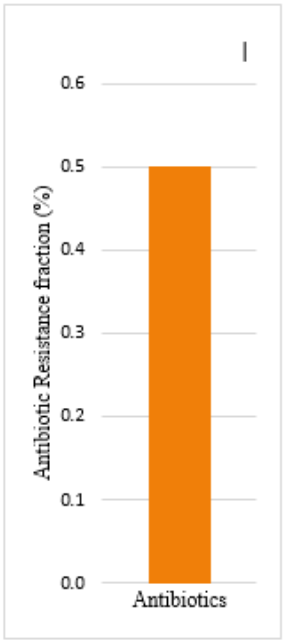
GEN,SXT,CIP,TET,AMP GEN,ERY,CIP,RIF,AMP
GEN,SXT,TET,CHL,RIF GEN,ERY,CIP,TET,AMP
VAN,SXT,TET,CHL,RIF GEN,ERY,CIP,TET,RIF
ERY,SXT,CIP,TET,RIF VAN,GEN,SXT,TET,RIF
GEN,SXT,TET,RIF,AMP GEN,ERY,SXT,CIP,TET
GEN,ERY,SXT,CIP,RIF VAN,GEN,ERY,SXT,TET
GEN,ERY,SXT,TET,RIF GEN,ERY,SXT,CHL,RIF
GEN,ERY,SXT,TET,CHL ERY,SXT,TET,CHL,RIF
GEN,SXT,CIP,TET,RIF

Figure 3.1: Antibiotic resistance patterns of *Enterococci* species showing nine antibiotic resistance patterns









F: Six Antibiotic Patterns

- GEN,ERY,SXT,TET,CHL,RIF
- GEN,ERY,SXT,CIP,TET,RIF
- ERY,SXT,CIP,TET,RIF,AMP
- GEN,ERY,CIP,CHL,RIF,AMP
- VAN,GEN,ERY,SXT,CIP,TET
- VAN,GEN,CIP,TET,RIF,AMP
- GEN,ERY,SXT,CIP,TET,CHL
- GEN,ERY,SXT,CIP,TET,AMP
- GEN,SXT,CIP,TET,RIF,AMP
- VAN,GEN,SXT,CIP,TET,RIF
- GEN,ERY,SXT,CIP,RIF,AMP

G: Seven Antibiotic Patterns

- GEN,ERY,SXT,CIP,TET,CHL,RIF
- VAN,GEN,SXT,CIP,TET,RIF,AMP
- VAN,GEN,SXT,CIP,TET,CHL,RIF
- GEN,ERY,SXT,CIP,TET,RIF,AMP
- VAN,GEN,ERY,SXT,CIP,TET,RIF

H: Eight Antibiotic Patterns

- VAN,GEN,ERY,SXT,CIP,TET,RIF,AMP
- GEN,ERY,SXT,CIP,TET,CHL,RIF,AMP

I: Nine Antibiotic Patterns

- VAN,GEN,ERY,SXT,CIP,TET,CHL,RIF,AMP

…Continuation of Figure 3.1

3.3.2. **Detection of tetracycline resistance genes**

Five tetracycline resistance genes were detected from 151 tetracycline resistant *Enterococci* isolates. The correct PCR product sizes of *tet* S, *tet* O, *tet* M, *tet* L and *tet* K genes were detected at 667 bp, 515bp, 406 bp, 267 bp and 169 bp, respectively (Figure 3.2).

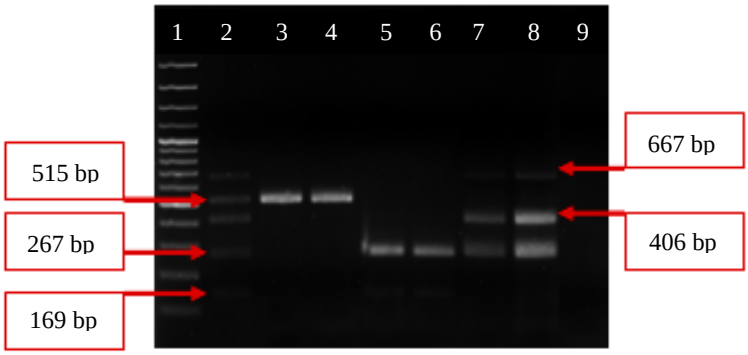


Figure 3.2: Amplicons from multiplex PCR assays of tetracycline resistance genes in selected *Enterococci* isolates. Lane1: 100 bp ladder; Lane 2: positive controls of *tet* S, *tet* O, *tet* M, *tet* L and *tet* K; Lane 3-4: *tet* O; Lane 5-6: *tet* L and *tet* K; Lane 7-8: *tet* S, *tet* M and *tet* L; Lane 9: negative control

The prevalence of the various tetracycline genes tested in the 151 tetracycline resistant *Enterococci* isolates is depicted in Figure 3.3. The *tet* M gene was the most prevalent gene found in 49 % of the isolates. The second highest was the *tet* L gene, found in 32 % of the isolates and the third most prevalent gene was the *tet* K genes (12 %). Only 3 % of the isolates showed to harbour the *tet* O gene and 4 % contained the *tet* S genes. The combinations of tetracycline resistance genes detected in the isolates can be seen in Table 3.4. Sixteen different combinations of one, two, three or four tetracycline resistance genes can be noted in Table 3.4. Combinations of one to four genes were

detected in the isolates, with the *tet* L/*tet* M combination being most prevalent (58 isolates). This was followed by isolates containing the *tet* M gene (43 isolates) and combination of *tet* K/*tet* L/*tet* M genes (21 isolates). The remaining 13 phenotypes were each under 10 isolates. None of the isolates had a combination of all five genes.

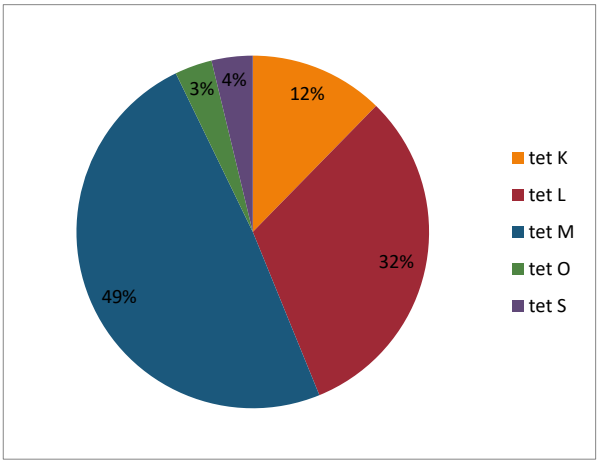


Figure 3.3: Distribution of tetracycline resistance genes among tetracycline resistant *Enterococci* isolates

Table 3.4: Combinations of tetracycline resistance genes detected in *Enterococci* species

		No. of Isolates				
Gene Profile	**No. of Genes**	***E. faecalis***	***E. faecium***	***E. hirea***	***Enterococci spp.***	**Total (n=151)**
***tet* L**	1	0	1	0	1	2
***tet* M**	1	17	10	12	4	43
***tet* O**	1	1	1	0	0	2
***tet* K,L**	2	0	1	0	1	2
***tet* K,M**	2	6	0	0	0	6
***tet* L,M**	2	17	19	18	4	58
***tet* M,O**	2	2	0	1	0	3
***tet* O,S**	2	0	0	0	1	1
***tet* M,S**	2	0	1	0	0	1
***tet* K,L,M**	3	14	4	1	2	21
***tet* K,M,O**	3	1	0	0	0	1
***tet* K,M,S**	3	3	0	0	0	3
***tet* L,M,O**	3	1	0	0	0	1
***tet* L,M,S**	3	0	3	2	0	5
***tet* K,L,M,O**	4	1	0	0	0	1
***tet* K,L,O,S**	4	0	0	0	1	1

3.3.3. Genetic fingerprint of *Enterococci* isolates

Patterns of the RAPD-PCR obtained for the 200 isolates, using the primer M13R2 showing 14 different clusters, is shown in Figure 3.4. The first cluster consisted of 11 isolates. Majority of these isolates were *E. faecium* and contained a combination of both *tet* L and *tet* M genes. Most of the isolates (91%) in cluster 1 were resistant to gentamicin (10 µg) and trimethoprim-sulfamethoxazole while, 82% of them are resistant to erythromycin and tetracycline. Also, 91% of the isolates, in this cluster, were susceptible to vancomycin. Cluster 2 contains 21 isolates that consisted mainly of *E. faecalis*. Seventy one percent of the isolates in this cluster possessed one or more tetracycline resistance genes. Cluster 2 contains isolates that were highly susceptible to ampicillin, gentamicin (120 µg), vancomycin and chloramphenicol at 90%, 86%, 81% and 76%, respectively. Cluster 3 consists of 9 isolates. Majority of these isolates belonged to *E. faecalis*. All

isolates (100 %) in this cluster were susceptible to vancomycin while, 89% and 78 % of the isolates were susceptible to gentamicin (120 µg) and ampicillin, respectively. The isolates in this cluster also showed 100% resistance to rifampicin with 78% containing resistance to trimethoprim-sulfamethoxazole and gentamicin (10 µg). Cluster 4 encompasses 19 isolates with majority of the isolates being *E. faecalis*. Fifty six percent and 16 % of the isolates contained the *tet* M and *tet* O gene, respectively. This cluster had the highest number of isolates containing the *tet* O gene. Majority of the isolates in this cluster were susceptible to ampicillin (95%) and vancomycin (84%) while, 68 % of the isolates were resistant to trimethoprim-sulfamethoxazole and gentamicin, and 63 % were resistant to tetracycline. This cluster showed lower levels of antibiotic resistance, overall. Cluster 5 consisted of 16 isolates with majority of them being *E. faecalis.* Eighty one percent of the isolates contained the *tet* M gene and 50% contained the *tet* L gene. The isolates were resistant to trimethoprim-sulfamethoxazole (94%), tetracycline (88%), rifampicin (81%) and gentamicin-10 µg (75%). The isolates in this cluster contained 100% susceptibility to gentamicin (120 µg).Cluster 6 consists of 9 isolates with most being *E. hirea.* In this cluster, 78% of the isolates contained the *tet* M gene and majority of the isolates were susceptibility to vancomycin and ampicillin. Also, all the isolates were resistant to gentamicin (10 µg) and trimethoprim-sulfamethoxazole and 89 % were resistant to tetracycline. Cluster 7 consisted of 17 isolates, mainly being *E. faecalis* (71%). As with cluster 6, cluster 7 contains isolates that were also most susceptible to vancomycin, gentamicin (120 µg) and ampicillin, but also susceptible to trimethoprim-sulfamethoxazole. The isolates from cluster 7 were highly resistant to tetracycline (82%). Cluster 8 consists of 16 isolates with majority of it being *E. hirea*. Most of these isolates contained a MAR index of 0.4 and 0.5, and showed to contain the combination of both *tet* L and *tet* M genes. This cluster showed high susceptibility to vancomycin, gentamicin (120 µg) and

ampicillin. Ninety four percent of the isolates were resistant to trimethoprim-sulfamethoxazole, tetracycline (88%) and gentamicin-10 µg (81%). Cluster 9 consisted of 12 isolates with 83% being *E. faecium.* Majority of these isolates showed susceptibility to vancomycin (92%) and chloramphenicol (83%). High resistance was observed to trimethoprim-sulfamethoxazole (83%), gentamicin- 10 µg (67%) and tetracycline (67%). Sixty five percent of the isolates in cluster 10 were all *E. hirea.* Majority of the isolates in this cluster contained the *tet* M and *tet* M/ *tet* L combination genes. These isolates were highly resistant to gentamicin (10 µg) and trimethoprim-sulfamethoxazole at 96% and 83% respectively. Susceptibility was shown to vancomycin (91%), gentamicin- 120 µg (74%), ampicillin (74%) and chloramphenicol (70%). Cluster 11 consists of 17 isolates. Most of these isolates were *E. faecalis* and contained an MAR index of 0.6. Eighty eight percent of the isolates contained tetracycline resistance gene, with majority of them containing either *tet* M gene or a combination of *tet* L and *tet* M genes. This cluster showed the highest susceptibility to vancomycin, gentamicin (120 µg) and ampicillin, with the highest resistance to gentamicin- 10 µg (94%), tetracycline (94%) and erythromycin (65%). The twelfth cluster consisted of 7 isolates with 71% being *E. hirea.* All isolates in this cluster contained the *tet* L or *tet* M gene or a combination of the two genes. All the isolates were also susceptible to vancomycin, gentamicin (120 µg) and ampicillin and resistant to trimethoprim-sulfamethoxazole and tetracycline. Cluster 13 consists mostly of *E. hirea* (53%) and have an MAR index of 0.3. Fifty three percent of the isolates contain a combination of the *tet* L and *tet* M genes. This cluster shows high resistance to both gentamicin (10 µg) and rifampicin at 87%. The final cluster, consists of 88% *E. faecium* with no isolate showing any susceptibility to erythromycin or gentamicin (10 µg). High resistance was observed for trimethoprim-sulfamethoxazole (88%).

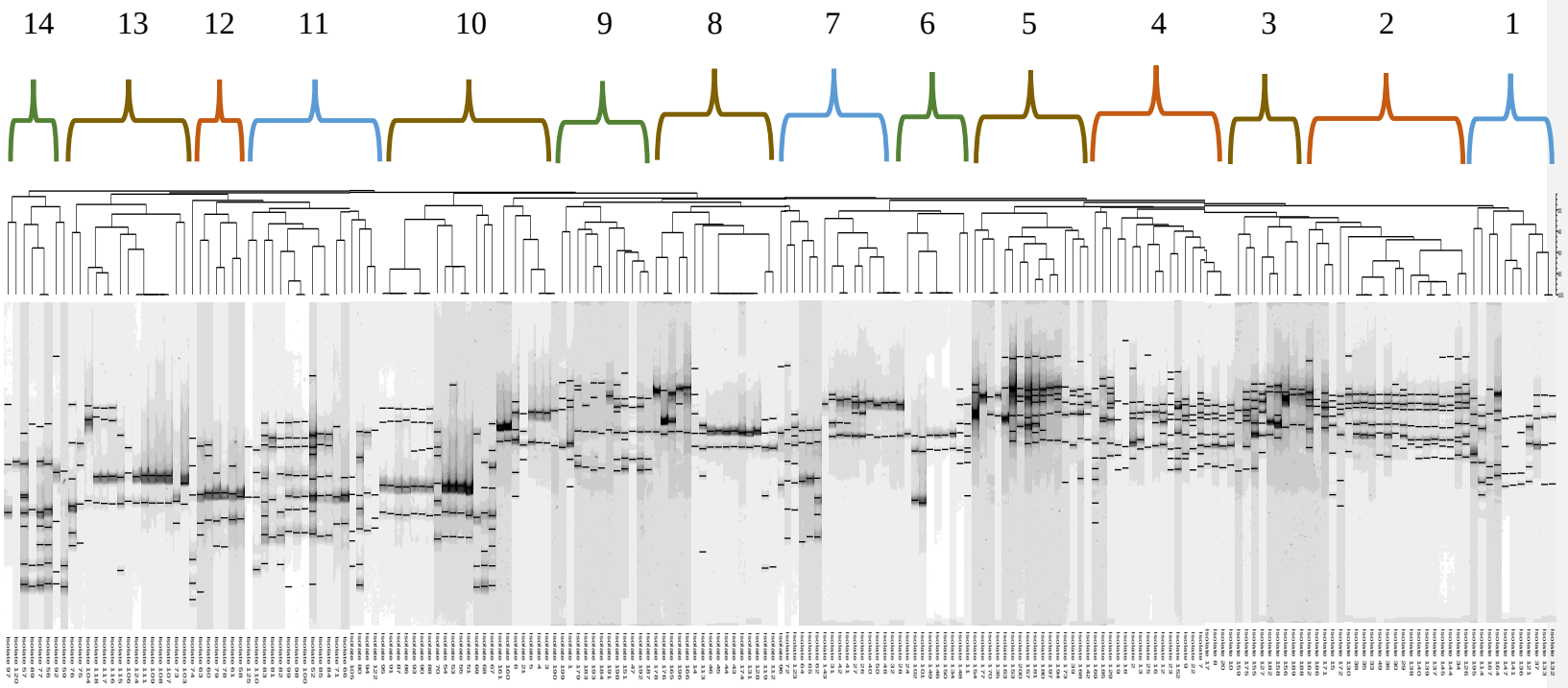


Figure 3.4: RAPD band patterns and corresponding dendrogram of 200 *Enterococci* isolates separated into 14 clusters

3.3.4. **Absolute quantification of selected tetracycline resistance genes**

The concentrations of the tetracycline resistance genes (copies/µl) at the two sampling points, obtained from the droplet digital PCR assays, are shown in Table 3.5. The concentrations decreased from influent to final effluent samples for both WWTPs for both sampling months, showing a high removal efficiency of all three tetracycline resistance genes. A decrease of 1141 copies/µl and 2112.9 copies/µl of the *tet* O gene in August 2014 was observed in WWTP1 and WWTP2, respectively. A greater decrease in tetracycline resistance genes is noted in August than June 2014. Influent concentrations were higher in all samples in WWTP2 compared to WWTP1. The concentrations ranged from 23 to 93 copies/µl for the influent samples, while the range for final fluent was as low as 1.3 to 13.35 copies/µl. Quantification of the *tet* O genes was highest, with values for influent ranging from 576 to 2116.5 copies/µl, while higher concentrations of this gene was also found in the final effluent samples ranging between 3.6 to 102.8 copies/µl. The high concentration of the *tet* O gene was followed by the *tet* M gene, with the *tet* L showing the lowest concentrations.

Figure 3.5 shows that both WWTPs were very efficient in their removal of the various tetracycline resistance genes. These values were all greater than 82 % except for the *tet* L gene in WWTP1 (June 2014) with only 42% removal. The removal efficiency for the month of August 2014 was higher in both plants. With values exceeding 98%, WWTP2 showed to have a far greater removal efficiency than WWTP1. For WWTP1 the *tet* L, *tet* M and *tet* O genes showed a higher removal efficiency in August 2014. The removal efficiency for WWTP2 for *tet* L was higher in June 2014, while that for *tet* M and *tet* O was in August.

Table 3.5: Absolute quantification concentrations of *tet* L, *tet* M and *tet* O in influent and final effluent samples for June and August 2014

		WWTP1		WWTP2	
		Influent	**AC**	**Influent**	**AC**
		Conc (copies/µl) ± SD			
June 2014	***tet* L**	23.05 ± 3.29	13.35 ± 0.87	93.5 ± 6.47	1.3 ± 0
	***tet* M**	137 ± 35.80	24.2 ± 0.69	521.5 ± 38.68	7.3 ± 0.46
	***tet* O**	576 ± 5.77	102.8 ± 3.70	1267.5 ± 28.29	15.65 ± 0.17
August 2014	***tet* L**	38.95 ± 0.87	3.45 ± 0.17	68.6 ± 2.77	1.31 ± 0.08
	***tet* M**	192.5 ± 15.59	12.15 ± 0.29	339.5 ± 15.59	1.085 ± 0.13
	***tet* O**	1201.5 ± 71.01	60.5 ± 0.35	2116.5 ± 76.79	3.6 ± 0.23

AC: After Chlorination

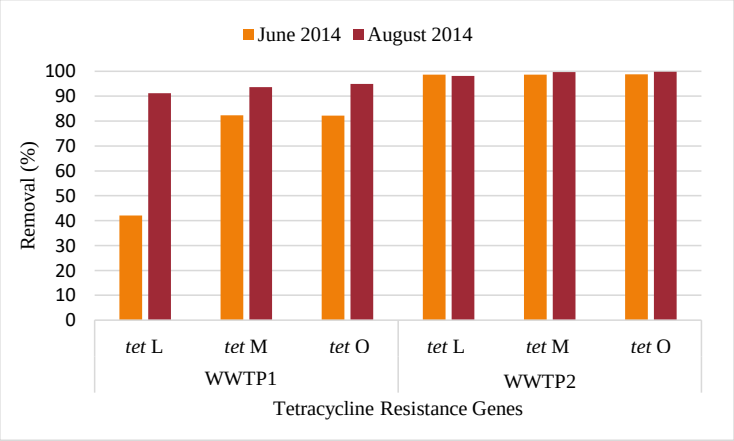


Figure 3.5: Removal of *tet* L, *tet* M and *tet* O during the treatment process at both WWTPs

3.4. Discussion

Antibiotics are used in the treatment and prevention of infectious disease by killing or inhibiting the growth of certain bacteria. Global antibiotic consumption increased by 36% between the years 2000-2010 (Van Boeckel *et al.*, 2014). The five fast growing countries under the association name 'BRICS'(Brazil, Russia, India, China and South Africa) have been reported to have the greatest spike in antibiotic use from 2000-2010. These countries held more than three-quarters of the total increase in global consumption (76%) despite the fact that they only accounted for one-third of the world's population increase (Van Boeckel *et al.*, 2014). Bacteria capable of naturally producing antibiotics are intrinsically resistant to the antibiotics they synthesize, while non-antibiotic producers survive by acquiring antibiotic resistance. Bacteria gain such a selective advantage by altering membrane permeability, preventing transport carriers, modifying the target's binding sites and obtaining the ability to degrade the antimicrobial agent (Jury *et al.*, 2010). Antibiotic resistance amongst *Enterococci* are clinically important as this contributes to untreatable serious infections and colonization (Goel *et al.*, 2016).

The ten antibiotics chosen for the antibiotic susceptibility test were based on their clinical significance and as they are commonly reported in literature. These antibiotics are commonly used alone or in combination with each other in enterococcal infections. The antibiotic susceptibility profile obtained in the present study showed that every *Enterococci* isolate displayed resistance to at least one antibiotic tested. This genera of bacteria are intrinsically resistant to some commonly used antibiotics and have a high rate of antibiotic resistance acquisition. Intrinsic resistance to β-lactams, such as ampicillin is a characteristic of

Enterococci (Arias *et al*., 2010). Ampicillin is the preferred drug in enterococcal infections. It is therefore expected for this microorganism to show a high level of resistance to this antibiotic. However, from the results obtained in the study, most of the isolates were susceptible to ampicillin, with only 21% of the isolates showing resistance. This was in accordance with another study that also found low level of ampicillin resistance in *Enterococci* isolated from wastewater (Leclercq *et al.,* 2013). Resistance to ampicillin in *Enterococci* are generalized as being non-transferable as it is commonly linked to mutations in the chromosomal genes. While, resistance to other antibiotics such as macrolides and tetracyclines are easily transferred by mobile genetic elements (Petsaris *et al.,* 2005; Hegstad *et al.,* 2010). The low level of resistance to this antibiotic is therefore promising for its confirmed use in the treatment of *E. faecalis* infections.

The introduction of clinically useful antibiotics (in large amounts) such as tetracycline, chloramphenicol and erythromycin, resulted in *Enterococci* quickly gaining resistance to them (Kristich *et al.,* 2014). High tetracycline and erythromycin resistance among the *Enterococci* isolates tested in this study corroborates the findings observed in recent studies (Ayeni *et al.,* 2016). Resistance to low-level gentamicin was very high, however this was expected as *Enterococci* contain intrinsic resistance to low level concentrations of gentamicin. To further check the severity of resistance to this antibiotic, high level gentamicin (120 µg) was tested and this resulted in low resistance (12%). High resistance was also observed for trimethoprim-sulfamethoxazole and rifampicin. Two classes of antibiotics, tetracyclines and sulfonamides, have prophylactic, agricultural and clinical applications and have been known to be found in sludge and wastewater at WWTPs (Ding *et al.,* 2011). This is possibly why high tetracycline (79%) and sulfonamide (78%) resistance was observed among the *Enterococci* isolates tested in this study. This also correlates with other studies that found high levels of tetracycline and

sulfonamide resistant bacteria and resistance genes in wastewater samples (Reinthaler *et al.*, 2003).

Based on MAR indices, it was noted that most isolates were resistant to 4 or 5 classes of antibiotics, with a large number having resistance to 6 or 2 different classes of antibiotics. This result revealed that 83% of the isolates were multidrug resistant, which is a cause for concern as this would pose a serious threat to public health. South Africa, as with many other countries, continually face the heavy burden of infectious disease. This problem is heightened by the notorious existence of multidrug resistant bacteria. Previous studies have shown that WWTPs in South Africa house large levels of multidrug resistant *Enterococci* (Iweriebor *et al.*, 2015). Due to multidrug resistance, microorganisms fail to respond to first line antibiotics and results in extended treatment which adds to increased hospital stay, health care costs and contributes to severe side effects from alternative treatment (Tanwar *et al.*, 2014).

Tetracyclines are a common drug of choice in many bacterial infections and have been extensively used for decades due to its broad-spectrum activity in human and animal infections. It is also used for its countless applications in agricultural practices such as feed additives for growth promotion (Chopra and Roberts, 2001). Multidrug resistant bacteria are commonly resistant to tetracycline (Levy *et al.*, 1999). Tetracycline resistant *Enterococci* were found to be the most prevalent (overall) among the 200 *Enterococci* isolates obtained from the secondary and final effluent and receiving river samples. Because of its clinical importance and observed high prevalence in this study, PCR detection of five tetracycline resistance genes were conducted to determine the mode of tetracycline resistance in these isolates. According to the presence of individual or combination genes, the mechanism of resistance is often associated

with tetracycline binding to the bacterial ribosomes, subsequently reducing the ribosome-tRNA interaction and preventing protein synthesis or an efflux protein which transports the antibiotic out of the cell (Schnappinger and Hillen, 1996; Guillaume *et al*., 2004). Resistance genes associated with an efflux mechanism are the *tet* K and *tet* L genes. Bacteria that confer resistance to tetracycline by ribosomal protection contain the *tet* M, *tet* O and/or *tet* S genes. The *tet* M is known to be the most prevalent in *Enterococci* and this was seen in the present study in which 49% of the isolates contained this gene (Poeta *et al*., 2006; Jackson *et al*., 2010). Previous reports stated that *tet* M can be found either chromosomally or on plamsids, while the *tet* K gene has only been found on plasmids (Warsa *et al.,* 1996). This may be the reason why some isolates carry both genes, while others carry only one. From the results obtained, the *tet* L gene was found in 32 % of the isolates (second highest tetracycline resistant gene detected). This was in accordance with previous studies, where the *tet* L gene was frequently detected tetracycline efflux gene in *Enterococci* (Platteeuw *et al*. 1995; Jackson *et al.,* 2010). Combinations of tetracycline resistant genes are common, as *Enterococci* are able to confer resistance by both the efflux mechanism and ribosomal protection (Jackson *et al.,* 2010). Combinations of one to four genes were detected in the isolates, with the *tet* L/*tet* M combination being most prevalent. This high combination corroborates with a previous study that also reported a high level of the *tet* L/*tet* M combination (Huys *et al.,* 2004). This may suggest that a greater level of tetracycline resistance is achieved when an organism has both the efflux mechanism and ribosomal protection mechanism (Huys *et al.,* 2004). This effect has also been reported in methicillin-resistant *Staphylococcus aureus* that contained a combination of tetracycline resistant genes, resulting in a greater level of resistance to the antibiotic (Trzcinski *et al.,* 2000).

The RAPD-PCR method allowed for the *Enterococci* isolates to be distinguished from each other by means of the positions of the amplified DNA fragments following PCR. Differentiation of the isolates were obtained by the presence or absence of the DNA fragments. Isolates were clustered into groups based on the differences in the individual patterns by separating isolates based on how related they are from each other. This method was used in the genotypic typing of the isolates in order to cluster then according to their phenotypic attributes. This allowed for the determination of isolates that shared any clonal relationships. Clustering analysis revealed 14 different genetic diversity groups of the *Enterococci* isolates. The clusters contained many sub-clusters showing that there were many polymorphisms amongst the isolates in each group. The RAPD analysis grouped majority of *E. faecalis* in 6 clusters, followed by *E. hirae* in 5 clusters and finally *E. faecium* in 3 clusters. These findings show that the environmental *Enterococci* isolates were genetically and phenotypically diverse. The clusters showed specific *Enterococci* species, antibiotic resistance profiles and MAR indices. This categorizing revealed the transfer of particular ARGs and subsequent resistance patterns between specific species. Additionally, the transmission of tetracycline resistance genes or combinations of it between specific species were noted in the different clusters.

New global emerging pollutants that have recently been reported as a threat to public health are antibiotics, ARB and ARGs (WHO, 2014b; Gao *et al.*, 2012). A potential reservoir that promotes the prevalence and spread of antibiotic resistance are WWTPs. In this study, the concentrations of 3 tetracycline resistance genes in influent and final treated effluent samples were quantified in order to determine the efficiency of the WWTPs in removing these genes from the received influent. Tetracycline was listed as an essential medicine by the World Health Organization (2014b), mainly due to its broad-spectrum antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria. Tetracycline is used as first-line therapy

for many diseases, including rickettsia, cholera and pneumonia and they are favourable antimicrobial agents due to the absence of major adverse side effects.

From previous studies, tetracycline resistance genes have been found in many WWTP samples (Martinez, 2009; Ding *et al*., 2011). The high prevalence of these genes may be linked to the prolonged clinical application of tetracycline (Pruden *et al.*, 2006). Despite high concentrations of these genes being found in influent samples, many treatment plants have shown poor efficiency in the removal and are influenced by operating conditions (Reinthaler *et al.*, 2003; Novo and Manaia, 2010). The present study, however, shows contrasting results in which both WWTPs had a removal efficiency of more than 82% in most cases. The *tet* O gene which is usually found in Gram-positive bacteria (Luna and Roberts, 1998) was found at high concentrations in the present study (576 to 2116.5 copies/µl). From previous studies, it has been shown that a significant reduction in *tet* O genes have been observed in WWTPs that conduct an activated sludge treatment process (Gao *et al.*, 2012). This is in accordance with the present study, that also utilizes activated sludge, as a reduction of 94.6% and 99.8% of *tet* O was obtained in WWTP1 and WWTP2 (August 2014), respectively. The *tet* O gene is a ribosomal protection gene. It has been described in *Streptococcus mutans*, *S. milleri*, group B *Streptococcus* species, and *Enterococcus faecalis* (Zilhao *et al.*, 1988).

Results obtained in this study revealed that the concentrations, of the tetracycline resistance genes, decreased from influent to final effluent samples for both WWTPs at both sampling times. Concentrations were higher in all samples in WWTP2 compared to WWTP1, which may be attributed to WWTP2 receiving both domestic and hospital influent, while WWTP1 receives domestic and industrial influent. Clinical isolates are known to possess higher tetracycline resistance (Mondino *et al.*, 2003). The treatment process of both WWTPs utilize activated sludge, mechanical aeration, anaerobic digestion, belt press and chlorine tertiary treatment.

Therefore, the elevated concentrations observed in WWTP2 may be attributed to its influent source. The *tet* M concentrations were much higher than the *tet* L genes found in influent. The *tet* O genes were found to be most prevalent, having the highest concentrations compared to the other two genes. Both WWTPs were very efficient in their removal of the various tetracycline resistance genes, with high removal efficiency of all 3 genes. These high levels of removal may be due to the secondary treatment process and not entirely to tertiary treatment. Studies have shown a significant reduction in ARGs during the secondary treatment stage, while the tertiary stages (chlorination) did not have much effect on the removal efficiency (Munir *et al.*, 2011; Gao *et al.*, 2012).

3.5. Conclusion

This chapter studied the antibiotic resistance profile of the *Enterococci* isolates and established the genetic diversity of isolates that were recovered from the final effluent and receiving surface water. In addition, concentration of selected tetracycline resistance genes were evaluated in order to investigate the efficiency of the treatment process for the removal of these genes from the influent. The results revealed that WWTPs and surface waters are substantial contributors to the high prevalence of multidrug resistant *Enterococci.* Additionally, *Enterococci* isolates have shown high levels of resistance to the popular drug of choice (tetracycline) and possess one or more tetracycline resistance genes. Despite WWTPs housing many tetracycline resistant bacteria, the treatment processes were efficient in removing extensive quantities of selected tetracycline resistance genes.

CHAPTER FOUR

GENERAL DISCUSSION AND CONCLUSION

4.1. Research in perspective

Enterococci are a complex group of bacteria and common inhabitants of the gastrointestinal tracts, skin and oral cavities of most mammals. They are also residents of different environments including water, soil and some food sources (Daniel *et al.,* 2015). Due to their ability to survive in a wide range of temperature and pH levels, it is usually challenging to control the spread of pathogenic *Enterococci* species, which may cause serious infections in both humans and animals. An additional problem, is its superior ability to easily acquire resistance to antibiotics (Daniel *et al.,* 2015). Wastewater treatment plants (WWTPs) are implicated as the leading reservoir for antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Munck *et al.,* 2015). The release of ARGs from WWTPs into the environment are worrisome, as this promotes the spread and transmission of antibiotic resistance among bacteria. Antibiotic resistant bacteria and ARGs have frequently been reported in numerous environmental sources (Knapp *et al.,* 2010; Korzeniewska *et al.,* 2013; Odjadjare and Olaniran, 2015). Studies, conducted in South Africa, have shown that treated effluent released from WWTPs negatively impact the environment by discharging substantial amounts of both ARB and ARGs into receiving surface bodies (Odjadjare and Olaniran, 2015; Iweriebor *et al.,* 2015; Pillay and Olaniran, 2016). Their release into the environment may pose serious health threats to communities that rely on the river water for domestic and recreational purposes (Odjadjare and Olaniran, 2015).

The temperature and pH profiles of all the water samples (within the WWTPs and receiving rivers) tested in this study, ranged between 15-26 ºC and 4.92-7.65, respectively. These values were within the temperature and pH growth range for *Enterococci* (temperature: 5 to 50 ºC and

pH: 4.6 to 9.9), thus promoting survival and proliferation of the organisms (Fisher and Phillips, 2009; Van den Berghe *et al.,* 2006). This was evident by consistent presence of *Enterococci* in all samples from both WWTPs and the receiving rivers. Antibiotic resistant *Enterococci* (ARE) were enumerated in all water samples obtained during the study period in order to determine the efficiency of the WWTPs in removing these organisms. Wastewater treatment plants serve as an important interface between society and the environment. They contain bacteria, organic and inorganic material as well as antibiotics from household and clinical settings, possibly acting as a breeding ground for ARB and ARGs (Martinez, 2009). A comparison of values obtained in samples before chlorination and after chlorination (final treated effluent) were conducted in order to determine the effect of the chlorination process. A great decrease in average CFU/100ml was noted from samples before chlorination to the ones after chlorination. The values for WWTP1 ranged from 700 - 8700 CFU/100ml (before chlorination) to 0 - 80 CFU/100ml (after chlorination), with similar values obtained for WWTP2. This showed that the chlorination step was effective in reducing the ARE prevalence. An additional comparison was also made between the final effluent values to the upstream and downstream river sample counts to determine the effect of the treated effluent discharge on the environment. A decrease in average CFU/100ml values was observed from the upstream to the downstream of the river samples. Lower values were obtained upstream and downstream of the first receiving river with values ranging from 0 - 200 CFU/100ml and 0 - 100 CFU/100ml, respectively. Due the increased stress of water shortage, reclaimed water has become a popular solution for agricultural, industrial and domestic uses (Mosteo *et al.,* 2013). This alternative is only viable if WWTPs are effective in the removal of pathogens from treated effluent. Tertiary treatment, followed by most WWTPs, are generally able to minimize the spread of pathogens to receiving environments (Mosteo *et al.,* 2013). It is therefore important to decipher how efficient the treatment process is in removing ARB, as these organisms pose a public health threat.

Higher prevalence of tetracycline resistant *Enterococci* was obtained in WWTP2 than in WWTP1, possibly due to the hospital influent received by WWTP2. High levels of erythromycin resistant *Enterococci* obtained in the influent and activated sludge samples of both WWTPs, is in accordance with another study, conducted in South Africa during the same sampling period as the present study (Iweriebor *et al.,* 2015). Iweriebor *et al.* (2015) reported high levels of tetracycline and erythromycin resistant *Enterococci* in wastewater received from both hospital and domestic sources, with a higher level of resistance obtained in wastewater received from hospitals. Also, higher resistance to vancomycin was frequently observed in the *Enterococci* recovered from samples, collected from WWTP2, probably because it received a portion of its waste from hospital settings. It has been shown that hospital waste contains VRE (Novais *et al.,* 2005). Of the 200 *Enterococci* isolates identified in this study, 34.5%, 26% and 25.5% were *E. faecalis*, *E. faecium* and *E. hirea*, respectively. These results are in accordance with other studies where, *E. faecalis* and *E. faecium* were reported to be predominantly found in human and animal faeces and sewage (Manero *et al*., 2002; Byappanahalli *et al.,* 2012).

The antibiotic susceptibility profile revealed that most of the 200 *Enterococci* isolates were resistant to 4 or 5 classes of antibiotics, with a large number having resistance to 6 different classes of antibiotics. In agreement with other reports (Reinthaler *et al.,* 2003; Ayeni *et al.,* 2016), high level of resistance against tetracycline, erythromycin and sulfonamide was observed among the *Enterococci* isolates in this study. An alarmingly high number of multidrug resistant *Enterococci* (83%) was found in the study, which could pose a serious threat to public health.

Detection of tetracycline resistance genes among the resistant phenotypes of *Enterococci,* revealed *tet* M gene to be the dominant (49%) of the five tetracycline resistance genes detected. This is not surprising since this gene enable tetracycline resistance by ribosomal protection

(Poeta *et al*., 2006; Jackson *et al*., 2010). Combinations of one to four genes were detected in the isolates, with *tet* L/*tet* M combination being the most prevalent. This high combination corroborates a previous study that also reported a high level of the *tet* L/*tet* M combination (Huys *et al.*, 2004).

The RAPD-PCR method, conducted in this study, allowed for the determination of genetic diversity among the *Enterococci* isolates. From the observed differences in the individual banding patterns, isolates were separated based on how related they were to each other. This resulted in the clustering of 14 different groups. The clusters showed groupings according to specific *Enterococci* species, antibiotic resistance profiles and MAR indices, indicating a common linkage. This denotes a relationship between the isolates and possibly a common ancestral linkage. This relationship could possibly be a result of transfer of particular ARGs.

The presence of ARGs in treated effluents are critical in its transfer between bacteria in the environment (Rizzo *et al.*, 2013). Final treated effluent discharged into the environment, has shown to increase the loads of ARGs in river sediments downstream of the WWTPs (Pruden, *et al.*, 2012; Marti *et al.*, 2013). In this study, the concentrations of 3 tetracycline resistance genes (*tet* L, *tet* M and *tet* O) in influent and treated final effluent samples were quantified, using the droplet digital PCR technique, in order to determine the efficiency of the WWTPs in removing these genes from the received influent. Contrary to many studies (Pruden, *et al.*, 2012; Marti *et al.*, 2013), the results of the present study revealed efficient removal of the three tetracycline resistance genes with a removal efficiency of more than 82%, except for the *tet* L gene in WWTP1 (June 2014) with only 42% removal. The *tet* O gene was found to be most prevalent, having the highest concentrations (in both influent and final effluent samples) compared to the other two genes. The removal efficiency for the month of August 2014 was higher at both WWTPs with over 98% removal observed at WWTP2.

The overall findings of this study reveal the dire need for responsible antibiotic use and prevention of its misuse. The magnitude of multidrug resistant bacteria in WWTPs and subsequently in the environment, are cause for concern. This study highlights the need to prevent an increase in VRE and resistance towards broad spectrum antibiotics, such as tetracycline, trimethoprim-sulfamethoxazole and gentamicin. This can be achieved by responsible clinical and agricultural use of broad spectrum antibiotics, implementing prudent vancomycin use, preventing and controlling nosocomial transmission of VRE, improved surveillance of antibiotic resistant infections and implementing regulations to prevent inappropriate disposal of antibiotics into sewage systems. Despite the high presence of ARE and tetracycline resistance genes in wastewater influent, the WWTPs proved to be efficient in their treatment process and removal of these organisms and the resistance genes. Regardless of the high removal rate of ARE and tetracycline resistance genes during the treatment process, as well as the decrease observed from upstream to downstream of the receiving rivers, their presence in the final treated effluent is a cause for concern. It is therefore important that continuous monitoring of WWTPs be conducted, to ensure the efficiency of the WWTPs in their treatment process, to safeguard public health.

4.2. Potential for future development of the study

With the world currently in the crux of life-threatening bacterial infections such as meningitis, pneumonia, tuberculosis, cholera and diarrheal illnesses, it is imperative that viable options are present in curbing disastrous epidemics. The recent tuberculosis, meningococcal and cholera outbreaks resulted in thousands of deaths across the world (WHO, 2014a; WHO, 2015b,c). Another disquieting outbreak was one that transpired in South African hospitals by vancomycin-resistant *Enterococci* (VRE) (SASCM, 2012). In order to prevent such catastrophe, antibiotics are highly relied upon in treating and eliminating deadly bacterial

diseases. However, as a result of antibiotic resistance, many bacterial infections become difficult to control. An antibiotic resistance analysis in South Africa, identified an increasing number of multidrug resistant bacterial outbreaks and thus identified an urgent need for action (Winters and Gellband, 2011). It is therefore necessary for future work to conduct testing of ARE to a broader range of antibiotics, including streptomycin, penicillin, daptomycin, tobramycin, amikacin, ceftriaxone, imipenem, linezolid, tigecycline and telavancin, in order to further examine the extent of multidrug resistance among these *Enterococci* isolates.

Wastewater treatment plants have been deemed as one of the leading reservoirs of ARB and ARGs that may be disseminated into the environment (Rizzo *et al.,* 2013). These hotspots are considered to be crucial in horizontal gene transfer, allowing for the spread of ARGs (Rizzo *et al.,* 2013). This is because WWTPs provide a selective pressure for ARB and ARGs, in terms of high bacterial populations, biofilms and the presence of chemical compounds, antibiotics and organic matter (Martinez, 2009). The distinctive environment may therefore result in the spread of antibiotic resistance. The present study focused on the detection and quantification of only selected tetracycline resistance genes. It would be of interest for future work to detect additional ARGs and to quantify them from metagenomic DNA obtained from the wastewater, in order to have a comprehensive understanding of the prevalence of ARGs and the efficiency of these WWTPs in removing them.

The removal of ARE and ARGs in wastewater is highly important and necessary before the release of treated effluent into the environment. Biological treatment processes alone are not effective in the removal of ARB (Adams *et al*., 2002). The disinfection process in wastewater treatment is essential to control pathogens and ARB in treated wastewater and receiving water bodies (Ge *et al*., 2012; Bouki *et al.,* 2013). Chlorination is the most popular form of disinfection in WWTPs as it is a well-established technology, inexpensive, requires simple

application and is readily available (Bouki *et al*., 2013). It has however, been reported that chlorination has fallen short of being efficient in removing ARB and ARGs during the treatment process (Karumathil *et al*., 2014). It is therefore essential to study alternative disinfection processes that do not pose a risk to the environment, human and animal health. Such alternatives include ultraviolet (UV) radiation, advanced oxidation processes and microfiltration (Hijnen *et al*., 2005; Breazeal *et al*., 2013; Rizzo *et al*., 2014). Many studies, including the present study, conducted analysis at WWTPs that utilized chlorination in the tertiary treatment step (Samie *et al.*, 2009; Odjadjare and Olaniran, 2015; Pillay and Olaniran, 2016). It would be interesting for future studies to analyse the removal efficiency of ARB and ARGs in WWTPs that utilizes a tertiary treatment step that does not involve chlorination to ascertain the best disinfection process for effective removal of ARB and ARGs.

Enterococci are commonly responsible for antibiotic resistant hospital-acquired infections, such as bacteraemia, urinary tract infections, endocarditis and wound infections (Sava *et al.*, 2010). These pathogens are often associated with nosocomial infections and are able to survive in adverse conditions due to their increased ability to acquire and share extra-chromosomal elements encoding virulence traits or antibiotic resistance (Mundy *et al.*, 2000; Olawale *et al*., 2011). *Enterococci* associated with nosocomial infections are known to contain many virulence genes, including *cylA*, *gelE*, *esp*, *asa1* and *hyl* which code for haemolytic cytolysin, gelatinase, enterococcal surface protein, aggregation substance and glycoside-hydrolase, respectively. The prevalence of these genes in enterococcal isolates has been found in aquatic environments (Creti *et al.*, 2004). Future work on the *Enterococci* isolates can evaluate the prevalence of selected virulence genes to assess the pathogenic potential of environmental strains.

REFERENCES

Abamecha, A., B. Wondafrash and A. Abdissa. 2015. Antimicrobial resistance profile of *Enterococcus* species isolated from intestinal tracts of hospitalized patients in Jimma, Ethiopia. *BMC Research Notes*. **8**: 213

Abdel-Raouf, N., A.A. Al-Homaidan and I.B.M. Ibraheem. 2012. Microalgae and wastewater treatment. *Saudi Journal of Biological Sciences*. **19**: 257–275

Adams, C., Y. Wang, K. Loftin and M. Meyer. 2002. Removal of antibiotics from surface and distilled water in conventional water treatment processes. *Journal of Environmental Engineering*. **128**: 253–260

Ahrberg, C.D., B.R. Ilic, A. Manza and P. Neuzil. 2016. Handheld real-time PCR device. *Lab Chip*. **16**: 586–592

Almagro-Moreno, S., M.G. Napolitano and E.F. Boyd. 2010. Excision dynamics of *Vibrio* pathogenicity island-2 from *Vibrio cholerae*: role of a recombination directionality factor VefA. *BMC Microbiology*. **10**: 306

Almeida, T., A. Brandão, E. Muñoz-Atienza, A. Gonçalves, C. Torres, G. Igrejas, P.E. Hernández, C. Herranz, L.M. Cintas and P. Poeta. 2011. Identification of bacteriocin genes in *Enterococci* isolated from game animals and saltwater fish. *Journal of Food Protection*. **74**: 1252–1260

Aminov, R.I. 2009. The role of antibiotics and antibiotic resistance in nature. *Environmental Microbiology*. **11**: 2970–2988

Andersen, S. R. 1993. Effects of waste water treatment on the species composition and antibiotic resistance of coliform bacteria. *Current Microbiology*. **26**: 97–103

Anwar, H., M.K. Dasgupta and J.W. Costerton. 1990. Testing the susceptibility of bacteria in biofilms to antibacterial agents. *Antimicrobial Agents and Chemotherapy*. **34**: 2043–2046

Arana, I. and I. Barcina. 2008. Ecological significance and posible risks of nonculturable intestinal bacteria in water systems. In: Van Dijk T, ed. Microbial Ecology Research Trends. New York, NY: Nova Science Publishers Inc. pp. 115-137

Arias, C.A., G.A. Contreras and B.E. Murray. 2010. Management of multidrug-resistant enterococcal infections. *Clinical Microbiology and Infection*. **16**: 555–562

Armstrong, J.L., J.J. Calomaris and R.J. Seidler. 1982. Selection of antibiotic-resistant standard plate count bacteria during water treatment. *Applied and Environmental Microbiology*. **44**: 308–316

Asano, T., F.L. Burton, H.L. Leverenz, R. Tsuchihashi and G. Tchobanoglous. 2007. Water Reuse: issues, technologies, and applications, 1st, ed.; McGraw-Hill: New York, NY, USA. pp. 954–955

Auerbach, E. A., E.E. Seyfried and K.D. McMahon. 2007. Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Research*. **41**: 1143–1151

Ayeni, F. A., B.T. Odumosu, A.E. Oluseyi and W. Ruppitsch. 2016. Identification and prevalence of tetracycline resistance in *Enterococci* isolated from poultry in Ilishan, Ogun State, Nigeria. *Journal of Pharmacy and Bioallied Sciences*. **8**: 69-73

Baddour, L.M., W.R. Wilson, A.S. Bayer, V.G. Fowler, I.M. Tleyjeh, M.J. Rybak, B. Barsic, P.B. Lockhart, M.H. Gewitz, M.E. Levison, A.F. Bolger, J.M. Steckelberg, R.S. Baltimore, A.M. Fink, P. O'Gara and K.A. Taubert. 2015. Infective endocarditis in adults: diagnosis, antimicrobial therapy, and management of complications: a scientific statement for healthcare professionals from the American heart association. *Circulation*. **132**:1435-1486

Baquero, F., J. L. Martínez and R. Cantón. 2008. Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology*. **19**: 260–265

Batt, A.L., D.D. Snow and D.S. Aga. 2006. Occurrence of sulfonamide antimicrobials in private water wells in Washington County, Idaho, USA. *Chemosphere.* **64**: 1963–1971

Bekele, B. and M. Ashenafi. 2010. Distribution of drug resistance among *Enterococci* and *Salmonella* from poultry and cattle in Ethiopia. *Tropical Animal Health and Production*. **42**: 857–864

Bik, E.M., P.B. Eckburg, S.R. Gill, K.E. Nelson, E.A. Purdom, F. Francois, G. Perez-Perez, M.J. Blaser and D.A. Relman. 2005. Molecular analysis of the bacterial microbiota in the human stomach. *Proceedings of the National Academy of Sciences*. **103**: 732–737

Billington, S.J., J.G. Songer and B.H. Jost. 2002. Widespread distribution of a *tet* W determinant among tetracycline-resistant isolates of the animal pathogen *Arcanobacterium pyogenes*. *Antimicrobial Agents and Chemotherapy.* **46**: 1281- 1287

Biowatch. 2016. Fact Sheet: Drought crisis. http://www.biowatch.org.za/docs/fs/2016/Drought%20Crisis%20March%202016%20website%20copy.pdf

Biswal, B.K., R. Khairallah, K. Bibi, A. Mazza, R. Gehr, L. Masson and D. Frigon. 2014. Impact of UV and peracetic acid disinfection on the prevalence of virulence and antimicrobial resistance genes in uropathogenic *Escherichia coli* in wastewater effluents. *Applied and Environmental Microbiology*. **80**: 3656-3666

Blasco, M.D., C. Esteve and E. Alcaide. 2008. Multiresistant waterborne pathogens isolated from water reservoirs and cooling systems. *Journal of Applied Microbiology*. **105**: 469–475

Bonilla, H.F., M.J. Zervos and C.A. Kauffman. 1996. Long-term survival of vancomycin-resistant *Enterococcus faecium* on a contaminated surface. *Infection Control* and *Hospital Epidemiology*. **17**: 770-772

Bonjoch, X., C. García-Aljaro and A. R. Blanch. 2011. Persistence and diversity of faecal coliform and *Enterococci* populations in faecally polluted waters. *Journal of Applied Microbiology*. **111**: 209–215

Bonten, M.J.M., M.K. Hayden, C. Nathan C, J. van Voorhis, M. Matushek, S. Slaughter, T. Rice and R.A. Weinstein. 1996. Epidemiology of colonization of patients and environment with vancomycin-resistant *Enterococci*. *Lancet.* **348**: 1615- 1619

Bouki, C., D. Venieri and E. Diamadopoulos. 2013. Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: A review. *Ecotoxicology and Environmental Safety*. **91**: 1–9

Breazeal, M.V., J.T. Novak, P.J. Vikesland and A. Pruden. 2013. Effect of wastewater colloids on membrane removal of antibiotic resistance genes. *Water Research*. **47**: 130-40

Brooks, J.P., S.L. Maxwell, C. Rensing, C.P. Gerba and I.L. Pepper. 2007. Occurrence of antibiotic resistant bacteria and endotoxin associated with the land application of biosolids. *Canadian Journal of Microbiology*. **53**: 616–622

Burmeister, A. R. 2015**.** Horizontal gene transfer. *Evolution, medicine and public Health.* **1**: 193-194

Byappanahalli, M.N., M.B. Nevers, A. Korajkic, Z.R. Staley and V.J. Harwood. 2012. *Enterococci* in the environment. *Microbiology and Molecular Biology Reviews*. **76**: 685–706

Carr, F.J., D. Chill and N. Maida. 2002. The lactic acid bacteria: a literature survey. *Critical Reviews in Microbiology*. **28**: 281-370

Carvalho, E.M.R., R.A. Costa, A.J.G. Araújo, F.C.T. Carvalho, S.P.P. Pereira, O.V. Sousa and R.H.S.F. Vieira. 2014. Multiple antibiotic resistance of *Enterococcus* isolated from coastal water near an outfall Brazil. *African Journal of Microbiology Research*. **8**: 1825-1831

Chajęcka-Wierzchowska, W., A. Zadernowska, B. Nalepa and L. Laniewska-Trokenheim. 2012. Occurrence and antibiotic resistance of *Enterococci* in ready-to-eat food of animal origin. *African Journal of Microbiology Research.* **6**: 6773-6780

Chapman, D. 1996. Water quality assessments: A guide to the use of biota, sediments and water in environmental monitoring 2nd. Ed. UNESCO, World Health Organization, United Nations Environment Programme, London

Chee-Sandford, J.C., R. I. Mackie, S. Koike, I. G. Krapac, Y. Lin, A. C. Yannarell, S. Maxwell and R. I. Aminov. 2009. Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *Journal of Environmental Quality*. **38**: 1086-1108

Cheng, H.A, F.E. Lucy, M.A. Broaders, S.E. Mastitsky, C. Chen and A. Murray. 2012. Municipal wastewater treatment plants as pathogen removal systems and as a contamination source of noroviruses and *Enterococcus faecalis*. *Journal of Water and Health*. **10**: 380-382

Chopra, I. and M. Roberts. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews*. **65**: 232- 260

Chuard, C. and L.B. Reller. 1998. Bile-esculin test for presumptive identification of *Enterococci* and *Streptococci*: effects of bile concentration, inoculation technique, and incubation time. *Journal of Clinical Microbiology*. **36**: 1135–1136

Ciftci, A., A. Findik, T. Ica, B. Bas, E.E. Onuk and S.Gungordu. 2009. Slime production and antibiotic resistance of *Enterococcus faecalis* isolated from arthritis in chickens. *International Journal of Veterinary Sciences and Medicine.* **71**: 849–853

Çitak, S., N. Yucel and S. Orhan. 2004. Antibiotic resistance and incidence of *Enterococcus* species in Turkish white cheese. *International Journal of Dairy Technology*. **57**: 27–31

Clara, M., B. Strenn, O. Gans, E. Martinez, N. Kreuzinger and H. Kroiss. 2005. Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane bioreactor and conventional wastewater treatment plants. *Water Research*. **39**: 4797–4807

Clevel, D. B. 1990. Movable genetic elements and antibiotic resistance in *Enterococci*. *European Journal of Clinical Microbiology and Infectious Disease*. **9**: 90–102

Clewell, D. B. 2014. Antibiotic Resistance Plasmids in Bacteria. In: eLS. John Wiley & Sons Ltd, Chichester

Clinical and Laboratory Standards Institute (CLSI). 2007. Performance standards for antimicrobial susceptibility testing; Seventeenth informational supplement, M100-S17. Clinical and laboratory standards institute, Wayne, PA

Clinical Laboratory Standards Institute (CLSI). 2006. Performance standards for antimicrobial disk susceptibility tests; Approved standard—9th ed. CLSI document M2-A9. 26:1. Clinical Laboratory Standards Institute, Wayne, PA

Colomer-Lluch, M., J. Jofre and M. Muniesa. 2011. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. *PLoS One*. **6**: e17549.

Courcelle, J., A. Khodursky, B. Peter, P.O. Brown and P.C. Hanawalt. 2001. Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient *Escherichia coli*. *Genetics*. **158**: 41–64

Creti, R., M. Imperi, L. Bertuccini, F. Fabretti, G. Orefici, R. Di Rosa and L. Baldassarri. 2004. Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. *Journal of Medical Microbiology.* **53**: 13–20

Cunha, B.A. 2006. Antimicrobial therapy of multidrug-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococci*, and methicillin-resistant *Staphylococcus aureus. Medical Clinics of North America*. **90**: 1165-1182

Czekalski, N., T. Berthold, S. Caucci, A. Egli and H. Burgmann. 2012. Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Genea, Switzerland. *Frontiers in Microbiology*. **3**: 1-13

da Costa, P.M., P. Vaz-Pires and F. Bernardo. 2006. Antimicrobial resistance in *Enterococcus* spp. isolated in inflow, effluent and sludge from municipal sewage water treatment plants. *Water Research*. **40**: 1735-1740

Daniel, D.S., S.M. Lee, G.A. Dykes and S. Rahman. 2015. Public health risks of multiple-drug-resistant *Enterococcus* spp. in Southeast Asia. *Applied and Environmental Microbiology*. **81**: 6090-6097

Darby, J., M. Heath, J. Jacangelo, F. Loge, P. Swaim and G. Tchobanoglous. 1995. Comparison of UV irradiation to chlorination: guidance for achieving optimal UV performance. Water Environment Research Foundation. Alexandria, Virginia. ISBN-10: 1572780029

Das, T.K. 2001. Ultraviolet disinfection application to a wastewater treatment plant. *Clean Technologies* and *Environmental* Policy. **3**: 69–80

Davies, C.M., D.J. Roser, A.J. Feitz and N.J. Ashbolt. 2009. Solar radiation disinfection of drinking water at temperate latitudes: inactivation rates for an optimised reactor configuration. *Water Research*. **43**: 643–652

Davies, J. 2012. Sanitation: sewage recycles antibiotic resistance. *Nature*. **487**: 302

de Perio, M.A., P.R. Yarnold and J. Warren. 2006. Risk factors and outcomes associated with non-*Enterococcus faecalis*, non-*Enterococcus faecium* enterococcal bacteremia. *Infection Control and Hospital Epidemiology*. **27**: 28-33

Deasy, B. M., M. C. Rea, G. F. Fitzgerald, T. M. Cogan, and T. P. Beresford. 2000. A rapid PCR based method to distinguish between *Lactococcus* and *Enterococcus. Systematic and Applied Microbiology*. **23**: 510–522

Department of Water Affairs (DWAF). 2004. Water Quality Management Series Sub-Series. Operational policy for the disposal of land-derived water containing waste to the marine environment of South Africa: Guidance on Implementation. Pretoria. Edition 1

Devriese, L.A., B. Pot, L. Van Damme, K. Kersters and F. Haesebrouck. 1994. Identification of *Enterococcus* species isolated from foods of animal origin. *International Journal of Food Microbiology*. **26**: 187–197

Devriese, L.A., J.I. Cruz Colque, P. De Herdt and F. Haesebrouck. 1992. Identification and composition of the tonsillar and anal enterococcal and streptococcal flora of dogs and cats. *Journal of Applied Bacteriology*. **73**: 421–425

Dhanalakshmi, T.A., S. Vijaya and S. Sharieff. 2015. Spontaneous enterococcal meningitis: A Case Report. *Journal of Medical Sciences and Health*. **1**: 27-29

Ding, Y., W. Zhang, C. Gu, I. Xagoraraki and H. Li. 2011. Determination of pharmaceuticals in biosolids using accelerated solvent extraction and liquid chromatography/tandem mass spectrometry. *Journal of Chromatography A*. **1218**:10–6

Dodd, M.C. 2012. Potential impacts of disinfection processes on elimination and deactivation of antibiotic resistance genes during water and wastewater treatment. *Journal of Environmental Monitoring*. **14**: 1754–1771

Donabedian, S.M., L.A. Thal, E. Hershberger, M.B. Perri, J.W. Chow, P. Bartlett, R. Jones, K. Joyce, S. Rossiter, K. Gay, J. Johnson, C. Mackinson, E. Debess, J. Madden, F. Angulo and M.J. Zervos. 2003. Molecular characterization of gentamicin-resistant *Enterococci* in the United States: Evidence of spread from animals to humans through food. *Journal of Clinical Microbiology*. **41**: 1109-1113

Downes, A. and T. P. Blunt. 1877. Researchers on the effect of light upon bacteria and other organisms. *Royal Society of London*. **26**: 488 – 500

Drews, S.J., S.E. Richardson, R. Wray, R. Freeman, C. Goldman, L. Streitenberger, D. Stevens, C. Goia, D. Kovach, J. Brophy and A.G. Matlow. 2008. An outbreak of vancomycin-resistant *Enterococcus faecium* in an acute care pediatric hospital: Lessons from environmental screening and a case-control study. *Canadian Journal* of Infectious Diseases and *Medical Microbiology*. **19**: 233–236

Dunlop, P.S.M., M. Ciavola, L. Rizzo and J.A. Byrne. 2011. Inactivation and injury assessment of *Escherichia coli* during solar and photocatalytic disinfection in LDPE bags. *Chemosphere*. **85**: 1160-1166

Dunlopa, P.S.M., M. Ciavola, L. Rizzo, D.A. McDowell and J.A. Byrne. 2015. Effect of photocatalysis on the transfer of antibiotic resistance genes in urban wastewater. *Catalysis Today*. **240**: 55–60

Duong, H.A., N.H. Pham, H.T. Nguyen, T.T. Hoang, H.V. Pham , V.C. Pham, M. Berg, W. Giger and A.C. Alder. 2008. Occurrence, fate and antibiotic resistance of fluoroquinolone antibacterials in hospital wastewaters in Hanoi, Vietnam. *Chemosphere*. **72**: 968-973

DWA. 2011. Green drop handbook. Version 1. Department of Water Affairs. Pretoria. RSA.

Eckburg, P.B., E.M. Bik, C.N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S.R. Gill, K.E. Nelson and D.A. Relman. 2005. Diversity of the human intestinal microbial flora. *Science*. **308**: 1635–1638

Eliasson, J. 2015. The rising pressure of global water shortages. *Nature.* **517**: 6

Esiobu, N., L. Armenta and J. Ike. 2002. Antibiotic resistance in soil and water environments. *International Journal of Environmental Health Research.* **12**: 133-144

Eyewitness News. 2015 (November). Government dealing with provinces affected by drought. Edited by Koyana, K. and L. Isaacs.

Fanaro, S., R. Chierici, P. Guerrini and V. Vigi. 2003. Intestinal microflora in early infancy: composition and development. *Acta Paediatrica supplement.* **441:** 48-55

Fernandes, S.C. and B. Dhanashree. 2013. Drug resistance & virulence determinants in clinical isolates of *Enterococcus* species. *Indian Journal of Medical Research.* **137**: 981–985

Ferreira da Silva, M., I. Tiago, A. Verıssimo, R.A.R. Boaventura, O.C. Nunes and C.M. Manaia. 2006. Antibiotic resistance of *Enterococci* and related bacteria in an urban wastewater treatment plant. *FEMS Microbiology Ecology.* **55**: 322–329

Feuerpfeil, I., J. Lopez-Pila, R. Schmidt, E. Schneider and R. Szewzyk. 1999. Antibiotic resistant bacteria and antibiotics in the environment. *Bundesgesundheitsblatt.-Gesundheitsforschung-Gesundheitsschutz*. **42**: 37-50

Fisher, K. and C. Phillips. 2009. The ecology, epidemiology and virulence of *Enterococcus*. *Microbiology.* **155**: 1749–1757

Fluit, A.C., M.E. Jones, F.J. Schmitz, J. Acar, R. Gupta and J. Verhoef. 2000. Antimicrobial resistance among urinary tract infection (UTI) isolates in Europe: results from the SENTRY Antimicrobial Surveillance Program 1997 and 1998. *Clinical Infectious Diseases*. **30**: 454-60

Food and Drug Administration. 2014. Antimicrobials sold or distributed for use in food-producing animals. 2009 Summary Report

Forslund, K., S. Sunagawa, J.R. Kultima, D.R. Mende, M. Arumugam, A. Typas and P. Bork. 2013. Country-specific antibiotic use practices impact the human gut resistome. *Genome Research.* **23**: 1163-1169

Foulquié Moreno, M.R., P. Sarantinopoulos, E. Tsakalidou and L. De Vuyst. 2006. The role and application of *Enterococci* in food and health. *International Journal of Food Microbiology*. **106**: 1-24

Fraise, A.P. 2002. Susceptibility of antibiotic-resistant cocci to biocides. *Journal of Applied Microbiology.* **92**: 158–162

Franz, C.M.A.P., W.H. Holzapfel and M.E. Stiles. 1999. *Enterococci* at the crossroads of food safety. *International Journal of Food Microbiology*. **47**: 1-24

Fraser, S.L. 2016. Enterococcal Infections. MedScape

Frigon, D., B.K. Biswal, A. Mazza, L. Masson and R. Gehr. 2013. Biological and physicochemical wastewater treatment processes reduce the prevalence of virulent *Escherichia coli*. *Applied and Environmental Microbiology*. **79**: 835–844

Furlaneto-Maia, L., K.R. Rocha, F.C. Henrique, A. Giazzi and M.C. Furlaneto. 2014. Antimicrobial resistance in *Enterococcus* sp isolated from soft cheese in Southern Brazil. *Advances in Microbiology*. **4**: 175-181

Gallert, C., K. Fund and J. Winter. 2005. Antibiotic resistance of bacteria in raw and biologically treated sewage and in groundwater below leaking sewers. *Applied Microbiology and Biotechnology*. **69**: 106–112

Gao, P., M. Munir and I. Xagoraraki. 2012. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Science of the Total Environment.* **421**: 173–183

Garcia, A.H.M., J. Rivas, L. Figueroa and A.L. Monroe. 1995. Case history: pharmaceutical wastewater treatment plant upgrade, SmithKline Beecham Pharmaceuticals Company. *Desalination*. **102**: 255–263

Garcia, S., B. Wade, C. Bauer, C. Craig, K. Nakaoka and W. Lorowitz. 2007. The effect of wastewater treatment on antibiotic resistance in *Escherichia coli* and *Enterococcus* sp. *Water Environment Research*. **79**: 2387-2395

Gavaldà, J., O. Len, J.M. Miró, P. Muñoz, M. Montejo, A. Alarcón, J. de la Torre-Cisneros, C. Peña, X. Martínez-Lacasa, C. Sarria, G. Bou, J.M. Aguado, E. Navas, J. Romeu, F. Marco, C. Torres, P. Tornos, A. Planes, V. Falcó, B. Almirante and A. Pahissa.2007. Brief communication: treatment of Enterococcus faecalis endocarditis with ampicillin plus ceftriaxone. Annals of Internal Medicine. **146**: 574-579

Ge, F., K. Guo, G. Zhou, H. Zhang, J. Liu and Y. Dai. 2012. Isolation and identification of bacteria in the activated sludge from four sewage treatment plants in Nanjing City and its antibiotic resistance analysis. *Journal of Environmental Sciences*. **33**: 1646–1651

Gelband, H., M. Miller-Petrie, S. Pant, S. Gandra, J. Levinson, D. Barter, A. White and R. Laxminarayan. 2015. State of the World's Antibiotics. 1st ed. Washington, D.C.: Center for Disease Dynamics, Economics & Policy.

Getachew, Y., L. Hassan, Z. Zakaria and A.A. Saleha. 2013. Genetic variability of vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* isolates from human, chickens, and pigs in Malaysia. *Applied and Environmental Microbiology*. **79**: 4528

Ghosh, G., S. Hanamoto, N. Yamashita, X. Huang and H. Tanaka. 2016. Antibiotics removal in biological sewage treatment plants. *Pollution.* **2**: 131-139

Ghosh, T.S., S.S. Gupta, G.B. Nair and S.S. Mande. 2013. In silico analysis of antibiotic resistance genes in the gut microflora of individuals from diverse geographies and age-groups. *PLoS One*. **8**

Gilmore, M.S., F. Lebreton and W. van Schaik. 2013. Genomic transition of *Enterococci* from gut commensals to leading causes of multidrug-resistant hospital infection in the antibiotic era. *Current Opinion in Microbiology*. **16**: 10–16

Giraffa, G. 2002. *Enterococci* from foods. *FEMS Microbiology Reviews*. **26**: 163-171

Goel, V., D. Kumar, R. Kumar, P. Mathur and S. Singh. 2016. Community acquired enterococcal urinary tract infections and antibiotic resistance profile in North India. *Journal of Laboratory Physicians*. **8**: 50-54

Gomes, B., C. Esteves, I. Palazzo, A. Darini, G. Felis, L. Sechi and E. De Martinis. 2008. Prevalence and characterization of *Enterococcus* spp. isolated from Brazilian foods. *Food Microbiology*. **25**: 668-675

Gordon, C.L. and M.H. Ahmad. 1991. Thermalsusceptibility of *Streptococcus faecium* strains isolated from frankfurters. *Canadian Journal of Microbiology*. **37**: 609-612

Government Gazette. 1984. No. 9225. Requirements for the Purification of Waste Water or Effluent. General and Special Standards. Minister of Environment Affairs and Fisheries.

Grassi, M., L. Rizzo and A. Farina. 2013. Endocrine disruptors compounds, pharmaceuticals and personal care products in urban wastewater: Implications for agricultural reuse and their removal by adsorption process. *Environmental Science and Pollution Research*. **20**: 3616-3628

Guardabassi L. and A. Dalsgaard. 2004. Occurrence, structure and mobility of Tn1546-like elements in environmental isolates of vancomycin-resistant *Enterococci*. *Applied and Environmental Microbiology*. **70**: 984-990

Guardabassi, L. and A. Dalsgaard. 2002. Occurrence and Fate of Antibiotic Resistant Bacteria in Sewage. Danish EPA Environmental Project Report. 722

Guardabassi, L., D.M.L.F Wong and A. Dalsgaard. 2002. The effects of tertiary wastewater treatment on the prevalence of antimicrobial resistant bacteria. *Water Research*. **36**: 1955–1964

Guillaume, G., V. Ledent, W. Moens and J. M. Collard. 2004. Phylogeny of efflux-mediated tetracycline resistance genes and related proteins revisited. *Microbial Drug Resistance*. **10**: 11–26

Gunasekera, S.P. and J. Perera. 2007. Drug resistant *Enterococci*: factors associated with gastrointestinal tract colonization. *The Ceylon Journal of Medical Science.* **50**: 9-14

Guo, M.T., Q.B. Yuan and J. Yang. 2015. Distinguishing effects of ultraviolet exposure and chlorination on the horizontal transfer of antibiotic resistance genes in municipal wastewater. *Environmental Science and Technology*. **49**: 5771–5778

Han, D., T. Unno, J. Jang, K. Lim, S.N. Lee, G. Ko, M.J. Sadowsky and H.G. Hur. 2011. The occurrence of virulence traits among high-level aminoglycosides resistant *Enterococcus* isolates obtained from feces of humans, animals, and birds in South Korea. *International Journal of Food Microbiology*. **144**: 387–392

Hasman H, A.G. Villadsen and F.M. Aarestrup. 2005. Diversity and stability of plasmids from glycopeptides resistant *Enterococcus faecium* (GRE) isolated from pigs in Denmark. *Microbial Drug Resistance*. **11**: 178–184

Hayashi, H., R. Takahashi, T. Nishi, M. Sakamoto and Y. Benno. 2005. Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. *Journal of Medical Microbiology*. **54**: 1093–1101

Hayes, J.R., L.L. English, P.J. Carter, T. Proescholdt, K.Y. Lee, D.D. Wagner and D.G. White. 2003. Prevalence and antimicrobial resistance of *Enterococcus* species isolated from retail meats. *Applied and Environmental Microbiology*. **69**: 7153-7160

Hegstad, K., T. Mikalsen, T.M. Coque, G. Werner and A. Sundsfjord. 2010. Mobile genetic elements and their contribution to the emergence of antimicrobial resistant *Enterococcus faecalis* and *Enterococcus faecium*. *Clinical Microbiology* and *Infection*. **16**: 541–554

Hendricks, R. and E.J. Pool. 2012. The effectiveness of sewage treatment processes to remove faecal pathogens and antibiotic residues. *Journal of Environmental Science and Health*. **47**: 289–297

Heuer, H. , E. Krögerrecklenfort, E.M.H. Wellington, S. Egan, J.D. van Elsas, L. van Overbeek, J.M. Collard, G. Guillaume, A.D. Karagouni, T.L. Nikolakopoulou and K. Smalla. 2002. Gentamicin resistance genes in environmental bacteria: prevalence and transfer. *FEMS Microbiology Ecology*. **42**: 289–302

Hidron, A.I., J.R. Edwards, J. Patel, T.C. Horan, D.M. Sievert, D.A. Pollock and S.K. Fridkin. 2008. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. *Infection Control and Hospital Epidemiology.* **29**: 996-1011

Hijnen, W.A.M., E.F. Beerendonk and G.J. Medema. 2005. Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: A review. *Water Research*. **40**: 3–22

Hindson, B.J., K.D. Ness, D.A. Masquelier, P. Belgrader, N.J. Heredia, A.J. Makarewicz, I.J. Bright, M.Y. Lucero, A.L. Hiddessen, T.C. Legler, T.K. Kitano, M.R. Hodel, J.F. Petersen, P.W. Wyatt, E.R. Steenblock, P.H. Shah, L.J. Bousse, C.B. Troup, J.C. Mellen, D.K. Wittmann, N.G. Erndt, T.H. Cauley, R.T. Koehler, A.P. So, S. Dube, K.A. Rose, L. Montesclaros, S. Wang, D.P. Stumbo, S.P. Hodges, S. Romine, F.P. Milanovich, H.E. White, J.F. Regan, G.A. Karlin-Neumann, C.M. Hindson, S. Saxonov and B.W. Colston. 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Analytical Chemistry*. **83**: 8604–8610

Hollenbeck, B.L. and L. B. Rice. 2012. Intrinsic and acquired resistance mechanisms in *Enterococcus*. *Virulence*. **3**: 421–433

Hong, P., N. Al-Jassim, M.I. Ansari and R.I. Mackie. 2013. Environmental and public health implications of water reuse: antibiotics, antibiotic resistant bacteria, and antibiotic resistance genes. *Antibiotics.* **2**: 367-399

Hu, Y., X. Yang, J. Qin, N. Lu, G. Cheng, N. Wu, Y. Pan, J. Li, L. Zhu, X. Wang, Z. Meng, F. Zhao, D. Liu, J. Ma, N. Qin, C. Xiang, Y. Xiao, L. Li, H. Yang, J. Wang, R. Yang, G.F. Gao, J. Wang and B. Zhu. 2013. Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. *Nature Communications*. **4**: 2151

Huang, J.J., H.Y. Hu, F. Tang, Y. Li, S.Q. Lu and Y. Lu. 2011. Inactivation and reactivation of antibiotic-resistant bacteria by chlorination in secondary effluents of a municipal wastewater treatment plant. *Water Research*. **45**: 2775–2781

Huang, J. J., H.Y. Hu, S.Q. Lu, Y. Li, F. Tang, Y. Lu and B. Wei. 2012. Monitoring and evaluation of antibiotic-resistant bacteria at a municipal wastewater treatment plant in China. *Environment International*. **42**: 31–36

Huddleston, J.R. 2014. Horizontal gene transfer in the human gastrointestinal tract: potential spread of antibiotic resistance genes. *Infection and Drug Resistance*. **7**: 167–176

Huycke, M.M., D.F. Sahm and M.S. Gilmore. 1998. **Multiple-drug resistant Enterococci: the nature of the problem and an agenda for the future.** *Emerging Infectious Diseases Journal.* **4:** 239-249

Huys, G., K. D'Haene, J. Collard and J. Swings. 2004. Prevalence and molecular characterization of tetracycline resistance in *Enterococcus* isolates from food *Applied and Environmental Microbiology*. **70**: 1555–1562

Igbinosa, E.O. and A.I. Okoh. 2009. Impact of discharge wastewater effluents on the physico-chemical qualities of a receiving watershed in a typical rural community. *International Journal of Environmental Science* and *Technology*. **6**: 175-182

Iversen, A., I. Kuhn, A. Franklin, and R. Mollby. 2002. High prevalence of vancomycin-resistant *Enterococci* in Swedish sewage. *Applied and Environmental Microbiology*. **68**: 2838-2842

Iversen, A., I. Kühn, M. Rahman, A. Franklin, L.G. Burman, B. Olsson-Liljequist, E. Torell and R. Möllby. 2004. Evidence for transmission between humans and the environment of a nosocomial strain of *Enterococcus faecium. Environmental Microbiology*. **6**: 55-59

Iweriebor, B.C., S. Gaqavu, L. C. Obi, U.U. Nwodo and A.I. Okoh. 2015. Antibiotic susceptibilities of *Enterococcus* species isolated from hospital and domestic wastewater effluents in Alice, Eastern Cape province of South Africa. *International Journal of Environmental Research and Public Health*. **12**: 4231-4246

Jacangelo, J.G. and C.A. Buckley. 1996. Microfiltration, in: J. Mallevialle, P. E. Odendaal,M. R. Wiesner (Ed.). Water Treatment: Membrane Processes, McGrawHill, New York, 11.11-11.39

Jackson, C. R., P. J. Fedorka-Cray and J. B. Barrett. 2004. Use of a genus- and species-specific multiplex PCR for identification of *Enterococci. Journal of Clinical Microbiology*. **42**: 3558

Jackson, C.R., P.J. Fedorka-Cray, J.A. Davis, J.B. Barrett, J.H. Brousse, J. Gustafson and M. Kucher. 2010. Mechanisms of antimicrobial resistance and genetic relatedness among *Enterococci* isolated from dogs and cats in the United States. *Journal of Applied Microbiology*. **108**: 2171–2179

Jaji, M.O., O. Bamgbose, O.O. Odukoya and T.A. Arowlo. 2007. Water quality assessment of Ogun River, South West Nigeria. *Environmental Monitoring and Assessment*. **133**: 447-482

Jhansi, S.C. and S.K. Mishra. 2013. Wastewater treatment and reuse: sustainability options. *Consilience: The Journal of Sustainable Development.* **10**: 1 – 15

Jimenez, B. 2008. Water reuse: an international survey of current practice, issues and needs. IWA Publishing: London, UK. pp. 241-249

Jones, M., J. Williams, K. Gärtner, R. Phillips, J. Hurst and J. Frater. 2014. Low copy target detection by Droplet Digital PCR through application of a novel open access bioinformatic pipeline, 'definetherain'. *Journal of Virological Methods*. **202:** 46–53

Jury, K.L., T. Vancov, R.M. Stuetz and S.J. Khan. 2010. Antibiotic resistance dissemination and sewage treatment plants. In current research, technology and education topics in *Applied Microbiology and Microbial Biotechnology*. **1**: 509–519

Kaltenthaler, E.C. and J.V. Pinfold. 1995. Microbiological methods for assessing hand washing practice in hygiene behaviour studies. *The American Journal of Tropical Medicine and Hygiene*. **98**: 101–106

Karki, S., K. Leder and A.C. Cheng. 2015. Should we continue to isolate patients with vancomycin-resistant *Enterococci* in hospitals. *Medical Journal of Australia*. **202**: 234–236

Karkman, A., T.A. Johnson, C. Lyra, R.D. Stedtfeld, M. Tamminen, J.M. Tiedje and M. Virta. 2016. High-throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant. *FEMS Microbiology Ecology*. **92**

Karumathil, D.P., H.B. Yin, A. Kollanoor-Johny and K. Venkitanarayanan. 2014. Effect of chlorine exposure on the survival and antibiotic gene expression of multidrug resistant *Acinetobacter baumannii* in water. *International Journal of* Environmental *Research* and *Public Health.* **11**: 1844-1854

Kibirige, I., R. Perissinotto and X. Thwala. 2006. A comparative study of zooplankton dynamics in two subtropical temporarily open/closed estuaries, South Africa. *Marine Biology*. **148**: 1307-1324

Kim, S., J.N. Jensen, D.S. Aga and A.S. Weber. 2006. Fate of tetracycline resistant bacteria as a function of activated sludge process organic loading and growth rate. *Water Science and Technology*. **55**: 291–297

Kim, S., J.N. Jensen, D.S. Aga and A.S. Weber. 2007. Tetracycline as a selector for resistant bacteria in activated sludge. *Chemosphere*. **66**: 1643–51

Kirkland, K.B., J.P. Briggs, S.L. Trivette, W.E. Wilkinson and D.J. Sexton. 1999. The impact of surgical-site infections in the 1990's: attributable mortality, excess length of hospitalization and extra costs. *Infection Control & Hospital Epidemiology*. **20**: 725–730

Klare, I., H. Heier, H. Claus, G. Böhme, S. Martin, S. Seltmann, R. Hakenbeck, V. Atanassova and W. Witte. 1995. *Enterococcus faecium* strains with vanA-mediated high-level glycopeptide resistance isolated from animal foodstuffs and fecal samples of humans in the community. *Microbial Drug Resistance*. **1**: 265-272

Klein, G. 2003. Taxonomy, ecology and antibiotic resistance of *Enterococci* from food and the gastro-intestinal tract. *International Journal of Food Microbiology*. **88**: 123–131

Knapp, C.W., J. Dolfing, P.A.I. Ehlert and D.W. Graham. 2010. Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environmental Science and Technology*. **44**: 580–587

Kohanski, M.A., M.A. DePristo and J.J. Collins. 2010. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Molecular Cell*. **37**: 311-320

Korzeniewska, E., A. Korzeniewska and M. Harnisz. 2013. Antibiotic resistant *Escherichia coli* in hospital and municipal sewage and their emission to the environment. *Ecotoxicology and Environmental Safety*. **91**: 96–102

Kristich, C.J., L.B. Rice and C.A. Arias. 2014. Enterococcal infection—treatment and antibiotic resistance. *Enterococci*: From commensals to leading causes of drug resistant infection. Gilmore MS, Clewell DB, Ike Y, et al., editors. http://www.ncbi.nlm.nih.gov/books/NBK190420/

Kubacka, A., M.S. Diez, D. Rojo, R. Bargiela, S. Ciordia, I. Zapico, J.P. Albar, C.Barbas, V.A.P. Martins dos Santos, M. Fernandez-Garcia and M. Ferrer. 2014. Understanding the antimicrobial mechanism of TiO2-based nanocomposite films in a pathogenic bacterium. Scientific Reports. **4**: 4134

Kuhn, I., A. Iversen, L.G. Burman, B. Olsson-Liljequist, A. Franklin, M. Finn, F. Aarestrup, A.M. Seyfarth, A.R. Blanch, H. Taylor, J. Caplin, M.A. Moreno, L. Dominguez and R. Mollby.2000. Epidemiology and ecology of *Enterococci*, with special reference to antibiotic resistant strains, in animals, humans and the environment. Example of an ongoing project within the European Research Programme. *International Journal of Antimicrobial Agents*. **14**: 337–342

Kümmerer, K. 2009. Antibiotics in the aquatic environment, a review, part II. *Chemosphere*. **75**: 435–441

LaPara, T. and T. Burch. 2012. Municipal wastewater as a reservoir of antibiotic resistance. In: PL KeenM. Montforts. Antimicrobial Resistance in the environment. Hoboken, NJ: Wiley-Blackwell. pp. 241–250

Layton, B.A., S.P. Walters, L.H. Lam and A.B. Boehm. 2010. *Enterococcus* species distribution among human and animal hosts using multiplex PCR. *Journal of Applied Microbiology*. **109**: 539–547

Lebreton, F., R.J.L. Willems and M.S. Gilmore. 2014. *Enterococcus* diversity, origins in nature, and gut colonization. In: Gilmore MS, Clewell DB, Ike Y, Shankar N, editors. *Enterococci*: From commensals to leading causes of drug resistant infection. Boston.

Leclercq, R. and P. Courvalin. 1997. Resistance to glycopeptides in *Enterococci*. *Clinical Infectious Diseases*. **24**: 545-54

Leclercq, R., K. Oberlé, S. Galopin, V. Cattoir, H. Budzinski and F. Petit. 2013. Changes in enterococcal populations and related antibiotic resistance along a medical center-wastewater treatment plant-river continuum. *Applied and Environmental Microbiology*. **79**: 2428–2434

Leclercq, R. 2009. Epidemiological and resistance issues in multidrug-resistant staphylococci and enterococci. *Clinical Microbiology and Infection.* **15**: 224–231

Lester, C.H., N. Frimodt-Møller, T.L. Sørensen, D.L. Monnet and A.M. Hammerum. 2006. In vivo transfer of the *vanA* resistance gene from an *Enterococcus faecium* isolate of animal origin to an *E. faecium* isolate of human origin in the intestines of human volunteers. *Antimicrobial Agents and Chemotherapy*. **50**: 596-599

Levy, S. B., L.M. McMurray, T.M. Barbosa, V. Burdett, P. Courvalin, W. Hillen, M.C. Roberts, J.I. Rood and D.E. Taylor. 1999. Nomenclature for new tetracycline resistance determinants. *Antimicrobial Agents and Chemotherapy.* **43**: 1523–1524

Ley, R. E., D.A. Peterson and J.I. Gordon. 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*. **124**: 837–848

Li, D., M. Yang, J. Hu, J. Zhang, R. Liu, X. Gu, Y. Zhang, and Z. Wang.2009. Antibiotic-resistance profile in environmental bacteria isolated from penicillin production wastewater treatment plant and the receiving river. *Environmental Microbiology*. **11**: 1506-1517

Liu, P., H. Zhang, Y. Feng , F. Yang and J. Zhang. 2014. Removal of trace antibiotics from wastewater: A systematic study of nanofiltration combined with ozone-based advanced oxidation processes. *Chemical Engineering Journal*. **240**: 211–220

Luczkiewicz, A., K. Jankowska, S. Fudala-Ksiazek and K. Olanczuk-Neyman. 2010. Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. *Water Research*. **44**: 5089-5095

Luna, V.A. and M.C. Roberts. 1998. The presence of the *tet* O gene in a variety of tetracycline resistant *Streptococcus pneumoniae* serotypes from Washington State. *Journal of Antimicrobial Chemotherapy*. **42**: 613–619

Lupo, A., S. Coyne and T. Berendonk. 2012. Origin and evolution of antibiotic resistance: the common mechanism of emergence and spread in water bodies. *Frontiers in Microbiology*. **3**: 1-13

Mackie, R.I., A. Sghir and H.R. Gaskin.1999. **Developmental microbial ecology of the neonatal gastrointestinal tract.** *The American Journal of Clinical Nutrition.* **69:** 1035-1045

Maier, R.M., I.L. Pepper and C.P. Gerba. 2009. Environmental Microbiology, second ed. (Ed.). Academic Press, Elsevier. Ch 26: pp. 539–545

Majlesinasr, M. 1998. Study of wastewater disposal status and effluent quality in hospitals of Shahid Beheshti. *Iranian Journal of Public Health*. **6**: 371 – 375

Malato, S., P. Fernández-Ibáñez, M.I. Maldonado, J. Blanco and W. Gernjak. 2009. Decontamination and disinfection of water by solar photocatalysis: Recent overview and trends. *Catalysis Today*. **147**: 1-59

Manero, A., X. Vilanova, M. Cerda-Cuellar and A. R. Blanch. 2002. Characterization of sewage waters by biochemical fingerprinting of *Enterococci*. *Water Research*. **36**: 2831–2835.

Marshall, B.M. and S.B. Levy. 2011. Food animals and antimicrobials: impacts on human health. *Clinical Microbiology Reviews*. **24**: 718–733

Marti, E., J. Jofre and J.L. Balcazar. 2013. Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment plant. *PLoS One.* **8**: 1-8

Martin, B., L. Corominas, M. Garriga and T. Aymerich. 2009. Identification and tracing of *Enterococcus* spp. by RAPD-PCR in traditional fermented sausages and meat environment. *Journal of Applied Microbiology*. **106**: 66–77

Martinez, J.L. 2009. Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environmental Pollution.* **157**: 2893–2902

Masschelein, W.J. 2002. Ultraviolet Light in Water and Wastewater Sanitation. Boca Raton, FL: Lewis Publishers

Matz, C. and S. Kjelleberg. 2005. Off the hook-how bacteria survive protozoan grazing. *Trends in Microbiology*. **13**: 302-307

McBride, S.M., V.A. Fischetti, D.J. Leblanc, R.C. Jr Moellering and M.S. Gilmore. 2007. Genetic diversity among *Enterococcus faecalis. PLoS One*. **2**: 582

McCullagh, C., J.M.C. Robertson, D.W. Bahnemann and P.K.J. Robertson. 2007. The application of TiO_2 photocatalysis for disinfection of water contaminated with pathogenic micro-organisms: A review. *Research on Chemical Intermediates*. **33**: 359-375

McKinney, C., Y. Ma, J.T. Novak and A. Pruden. 2009. Disinfection of microconstituent antibiotic resistance genes by UV light and sludge digestion. *Proceedings of the Water Environment Federation*. **13**: 577–589

McKinney, C.W. and A. Pruden. 2012. Ultraviolet disinfection of antibiotic resistant bacteria (ARBS) and their antibiotic resistance genes (ARGS) in water and wastewater. *Environmental Science and Technology.* **46**: 13393–13400

McMahon, M.A.S., J. Xu, J.E. Moore, I.S. Blair and D.A. McDowell. 2007. Environmental stress and antibiotic resistance in food-related pathogens. *Applied and Environmental Microbiology*. **73**: 211-217

Medeiros, A.W., R.I. Pereira, D.V. Oliveira, P.D. Martins, P.A. d'Azevedo, S. van der Sand, J. Frazzon and A.P.G. Frazzon. 2014. Molecular detection of virulence factors among food and clinical *Enterococcus faecalis* strains in south Brazil. *Brazilian Journal of Microbiology*. **332**: 327–332

Mema, V. 2010. Impact of poorly maintained waste water and sewage treatment plants: Lessons from South Africa. *ReSource*. 12: 60–65

Michael, I., L. Rizzo, C.S. McArdell, C.M. Manaia, C. Merlin, T. Schwartz, C. Dagot and D. Fatta-Kassinos. 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review. *Water Research.* **47**: 957–995

Mikalsen, T., T. Pedersen, R. Willems, T.M. Coque, G. Werner, E. Sadowy, W. van Schaik, L.B. Jensen, A. Sundsfjord and K. Hegstad. 2015. Investigating the mobilome in clinically important lineages of *Enterococcus faecium* and *Enterococcus faecalis*. *BMC Genomics.* **16**: 2-16

Mills, S., F. Shanahan, C. Stanton, C. Hill, A. Coffey and R.P. Ross. 2013. Movers and shakers: influence of bacteriophages in shaping the mammalian gut microbiota. *Gut Microbes*. **4**: 4–16

Miotke, L., B.T. Lau, R. T. Rumma and H.P. Ji. 2014. High sensitivity detection and quantitation of DNA copy number and single nucleotide variants with single color droplet digital PCR. *Analytical Chemistry*. **86**: 2618–2624

Mohanty, S., B. Dhawan, R. S. Gadepalli, R. Lodha and A. Kapil. 2006. Case report of vancomycin resistant *Enterococcus faecium* vanA phenotype: First documented isolation in India. *The Southeast Asian Journal of Tropical Medicine and Public Health*. **37**: 335-337

Mondino, S.S., A.C. Castro, P.J. Mondino, M.G. Carvalho, K.M. Silva and L.M. Teixeira. 2003. Phenotypic and genotypic characterization of clinical and intestinal *Enterococci* isolated from inpatients and outpatients in two Brazilian hospitals. *Microbial Drug Resistance*. **9**: 167–174

Morozzi, G., R. Sportolari, G. Caldini, G. Cenci and A. Morosi. 1988. The effect of anaerobic and aerobic wastewater treatment on faecal coliforms and antibiotic-resistant faecal coliforms. Zentralblatt für Bakteriologie, *Mikrobiologie und Hygiene*. **185**: 340–349

Mosteo, R., M.P. Ormad and P. Goni. 2013. Identification of pathogen bacteria and protozoa in treated urban wastewaters discharged in the Ebro River (Spain): Water reuse possibilities. *Water Science and Technology*. **68**: 575–583

Mpenyana-Monyatsi, L., M.S. Onyango and M.N.B. Momba. 2012. Groundwater quality in a South African rural community: A possible threat to public health. *Polish Journal of Environmental Studies*. **21**: 1349–1358

Mulvey, M.R. and A.E. Simor. 2009. Antimicrobial resistance in hospitals: How concerned should we be. *Canadian Medical Association Journal*. **180:** 408-415

Munck,C., M. Albertsen, A. Telke, M. Ellabaan, P.H. Nielsen and M.O.A. Sommer. 2015. Limited dissemination of the wastewater treatment plant core resistome. *Nature Communications*. **6**: 1-10

Mundy, L. M., D.F. Sahm, and M. Gilmore. 2000. Relationships between enterococcal virulence and antimicrobial resistance. *Clinical Microbiology Reviews*. **13**: 513–515

Munir, M., K. Wong and I. Xagoraraki. 2011. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Research*. **45**: 681–693

Muruleedhara N. Byappanahalli, M.N., M.B. Nevers, A. Korajkic, Z.R. Staley and V. J. Harwood. 2012. *Enterococci* in the environment. *Microbiology and Molecular Biology Reviews*. **76**: 685–706

Mutters, N.T., V. Mersch-Sundermann, R. Mutters, C. Brandt, W. Schneider-Brachert and U. Frank. 2013. Control of the Spread of Vancomycin-Resistant *Enterococci* in Hospitals: Epidemiology and Clinical Relevance. *Deutsches Ärzteblatt International*. **110**: 725–731

Myers, D. N. and M. A. Sylvester. 1997. Fecal indicator bacteria. U.S. Geological Survey TWRI

Nagulapally, S.R., A. Ahmad, A. Henry, G.L. Marchin., L. Zurek and A. Bhandari. 2009. Occurrence of ciprofloxacin-, trimethoprimesulfamethoxazole-, and vancomycin-resistant bacteria in a municipal wastewater treatment plant. *Water Environment Research*. **81**: 82–90

Naidoo, S. and A.O. Olaniran. 2014. Treated wastewater effluent as a source of microbial pollution of surface water resources. *International Journal of* Environmental Research and *Public Health.* **11**: 249-270

Nakajo, K., Y. Iwami, R. Komori, S. Ishikawa, T. Ueno, Y. Suzuki and N. Takahashi. 2005. The resistance to acidic and alkaline environments of endodontic pathogen *Enterococcus faecalis*. *International Congress Series*. **1284**: 191–192

Neely, A. N. and M.P. Maley. 2000. Survival of *Enterococci* and *Staphylococci* on hospital fabrics and plastic. *Journal of Clinical Microbiology*. **38**: 724–726

Ng, L. K., I. Martin, M. Alfa and M. Mulvey. 2001. Multiplex PCR for the detection of tetracycline resistant genes. *Molecular and Cellular Probes*. **15**: 209–215

Noble, C.J. 1978. **Carriage of group D *Streptococci* in the human bowel.** *Journal of Clinical Pathology.* **31:** 1182-1186

Norton, S.E., J.M. Lechner, T. Williams and M. R. Fernando. 2013. A stabilizing reagent prevents cell-free DNA contamination by cellular DNA in plasma during blood sample storage and shipping as determined by digital PCR. *Clinical Biochemistry*. **46**: 1561–1565

Noskin, G.A., V. Stosor, I. Cooper and L.R. Peterson. 1995. Recovery of vancomycin-resistant *Enterococci* on fingertips and environmental surfaces. *Infection Control* and *Hospital Epidemiology*. **16**: 577-581

Novais, C., T.M. Coque, H. Ferreira, J.C. Sousa and L. Peixe. 2005. Environmental contamination with vancomycin-resistant *Enterococci* from hospital sewage in Portugal. *Applied and Environmental Microbiology.* **71**: 3364-3368

Novo, A. and C.M. Manaia. 2010. Factors influencing antibiotic resistance burden in municipal wastewater treatment plants. *Applied Microbiology and Biotechnology*. **87**: 1157–1166

Oberoi, L. and A. Aggarwal. 2010. Multidrug resistant *Enterococci* in a rural tertiary care hospital—a cause of concern. *JK Science*. **12**: 157-158

Odjadjare, E.C. and A.O. Olaniran. 2015. Prevalence of antimicrobial resistant and virulent *Salmonella* spp. in treated effluent and receiving aquatic milieu of wastewater treatment plants in Durban, South Africa. *International Journal of Environmental Research and Public Health***. 12**: 9692-9713

Olańczuk-Neyman, K., H. Stosik-Fleszar and S. Mikołajski. 2001. Evaluation of indicator bacteria removal in wastewater treatment processes. *Polish Journal of Environmental Studies.* **10**: 457-461

Olaniran, A.O., S. Naidoo and B. Pillay. 2012. Surveillance of invasive bacterial pathogens and human enteric viruses in wastewater final effluents and receiving water bodies – a case study from Durban, South Africa. *CLEAN – Soil, Air, Water*. **40**: 681–691

Olawale, K.O., S.O. Fadiora and S.S. Taiwo. 2011. Prevalence of hospital-acquired *Enterococci* infections in two primary-care hospitals in Osogbo, Southwestern Nigeria. *African Journal of Infectious Diseases*. **5**: 40-46

Oliveira, A.J.F.C. and J.M.W. Pinata. 2008. Antimicrobial resistance and species composition of *Enterococcus* spp. isolated from waters and sands of marinen recreational beaches in Southeastern Brazil. *Water Research*. **42**: 2242-2250

Orrhage, K. and C.E. Nord. 1999. Factors controlling the bacterial colonization of the intestine in breastfed infants. *Acta Paediatrica supplement*. **430:** 47-57

Osuolale, O. and A. Okoh. 2015. Assessment of the physicochemical qualities and prevalence of *Escherichia coli* and Vibrios in the final effluents of two wastewater treatment plants in South Africa: Ecological and Public Health Implications. *International Journal of Environmental Research and Public Health*. **12***:* 13399-13412

Patel, R. 2003. Clinical impact of vancomycin-resistant *Enterococci*. *Journal of Antimicrobial Chemotherapy*. **51**: 13–21

Pei, R., S. Kim, K.H. Carlson and A. Pruden. 2006. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Research*. **40**: 2427-2435

Pernthaler, J. 2005. Predation on prokaryotes in the water column and its ecological implications. *Nature Reviews Microbiology*. **3**: 537-546

Peters, J., K. Mac, H. Wichmann-Schauer, G. Klein and L. Ellerbroek. 2003. Species distribution and antibiotic resistance patterns of *Enterococci* isolated from food of animal origin in Germany. *International Journal of Food Microbiology*. **88**: 311-314

Petsaris, O., F. Miszczak, M. Gicquel-Bruneau, A. Perrin-Guyomard, F. Humbert, P. Sanders and R. Leclercq. 2005. Combined antimicrobial resistance in *Enterococcus faecium* isolated from chickens. *Applied and Environmental Microbiology*. **71**: 2796 –2799

Pillay, L. and A.O. Olaniran. 2016. Assessment of physicochemical parameters and prevalence of virulent and multiple-antibiotic-resistant *Escherichia coli* in treated effluent of two wastewater treatment plants and receiving aquatic milieu in Durban, South Africa. *Environmental Monitoring and Assessment*. **188**: 260

Platteeuw, C., F. Michiels, H. Joos, J. Seurinck and W. M. de Vos. 1995. Characterization and heterologous expression of the *tetL* gene and identification of iso-ISS1 elements from *Enterococcus faecalis* plasmid pJH1. *Gene*. **160**: 89-93

Poeta, P., D. Costa, J. Rodrigues and C. Torres. 2006. Antimicrobial resistance and the mechanisms implicated in faecal *Enterococci* from healthy humans, poultry and pets in Portugal. *International Journal of Antimicrobial Agents.* **27**: 131–137

Ponce-de-Leon, S. 1991. The needs of developing countries and the resources required. *Journal of Hospital Infection.* **18**: 376–381

Porwancher, R., A. Sheth, S. Remphrey, E. Taylor, C. Hinkle and M. Zervo. 1997. Epidemiological study of hospital-acquired infection with vancomycin-resistant *Enterococcus faecium*: possible transmission by an electronic ear-probe thermometer. *Infection Control* and *Hospital Epidemiology.* **18**: 771-773

Praharaj, I., S. Sujatha and S.C. Parija. 2013. Phenotypic & genotypic characterization of vancomycin resistant *Enterococcus* isolates from clinical specimens. *Indian Journal of Medical Research.* **138**: 549-556

Proia, L., D. von Schiller, A. Sànchez-Melsió, S. Sabater, C.M. Borrego, S. Rodríguez-Mozaz and J.L. Balcázar. 2015. Occurrence and persistence of antibiotic resistance genes in river biofilms after wastewater inputs in small rivers. *Environmental Pollution*. **210**: 121-128

Pruden, A., M. Arabi and H.N. Storteboom. 2012. Correlation between upstream human activities and riverine antibiotic resistance genes. *Environmental Science and Technology.* **46**: 11541–11549

Pruden, A., R. Pei, H. Storteboom and K.H. Carlson. 2006. Antibiotic resistance genes as emerging contaminants: studies in Northern Colorado. *Environmental Science* and *Technology*. **40**: 7445–7450

Qasim, S.R. 1999. Waste-water treatment plants: planning, design, and operation, second edition. Lancaster, Pennsylvania: Technomic

Quiroz, T.S., P.A. Nieto, H.E. Tobar, F.J. Salazar-Echegarai, R.J. Lizana, C.P. Quezada, C.A. Santiviago, D.V. Araya, C.A. Riedel, A.M. Kalergis and S.M. Bueno. 2011. Excision of an unstable pathogenicity island in *Salmonella enterica* serovar Enteritidis is induced during infection of phagocytic cells. *PLoS One*. **6**

Radha, K.V. 2009. A case study of biomedical waste management in hospitals. *Global Journal of Health Science.* **1**: 82 – 88

Reinthaler, F.F., J. Posch, G. Feierl, G. Wust, D. Haas, G. Ruckenbauer, F. Mascher and E. Marth. 2003. Antibiotic resistance of *E. coli* in sewage and sludge. *Water Research*. **37**: 1685–1690

Riboldi, G.P., J. Frazzon, P.A. D'Azevedo and A.P.G. Frazzon. 2009. Antimicrobial resistance profile of *Enterococcus* spp isolated from food in Southern Brazil. *Brazilian Journal of Microbiology*. **40**: 125- 128

Rizzo, L., C. Manaia, C. Merlin, T. Schwartz, C. Dagot, M. C. Ploy, I. Michael and D. Fatta-Kassinos. 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Science of the Total Environment.* **447**: 345–360

Rizzo, L., G. Ferro and C.M. Manaia. 2014. Wastewater disinfection by solar heterogeneous photocatalysis: effect on tetracycline resistant/sensitive *Enterococcus* strains. *Global NEST Journal*. **16**: 455-462

Robertson, P.K.J., J.M.C. Robertson and D.W. Bahnemann. 2012. Removal of microorganisms and their chemical metabolites from water using semiconductor photocatalysis. *Journal of Hazardous Materials*. **211–212**: 161-171

Russo, T., K. Alfredo and J. Fisher. 2014. Sustainable water management in urban, agricultural, and natural systems. *Water.* **6**: 3934-3956

Rutala, W.A., M.M. Stiegel, F.A. Sarubbi and D.J. Weber. 1997. Susceptibility of antibiotic-susceptible and antibiotic-resistant hospital bacteria to disinfectants. Infect. *Infection Control and Hospital Epidemiology*. **18**: 417–421

Sahlström, L., V. Rehbinder, A. Albihn, A. Aspan and B. Bengtsson. 2009. Vancomycin resistant *Enterococci* (VRE) in Swedish sewage sludge**.** *Acta Veterinaria Scandinavica*. **51**: 24

Salyers, A.A., A. Gupta and Y. Wang. 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends in Microbiology*. **12**: 412– 416

Samie, A. and P. Ntekele. 2014. Genotypic detection and evaluation of the removal efficiency of Giardia duodenalis at municipal wastewater treatment plants in Northern South Africa. *Tropical Biomedicine.* **31**: 122–133

Samie, A., C.L. Obi, J.O. Igumbor and M.N.B. Momba. 2009. Focus on 14 sewage treatment plants in the Mpumalanga Province, South Africa in order to gauge the efficiency of wastewater treatment. *African Journal of Biotechnology.* **8**: 3276-3285

Sarmah, A.K., M.T. Meyer and A.B. Boxall. 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere*. **65**: 725–59

Sartor, R.B. 2008. Microbial influences in inflammatory bowel diseases. *Gastroenterology*. **134**: 577–594

Sava, G., E. Heikens and J. Huebner. 2010. Pathogenesis and Immunity in enterococcal Infections. *Clinical Microbiology and Infection*. **16**: 533-540

Schjorring, S. and K.A. Krogfelt. 2011. Assessment of bacterial antibiotic resistance transfer in the gut. *International Journal of Microbiology*. **2011**: 1–10

Schnappinger, D. and W. Hillen. 1996. Tetracyclines: antibiotic action, uptake, and resistance mechanisms. *Archives of Microbiology.* **165**: 359-369

Schwartz, T., W. Kohen, B. Jansen and U. Obst. 2003. Detection of antibiotic resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiology Ecology.* **43**: 325-335

Servais, P. and J. Passerat. 2009. Antimicrobial resistance of fecal bacteria in waters of Seine river watershed (France). *Science of the Total Environment*. **408**: 365–372

Shankar, N., A.S. Baghdayan and M.S. Gilmore. 2002. Modulation of virulence within a pathogenicity island in vancomycin-resistant *Enterococcus faecalis. Nature*. **417**: 746-750

Shepard, B.D. and M.S. Gilmore. 2002. Antibiotic-resistant *Enterococci*: the mechanisms and dynamics of drug introduction and resistance. Microbes and Infection. **4**: 215–224

Shi, P., S. Jia, X.X. Zhang, T. Zhang, S. Cheng and A. Li. 2013. Metagenomic Insights into chlorination effects on microbial antibiotic resistance in drinking water. *Water Research*. **47**: 111–120

Shrivastava, R., R.K. Upreti, S.R. Jain, K.N. Prasad, P.K. Seth U.C. Chaturvedia. 2004. Sub optimal chlorine treatment of drinking water leads to selection of multidrug-resistant *Pseudomonas aeruginosa*. *Ecotoxicology and Environmental Safety*. **58**: 277–283

Sievert, D.M., P. Ricks, J.R. Edwards, A. Schneider, J. Patel, A. Srinivasan, A. Kallen, B. Limbago and S. Fridkin. 2013. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. *Infection Control and Hospital Epidemiology.* **34**: 1-14

Silva, N., G. Igrejas, A. Gonçalves and P. Poeta. 2011. Commensal gut bacteria: distribution of *Enterococcus* species and prevalence of *Escherichia coli* phylogenetic groups in animals and humans in Portugal. *Annals of Microbiology*. **62**: 449-459

Silva, N., G. Igrejas, N. Figueireido, A. Gonçalves, H. Radhouani, J. Rodrigues and P. Poeta. 2010. Molecular characterization of antimicrobial resistance in *Enterococci* and *Escherichia coli* isolates from European wild rabbit (Oryctolagus cuniculus). *Science of the Total Environment*. **408**: 4871–4876

Sinthuchai, D., S.K. Boontanon, N. Boontanon and C. Polprasert. 2016. Evaluation of removal efficiency of human antibiotics in wastewater treatment plants in Bangkok, Thailand. *Water Science* and *Technology*. **73**: 182-91

Smyth, C.J., H. Matthews, M.K. Halpenny, H. Brandis and G. Colman. 1987. Biotyping, serotyping and phage typing of *Streptococcus faecalis* isolated from dental plaque in the human mouth. *Journal of Medical Microbiology*. **23**: 45–54

Sobsey, M.D., M.J. Casteel, H. Chung, G. Lovelace, O.D. Simmons III and J.S. Meschke. 1998. Innovative technologies for waste water disinfection and pathogen detection. *Proceedings of Disinfection*. pp. 483-493

Sommer, M.O., G. Dantas and G.M. Church. 2009. Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science*. **325**: 1128-1131

Soto, S.M., M.T. Jimenez de Anta and J. Vila. 2006. Quinolones induce partial or total loss of pathogenicity islands in uropathogenic *Escherichia coli* by SOS-dependent or -independent pathways, respectively. *Antimicrobial Agents and Chemotherapy*. **50**: 649–653

South African River Health Programme (RHP). State of the Rivers Report. 2011. Umgeni River and Neighbouring Rivers and Streams.

South African Society for Clinical Microbiology (SASCM) Laboratory Surveillance data, Private Sector, Jan-Jun 2012

Spencer, R. 1996. Predominant pathogens found in the European prevalence of infections in intensive care study. *European Journal of Clinical Microbiology & Infectious Disease*. **15**: 3-12

Stalder, T., O. Barraud, M. Casellas, C. Dagot and M. Ploy. 2012. Integron involvement in environmental spread of antibiotic resistance. *Frontiers in Microbiology*. **3**: 9

Stalder, T., O. Barraud, T. Jové, M. Casellas, M. Gaschet, C. Dagot and M.C. Ploy. 2014. Quantitative and qualitative impact of hospital effluent on dissemination of the integron pool. *The ISME Journal*. **8**: 768-777

Tannock, G. W. and G. Cook. 2002. *Enterococci* as members of the intestinal microflora of humans. In the *Enterococci*: Pathogenesis, molecular biology, and antibiotic resistance (ed M. S. Gilmore, D. B. Clewell, P. Courvalin, G. M. Dunny, B. E. Murray and L. B. Rice). Washington, D. C.: ASM Press. pp. 101-132

Tanwar, J., S. Das, Z. Fatima and S. Hameed. 2014. Multidrug resistance: an emerging crisis. *Interdisciplinary Perspectives on Infectious Diseases.* **2014**: 1-7

Tao, R., G.G. Ying, H.C. Su, H.W. Zhou, J.P.S. Sidhu. 2010**.** Detection of antibiotic resistance and tetracycline resistance genes in *Enterobacteriaceae* isolated from the Pearl Rivers in South China. *Environmental Pollution.* **158**: 2101-2109

Tchobanoglous, G. and F.L. Burton. 1991. Inc. Wastewater engineering: treatment disposal and reuse, third edition. New York:McGraw-Hill, 1991

Tchobanoglous, G., F.L. Burton and H.D. Stensel. 2003. Wastewater engineering: treatment and reuse, 4th edn. Metcalf & Eddy, Inc, McGraw-Hill, New York

Templeton, M.R., F. Oddy, W. Leung and M. Rogers. 2009. Clorine and UV disinfection of ampicillin-resistant and trimethoprim-resistant Escherichia coli. *Canadian Journal of Civil Engineering*. **36**: 889–894

Ternes, T. 1998. Occurrence of drugs in German sewage treatment plants and rivers. *Water Research*. **32**: 3245–3260

Teuber, M., L. Meile and F. Schwarz. 1999. Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie van Leeuwenhoek*. **76**: 115-137

Teuber, M., V. Perreten and F. Wirsching. 1996. Antibiotic-resistant bacteria: a new dimension in food microbiology. *LWT - Food Science and* `Technology`. **29**: 182-199

Torres, O.R., R.Z. Korman, S.A. Zahler and G.M. Dunny. 1991. The conjugative transposon Tn925 - enhancement of conjugal transfer by tetracycline in *Enterococcus-faecalis* and mobilization of chromosomal genes in *Bacillus subtilis* and *E.faecalis*. *Molecular and General Genetics*. **225**: 395-400

Tree, J.A., M.R. Adams and D.N. Lees. 2003. Chlorination of indicator bacteria and viruses in primary sewage effluent. *Applied and Environmental Microbiology*. **69**: 2038–2043

Trzcinski, K., B.S. Cooper, W. Hryniewicz, and C.G. Dowson. 2000. Expression of resistance to tetracyclines in strains of methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*. **45:** 763–770

Union of Concerned Scientists (UCS). 2001. Hogging it: Estimates of Antimicrobial Abuse in Livestock.UCS Publishing: Cambridge, MA, USA. 109

United Nations. 2015. Water for life decade 2005-2015. The human right to water and sanitation. http://www.un.org/waterforlifedecade/ human_right_to_water.shtml

United States Environmental Protection Agency (EPA). 1999. Wastewater technology fact sheet chlorine disinfection

United States Environmental Protection Agency (EPA). 2004. Primer for municipal wastewater treatment systems

Van Boeckel, T.P., S. Gandra, A. Ashok, Q. Caudron, B.T. Grenfell, S.A. Levin and R. Laxminarayan. 2014. Global antibiotic consumption 2000 to 2010: An analysis of national pharmaceutical sales data. *The Lancet Infectious Diseases*. **14**: 742 – 750

Van den Berghe, E., T. De Winter and L. De Vuyst. 2006. Enterocin A production by *Enterococcus faecium* FAIR-E 406 is characterised by a temperature- and pH-dependent switch-off mechanism when growth is limited due to nutrient depletion. *International Journal of Food Microbiology*. **107**: 159–170

Van den Bogaard, A.E. and E.E. Stobberingh. 2000. Epidemiology of resistance to antibiotics. Links between animals and humans. *International Journal of Antimicrobial Agents*. **14**: 327-335

Varela, A.R., G. Ferro, J. Vredenburg, M. Yanık, L. Vieira, L. Rizzo, C. Lameiras and C.M. Manaia. 2013. Vancomycin resistant *Enterococci*: From the hospital effluent to the urban wastewater treatment plant. *Science of the total environment*. **451**: 155-161

Vilanova, R., R. Katebi and N. Wahab. 2011. N-removal on wastewater treatment plants: a process control approach. *Journal of Water Resource and Protection*. **3**: 1-11

Vilanova, X., A. Manero, M. Cerdà-Cuéllar and A.R. Blanch. 2004. The composition and persistence of faecal coliforms and enterococcal populations in sewage treatment plants. *Journal of Applied Microbiology*. **96**: 279-288

Wagner, M. and A. Loy. 2002. Bacterial community composition and function in sewage treatment systems. *Current Opinion in Biotechnology*. **13**: 218–227

Wang, H.H. and D.W. Schaffner. 2011. Antibiotic resistance: how much do we know and where to go from here? *Applied and Environmental Microbiology*. **77**: 7093–7095

Warsa, U.C., M. Nonoyama, T. Ida, R. Okamoto, T. Okubo, C. Shimauchi, A. Kuga and M. Inoue. 1996. Detection of *tet*(K) and *tet*(M) in *Staphylococcus aureus* of Asian countries by the polymerase chain reaction. *Journal of Antibiotics.* **49**: 1127–1132

Whitman, R.L., D.A. Shively, H.P.M.B. Nevers and M.N. Byappanahalli. 2003. Occurrence of *Escherichia coli* and *Enterococci* in Cladophora (Chlorophyta) in nearshore water and beach sand of Lake Michigan. *Applied and Environmental Microbiology*. **69**: 4714-4719

Winters, C. and H. Gellband. 2011. Part 1. The Global Antibiotic Resistance Partnership (GARP). *South African Medical Journal*. **101**: 566-567

Wise, R. 2002. Antimicrobial resistance: Priorities for action. *Journal of Antimicrobial Chemotherapy*. **49**: 585–586

Witte, W. 1998. Medical consequences of antibiotic use in agriculture. *Science*. **279**: 996-997

Wong, M.L. and J.F. Medrano. 2005. Real-time PCR for mRNA quantitation. *BioTechniques*. **39**: 75-85

World Health Organization (WHO) and UNICEF. 2013. Progress on sanitation and drinking-water: 2012 update. World Health Organisation/United Nations Children's Fund, Geneva. ISBN: 978-92-806-4632-0

World Health Organization (WHO). 1989. Health guidelines for use of wastewater in agriculture and aquaculture. Technical Report Series 778. Geneva, Switzerland

World Health Organization (WHO). 2002. Prevention of hospital-acquired infections. A practical guide. 2nd edition

World Health Organization (WHO). 2014a. Global Tuberculosis Report

World Health Organization (WHO). 2014b. Antimicrobial resistance, Global Report on Surveillance.

World Health Organization (WHO). 2015a. Water and Sanitation Health (WaSH). Content of the human right to water. http://www.who.int/water_sanitation_health/humanrights/en/index2.html

World Health Organization (WHO). 2015b. Meningococcal disease – Niger (update). Disease outbreak news

World Health Organization (WHO). 2015c. Cholera – United Republic of Tanzania. Disease Outbreak News

Wright, G. D. 2010. Antibiotic resistance in the environment: a link to the clinic. *Current Opinion in Microbiology*. **13**: 589-594

Xi, C., Y. Zhang, C.F. Marrs, W. Ye, C. Simon, B. Foxman and J. Nriagu. 2009. Prevalence of antibiotic resistance in drinking water treatment and distribution systems. *Applied and Environmental Microbiology*. **75**: 5714–5718

Yuan, Q. B., M. T. Guo and J. Yang. 2015. Fate of antibiotic resistant bacteria and genes during wastewater chlorination: implication for antibiotic resistance control. *PLoS ONE.* **10**

Zapata, A., I. Oller, L. Rizzo, S. Hilgert, M.I. Maldonado, J.A. Sánchez-Pérez and S. Malato. 2010. Evaluation of operating parameters involved in solar photo-Fenton treatment of wastewater: interdependence of initial pollutant concentration, temperature and iron concentration. *Applied Catalysis B: Environmental*. **97**: 292-298

Zhang, K. and K. Farahbakhsh. 2007. Removal of native coliphages and coliform bacteria from municipal wastewater by various wastewater treatment processes: implications to water reuse. *Water Research*. **41**: 2816–2824

Zhang, X., B. Wu, Y. Zhang, T. Zhang, L. Yang, H. H. Fang, T. Ford, S. Cheng and S. 2009a. Cla**ss** 1 integronase gene and tetracycline resistance genes *tet*A and *tet*C in different water environments of Jiangsu province, China. *Ecotoxicology*. **18**: 652–660

Zhang, X.X., T. Zhang, M. Zhang, H. H. P. Fang and S. P. Cheng. 2009b. Characterization and quantification of class 1 integrons and associated gene cassettes in sewage treatment plants. *Applied Microbiology* and *Biotechnology*. **82:**1169–77

Zilhao, R., B. Papadopoulou, and P. Courvalin. 1988. Occurrence of the Campylobacter resistance gene *tet* O in *Enterococcus* and *Streptococcus* spp. *Antimicrobial Agents and Chemotherapy*. **32**: 1793–1796