Molecular quantification and characterisation of aminoglycoside resistant bacteria and genes from aquatic environments

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DECLARATION

I declare that the dissertation submitted by me for the degree Magister Scientiae in Environmental studies at the North-West University (Potchefstroom Campus), Potchefstroom, North-West, South Africa, is my own independent work and has not previously been submitted by me at another university.

Signed in Potchefstroom, South Africa

Signature:

Date:

Tamryn van der Merwe

ABSTRACT

Antibiotic resistance bacteria and antibiotic resistance genes (ARGs) could be disseminated and selected for in the environment, particularly aquatic ecosystems. This is due to the interplay between humans, animals and this ecosystem. The aim of this study was to investigate the levels and characteristics of aminoglycoside resistant bacteria and associated ARGs, isolated from surface water and sediment of the Crocodile and Marico Rivers. Levels of kanamycin resistant bacteria were determined by plating samples on nutrient agar, supplemented with kanamycin. Isolates were purified and resistance profiles to three other aminoglycosides were determined. Multi-aminoglycoside resistant isolates were identified using Gram staining and 16S rRNA gene sequencing. These were screened for *nptII* and related ARGs (*intI 1, ampC* and *msrA/B* efflux pump). Kanamycin resistant bacteria levels ranged from a few (10 CFU/ml) to very high (2.0×10^6 CFU/ml) in both river systems. No *nptII* genes were detected using this method. However, the efflux pump gene (*msrA/B*) were detected among some of the isolates. Additionally, the microbial populations at various sites were screened for these selected ARGs using culture-dependent and culture-independent methods. The culture-dependent method involved enrichment either supplemented with or without kanamycin. Plasmid, as well as genomic DNA, was extracted. Environmental DNA was also extracted directly from filtered water samples (eDNA). This DNA (enriched plasmid, as well as genomic DNA and eDNA) was analysed by end-point PCR, real-time PCR (qPCR), as well as droplet digital PCR (ddPCR). Results indicated that *nptII* could be quantified in plasmid and genomic DNA of the samples (both with and without kanamycin). Levels determined by qPCR ranged from undetectable to 1.58×10^4 copies per nanogram of input DNA. ddPCR yielded copy numbers ranging from undetectable to 3.70×10^{-5} copies per nanogram of input DNA. In the case of *ampC* quantification in plasmid DNA, qPCR results indicated levels ranging from undetectable to 4.90×10^9 copies per nanogram of input DNA, whereas ddPCR ranged from undetectable to 6.55×10^{-3} copies per nanogram of input DNA. Quantification of *nptII* using the eDNA, qPCR results indicated levels ranging from undetectable to 1.23×10^5 copies per nanogram of input DNA. No samples were quantifiable using ddPRC. Relevant ARGs (*msrA/B* efflux pump, β-lactam *ampC* and integrase class one (*intI*), were detected using the culture-dependent, as well as culture-independent approaches. This is significant, since the class 1 integrase gene is the most ubiquitous among multidrug resistant bacteria. Bacteria containing this gene are able to harbour multiple resistant gene cassettes and could serve as a proxy for anthropogenic pollution. Overall, the results from this study indicated that the culture-based enrichment method provided the best resolution of resistance gene diversity in the two Rivers; however, the culture-independent method indicated ubiquity of the *intI 1* gene, demonstrating the

potential transferability of ARGs. This study emphasizes the importance of examining antibiotic resistance in the environment.

Key words: Antibiotic Resistance Genes, aminoglycosides, kanamycin, *nptII,* droplet digital PCR, *intI 1*, efflux pumps, *ampC*.

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ABBREVIATIONS

(APH)-II	3'-phosphotransferase
AACs	acetyltransferase
AMEs	aminoglycoside modifying enzymes
ANTs	nucleotidyltransferases
APHs	phosphotransferases
ARB	antibiotic resistant bacteria
ARGs	antibiotic resistance genes
BLAST	basic local alignment search tool
CFU	colony forming units
CI	chromosomal integrons
ddPCR	digital droplet PCR
DNA	deoxyribo nucleic acid
EtBr	ethidium bromide
GCs	gene cassettes
GI	gastro intestinal
HGT	horizontal gene transfer
HPC	heterotrophic plate count
IS’s	insertion sequences
MDR	multidrug resistance
MDR-TB	multidrug-resistant

MGEs	mobile genetic elements
MI	mobile integrons
NGS	next generation sequencing
NTC	no template control
ORF’s	open reading frames
PCR	polymerase chain reaction
qPCR	real-time PCR
ROS	reactive oxygen species
R plasmids	resistance plasmids
rRNA	ribosomal ribonucleic acid
TWQR	target water quality rate
WMA	water management area
WWTPs	wastewater treatment plants
XDR-TB	extensively drug-resistant tuberculosis

CHAPTER 1: GENERAL INTRODUCTION AND RATIONALE

1.1 Introduction

Antibiotics are considered to be one of the greatest discoveries of the 20th century (Carvalho & Santos, 2016). These miraculous compounds have revolutionized medicinal and production industries. However, these substances are now being associated as a rising class of environmental contaminants (Zhang *et al.,* 2009; Manaia *et al.,* 2016).

According to Iglesias *et al.* (2013), production and consumption of antibiotics have increased globally. Between 2000 and 2010, a 36% increase in the use of antibiotics in countries like Brazil, Russia, India, China and South Africa have occurred (Manaia *et al.,* 2016). Antibiotics enter the environment through various anthropogenic sources and persist via transformation and bioaccumulation processes (Kümmerer, 2009; Carvalho & Santos, 2016). Not only do these substances have ecological effects, but also create a selective pressure on microorganisms to develop antibiotic resistance genes (ARGs) (Kümmerer, 2004, Xu *et. al.,* 2015).

The development of antibiotic resistant bacteria (ARB) and ARGs is of global concern, because these auto-replicative pollutants can travel vast distances and their spread to humans and animals is a potential health risk (Kemper, 2008; Zhang *et al.,* 2009; Xu *et al*., 2015, Martínez, 2017). Without antibiotic therapy, many procedures would be a waste of time and resources, since the risk for bacterial infection is too high (Bennet, 2008; Pruden *et al.,* 2013). Antibiotic resistance development is an adaptive trait that can be genetically encoded or acquired by bacterial subpopulations (Carvalho & Santos, 2016).

ARGs have been reported in various aquatic environments (Zhang *et al.,* 2009; Xu *et al.,* 2015). In the past decade, intensification in studies on the antibiotic resistance epidemic has been done. However, there is a lack of knowledge concerning the occurrence of these determinants in the aquatic environment (Kümmerer, 2009; Grenni *et al.,* 2017).

According to Zhu (2007), studying abundance and dynamics of ARGs will help to better understand the potential risks to environmental and human health. When studying ARGs in any environment, there are two approaches: (1) culture based or (2) culture independent methods. Each method has its advantages and limitations. Information on the occurrence of ARB and ARGs in aquatic ecosystems is crucial to be able to make informed decisions about the future use of water.

1.2 Problem statement

Antibiotic resistance is considered to be one of the most significant health issues of the 21st century (Marshall & Levy, 2011; Rodriquez-Mozaz *et al.,* 2015). It threatens the very foundation of modern medicine, which affects healthcare, veterinary and agricultural industries (WHO, 2014; CDC, 2017). Microorganisms are developing mechanisms of resistance to antibiotics that are commonly used for medicinal purposes (Bennet, 2008). This phenomenon claims the lives of approximately 700,000 people every year and is estimated to reach an additional 10 million by 2050 (Carvalho & Santos, 2016).

Various techniques exist to study ARGs in the environment, but most are time consuming and or expensive (Deshmukh *et al*., 2016). Knowledge on how antibiotic resistance rises, ARGs spread, as well as the influence this has on human health is incomplete (Ju *et al.,* 2016). Since the risk of non-species specific ARG spread exists, research efforts need to include non-pathogenic bacteria from environmental settings and not only clinical isolates (Zhang *et al*., 2009). A rapid, but accurate, universal analysis method to detect and quantify ARGs from environmental samples is of profound importance (Li *et al.,* 2015a; Deshmukh *et al.,* 2016). This will provide a clearer picture on the overall state of total bacterial population and genes that may be present in any given environment.

1.3 Aim and objectives

The aim of this study was to quantify and characterise aminoglycoside resistant bacteria (ARB) and screen for relevant ARGs from aquatic environments using culture dependent and culture independent methods. The objectives used to reach this aim were as follows:

- To determine the levels of kanamycin resistance in the surface water and sediment of the Crocodile and Marico Rivers using culture based techniques.
- To identify multi-aminoglycoside resistant bacteria using 16S rRNA end-point polymerase chain reaction (PCR) and sequencing.
- To investigate the presence of *nptII* and other relevant resistance genes in the river systems using culture dependent and culture independent methods.
- To quantify aminoglycoside and other relevant resistance genes using real-time PCR (qPCR) and digital droplet PCR (ddPCR).
- The hypothesis for this study is that aminoglycoside resistance is present in the environment and culture dependent methods combined with molecular methods will yield the most accurate and thorough results regarding overall presence of antibiotic resistance phenotypes and genes in the River systems.

CHAPTER 2: LITERATURE REVIEW

2.1 Key point sources of antibiotic pollution

Water is a habitat abundant in microorganisms (Manaia *et al.,* 2016). It is therefore a major contributor in the propagation of bacteria and substances between environmental compartments (Vaz-Moreira *et al.,* 2014; Rodriguez-Mozaz *et al.,* 2015; Manaia *et al.,* 2016). The urban water cycle, composed of waste, surface and drinking water, was created through anthropogenic events (Vaz-Moreira *et al.,* 2014). Multiple sources of external antimicrobial contamination occur in terrestrial and aquatic environments (Kemper, 2008; Rodriguez-Mozaz *et al.,* 2015; Carvalho & Santos, 2016). The greatest application of antibiotics is in human and veterinary medicine. As illustrated in figure 1, human medicine mainly uses antibiotics as intervention to treat infections, where antibiotics applied in veterinary medicine are implemented as growth promoters, prevent illness and treat infections. The route of antibiotic flow between anthropogenic activities and how it flows into the natural aquatic environment can be seen in figure 1 (Carvalho & Santos, 2016).

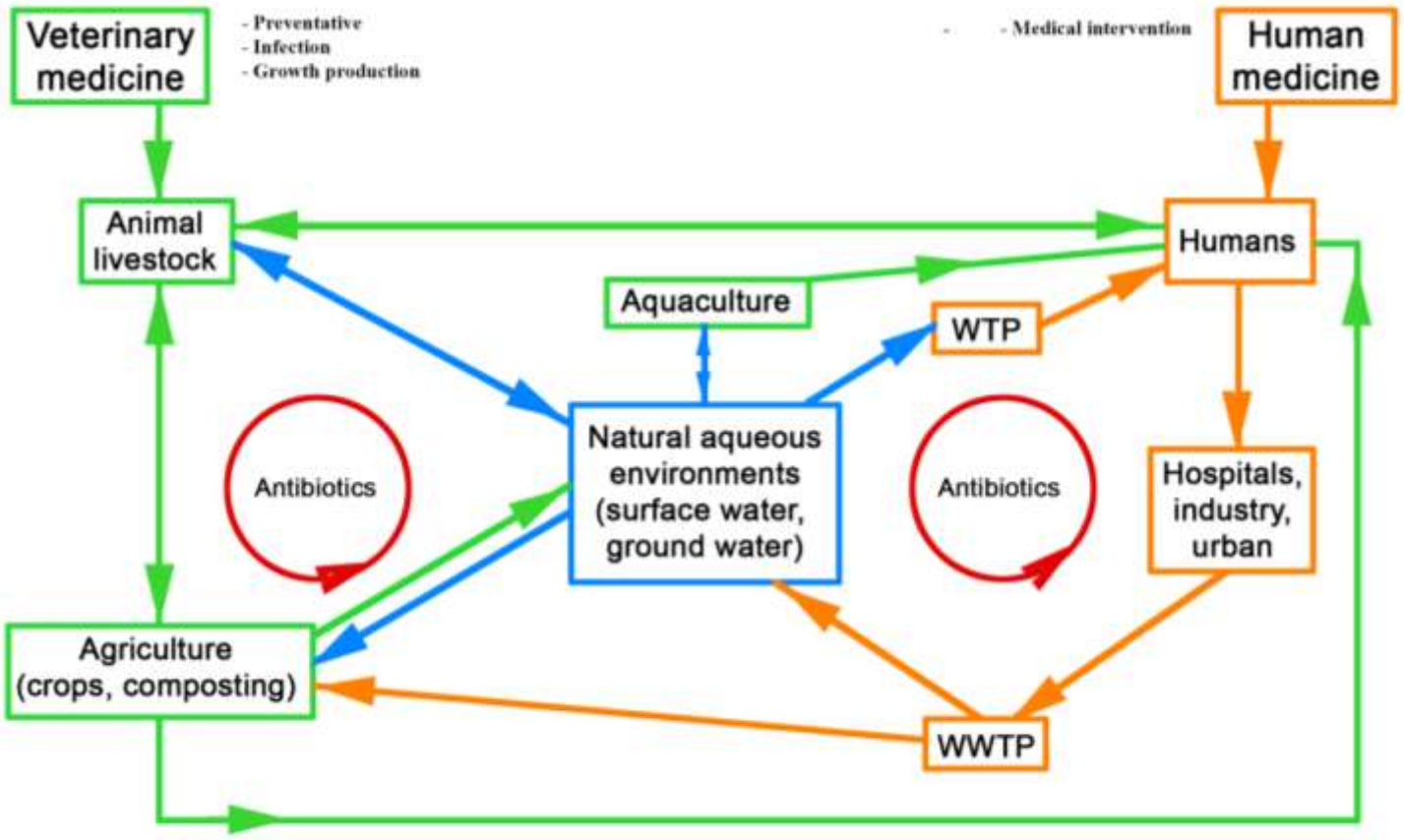

Figure 1: Schematic representation of possible environmental pathways of antibiotic dissemination when applied for human and veterinary medicine (adapted from Carvalho & Santos, 2016).

Naturally occurring antibiotics can be found in bacterial-fungi soil systems, which play a role in controlling population dynamics (Kümmerer, 2004; Gothwal & Shashidhar, 2015). However, antibiotics produced unnaturally are generally more stable and not easily broken down by bacteria. Antibiotics have been essential in various areas, including agriculture, aquaculture and beekeeping, to name a few and are also applied as growth promoters in livestock (Kümmerer, 2004; Gothwal & Shashidhar, 2015). These substances can enter the aquatic environment through various anthropogenic activities and the key point sources are described in the following section.

2.1.1 Human medicine and wastewater point sources

According to Carvalho and Santos (2016), substances used for medicinal purposes are ever present in the environment. Antimicrobials have been applied for several decades, thus the misuse and overconsumption has led to their manifestation in municipal wastewater (Gothwal & Shashidhar, 2015; Rodriguez-Mozaz *et al.,* 2015). It has been estimated that roughly 2.6 billion people do not have access to basic sanitation and could consequently lead to the direct flush of ARB and ARGs into the environment and receiving waters (Pruden *et al.,* 2013). According to Xu *et al.* (2015), the majority of antibiotic substances in the environment originated from sewage and is transported through wastewater systems to eventually end up in wastewater treatment plants (WWTPs).

WWTPs need to digest physical, chemical and biological contaminants from millions of cubic meters of sewage on a regular basis, which means there is no one specific process for each class of contaminant (Ju *et al.,* 2016). Wastewater is collected from multiple sources such as domestic houses, industrial plants and hospitals (Rodriguez-Mozaz *et al.,* 2015). Wastewater has therefore been recognised as an abundant source of antibiotic pollution (Duong *et al*., 2008; Kümmerer, 2009; Iglesias *et al.*, 2013; Rodriguez-Mozaz *et al.,* 2015).

Hospital wastewater is an important source of antimicrobial agents (Duong *et al*., 2008; Rodriguez-Mozaz *et al.,* 2015). Hospitals have the highest selective conditions for ARB, since antibiotic therapy, as well as metals such as arsenic and mercury, is abundant (Manaia *et al.,* 2016). A wide variety of antibiotics such as macrolides, aminoglycosides, tetracycline, sulphonamides and quinolones, to name a few, have been detected in hospital effluents up to a 1 g/L range (Duong *et al*., 2008; Kümmerer, 2009). These antibiotic substances are still active compounds, because after administration of an antibiotic, a non-metabolized fraction is excreted into the effluent (Kümmerer, 2009; Gothwal & Shashidhar, 2015; Grenni *et al.,* 2017). This is of

concern, because these active substances interact with environmental and pathogenic bacteria, which lead to numerous biological transformations (Kümmerer, 2009; Manaia *et al.,* 2016).

Wastewater is considered to be a rich habitat of pathogens and ARGs associated with human health (Vaz-Moreira *et al.,* 2014; Becerra-Castro *et al.,* 2015; Li *et al.,* 2015b; Manaia *et al.,* 2016). Bacterial proliferation is common in WWTPs, because a wide variety of antibiotics induce selection pressure on the bacteria (Pruden *et al.,* 2013; Grenni *et al.,* 2017). The formation of biofilms and abundance in nutrients offer the ideal setting to develop increased fitness (Xu *et al.,* 2015; Manaia *et al.,* 2016).

According to Vaz-Moreira *et al.* (2014), about one billion cultivable antibiotic resistant coliforms are discharged per minute. This happens in different world regions, using various wastewater treatment processes (Vaz-Moreira *et al.,* 2014). Conventional treatment processes are not equipped to remove these active compounds (Carvalho & Santos, 2016; Grenni *et al.,* 2017). Substances that are not eradicated during the treatment process enter the aquatic environment via effluent discharge from the WWTP (Gothwal & Shashidhar, 2015; Carvalho & Santos, 2016; Grenni *et al.,* 2017). The accidental breaking of sewer or effluent pipes could be another source of antibiotic and all associated determinants release into the environment (Gothwal & Shashidhar, 2015).

Anthropogenic point sources contribute to the total antibiotic concentration found in sewage, surface water and sediment (Singer *et al.,* 2016; Grenni *et al.,* 2017). Most antibiotics are water soluble and have direct contact to bacteria, since they are ubiquitous in the environment. This exposure to sub-therapeutic concentrations, over extended periods, is considered to aid in the development of resistant bacterial strains (Kümmerer, 2004; Singer *et al.,* 2016; Grenni *et al.,* 2017). Little is known of the possible impact these substances may have on the environment. What is certain is that a health risk exists when antibiotics and subsequent resistance determinants are released into the receiving rivers and the potential route of dissemination can be seen in figure 1 (Rodriguez-Mozaz *et al.,* 2015; Ju *et al.,* 2016).

2.1.2 Agriculture and animal husbandry

Water is a scarce resource in many countries. There is an increasing demand for usable water because of economic development, population growth, climate change and pollution to name a few (DWA, 2013b; Becerra-Castro *et al*., 2015). The reuse of wastewater for irrigation purposes (as illustrated in figure 1) is common practice, since irrigation and environmental applications diminishes up to 70% of the little usable water available for human consumption (Becerra-

Castro *et al*., 2015). Although this method has its advantages, it is a major health and environmental risk (Ju *et al.,* 2016; Singer *et al.,* 2016). According to Kemper (2008), multi-resistant bacteria have been detected in the wastewater being used as fertilizer or for irrigation purposes. This could result in direct depositing of antibiotics in groundwater and subsurface draining networks (Gothwal & Shashidhar, 2015). The antibiotic link between wastewater and agriculture is displayed in figure 1.

Besides the health risk to humans, using wastewater for irrigation can have other negative effects (Vaz-Moreira *et al.,* 2014). Wastewater is extremely rich in contaminants such as metals and micro pollutants (Manaia *et al*., 2016; Singer *et al.,* 2016). These compounds accumulate, causing soil particles to aggregate and decrease soil permeability (Becerra-Castro *et al.,* 2015). This change in structure could influence plant growth and soil micro biota (Singer *et al.,* 2016). For example, an increase in salinity can reduce fungal counts, consequently impacting microbial diversity (Becerra-Castro *et al.,* 2015). Metals also enhance antibiotic accumulation and may aid in selection of antibiotic resistance determinants (Pruden *et al*., 2013; Vaz-Moreira *et al.,* 2014; Manaia *et al.,* 2016; Singer *et al.,* 2016; Grenni *et al.,* 2017). Soil is a heterogeneous environment and plays a central role in the dissemination of resistance determinants between bacteria and ultimately human pathogens (Nesme & Simonet, 2015).

Animals, especially livestock production, have also been recognized as a source of antimicrobial discharge into the environment (Iglesias *et al.,* 2013; Singer *et al*., 2016). According to Kemper (2008), the application of antibiotics for livestock farming has been implemented since the 1950s. Cattle are mainly treated for mastitis, whereas pigs are treated for gastro intestinal (GI) disorders (Kemper, 2008). Antibiotics like aminoglycosides are often used in combination with a β-lactam for prevention or treatment of pathogenic infections (Sundsfjord *et al.,* 2004; Ramirez & Tolmasky, 2010). However, antibiotics are often misused and applied for growth and product enhancement, by adding low doses in the feedlots (Summers, 2006; Kümmerer, 2009; Zhang *et al*., 2009; Kwon-Rae *et al.,* 2011). This ensures a better product is derived, since antibiotics lower the amount of fat and enhance protein percentage (Kümmerer, 2009). This can also influence the microbiota of the animals' gut (Grenni *et al.,* 2017).

Additional environmental contamination occurs from manure fertilizers and pasture-reared animals excreting directly onto the land (Kemper, 2008; Iglesias *et al.,* 2013; Gothwal & Sashidhar, 2015). Applying animal manure as soil fertilization is a major contributor to veterinary medicine complications (Carvalho & Santos, 2016). As in humans, antibiotics are not completely broken down in the animal gastro intestinal (GI) tract and are excreted as bioactive substances (Kemper, 2008; Zhang *et al*., 2009; Singer *et al.,* 2016). These substances, in their unaltered

state, can enter the aquatic environment via surface runoff, leaching into ground and drinking water (Kemper, 2008; Kümmerer, 2009; Zhang *et al.,* 2009; Kwon-Rae *et al.,* 2011; Carvalho & Santos, 2016).

Another route of antibiotic dissemination is through application of antibiotics for aquaculture, typically for therapeutic and preventative reasons (Kümmerer, 2009; Gothwal & Shashidhar, 2015). Antibiotics are usually added to the water directly (Vaz-Moreire *et al.,* 2014). Incident spills, industrial effluent and negligent disposal of unused drugs into aquatic environments are also of concern (Gothwal & Shashidhar, 2015; Carvalho & Santos, 2016). Antibiotics can persist in the environment, aid in selection pressure of ARB and ARGs (Kwon-Rae *et al.,* 2011; Pruden *et al*., 2013; Vaz-Moreira *et al.,* 2014). These substances affect organisms on different trophic levels (Kemper, 2008; Kümmerer, 2009). Basic nitrification processes can be influenced and undermine the entire balance of the aquatic ecosystem (Kümmerer, 2009; Becerra-Castro *et al.,* 2015). Physico-chemical properties play a role in antibiotic resistance bacterial proliferation. Temperature, pH, dissolved oxygen (DO), biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS), nitrate and nitrites, sulphates and sulphides are some of the parameters used to study water quality and the effect it may have on the bacterial population.

Figure 1 illustrates how this cycling of antibiotics and subsequent determinants in the aquatic environment can affect not only livestock and crops, but also human health via drinking water (Iglesias *et al.,* 2013).

2.2 Antibiotic resistance development

The production of antibiotics is a natural development; therefore antibiotic resistance predates the phenomenon faced currently (Vaz-Moreira *et al.,* 2014; Ma *et al.,* 2016). Antibiotic producing bacteria have the potential to modify their inhibitory biochemical products (Vaz-Moreira *et al.,* 2014). This mechanism is a possible self-protection system, particularly advantageous in soil and water environments (Forsberg *et al.,* 2012; Vaz-Moreira *et al.,* 2014). It is also referred to as natural resistance, illustrating why antibiotic resistance is referred to as an ancient occurrence (Vaz-Moreira *et al.,* 2014).

According to Vaz-Moreira *et al.* (2014), increased levels of pathogenic and opportunistic bacteria have arisen in the last 70 years, specifically in areas with anthropogenic influence. This is explained by the fact that bacterial population enhancement has occurred, because these organisms were either resistant to or attained resistance to antibiotics used via selective pressure (Heuer *et al.,* 2002; Kümmerer, 2009).

The difference between natural resistance and the current resistance phenomenon is the modern selective pressure in diverse environments from the aggravated antibiotic use (Ma *et al*., 2016). According to Davies (1994), this profuse use has stimulated pressure for antibiotic trait selection. The selective pressure arising from the disuse of antibiotics, cause bacteria to spend much time and energy to regulate genes and actively resist these toxic compounds (Wright, 2005).

2.3 Strategies of antibiotic resistance by microorganisms

Bacteria have very impressive potential for adaptation and are thus able to easily colonize inhospitable parts of the planet. They have become adept at developing DNA modifying strategies to help them adapt and, subsequently, evolve (Bennet, 2008). According to Wright (2005), resistance can be classified as active (selective pressure causing transformation) or passive (adaptation by chance). Bacteria attain active resistance by means of three main mechanisms (Wright, 2005; Vaz-Moreira *et al.,* 2014; Grenni *et al.,* 2017):

1. Efflux of toxic substances by means of pumping proteins in the membrane,
2. modification of antibiotic binding site, and
3. production of modifying enzymes, which degrades the antibiotic.

Genes that encode the various enzymatic strategies for antibiotic resistance are usually associated with mobile genetic elements (MGEs) like resistance plasmids (R plasmids), transposons and integrons (Stalder *et al.,* 2012; Grenni *et al.,* 2017). Consequently, these resistance encoding genes are prevalent in bacterial populations even if antibiotics are not frequently employed (Wright, 2005; Zhang *et al.,* 2009).

According to Bennet (2008), changes of bacterial inheritance can be made in two ways:
1. Random changes to existing DNA, or
2. acquisition of new genetic material and expanding the genome.

Changes made to confer resistance, were not necessarily by design, but rather at random. Not all changes are useful or even kept, but when they help the organism to survive, changes are conserved and amplified (Bennet, 2008). This supports the Darwinian hypothesis of ‘survival of the fittest’ (Bennet, 2008).

2.4 ARG: acquisition and spread

In the early studies of spontaneous streptomycin resistant mutants, development of antibiotic resistant strains during treatment was considered unlikely (Davies, 1994). Needless to say, it was surprizing when research suggested environmental bacteria could acquire and exchange genetic information so efficiently, with little species specificity (Zhu, 2007). Resistance to the vast variety of antibiotics is genetically induced by hundreds of ARGs, which are being detected in water environments (Zhang *et al*., 2009; Xu *et al.,* 2015). The environment can thus be viewed as an unlimited reservoir of resistance genes (Forsberg *et al.,* 2012).

According to Vaz-Moreira *et al.* (2014), acquired antibiotic resistance is just a form of biological evolution, where genetic variability occurs, and impacts physiology and ecology of bacteria. Acquired resistance is the product of random mutation and genetic recombination or exchange via horizontal gene transfer (HGT) (Vaz-Moreira *et al.,* 2014). This is the genetic basis for antimicrobial resistance (Sundsfjord *et al.,* 2004). When exposed to selective pressures, organisms with the potential to attain acquired resistance, will have improved fitness, therefore survive and reproduce (Vaz-Moreira *et al.,* 2014; Grenni *et al.,* 2017). Intrinsic resistance, which is the natural resistance found in organism could possibly spread to areas like water that are abundant in bacteria (Grenni *et al.,* 2017). This can lead to acquired resistance by receiving bacteria under stress from environmental contaminants and so the cycle continues.

When referring to the process of gene acquisition, it implies that genes from external sources (usually other bacteria) are transferred into different bacteria. The three most common methods of bacterial genetic exchange are transformation, transduction and conjugation (Davies, 1994; Sundsfjord *et al.,* 2004; Jana & Deb, 2006; Bennet, 2008; Kemper, 2008; Vaz-Moreira *et al.,* 2014; Becerra-Castro *et al.,* 2015). Most bacteria have at least one of these approaches at their disposal to exchange genetic material via HGT (Summers, 2006). Bacterial plasmids (platform of gene assemblage) primarily support these methods (Bennet, 2008). Bacteria are able to thrive in hazardous environments by utilizing these platforms to expand their adaptive potential (Davies, 1994). Multi-drug resistance development is a good example (Bennet, 2008). This phenomenon is of global concern, since it is the reason that treatment of infectious diseases is failing (Pruden *et al.,* 2013).

According to Bennet (2008), MGEs can be classified into elements that are transferred from one cell to another (plasmids and conjugative resistance transposons) or elements moving genetic information within the same cell (gene cassettes, resistance transposons). According to Zhang *et al.* (2009), transformation by naked DNA, induced competence when calcium is abundant and transduction caused by bacteriophages are other means of HGT and recombination utilized

by environmental bacteria. These recombination systems allow the dissemination and accumulation of ARGs. According to Kemper (2008), approximately 95% of antibiotic resistance is due to these mobile elements, and not necessarily chromosomal based determinants. Relevant MGEs and their role in resistance dissemination will be explained in the following subsections.

2.4.1 Bacterial plasmids

Antimicrobial resistance gene spread, especially among Gram-negative bacteria is largely because of non-species specific DNA exchange from plasmid-located resistance genes (Carattoli, 2013). These elements are thought of as dispensable chromosomes and associated with HGT (Carattoli, 2013; Gothwal & Shashidhar, 2015; Li *et al.,* 2015b). Plasmids are extra-chromosomal DNA that replicate independently from the chromosomal DNA (Bennet, 2008; Carattoli, 2013; Li e*t al.,* 2015b). They are able to carry genes, which allow the cell to exploit environmental stress. These genes typically include genes such as virulence factors and ARGs (Bennet, 2008; Ramirez *et al.,* 2014; Li *et al.,* 2015b).

R plasmids contain genes capable of resisting most classes of antibiotics used in antibiotic therapy, which include β-lactams, aminoglycosides, tetracycline, fluoroquinolones etc. (Bennet, 2008; Carattoli, 2013). Some R plasmids can be classified as conjugative (30kb or larger), which promote cell-to-cell coupling and thereby transfer of genes and themselves (Bennet, 2008). These plasmids can be narrow range (specific species) or broad range (Carattoli, 2013). The latter allows elements to transfer to an extensive variety of species with the same Gram staining capability, such as plasmid RP1 (Bennet, 2008).

When plasmids are coupled with other resistance determinants such as transposons and integrons, there is no barrier between species that can hinder dissemination of resistance (Ramirez *et al.,* 2014). It is alarming how common plasmids are in aquatic environments (Zhang *et al.,* 2009), and how most environmental bacteria can utilize the potential pool of mobile genes available for bacterial transformation and resistance development.

2.4.2 Transposons

Another important gene transport system or mobile element is the transposons or ‘jumping genes’ (Zhang *et al.,* 2009). Transposons have the potential to incorporate resistance genes in small cryptic elements (insertion sequences). These elements can ‘jump’ from one plasmid to another or even to chromosomal DNA, since conjugative transposons are chromosomally

located (Sundsfjord *et al.,* 2004; Bennet, 2008; Zhang *et al.,* 2009). According to Summers (2006), three classes of transposons exist, but each contain inverted repeats (25 bp to 50 bp) at their ends. They also contain transposase, which allows the smaller transposons or insertion sequences (IS's) to be incorporated with little site-specificity (Summers, 2006). Conjugative transposons are able to encode their own excision and intercellular transfer functions (Sundsfjord *et al.,* 2004). It is distressing that in some situations, no DNA homology between the elements incorporated and the site of insertion is necessary for this process to be successful. Transposons carrying resistance gene IS's can therefore randomly 'jump' onto plasmids and form new R plasmids (Zhang *et al.,* 2009).

The transposon Tn*5* encodes resistance to important aminoglycosides like kanamycin, neomycin and streptomycin (Smalla *et al.,* 1993). Bacteria containing this MGE are typically members of the *Enterobacteriaceae* family (Bennet, 2008). According to Bennet (2008), these resistance elements are created by chance, but become established in the organism when exposed to antibiotics. As a result, these elements provide a survival advantage and also support the Darwinian hypothesis of 'survival of the fittest' (Bennet, 2008).

2.4.3 Integrons

Integrons are genetic elements capable of acquiring and expressing genes (Stalder *et al.,* 2012). They have been identified as key MGEs and natural cloning systems, consisting of two conserved areas that flank a central segment or open reading frames (ORF's) (Sundsfjord *et al.,* 2004). These segments are also referred to as gene cassettes, which typically transcribe functions like antibiotic resistance (Davies, 1994; Sundsfjord *et al.,* 2004). According to Stalder *et al.* (2012), gene cassettes contain most genes conferring resistance to almost all antibiotic families, including aminoglycosides, *β*-lactams, chloramphenicol and macrolides to name a few. Integrons consist of three key elements; an integrase gene, referred to as *intI*, a recombination site (*attI*) and a promoter (Sundsfjord *et al.,* 2004; Stalder *et al.,* 2012). Consequently, integrons undergo site specific recombination, excision and insertion (illustrated in figure 2) and all that is required is a slightly similar *attC* region of 59 to 120 bases (Sundsfjord *et al.,* 2004; Summers, 2006). By this means, new transposable elements, with various combinations of antibiotic resistance genes, are accessible.

According to Stalder *et al.* (2012), two groups of integrons exist; namely the chromosomal integrons (CI) and mobile integrons (MI), which are usually located on MGEs. CIs can carry up to 200 cassettes encoding various functions; whereas MIs will carry only about 10, but they usually encode antibiotic resistance determinants (Stalder *et al.,* 2012). Analysis of the three

classes of integrons has revealed gene cassettes that contain ARGs conferring resistance to aminoglycosides and trimethoprim (Leverstein-van Hall *et al.,* 2003; Sundsfjord *et al.,* 2004).

Class 1 integrons, whose integrase is called *intI1*, are of particular interest since studies have revealed the presence of multiple co-expressed resistance determinants when sequencing gene cassettes (Sundsfjord *et al.,* 2004). This could be because it is frequently associated with transposons (specifically Tn*21*) (Summers, 2006). When a combination of resistance integrons in mobile genetic elements like plasmids or transposons exists, the possibility of intra- and interspecies transfer of antibiotic resistance determinants is a reality (Rowe-Magnus & Mazel, 2002; Sundsfjord *et al.,* 2004; Hall *et al.,* 2017). Figure 2 is an illustration of the excision and insertion of resistance genes that can occur and be transferred to mobile elements.

In figure 2 the P is for the promoter, GC represents a gene cassette and the double slash represents the mobile element like plasmids, where the integron can be incorporated and transported. This figure illustrates how various ARGs can be incorporated into integrons with little specificity required. The genes *nptII* and *ampC* were used in this illustration, since they are important to the study, but also because β-lactam and aminoglycoside ARGs are often grouped together on integrons (Ju *et al.,* 2016).

When a combination of resistance integrons in mobile genetic elements like plasmids or transposons exists, the possibility of intra- and interspecies transfer of antibiotic resistance determinants is a reality (Sundsfjord *et al.,* 2004; Hall *et al.,* 2017). This is of concern, because the acquisition of resistance determinants in non-pathogenic environmental bacteria could lead to transformation and sharing of these determinants to pathogenic bacteria (Bennet, 2008). Figure 3 is an illustration of this scenario.

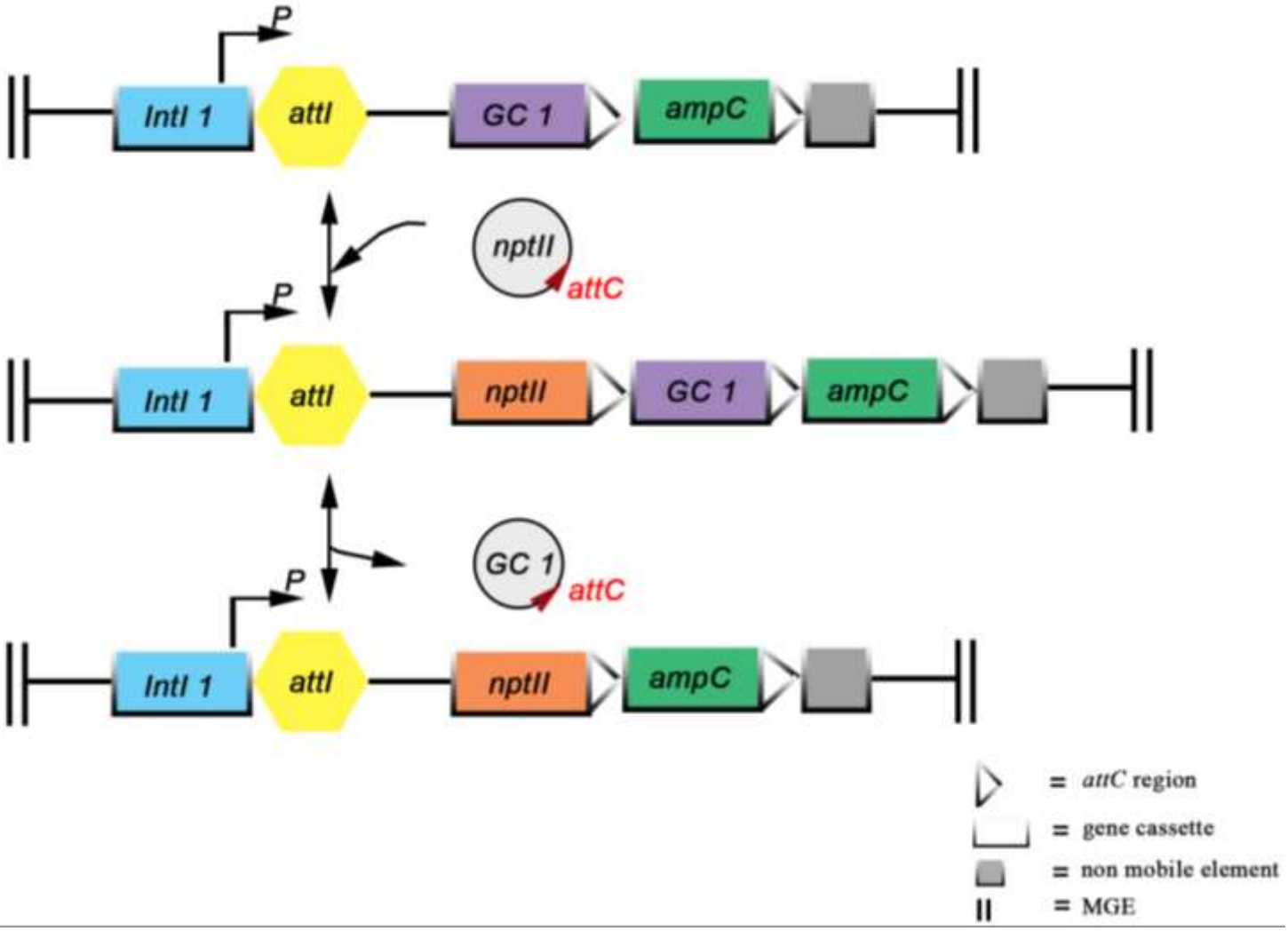

Figure 2: Schematic representation of integron and gene cassette organization when excision and insertion of antibiotic resistance genes occur (adapted from Summers, 2006; Stalder *et al*., 2012).

Figure 3 is a schematic representation showing how bacteria can take up free DNA or ARGs from the resistance gene pool. The resistance gene pool represents all potential sources of antibiotic resistance determinants available for bacteria to take up and incorporate into gene cassettes (GCs). These GCs, with their resistance determinants are incorporated into integrons, as illustrated in figure 2. This leads to the incorporation of these resistant containing GCs into stable replicons and become mobile when taken up by carriers. MDR pathogens can therefore acquire increased fitness, via gene uptake from resistance gene pools, carriers (mainly environmental bacteria carrying MGEs with ARGs) and/or vectors. These MDR pathogens and resistance determinants can thus be transmitted to the food chain (as illustrated in figure 3) and end up in the aquatic environment. This will consequently contribute to the resistance gene pool and the cycle continues as illustrated in figure 3.

The efficiency of resistance determinant transmission, from the chromosome to the plasmid, and between species may vary depending on bacterial genera (in terms of their complexity in regulation), population dynamics (ecological niche) and are considered to be non-species-specific (Hall *et al.,* 2017). Even though it is impossible to replicate the conditions of natural environments, it is reasonably certain that when selection pressure is present DNA readily transfers in MGEs and bacterial transformation is likely to occur.

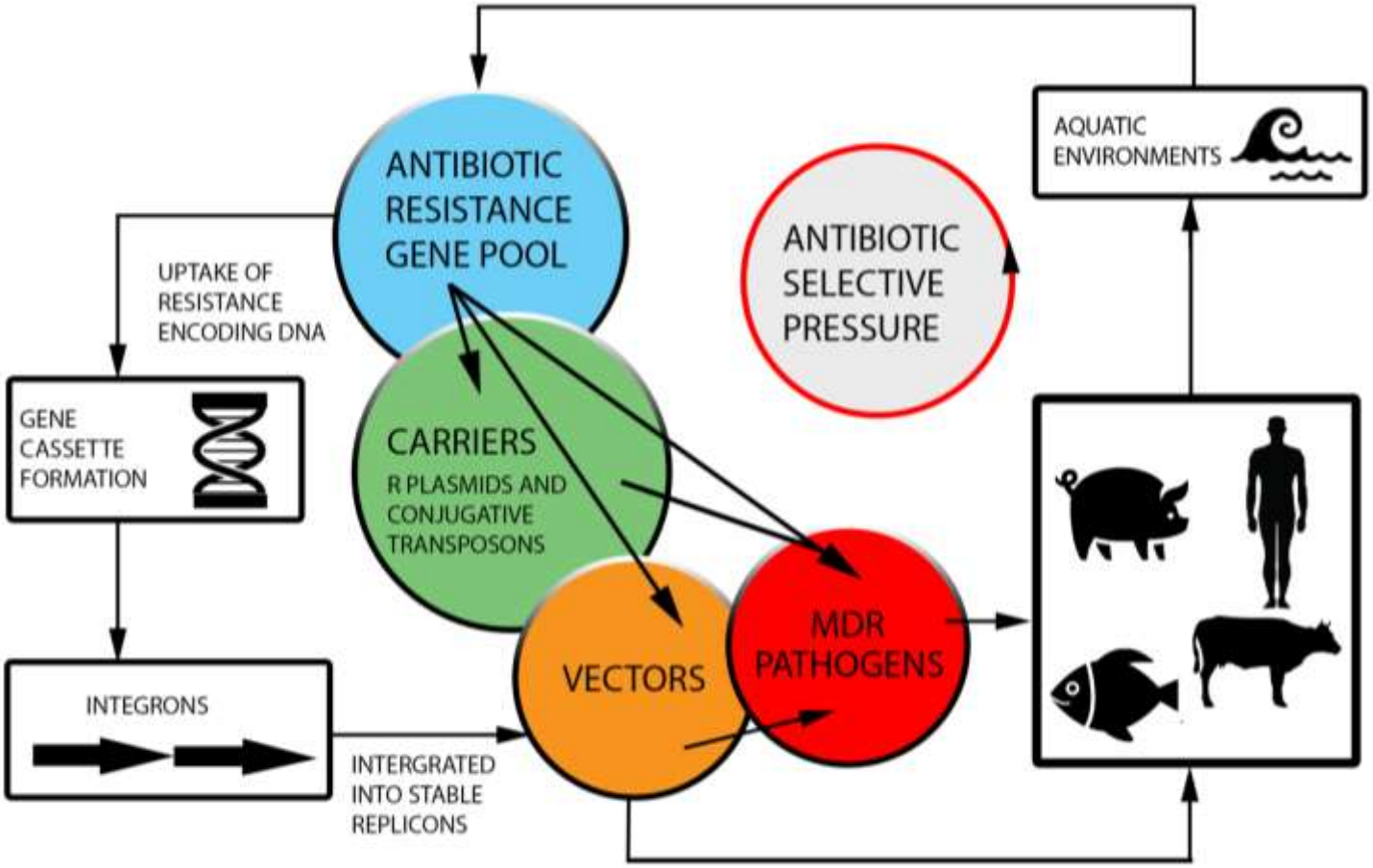

Figure 3: Illustration of the antibiotic resistance gene pool (adapted from Davies, 1994; Manaia, 2017).

Figure 3 is a schematic representation showing how bacteria can take up free DNA or ARGs from the resistance gene pool. The resistance gene pool represents all potential sources of antibiotic resistance determinants available for bacteria to take up and incorporate into gene cassettes (GCs). These GCs, with their resistance determinants are incorporated into integrons, as illustrated in figure 2. This leads to the incorporation of these resistant containing GCs into stable replicons and become mobile when taken up by carriers. MDR pathogens can therefore acquire increased fitness, via gene uptake from resistance gene pools, carriers (mainly

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2.5 Overview of aminoglycosides

Antibiotics can be subdivided into many families, according to their chemical structure or mechanism of action (Kümmerer, 2009). Aminoglycosides are one of the families known for having an aminocyclitol ring (streptamine, 2-deoxystreptamine or streptidine) that is linked with two or more amino sugars and by glycosidic bonds (Jana & Deb, 2006; Ramirez & Tolmasky, 2010). Aminoglycosides were first characterized in 1944 and are considered to be potent bactericidal agents that were widely applied to treat various infections, including tuberculosis (Toth *et al*., 2013). They inhibit infection causing bacteria by disrupting protein synthesis by binding to the 30S subunit of prokaryotic ribosomes, leading to obstruction of translation and ultimately cell death (Vakulenko & Mobashery, 2003; Jana & Deb, 2006; Foughy *et al*., 2014).

Streptomycin was the first aminoglycoside discovered and is still used as a first-line drug combination for the treatment of tuberculosis (Vakulenko & Mobashery, 2003; Thaver & Ogunbanjo, 2006). Kanamycin, amikacin, viomycin and cepreomycin are also applied second line drug treatment in multidrug-resistant (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) cases (Thaver & Ogunbanjo, 2006; Bardien *et al.,* 2009).

This family of antibiotics is used in combination with other classes of antibiotics like β-lactams to inhibit invasive infections caused by organisms like α-hemolytic streptococci, staphylococci and enterococci (Sundsfjord *et al.,* 2004; Jana & Deb, 2006; Ramirez & Tolmasky, 2010). As previously mentioned, antibiotics are added to feedlots of animals. Kanamycin and neomycin are applied for this purpose and also for veterinary medicine (Smalla *et al.,* 1993).

In human medicine aminoglycosides are often used to treat severe infections of the abdomen, secondary urinary tract infections or septicaemia and are applied as a prophylaxis for

endocarditis (Jana & Deb, 2006; Toth *et al.,* 2013). Because aminoglycosides have such a broad spectrum, are fast acting and have synergistic effects with other antibiotics, they are considered to be useful when treating serious nosocomial infections (Jana & Deb, 2006).

Unfortunately, aminoglycosides have serious health implications, since they are nephro- and ototoxic (Bardien *et al.,* 2009; Toth *et al.,* 2013). The use of these antibiotics can thus lead to permanent hearing loss, but are still used, since there is a lack of available antimicrobials that are effective (Toth *et al.,* 2013; Petersen & Rogers, 2015). Resistance to aminoglycosides is a health threat worldwide (Jana & Deb, 2006).

2.5.1 Aminoglycoside associated resistance mechanisms

As with all antibiotics, frequent use of aminoglycosides causes selective pressure to induce mutations, leading to altered expression (Jana & Deb, 2006). According to Gad *et al.* (2011), there are several mechanisms that contribute to the resistance of this family of antibiotics. These mechanisms include the following (Vakulenko & Mobashery, 2003):

1. Efflux systems,
2. inactivation of drug through aminoglycoside modifying enzymes (AMEs),
3. alteration of target site and
4. decreased permeability of cell membrane.

According to Ardic *et al*. (2006), most aminoglycoside resistance is achieved by inactivation via AMEs. More than 50 modification enzymes have been identified (Zhang *et al*., 2009). Most AMEs are located on plasmids and several are included in transposons and integrons (Jana & Deb, 2006). The enzymes that confer this resistance fall under the category of group transfer mechanisms (Wright, 2005).

The biochemical action of the enzymes determines in which of the sub-groups it is categorized (Fernández-Martínez *et al.,* 2015). According to Wright (2005), the largest and most diverse sub-group of resistance enzymes are the transferases group. This group is responsible for the modification of target binding sites and uses chemical strategies such as acetylation and phosphorylation. Typical examples are the aminoglycoside acetyltransferase (AACs) and aminoglycoside kinases or phosphotransferases (APHs) (Zhang *et al.,* 2009; Foughy *et al.,* 2014). The bi-functional enzyme gene aac(6')/aph(2'), which is a combination of these two groups, is often encountered in multi-resistant Gram positive enterococci and staphylococci (Ardic *et al.,* 2006; Toth *et al.,* 2013). Nucleotidyltransferases (ANTs) is another example of group transfer enzymes that modify aminoglycosides. ANTs are the minor class of inactivating

enzymes, but they confer resistance to crucial clinical aminoglycosides like gentamicin, kanamycin and tobramycin (Wright, 2005). According to Zhang *et al.* (2009), these three groups have been detected in various genera commonly found in polluted water environments. This includes genera such as *Aeromonas, Escherichia, Vibrio, Salmonella* and *Listeria* spp. (Zhang *et al.,* 2009). Many of these modifying enzymes have been detected in clinical environments (Zhang *et al.*, 2009; Fernández-Martínez *et al.,* 2015).

According to Zhu (2007), environmental bacteria commonly confer resistance to the aminoglycoside kanamycin. The aminoglycoside 3'-phosphotransferase (APH)-II gene, also known as *nptII,* is one of the main mechanisms of kanamycin resistance (Beck *et al.,* 1982; Zhu, 2007). This gene was first detected as a region, consisting of 264 amino acid residues, localised on the Gram-negative Tn*5* transposon (Beck *et al.,* 1982; Smalla *et al.,* 1993). The *nptII* gene plays an important part in synthetic biology as a selection marker (Smalla *et al*., 1993).

Kanamycin is an important antibiotic, especially considering its role in the treatment of XDR-TB (Bardien *et al.,* 2009). Therefore, monitoring the presence of the kanamycin resistance is extremely important, considering the potential for resistance gene spread as explained in the previous section. However, previous studies on cultured kanamycin resistant bacteria have found few isolates that contain the *nptII* gene (Smalla *et al.,* 1993; Leff *et al.,* 1993; Zhu, 2007). Studies conducted by Zhu (2007) detected *nptII* in Canada river water by using qPCR to overcome detection limitations of environmental samples (Zhang *et al.,* 2009). Results indicated that *nptII* homolog sequence levels ranged from undetectable to 4.36 x 10^6 copies per litre of river water (Zhu, 2007).

In another study conducted by Padmasini e*t al.* (2014), enterococci isolates showed high levels of resistance to aminoglycosides, streptomycin and gentamicin, but could not detect their associated AMEs. It is important to consider the process of gene detection and the potential of multi-resistance in the environment. Even though only a fraction of kanamycin resistant bacteria contain *nptII*, there are several other resistance mechanisms that can confer this phenotypic resistance (Smalla *et al.,* 1993). This can be explained by the concept of co-resistance and cross resistance. In the case of co-resistance, one mobile genetic element, like plasmids or integrons, may carry several ARGs that each confers resistance to a different antimicrobial determinant (Blanco *et al.,* 2016), whereas cross resistance is capable of conferring resistance to various drug families (Sundsfjord *et al.,* 2004; Blanco *et al.,* 2016). One well known example of cross resistance is multidrug resistance (MDR) efflux pumps that are present in most organisms, including bacterial pathogens (Sundsfjord *et al.,* 2004; Nikaido, 2009; Blanco *et al.,* 2016).

According to Blanco *et al.* (2016), efflux pumps are highly conserved in different species and well regulated. They can be acquired via HGT and contribute to intrinsic resistance of various multidrug resistant pathogens (Jana & Deb, 2006; Nikaido, 2009; Blanco *et al.,* 2016). According to Blanco *et al.* (2016), this resistance mechanism is able to inactivate various antibiotic families, as well as other toxic substances. MDR efflux pumps can therefore decrease the concentration of aminoglycosides in the cell and affect the susceptibility to the entire class of aminoglycoside compounds (Jana & Deb, 2006). It also contributes to phenotypic resistance, depending on the level of expression (Blanko *et al.,* 2016).

It is crucial to bear in mind that resistance capability to an antibiotic can be caused by several ARGs that use a variety of mechanisms (Zhang *et al.,* 2009). Genes that confer resistance to aminoglycosides are usually encoded within plasmids, integrons and transposons (Sundsfjord *et al.,* 2004; Jana & Deb, 2006; Ardic *et al.,* 2006). Hypothetically resistant bacteria can initially use one ARG mechanism and then 'abandon' it as the cell stabilises. Once the cell has overcome the stressor it can then utilise another gene that requires less energy.

Interestingly, antibiotics like β-lactams, quinolones and aminoglycosides have the potential to stimulate reactive oxygen species (ROS), which in turn stimulate bacterial error-correcting response (Singer *et al.,* 2016). This response is a repair system induced by exposure to low levels of antibiotics. Continued exposure leads to cascading mutation rates that can aid in the formation of multidrug resistance (Singer *et al.,* 2016).

2.6 Antibiotic resistance associated health risks

Since the discovery of antibiotics, bacterial infections were viewed as little more than an annoyance. Antibiotic resistance is, however, now viewed as a significant health issue and threatens the way of modern medicine for present and future treatment of pathogenic infections (Bennet, 2008; Marshal & Levy, 2011). Rising multi-resistance and the fact that fewer antibiotics are available or being developed, may only be the beginning of the post antibiotic era (Bennet, 2008).

There is also concern for the biotic environment that comes into contact with these substances, since antibiotics can be taken up by vegetable crops and animals, which is a great food safety concern (Gothwal & Sashidhar, 2015). With various resistance mechanisms available to bacteria, the main concern with regard to antibiotic pollution, is the selective pressure it places on bacteria to utilize these mechanisms and disseminate ARGs (Kümmerer, 2004; Xu *et al.,* 2015).

Antibiotic polluted environmental settings create opportunities for resistant bacteria and genes to cross the border from environment to humans, animals and vice versa (Manaia *et al.,* 2016). ARB and/or resistance determinants have the potential to transfer by direct contact or via the food chain (Kemper, 2008; Zhang *et al.,* 2009; Manaia *et al.,* 2016). The effect these determinants have on the host greatly depends on the condition of the receiving host and the ability of the vector to invade the host tissue, organs etc. (Manaia, 2017).

Antibiotics like aminoglycosides are crucial in the treatment of serious illnesses like septicaemia and MDR-TB (Peterson & Rogers, 2015). Therefore, in a country like South Africa, one of the highest TB burdened countries in the world, aminoglycoside resistance could mean catastrophe (Bardien *et al.,* 2009). South Africa's public health system is also burdened with HIV/AIDS, which often goes hand in hand with XDR-TB (Thaver & Ogunbanjo, 2006). The TB epidemic in South Africa claims the lives of approximately 100,000 a year and three million globally (Thaver & Ogunbanjo, 2006; Bardien *et al.,* 2009).

Immoderate application of antibiotics, poor drug compliance, poverty etc., has an effect on human health via potential for resistance development, as well as the organ damage associated with taking these substances (Kemper, 2008). Other relevant resistance is that of enterococci, which are relevant nosocomial pathogens. These pathogens are displaying MDR to various antibiotics, especially aminoglycosides, β-lactams and glycopeptides (Padmasini e*t al.,* 2014). Aminoglycoside resistance in *E.coli*, isolated from blood has shown an increase of 6.8% (2001) to 15.6% (2012) in a surveillance study done in Spain (Fernández-Martínez *et al.,* 2015).

The danger is that antibiotics used for animal husbandry are also critical in human medicine (WHO, 2012). The continued misuse of antibiotics and subsequent dissemination into the environment will lead to promotion of resistant bacteria (Kemper, 2008; Singer *et al.,* 2016). Since rivers are the source for water consumption, the threat of resistance intake via direct contact or the food chain is a reality (Zhang *et al*., 2009; Rodriguez-Mozaz *et al.,* 2015; Manaia *et al.,* 2016).

2.7 Rivers under investigation

South Africa is a water scarce country and water is vital for economic and social development (DWA, 2013b), therefore research efforts need to be implemented to contribute to the state of the aquatic ecosystem on a regular basis. The Crocodile and “Groot” Marico river systems were chosen for this study. These two rivers stretch over three provinces: Gauteng, North West and Limpopo and are also collectively known as the Crocodile West Marico water system (River Health Program, 2005). The Crocodile West and Marico water management areas are important contributors to South Africa’s economy and are under stress (DWAF, 2004). The Marico River is dominated by agricultural activities, whereas the Crocodile River is subject to a variety of mining activities (DWA, 2012a). Major collective sources of pollution in these river systems include the following (DWAF, 2004):

- Agricultural drainage and wash off (irrigation, fertilizers, pesticides and runoff from feedlots).
- Urban wash-off and effluent return (bacteriological contamination, nutrients and salts).
- Industrial runoff (chemicals, acids and salts).
- Insufficient wastewater treatment.

These river systems eventually flow together to form the Limpopo River, which flows eastwards to the Indian Ocean (River Health Program, 2005). A summary of each river and subsequent catchments will be described in the following section.

2.7.1 The Marico River system

The Marico River system has a length of 250 km, where the upper tributaries are virtually undisturbed from anthropogenic activities (DWA, 2012a). It is considered to be a flat basin with little rainfall, thus water is limited (DWA, 2013b). The lower catchment area of the Marico River system is subject to agricultural activities and urban growth (DWA, 2012a; Bezuidenhout *et al.,* 2017).

The river flow is variable and regulated by the Marico Bosveld, Molatedi Dam and the Klein Maricopoort Dam (River Health Program, 2005; Bezuidenhout *et al.,* 2017). The Marico is fed with springs and the Marico Eye is considered to be the source of the river (River Health Program, 2005). This catchment was divided into eight sub-catchments in order to study the state of the river at various sampling points, which was determined according to the analysis done by Bezuidenhout *et al.* (2017). Figure 4 gives a visual illustration of the Marico River catchment and eight sub-catchments.

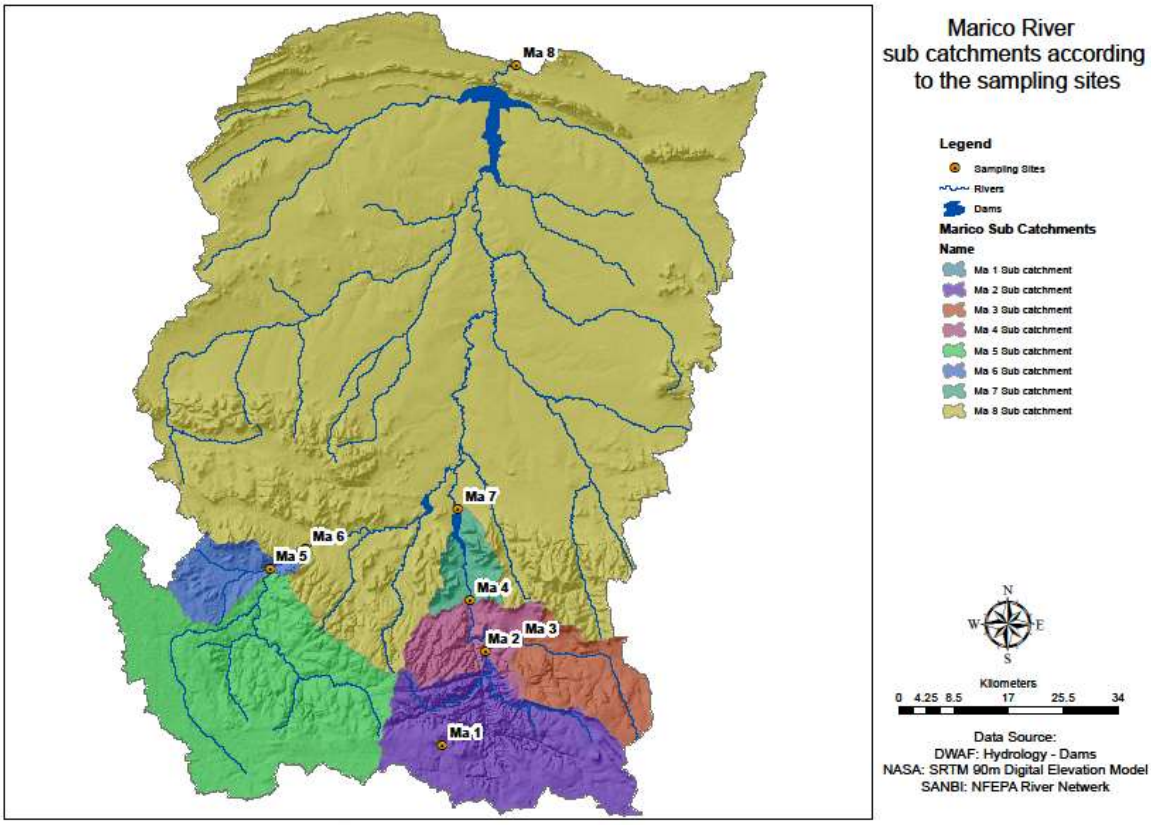

Figure 4: Map of Marico River catchment, divided into eight sub-catchments according to the sampling sites (Bezuidenhout *et al.,* 2017).

Table 1 provides a summary of the sub-catchments and the overall state of the sampling site chosen for the study in order to better understand what could be the cause possible problems at certain sites.

Table 1: Summary of Marico sub-catchments and state of sampling sites (Bezuidenhout *et al.*, 2017).

Site description	Overall state description and influences on sites
Site 1 – Marico Eye (M1)	This sub-catchment is a small area surrounding the source of the Marico River. It is a natural area, not influenced by anthropogenic pollution (River Health Programme, 2005).
Site 2 – Before confluence with Sterkstroom (M2)	This sub-catchment is also a natural area, but agricultural activities cover about 13% of the natural land cover. Influence of mines, wetlands and erosion increase with about 0.5% each, which could have a significant impact on water quality in the future as human activities increase.
Site 3 – Sterkstroom before confluence with Marico (M3)	This sub-catchment has a greater natural land cover than that of site 2. Agricultural activities are less (decreases by 8%), but erosion increases to about 2%. Erosion may have an influence on turbidity and sedimentation, which are also contributors to eutrophication (DWA, 2013b)
Site 4 – Before Marico Bosveld (M4)	This sub-catchment is also a natural area (about 93%). Agriculture activities are minimal (about 4%) and some human activities and roads are near the river. Erosion and bare ground cover are less (about 1%), but still poses a risk for turbidity and sedimentation issues on water quality (DWA, 2013b).
Site 5 – Klein Marico 5 km above Klein-Maricopoort Dam (M5)	This sub-catchment does not really differ from site 4 in terms of natural land cover and agricultural activities, but it does have urban areas which take up about 0.6% of the land cover.
Site 6 – Klein Marico 1 km below Klein-Maricopoort Dam (M6)	This sub-catchment has a decrease in natural land cover, with agricultural activities at about 8% and the irrigation thereof influences the river catchment running alongside these activities. The water land cover increases to about 0.7% and is mainly due to the Klein Maricopoort Dam situated in this sub-catchment. Urban land cover also increases by about 4% and there is a WWTP located in the town Zeerust. WWTP and urban activities could be a non-point pollution source that may have an effect on water quality in terms of eutrophication, microbial contamination and salinization (DWA, 2013b)
Site 7 – Marico River immediately below Marico Bosveld Dam (M7)	This sub-catchment is also mainly a natural land cover area (about 86%). Agricultural activities are slightly less at about 6%. Water land cover is more at about 2%, mainly due to the Bosveld Dam situated in this area. The most significant increase is the erosion at about 3%, which may increase turbidity and sedimentation, influencing water quality (DWA, 2013b).
Site 8 – Marico River at Derdepoort (M8)	This sub-catchment has a large natural land cover (about 91%), with erosion at about 2% and a decrease in agricultural activities (about 3%). Urban activities are little (about 2%), since this is considered to be a rural sub-catchment. The Molatedi Dam is located in this catchment and sites 6 (M6) and 7 (M7) flow into this sub-catchment.

2.7.2 The Crocodile West River system

According to the River Health Programme (2005), about 75% of the total surface runoff from the Crocodile West Marico water management area (WMA) flows into the Crocodile River. This management area is also the second most populous water management area in South Africa. More than half of the available water is used for industrial and mining activities, a third for irrigation, and the rest is used for rural water supplies and power generation (River Health Program, 2005). The Crocodile River has many rivers that contribute to its flow including Pienaars, Apies, Moretele, Hennoos, Jukskei, Magalies, and Elands River (DWAF, 2004; Bezuidenhout *et al.,* 2017). This River stretches across the Gauteng Provence (upper part of the catchment) and the North-West Province (central or western sections of catchment) (DWAF, 2004; Bezuidenhout *et al.,* 2017).

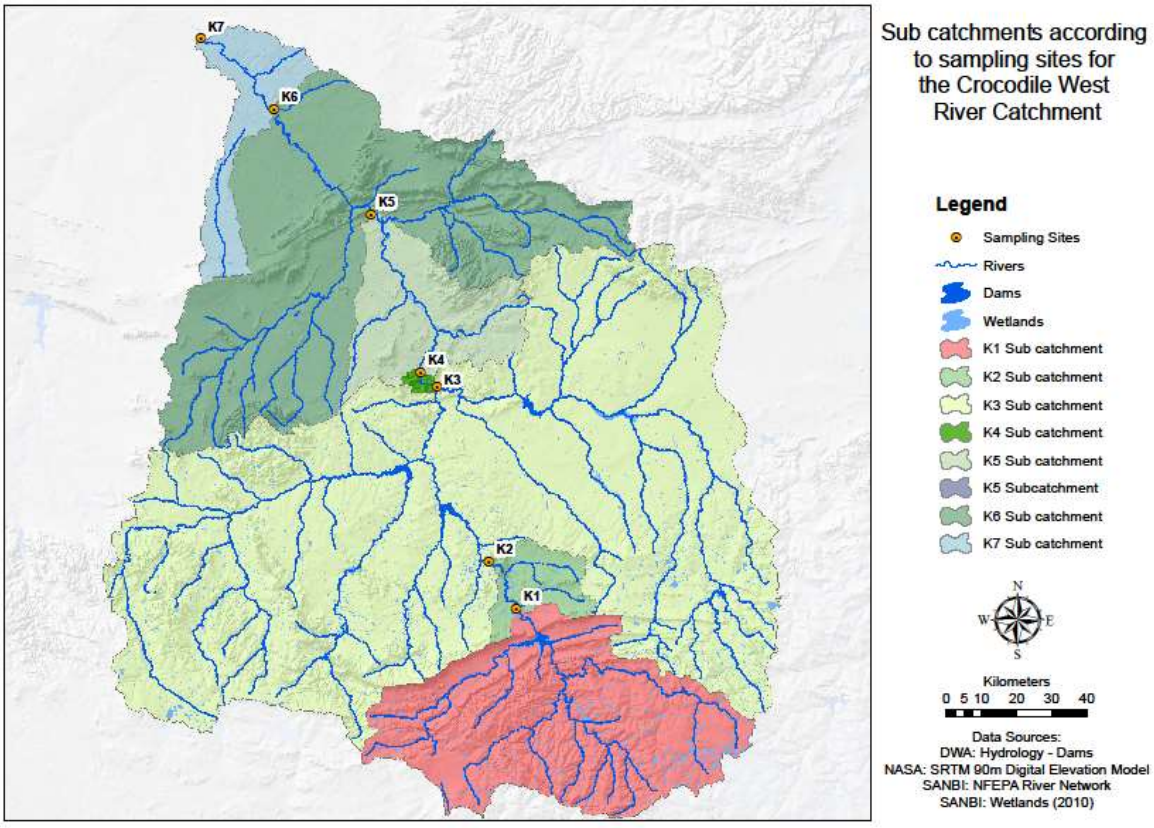

Figure 5: Map of Crocodile (West) River catchment, divided into eight sub-catchments according to the sampling sites (Bezuidenhout *et al.,* 2017).

This catchment was divided into seven sub-catchments in order to study the state of the river at various sampling points, which was determined according to the analysis done by Bezuidenhout *et al.* (2017). Figure 5 gives a visual illustration of the Crocodile (West) River catchment and eight sub-catchments and Table 2 provides a summary of the sub-catchments and the overall state of the sampling site chosen for the study.

Table 2 gives a summary of the overall state of the Crocodile River at certain sites in order to identify what could be possible causes of results generated in the study at each site.

Table 2: Summary of the Crocodile (West) River and state of sampling sites (Bezuidenhout *et al.,* 2017).

Site description	Overall state description and influences on sites
Site 1 – Before Brits (C1)	This sub-catchment has many economic activities and thus has far less natural land cover (about 52%) compared to the Marico River. There is a plethora of urban activities, which use about 26% of the land cover and industrial development, especially in the Gauteng Province (DWAF, 2004). The north-western part of the catchment is where most of the land cover is dominated by agricultural activities (about 15%). Other land covers include plantations (about 3%) and wetlands (about 2.5%). Urbanisation is a source of non-point pollution for this sub-catchment, because of the surface runoff from streets. Point source pollution could be from out dated and/or inadequate infrastructure in WWTP. These activities could affect water quality in terms of eutrophication, microbial contamination and salinisation (DWA, 2013b).
Site 2 – After Brits (C2)	This sub-catchment has about 12% more natural land cover than that of site 1. Agricultural land cover increases in this sub-catchment by 5% and urbanization reduces by 16%, which makes it more rural than site 1. There is a 2% increase in mine land cover and there is also a WWTP present. The mine, WWTP and agricultural activities may have a collective influence on water quality in terms of eutrophication, microbial and metal contamination, acid mine drainage and salinisation (DWA, 2013b).
Site 3 – Pienaars River (C3)	This sub-catchment had a significant increase in natural land cover making it a total of about 76%, where the agricultural land cover decreased by about 9%. There was a decrease in mines (about 1.5%) and wetlands (about 1%). The sub-catchment may have more natural characteristics than site 2, but there are numerous WWTP present, which may have an influence on water quality in terms of eutrophication, microbial contamination and salinisation (DWA, 2013b).
Site 4 – Koedoeskop Bridge (C4)	This sub-catchment is dominated by natural land cover (about 98%). There was an overall decrease in other land cover activities, for instance agricultural activities that showed a 9% decrease and urban land cover showed a 10% decrease.
Site 5 – Croc Thaba (C5)	This sub-catchment shows an overall decrease in natural land cover of about 10% and an increase in agricultural land cover of 10%. Most of the agricultural activities are adjacent to the river and may influence water quality in terms of eutrophication and salinisation (DWA, 2013b).
Site 6 – Croc to Thabazimbi (C6)	This sub-catchment has a large natural area of about 90% and there is a decrease in agricultural land cover of about 6%. Urban land cover is about 3% and numerous WWTP are present in this sub-catchment. Water quality may be influenced by the urban activities and WWTP in terms of eutrophication, microbial contamination, surface runoff and salinisation (DWA, 2013b).
Site 7 – Croc Draai (C7)	This sub-catchment is also mainly natural land cover (about 86%). The agricultural land cover increased by about 6%. Urban land cover decreased by about 1.5% and wetlands increased by 0.5%. The decrease in urban activities and increase in wetlands could act as a buffer for water quality; however, the

	increased agricultural activities adjacent to the river may cancel out the buffering effect.

Since South Africa is a semi-arid region, water demand far exceeds what can be supplied (River Health Program, 2005). The little water that is available is subject to antibiotic pollution. The most alarming form of antibiotic introduction into the environment is via wastewater. There are several WWTPs that do not comply with 50% of the Target Water Quality Rate (TWQR) set by the Department of Water Affairs (2012b). This is of concern since antibiotics are not completely metabolized when used and are thus excreted as an active substance. These active compounds then interact with the microbes in the wastewater. Environmental organisms and potential pathogens will either die out or attain resistance, via natural selection and/or HGT as mentioned in previous sections. Studying the health of aquatic systems is therefore vital to ensure sustainable water resource management and access to safe drinking water.

2.8 Methods that can be used to study antibiotic resistance in the environment

ARB and ARGs from environmental settings are usually investigated using traditional culturing methods (culture-dependent), as well as molecular methods (culture- independent). These methods are either used individually or in combination to investigate levels of antibiotic resistance and corresponding ARGs (Gothwal & Sashidhar, 2015; Manaia *et al.,* 2016). Molecular techniques most commonly applied to detect ARG's in the environment include PCR, qPCR, ddPCR, microarray and other metagenomic approaches (Zhang *et al.,* 2009; Li *et al.,* 2015a; Gothwal & Sashidhar, 2015; Manaia, 2017). Relevant techniques for this study will be introduced in the following section.

2.8.1 Culture-dependent or enrichment

(i) General introduction

The culturing of microorganisms has been the golden standard for diagnostics in clinical laboratories (Hilton *et al.,* 2016). Culturing methods involve growing organisms on enriched or selective media and has been used for more than a century (Hilton *et al.,* 2016). According to Douterelo *et al.* (2014), culture based methods are standard procedure for water analysis, to assess the quality of drinking water and monitor faecal contamination. According to Manaia *et al.* (2016) culture dependent methods are commonly employed to screen for antibiotic resistant bacteria. Culturing techniques are a reliable way to document resistance frequency and identify resistant organisms (Leff *et al.,* 1993).

(ii) Limitations of culture based techniques

Antibiotic susceptibility testing requires the cultivation of samples, as well as purification of the isolates, before further testing can be done (Sundsfjord *et al.,* 2004). This requires multiple incubation periods, which are time consuming. Studies that aim to detect ARGs mostly have a culture-based step to screen for resistant isolates and follow with PCR-based approaches to identify ARGs and subsequent resistant organisms (Foughy *et al.,* 2014). Unfortunately, phenotypic resistance to a certain antibiotic does not always confirm that the gene of interest is the main mechanism used by microorganisms (Smalla *et al.,* 1993; Sundsfjord *et al.,* 2004; Blanco *et al.,* 2016). Also, this technique does not take into account that most bacteria (99%), from any environment, are not cultivable in laboratory settings (Smalla *et al.,* 1993; Foughy *et al.,* 2014). Culture based methods can only confirm the presence of organisms that are able to grow on selected media (Hilton *et al.,* 2016). This implies that a large percentage of organisms, potentially carrying ARGs, are overlooked. As a result, culture based strategies are subject to bias. It is also possible that various nucleotide sequences exist in a sample that can encode enzymes conferring resistance to similar phenotypes (Leff *et al.,* 1993).

Even though many limitations exist when using this technique, it is still a widely used diagnostic tool, because of its cost effectiveness, reliable validation and relatively quick answers when doing certain confirmation tests (Douterelo *et al.,* 2014; Hilton *et al.,* 2016). Culturing techniques, coupled with PCR based techniques can be an effective gene detection method.

2.8.2 Culture-independent

(i) General introduction

The continuous improvement and application of molecular techniques, has allowed new insights into various microbial environments, without the need to culture the bacteria first. As previously mentioned, culture-based techniques on environmental samples only give information on the small portion (<1%) of the microbial community that is viable under laboratory conditions. This provides limiting information and bias (Douterelo *et al.,* 2014). These limitations are, however, overcome when using culture-independent methods. Techniques used to investigate non-cultivable bacteria commonly involve the extraction of nucleic acid from the total environmental sample (Leff *et al.,* 1993). These techniques focus on the genetic profile of the sample instead of morphology and phenotypic features associated with culture based techniques (Hilton *et al.,* 2016).

When combining culture-independent techniques with PCR assays, quick, specific and relatively inexpensive results can be obtained (Hilton *et al.,* 2016). According to Sundsfjord *et al*. (2004), low level resistance is easier to detect when using culture independent methods. This detection method is not subject to phenotypic resistance profiles, therefore no predetermined assumptions of which ARG is present or absent can be made. Also, no culturing steps are needed, thus shorter time is needed to detect ARGs (Sundsfjord *et al.,* 2004). This also avoids the bias of culture dependent methods.

(ii) Limitations to culture independent methods

Detection of genetic determinants is based on the assumption that the extracted isolates will contain a certain gene and that this gene will be functional (Leff *et al.,* 1993). Screening is therefore also based on known resistance genes and does not take into account that variations of a gene can be present (Sundsfjord *et al.,* 2004). False-positive and false negative results may be obtained during PCR due to silent genes or mutations in primer binding sites, not to mention the inhibitory substances in the sample (Sundsfjord *et al.,* 2004). The time and cost-effectiveness of the PCR technique are thus void, since multiple PCR reactions per sample need to be done to overcome false-negative results through optimisation (Hilton *et al.,* 2016). Regulatory mutations affecting gene expression can be overlooked, which is important when detecting genes in multi-resistant bacteria (Sundsfjord *et al.,* 2004).

2.8.3 Relevant molecular techniques

(i) Polymerase Chain Reaction (PCR) based approaches

(a) End-point PCR

End-point PCR or conventional PCR is the most common molecular technique used to detect pathogens in aquatic environments (Deshmukh *et al.,* 2016). This technique is used to amplify specific extracted DNA or RNA segments or fragments in a cyclic three-step process (Douterelo *et al.,* 2014; Deshmukh *et al.,* 2016). Presence of amplified products is confirmed by gel electrophoresis (Deshmukh *et al.,* 2016). The PCR technique forms the basis for follow up fingerprinting and next generation sequencing methods (Douterelo *et al.,* 2014). According to Douterelo *et al.* (2014), the application of this technique has a multitude of advantages; however, amplification of ribosomal RNA to study phylogenetic relations and identify bacteria is the more common application of the technique (Douterelo *et al.,* 2014). PCR based techniques are useful when working with environmental DNA, which can be troublesome to extract high

DNA concentrations. This technique is able to amplify the target gene, if no inhibiting products are present in the DNA sample. According to Zhang *et al*. (2009), end-point PCR is frequently used to detect ARGs encoding resistance to antibiotics like aminoglycosides, tetracycline, β-lactams etc. in pure and mixed environmental samples. On the other hand, false positive results are a reality when using conventional end-point PCR approaches (Zhang *et al.,* 2009).

(b) Quantitative real-time PCR (qPCR)

Real-time PCR is another powerful tool used to quantify the amount of the target gene copies in a given sample (Zhang *et al.,* 2009; Douterelo *et al.,* 2014). The technique is based on the principle that the amount of product obtained from fluorescence, at the exponential phase of the cycles, can be compared to the initial fluorescence and thus obtain amount of target gene copies (Zhang *et al.,* 2009; Douterelo *et al.,* 2014; Deshmukh *et al.,* 2016). Samples can therefore be screened for the gene of interest and the amount of target gene is compared to a standard curve, with known concentrations of a positive control (Deshmukh *et al.,* 2016). PCR techniques like multiplex PCR (using several oligonucleotide probes) and qPCR are important when detecting ARB, pathogens and related genes in water environments (Deshmukh *et al.,* 2016; Ju *et al.,* 2016). According to Zhang *et al.* (2009), SYBR Green is one of the most common fluorescent reagents when using this technique, especially when quantifying ARGs in environmental samples. qPCR is fast, has a low risk of cross contamination, sensitive and no gel electrophoresis is required after amplification (Deshmukh *et al.,* 2016).

(c) Droplet digital PCR (ddPCR)

This digital PCR technique is a simple linear quantification tool that is based on water oil emulsion droplet technology (Pavšič *et al.,* 2016). It relies on PCR-based methods and Poisson's distribution statistics. This technique can be done on a microfluidic-based style or by a droplet based approach (Pavšič *et al.,* 2016). In each approach the sample, with all its mastermix, primers and probes, is divided into thousands of single reactions. The PCR reaction then takes place and the number of positive reactions, together with Poisson's distribution is used and compared to the original target concentration (Hayden *et al.,* 2013). This method is extremely convenient, since it does not require calibration curves, only a strong positive control (Hayden *et al.,* 2013). Therefore, the sample is analysed on a reader after the PCR reaction and the result is either positive or negative quantification. This technique has high repeatability and can measure very low DNA concentrations (Pavšič *et al.,* 2016). ddPCR is useful to detect pathogens, genetically modified organisms, ARB and ARG detection, rare-mutant detection, etc.

(Pavšič *et al.,* 2016). This technique is receiving much interest in research, but is still very expensive, compared to PCR.

(ii) Sequencing

According to Douterelo *et al. (*2014), the conventional and more widespread approach used to obtain detailed and accurate phylogenetic information is done using cloning and sequencing based approaches. Firstly, nucleic acids need to be extracted. The rRNA gene of interest is then amplified with specific primers and the nucleic acid sequence can be analysed in detail. Unfortunately, this technique is expensive and time consuming. Next generation sequencing (NGS) platforms like shotgun metagenomics are rather applied (Ju *et al.,* 2016). This technique can analyse DNA sequences from PCR amplicons, as well as environmental nucleic acids to give a parallel assessment of any given sample (Deshmukh *et al.,* 2016).

In this study culture-dependent techniques were employed, together with molecular methods to determine aminoglycoside antibiotic resistance profiles of environmental water samples and to detect subsequent ARGs. Culture-independent techniques were also employed to try and establish a fast and efficient screening method for ARGs in environmental water samples. These techniques, with their advantages and limitations, will be described in the following section.

Because of the ease, time and sensitivity of the culture-independent approaches, combined with molecular techniques such as quantitative real-time PCR (qPCR), it is a popular choice when detecting ARGs in environments. These techniques provide valuable insights, but also have disadvantages. There is a need for a rapid, but accurate, universal analysis method to detect and quantify ARGs from environmental samples (Vaz-Moreira *et al.,* 2014; Li *et al.,* 2015a).

CHAPTER 3: MATERIALS AND METHODS

In this study, three methods were chosen to study ARG occurrence in the Marico and Crocodile Rivers. The culture-dependent method applied in 2015 was used to characterise aminoglycoside resistance in the River systems and screen for ARGs. The enrichment method applied in 2017 involved enrichment of bacteria by media, as well as media supplemented with kanamycin, to place a selective pressure on the total bacteria present in the sample inoculated. ARGs were then screened and quantified. The culture-independent method involved screening for ARGs from eDNA that was isolated directly from a membrane filter. ARGs were also screened and quantified. In this Chapter each method will be described.

3.1 Sampling

Sediment and surface water of the Crocodile and Marico Rivers were sampled on two occasions throughout this study (wet run in March and dry run in July of 2015). In 2017 the surface water was sampled in the wet season. Sampling was done according to the Quality of domestic water supplies volume 2 sampling guide. Samples were kept on ice until analysis was conducted later on the same day.

Coordinates to sampling sites can be found in the appendix.

3.2 Culture-dependent methods of 2015

3.2.1 Preparation of stock solutions, media and inoculation of media

(a) Stock solutions

Stock solutions were prepared for the following aminoglycosides; kanamycin monosulphate, neomycin sulfate, streptomycin sulfate and gentamicin sulphate (Melford, UK). This was done by dissolving the antibiotics according to the manufacturer's instructions. The stock solutions were then filter sterilized using a sterile syringe and 0.22 µm bottle top filters. Antibiotic stock solutions were stored at -20ºC until they needed to me added to the media.

(b) Media preparation and inoculation of samples

Nutrient agar (Merck, Germany) was prepared and supplemented with kanamycin (50 µg/ml). A standard dilution series of 10^{-1} up to 10^{-4} of the water and sediment samples were made.

Spread plates were prepared onto supplemented kanamycin nutrient agar (50 µg/ml). This was done aseptically in triplicate. Plates were incubated at room temperature (24 – 28ºC) for three to four days, depending on growth. After incubation the colony forming units (CFU) per millilitre was determined.

3.2.2 Purification of isolates and establishing resistance profiles

Resistant colonies from the wet sampling run in 2015 were selected based on phenotypic morphological features and each was streaked onto kanamycin supplemented nutrient agar (50 µg/ml). This was done aseptically in triplicate. Isolates were then purified by means of the streak plate method (in triplicate) and final isolates were Gram stained to test their purity, as well as Gram staining ability.

To determine if the selected isolates were multi aminoglycoside resistant, they were lazy streaked onto streptomycin, gentamicin and neomycin supplemented nutrient agar (50 µg/ml) respectively. Plates were incubated at room temperature (24 – 28ºC) for three to four days, depending on growth. If growth occurred for a particular isolate on all of the antibiotic supplemented plates, the isolate was marked as multi-resistant to aminoglycosides.

3.2.3 DNA extraction of multi aminoglycoside resistant isolates

Multi-resistant isolates were inoculated in 9 ml of nutrient broth (Merck, Germany) and incubated at 37ºC for 24 hours. DNA was extracted using the NucleoSpin® Genomic DNA isolation kit (Macherey-Nagel, Germany). Plasmid extractions were done using the NucleoSpin® Plasmid EasyPure kit (Macherey-Nagel, Germany). The extraction procedures were done according to the manufacturer's instructions.

Quality and concentration of the DNA was determined using a Nanodrop Spetrophotometer 3300 (Thermo Scientific, US). Gel electrophoresis of the isolated DNA was performed by mixing 5 µl DNA and 3 µl 6x Orange Loading Dye (Thermo Scientific, US) and was loaded in a 1% agarose gel in 1 x TAE (Tris base, acetic acid and EDTA) buffer. Electrophoresis was performed at 80V for 45 minutes using a Bio-Rad electrophoresis tool. A 1kb ladder (Fermentas, US) was used as a molecular weight marker.

3.2.4 16S rRNA PCR and sequencing

Identification of 2015 multi-aminoglycoside resistant isolates was done using 16S rRNA PCR on a Thermocycler (Bio-Rad, US). Reaction volume was 25 µl and each reaction contained 12.5 µl

2 × DreamTaq PCR Master Mix, 0.5 µl of each primer (0.4 µM), 1 µl DNA template (20 - 50 ng/µl), 10.5 µl of Nuclease free water (Fermentas Life Sciences, USA).

PCR amplicons were sequenced by Inqaba biotec (RSA). Sequences were edited by using the FinchTV (version 1.4.0) program. Isolates were identified by comparing the sequences with those in the Basic Local Alignment Tool (BLAST) database (http://www.ncbi.nlm.nih.gov/BLAST).

Primers and conditions are shown in Table 3.

3.2.5 Gene amplification of ARGs

(i) End-point PCR

The *nptII* aminoglycoside resistance gene was targeted on genomic and plasmid DNA of multi aminoglycoside resistant isolates. Reaction volume was 20 µl and each reaction contained 10 µl 2 × DreamTaq PCR Master Mix, 1 µl of each primer (0.5 µM), 1 µl DNA template (20 - 50 ng/µl) and 7 µl of Nuclease free water (Fermentas Life Sciences, USA).

The *intI 1* and *ampC* genes were targeted on plasmid DNA of multi aminoglycoside resistant isolates. Reaction volume was 20 µl and each reaction contained 10 µl PCR Master Mix (Thermo Scientific, US), 1 µl of each primer (0.5 µM), 1 µl DNA template (20 - 50 ng/µl) and 7 µl of Nuclease free water (Fermentas Life Sciences, USA).

The efflux pump gene *msrA/B* was also targeted on plasmid DNA of multi aminoglycoside resistant isolates. Reaction volume was 20 µl and each reaction contained 12.5 µl 2 x DreamTaq PCR Master Mix, 0.5 µl of each primer (0.25 µM), 1 µl DNA template (20 - 50 ng/µl) and 5.5 µl of Nuclease free water (Fermentas Life Sciences, USA).

Gel electrophoresis was performed on the PCR products to see if the reaction was successful. Of each PCR product 3 µl, together with 2 µl 6x Orange Loading Dye (Thermo Scientific, US) was loaded into individual wells of a 2% Ethidium Bromide (EtBr) containing agarose gel. Electrophoresis was performed using Bio-Rad electrophoresis tool at 80V for 45 minutes. Two ladders (1kb and 100bp) (Fermentas, US) were used.

3.3 Enrichment methods in 2017

3.3.1 Preparation of media and inoculation

Two 9 ml Luria Burtani (Merck, Germany) broths were prepared per site sampled. One broth was supplemented with kanamycin (50 µg/ml). Each broth was inoculated with 1 ml of sample and incubated aerobically at 37°C for 24 hours or until growth occurred.

3.3.2 DNA extraction

Genomic and Plasmid DNA was extracted from total bacteria from each site. The samples were either supplemented with kanamycin or without, as described in 3.3.1. Genomic DNA was extracted using the Chemagic DNA Bacteria Kit (PerkinElmer, USA). Plasmid DNA was extracted using the NucleoSpin® Plasmid EasyPure (Macherey-Nagel, Germany) kit. The extraction procedures were done according to the manufacturer's instructions. Quality and concentration of the DNA was determined as in 3.2.3.

3.3.3 Gene amplification of ARGs

Primer sequences and PCR conditions are described in Table 3.

(i) End Point PCR:

The *nptII, intI 1* and *msrA/B* genes were targeted on the genomic and plasmid DNA of the samples. Reaction volumes were as in 3.2.5. The *ampC* gene was, however, only targeted on the plasmid DNA, since it is redundant to detect this gene in the genomic DNA. Reaction volumes, as well as visualisation of amplicons, were done as in 3.2.5.

(ii) Real-time PCR (qPCR):

Qubit fluorometric quantitation was done on samples testing positive for *nptII* and *ampC* during end-point PCR amplification. The dsDNA BR Assay kit and Qubit Fluorometer 3.0 (Invitrogen, US) were used to ensure the correct concentration was prepared for the reaction and quantification. All qPCR experiments were conducted using the CFX96^{TM} Real-Time System on a C1000^{TM} Thermal Cycler (Bio-Rad, US).

The *nptII* aminoglycoside resistance gene was targeted. A standard curve was prepared by using 10-fold dilutions (5 ng to 5 fg) of a cloned plasmid containing the *nptII* resistance gene

(reference gene V00618, as described in Woegerbauer *et al.,* 2014). Reaction volume was 10 µl and each reaction contained 5 µl SsoFast Supermix (Bio-Rad, US), 0.5 µl of each primer (0.5 µM), 1 µl DNA template (5 ng/µl) and 3 µl of Nuclease free water (Fermentas Life Sciences, USA).

The *ampC* gene was targeted on plasmid DNA that amplified for the gene during end-point PCR. A standard curve was prepared by making 10-fold dilutions (5ng to 50 fg) using *E.coli* ATCC-10536 strain. Reaction volume was 10 µl and each reaction contained 5 µl SsoFast Supermix (Bio-Rad, US), 0.5 µl of each primer (0.5 µM), 1 µl DNA template (5 ng/µl) and 3 µl of Nuclease free water (Fermentas Life Sciences, USA).

(iii) Droplet digital PCR (ddPCR):

A dilution series (1 ng to 1 pg) of plasmid containing *nptII* gene (positive control) and *E.coli* ATCC-10536 strain containing the *ampC* gene was prepared to ensure that the wells were not oversaturated. Samples that amplified for *nptII* and *ampC* during end-point PCR underwent quantification.

Reaction volume was 22 µl and each reaction contained 11 µl QX200^{TM}EvaGreen ddPCR^{TM} Supermix (Bio-Rad, US), 0.22 µl of each primer (0.45 µM), 2 µl DNA template (30 pg for *ampC* and 50 pg for *nptII*) and 8.56 µl of Nuclease free water (Fermentas Life Sciences, USA). PCR was done in a 96 well plate using a C1000 Touch Thermal cycler (Bio-Rad, US).

3.4 Culture independent 2017 (direct eDNA analysis)

3.4.1 Membrane filtration

One litre of water from each site (seven sites per River) was filtered through a membrane filter 0.45 µm (47 mm grid) (PALL Life Sciences, Mexico).

3.4.2 Total DNA extraction

DNA was extracted from the filters using a DNeasy® PowerWater® Kit. (QIAGEN, Germany). Extractions were performed according to manufacturer's instructions.

3.4.3 Gene amplification of ARGs

End-point PCR detection of *nptII*, *intI 1, ampC* and *msrA/B* genes were performed on the membrane extracted total eDNA. PCR was done in triplicate on all samples. The *nptII* and *ampC* genes were quantified on all sites of the two river systems using qPCR and ddPCR.
All reaction volume setups, PCR primer sequences and conditions were the same as for the culture-based method described in 3.2.5.

3.5 Statistics, analysis and primer design software

Microsoft Excel (2011) was used to calculate averages and standard deviations to calculate average CFU/ml of aminoglycoside resistant bacteria. The *ampC* short primer was designed in-house using Vector NTI 6.0 Suite Software as described by Guoqing and Moriyama (2004). Real-time PCR data was analysed using the Bio-Rad CFX Manager 3.1 version 3.1.1517.0823 software package (Bio-Rad, US). The ddPCR data was analysed using the Quantasoft-Analysis Pro version 1.0.596 (Bio-Rad, US).

Table 3: PCR oligonucleotide primes and PCR conditions used during this study.

Genes	Target/ Primer	Sequence (5'-3')	Size (bp)	PCR conditions	Reference
16S rRNA	*16S RNA-F*	AGAGTTTGATCMTGGCTCAG	1465	Denaturation 92ºC 120 s, 40 cycles at 94ºC for 30 s, 54ºC for 60 s, 72ºC 300 s	Lane *et al.,* 1991, cited by Frank *et al.,* 2008
	16S RNA-R	GGTTACCTTGTTACGACTT			
Amino-glycoside resistance	*nptII-F* (short)	GATCTCCTGTCATCTCACCTTGCT	129	**qPCR:** Denaturation 95ºC 180 s, 35 cycles at 94ºC for 60 s, 53ºC for 30 s, 72ºC 60 s Final extension 72ºC 300 s. Melt curve 65ºC 5 s, 95ºC for 50 s. **ddPCR:** Denaturation 95ºC 10 min, 40 cycles at 94ºC for 30 s, 58ºC for 60s. Final extension 98ºC for 10 min	Woegerbauer *et al.,* 2014
	nptII-R (short)	TCGCTCGATGCGATGTTTC			
	nptII-F (long)	ATGATTGAACAAGATGGATTGC	795	Denaturation 95ºC 180 s, 40 cycles at 94ºC for 60 s, 53ºC for 30 s, 72ºC 60 s Final extension 72ºC 300 s	Woegerbauer *et al.,* 2014
	nptII-R (long)	TCAGAAGAACTCGTCAAGAAGG			
Class I integrase	*intI 1-F*	CTGGATTTCGATCACGGCACG	500	Denaturation 95°C 300 s, 30 cycles at 94ºC for 30 s, 64ºC for 30 s, 72ºC 60 s Final extension 72ºC 300 s	Labbate *et al.,* 2008
	intI 1-R	ACATGCGTGTAAATCATCGTCG			
β-lactam resistance	*ampC-F* (short)	GCCACTCAAACTCAACCA	120	**qPCR:** Denaturation 95ºC 10 min, 30 cycles at 95ºC for 30 s, 58ºC for 30 s, 72ºC 30 s. Melt curve 65ºC 5 s, 95ºC for 50 s **ddPCR** Denaturation 95ºC 10 min, 40 cycles at 94ºC for 30 s, 58ºC for 60s. Final extension 98ºC for 10 min	This study
	ampC-R (short)	GCTTCAGCATCTAACGCCCC			
	ampC-F (long)	TTCTATCAAMACTGGCARCC	550	Denaturation 94ºC for 300 s, 33 cycles at 94ºC for 30 s, 49ºC for 30 s, 72ºC for 60 s. Final extension 72ºC 420 s	Schwartz *et al*., 2003
	ampC-R (long)	CCYTTTTATGTACCCAYGA			
Efflux pumps	*msrA/B-F*	GCAAATGGTGTAGGTAAGACAACT	400	Denaturation 95 °C 180 s, 35 cycles at 93 °C 30 s, 55 °C 120s, 72 °C 90 s.	Molale & Bezuidenhout, 2016
	msrA/B-R	ATCATGTGATGTAAACAAAAT			

CHAPTER 4: RESULTS

In this Chapter the results of the three methods applied to study aminoglycoside resistance and other relevant resistance genes are provided. Aminoglycoside resistant bacterial levels in the water and sediment are presented. Molecular quantification results of aminoglycoside resistance gene *nptII* and β-lactam resistance gene *ampC* are provided. Results of other relevant resistance genes detected using the various detection methods are also provided.

4.1 Culture-dependent method (single isolate selection in 2015)

4.1.1 Characterization of aminoglycoside resistant bacteria

Aminoglycoside resistant bacteria were isolated from water and sediment samples of the two river systems. Various sites were tested by means of the spread plate technique on kanamycin supplemented nutrient agar plates (50 µg/ml). Tables 4 and 5 are summaries of the levels of kanamycin resistant bacteria. These levels were also compared to the HPC levels (Bezuidenhout *et al.,* 2017) in the Marico and Crocodile River systems. Surface water was analysed for the heterotrophic plate count (HPC) and colony forming units (CFU) levels, where sediment was analysed only for kanamycin resistance level characterisation during the wet and dry season.

Table 4: Average levels of heterotrophic plate count bacteria compared to aminoglycoside resistant bacterial levels of the Marico River water samples during the wet and dry seasons (HPC data from Bezuidenhout *et al.,* 2017). Levels of kanamycin resistant bacteria in sediment are also illustrated.

	HPC (CFU/ml)		Kanamycin resistance (CFU/ml)			
	Water		Water		Sediment	
Site	**Wet**	**Dry**	**Wet**	**Dry**	**Wet**	**Dry**
M1	6.00×10^3	2.18×10^5	10	70	NA	NA
M2	8.90×10^4	1.13×10^5	3.48×10^2	20	1.35×10^4	NA
M3	2.54×10^5	2.19×10^5	9.36×10^2	10	5.16×10^4	5.7×10^3
M4	1.53×10^4	9.35×10^4	3.91×10^3	93.33	1.91×10^5	7.96×10^4
M5	Dry	Dry	Dry	Dry	Dry	NA
M6	4.06×10^6	3.13×10^5	3.33×10^2	1.03×10^2	3.15×10^5	2.0×10^6
M7	2.00×10^5	2.31×10^5	66.66	20	6.71×10^4	9.33×10^2
M8	2.14×10^5	1.39×10^5	81.66	1.83×10^2	4.94×10^4	2.42×10^3

[HPC – heterotrophic plate count bacteria; CFU – colony form units; NA - Not available for sampling]

The results of the Marico River water samples in Table 4 show that the levels of kanamycin resistant bacteria were higher during the wet season compared to the dry season. The highest level of resistant bacteria, during the wet season was observed at site M4 (3.91×10^3 CFU/ml). During the dry season, the highest levels of kanamycin resistant bacteria were detected at M6 (1.03×10^2 CFU/ml) and M8 (1.83×10^2 CFU/ml). Levels of kanamycin resistant bacteria were lower in every site of surface water compared to the HPC levels during the wet and dry season. However, during the wet season site M4 kanamycin resistance level (3.91×10^3 CFU/ml) differed with one log to the HPC levels (1.53×10^4 CFU/ml).

Results of the Marico River sediment samples had higher levels of kanamycin resistant bacteria, during the wet season. The highest level of growth from the sediment samples was detected at site M6 for the wet (3.15×10^5 CFU/ml) and dry (2.0×10^6 CFU/ml) season. Site M4 also showed high levels of kanamycin resistant bacteria during the wet (1.91×10^5 CFU/ml) and dry (7.96×10^4 CFU/ml) seasons.

Table 5: Average levels of heterotrophic plate count bacteria compared to aminoglycoside resistant bacterial levels of the Crocodile River water samples during the wet and dry seasons (HPC data from Bezuidenhout *et al.,* 2017). Levels of kanamycin resistant bacteria in sediment are also illustrated.

	HPC (CFU/ml)		Kanamycin resistance (CFU/ml)			
	Water		Water		Sediment	
Site	**Wet**	**Dry**	**Wet**	**Dry**	**Wet**	**Dry**
C1	1.26×10^5	8.32×10^5	5.0×10^2	2.02×10^3	2.43×10^5	NA
C2	3.69×10^5	4.93×10^5	4.3×10^3	2.42×10^3	8.26×10^4	5.61×10^3
C3	1.11×10^5	1.93×10^5	3.61×10^2	3.43×10^3	NA	NA
C4	3.40×10^4	3.22×10^5	2.15×10^2	1.75×10^3	3.41×10^4	9.0×10^4
C5	2.35×10^4	9.04×10^5	6.13×10^3	1.8×10^3	4.88×10^4	1.87×10^5
C6	2.45×10^4	5.45×10^5	1.25×10^2	1.36×10^3	6.3×10^4	1.43×10^4
C7	2.25×10^5	1.54×10^5	1.75×10^3	6.08×10^2	3.7×10^4	2.36×10^3

[HPC – heterotrophic plate count bacteria; CFU – colony form units; NA - Not available for sampling]

Surface water samples of the Crocodile River had the highest HPC levels at site C1 to C6 during the dry season; varying from 1.93×10^5 to 9.04×10^5 CFU/ml. Levels of kanamycin resistant bacteria were mostly higher during the dry season than the wet season. The highest level of kanamycin resistant bacteria was detected at site C5 (6.13×10^3 CFU/ml) during the wet season. The CFU count of kanamycin resistant bacteria differed by only one log (2.35×10^4 HPC/ml) for the same site.

The CFU results of the Crocodile River sediment samples show that more kanamycin resistant bacteria occurred during the wet season, varying from 3.41×10^4 to 2.43×10^5 CFU/ml. No sediment was sampled during the dry season at site C1. During the wet season, the highest level of kanamycin resistant bacteria was detected (2.43×10^5 CFU/ml), while site C5 had the highest level (1.87×10^5 CFU/ml) during the dry season.

4.1.2 Resistance profiles of multi aminoglycoside resistant bacteria

The resistance profiles of the pure isolates selected from the surface water and sediment of the two River systems, during the wet season, were determined by streaking selected isolates onto nutrient agar supplemented with aminoglycosides, neomycin, streptomycin and gentamicin (50 µg/ml) respectively. A total of 48 isolates from the Crocodile River water, 57 isolates from the sediment, a total of 44 isolates from the Marico River water and 53 isolates from the sediment were selected. Table 6 summarizes the number of bacteria that were resistant to the various aminoglycosides.

Table 6: Summary of resistance profiles of individual isolates purified from the two River systems' surface water and sediment.

River system	Neomycin	Streptomycin	Gentamicin	Multi-aminoglycoside resistant bacteria
Crocodile water	14	16	4	3
Crocodile sediment	25	15	19	9
Marico water	6	6	7	6
Marico sediment	30	25	26	21

*All aminoglycosides were supplemented into nutrient agar to yield a final concentration of 50 µg/ml.

Table 6 indicates that the most multi-aminoglycoside resistant isolates were from the Marico River sediment (n = 21). The Crocodile River sediment yielded 9 aminoglycoside resistant isolates. The Crocodile River surface water contained more aminoglycoside resistant isolates (n = 6) compared to the Marico River surface water (n = 3). These results indicate that overall the sediment has a higher level of multi-aminoglycoside resistant bacteria. This could mean that a substantial fraction of the kanamycin resistant bacteria in the sediment, detected during the wet season, summarised in Table 4 and 5 were potentially multi-aminoglycoside resistant bacteria.

4.1.3 DNA extractions

The genomic and plasmid DNA was extracted from multi-aminoglycoside resistant isolates, as well as from enriched media. DNA was also directly isolated from water using a membrane filtration method. The extracted DNA was of relative good quality

even if the yield was somewhat low. Concentrations varied from 2.5 to 216 ng/µl. If bands were present after gel electrophoresis, the DNA was used for PCR amplification and sequencing (2015 isolates). An example of a genomic DNA depicted on an EtBr stained agarose gel is illustrated in figure 6.

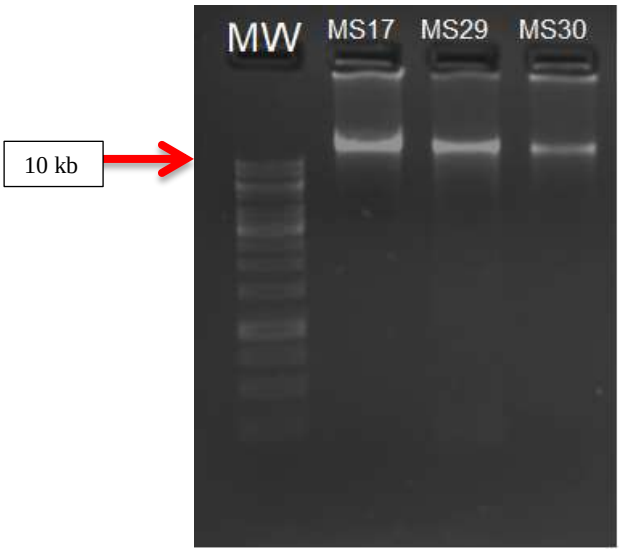

Figure 6: Image of an agarose (1%) genomic DNA electrophoresis gel of Marico River sediment isolates.

In figure 6 a 1 kb molecular weight marker was used. DNA bands in the gel are of a relatively high intensity. There are no smears present and the bands are clearly visible, with sizes larger than 10 kb. The results thus indicate that the DNA was of good integrity. The Nanodrop results indicate that the isolates contained usable DNA, since the 260/280 ratio varied from 1.7 – 2.2. Even though certain isolates had a higher DNA concentration than the recommended 1.8 for DNA, indicating RNA, phenol or other contaminants are present, products were still usable for PCR amplification, since the gel electrophoresis showed such good integrity of DNA bands present.

4.1.4 PCR analysis

(i) 16S rRNA and sequencing:

Figure 7 depicts examples of an agarose (2%) EtBr stained electrophoresis gel containing the amplified 16S rRNA products of multi-aminoglycoside resistant isolates.

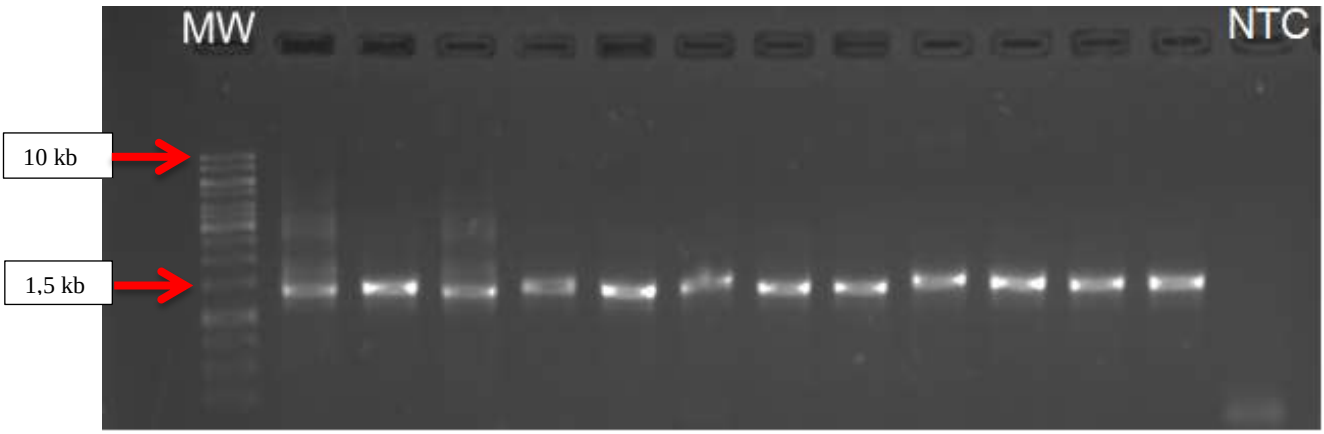

Figure 7: Illustration of an agarose (2%) EtBr stained electrophoresis gel of amplified 16S rRNA products of multi aminoglycoside resistant isolates.

A 1 kb ladder was used to determine the size of the PCR product. According to figure 7, the products are in the range of 1.5 kb in size. There are no bands present in the No template control (NTC). This indicates that the PCR reagents used were not contaminated by external DNA. The raw PCR products were sent to Inqaba Biotec (RSA) for sequencing. The sequences were compared to the known sequences found in the NCBI database, using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST). Identities of multi aminoglycoside resistant isolates identified are listed in Table 7.

Table 7 contains a list of the potential identities, according to the BLAST database, of isolates. The E-values (expected values) for all organisms were zero. The E value indicates the minimum error that could have occurred. The closer the value is to zero, the better the result (Madden, 2002). However, when doing identification, it is more important to consider the bit score, which shows the overall quality of the alignment in terms of gaps and sequence similarity (Madden, 2002). The bit score is an additional tool that gives a numerical value to the sequence similarity, independent from database size; thus, the higher the score, the better the similarity. The Max identity percentage shows how similar the sequence of the isolate is to the sequence in the database it is being compared to (Madden, 2002). Therefore, the closest similarity in sequence will score the highest percentage. Hypothetically, identity percentage that is in this range of 97%, the organism identified is likely to be of the genus identified, but not necessarily the species given. It is also important to consider that species name may change depending on the person or the program editing the sequences.

Table 7: Identities of the unknown isolate sequences using the Basic Local Alignment Search Tool (BLAST) and NCBI database.

Isolate	Name	Bit score	Max ID	Accession number	Resistance genes amplified
CS 2 (Site C1)	*Oerskovia turbata* strain 27, partial sequence	1589	100%	NR 029279.1	
CS 30 (Site C4)	*Chryseobacterium* Taiwanese strain BCRC 17412, partial sequence	2750	100%	NR 043715.1	
CS 35 (Site C4)	*Microbacterium arborescens* strain DSM 20754, partial sequence	1109	99%	NR 029265.1	
CS 51 (Site C7)	*Pontibacter rhizosphera strain* IMTB-1969, partial sequence	1423	96%	NR 132603.1	
CW 43 (Site C7)	*Sphingobacterium detergens* strain 6.25, partial sequence	1698	99%	NR 118238.1	
MS 17 (Site M3)	*Microbacterium saccharophilum* strain K-1, partial sequence	1668	99%	NR 114342.1	*msrA/B*
MS 29 (Site M6)	*Sphingobacterium alimentarium* strain WCC 4521, partial sequence (isolated from dairy)	952	96%	NR 108489.1	
MS 30 (Site M6)	*Bacillus subtilis* JCM 1465, partial sequence	870	100%	NR 118486.1	*msrA/B*
MS 31 (Site M6)	*Algoriphagus jejuensis* strain CNU040, partial sequence	929	99%	NR 108184.1	
MS 33 (Site M6)	*Sphingobacterium composti* Ten et al. T5-12, partial sequence	961	97%	NR 112559.1	*msrA/B*
MS 35 (Site M6)	*Microbacterium fluvii* strain YSL3-15	817	99%	NR 041561.1	
MS 36 (Site M6)	*Sphingobacterium hotanense* strain XH4, partial sequence	787	99%	NR 108440.1	
MS 37 (Site M6)	*Chryseobacterium humi* strain ECP37, partial sequence	906	97%	NR 104496.1	*intI 1, msrA/B*
MS 38 (Site M6)	*Sphingobacterium alimentarium* strain WCC 4521, partial sequence	673	97%	NR 108489.1	*msrA/B*

MS 39 (Site M6)	*Pseudoxanthomonas wuyuanensis* strain XC21-2, partial sequence	782	99%	NR 126229.1	
MS 40 (Site M6)	*Sphingobacterium composti* Ten et al. 2007 T5-12, partial sequence	1003	97%	NR 112559.1	
MS 45 (Site M7)	*Ensifer adhaerens*, strain NBRC 100388, partial sequence	1179	100%	NR 113893.1	
MS 52 (Site M8)	*Bacillus subtilis* JCM 1465, partial sequence	878	100%	NR 118486.1	
MW 31 (Site M6)	*Sanguibacter* soli strain DCY22, partial sequence	1094	99%	NR 044276.1	
MW 32 (Site M6)	*Chryseobacterium haifense* strain H38, partial sequence	859	99%	NR 044167.1	
MW 34 (Site M6)	*Sphingobacterium hotanense* strain XH4, partial sequence	1589	97%	NR 108440.1	
MW 38 (Site M6)	*Sphingobacterium composti* Ten et al. 2007 strain T5-12	1530	96%	NR 112559.1	*msrA/B*

[CS = Crocodile Sediment, CW = Crocodile Water, MS = Marico sediment, MW = Marico Water]

In total twenty-two multi-aminoglycoside resistant isolates were identified from the different sampling points. Table 7 indicates that most of the identified isolates were from the Marico sediment samples. Site M6 is also where most multi-aminoglycoside resistant isolates were originally isolated from. There are, however, some isolates that are of the same species at the same sampling site and could be clones. For example, isolate MS 29 and MS 38 were both identified as the *Sphingobacterium alimentarium* strain. This is plausible, because both isolates were selected from a dilution made from the sediment of site M6. Isolate MS 33 and MS 40 were both identified as *Sphingobacterium composti.* Both isolates were selected from the same dilution of sediment from site M6. Isolate MS 36 and MW 34 were both identified as *Sphingobacterium hotanense* strain XH4. Even though one isolate was originally from the Marico water and the other from sediment, both were isolated from site M6. These results indicate that selection via phenotypic morphology can give redundant duplicates.

(ii) Detection of *nptII* aminoglycoside resistance gene

The genomic and plasmid DNA of the multi-aminoglycoside resistant isolates from 2015 were tested for the presence of the *nptII* resistance gene, and were all negative.

(iii) Detection of the β-lactam *ampC* resistance gene

One isolate CW 26 of the multi-aminoglycoside resistant plasmid DNA was positive for the *ampC* gene.

(Iv) Detection of *intI 1* class one integrase gene

Plasmid DNA of the multi-aminoglycoside resistant isolates CW 26 and MS 37 were positive for the *intI 1* gene.

(v) Detection of efflux pump gene *msrA/B*

Plasmid DNA yielded positive results for the ms*rA/B* gene for multi-aminoglycoside resistant isolates and are summarised in Table 11. The Table indicates that *Sphingobacterium, Chryseobacterium* and *Microbacterium* possessed the *msrA/B* efflux pump.

4.2 Enrichment method 2017

(i) Detection of *nptII* aminoglycoside resistance gene

(a) End-point PCR

According to gel electrophoresis results, genomic DNA of site C1 and C3, which were originally supplemented with kanamycin (50 µg/ml), amplified for the *nptII* resistance gene. Genomic DNA from site C7 that was enriched with media, but did not contain kanamycin, was positive for the *nptII* gene. The plasmid DNA from site C7 and M3 that were originally supplemented with kanamycin (50 µg/ml), were also positive.

Results therefore indicated that very few samples contained the *nptII* resistance gene. An illustration of *nptII* amplification on an agarose gel (2%) stained with EtBr is shown in figure 8. A 100 bp molecular weight marker was used.

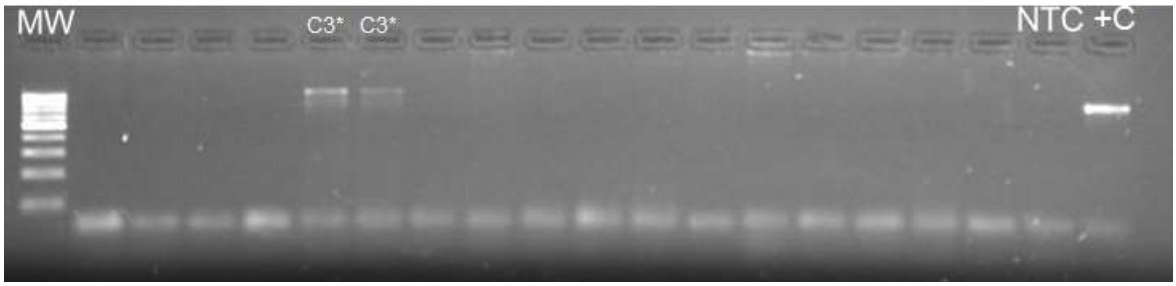

Figure 8: Image of an agarose gel (2%) stained with EtBr of the *nptII* resistance gene products from total genomic DNA extractions and sample C3 (enriched with kanamycin) amplifying.

The gel electrophoresis of the *nptII* resistance gene shows poor amplification, and the positive control is not perfectly aligned with the amplified samples. This could be an artefact of the electrophoresis.

(b) Real-time PCR and ddPCR

Molecular quantification using qPCR and ddPCR was performed on genomic and plasmid DNA samples that tested positive for *nptII* during end-point PCR analysis. Using the standard curves (figure 9) generated for the *nptII* gene, the copy number of the target genes could be calculated. Reactions were performed in triplicate and the PCR efficiency for this approach was 100.31%, with a slope of -3.315 and R^2 value of 0.998. To normalise the data, all starting quantities, as determined by the standard curve, were inserted into a copy number calculator (http://cels.uri.edu/gsc/cndna.html). This was used to determine the absolute quantity of copies detected according to the amplicon size.

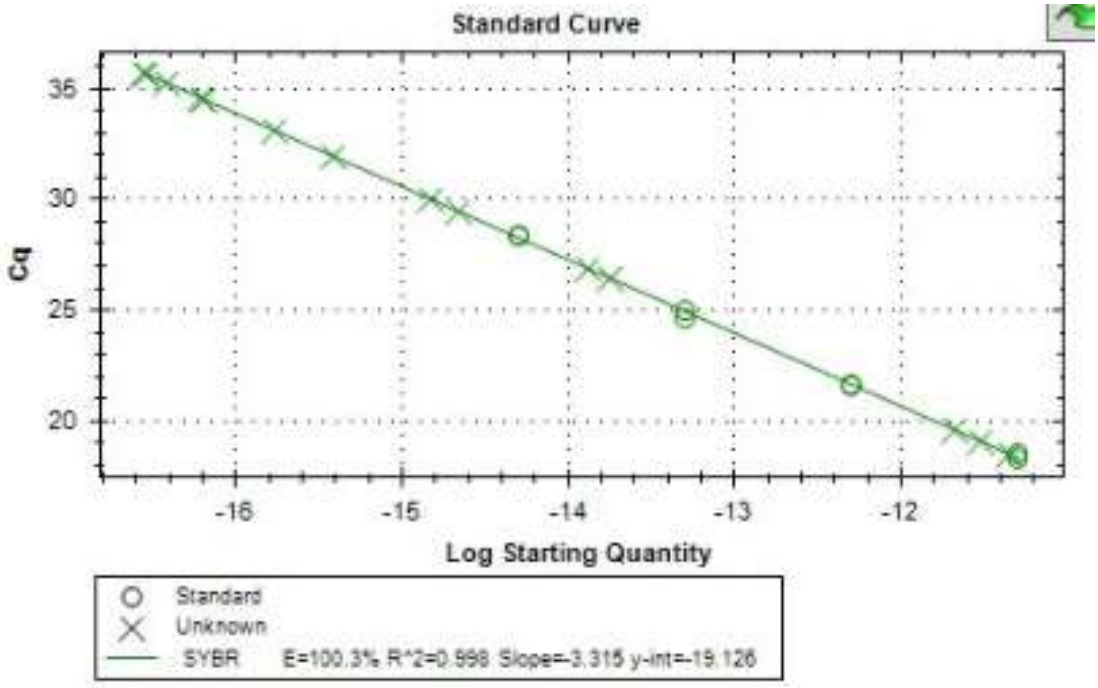

Figure 9: Standard curve of *nptII* gene for plasmid and genomic DNA samples.

Specificity of the qPCR products were determined by analysing the melt curves (figure 10 B). For the ddPCR a dilution of the positive control determined the threshold value for positive and negative droplet detection. The threshold was consistently applied for each sample to quantify the gene in each sample. The average copy number determined per sample is summarised in Table 8.

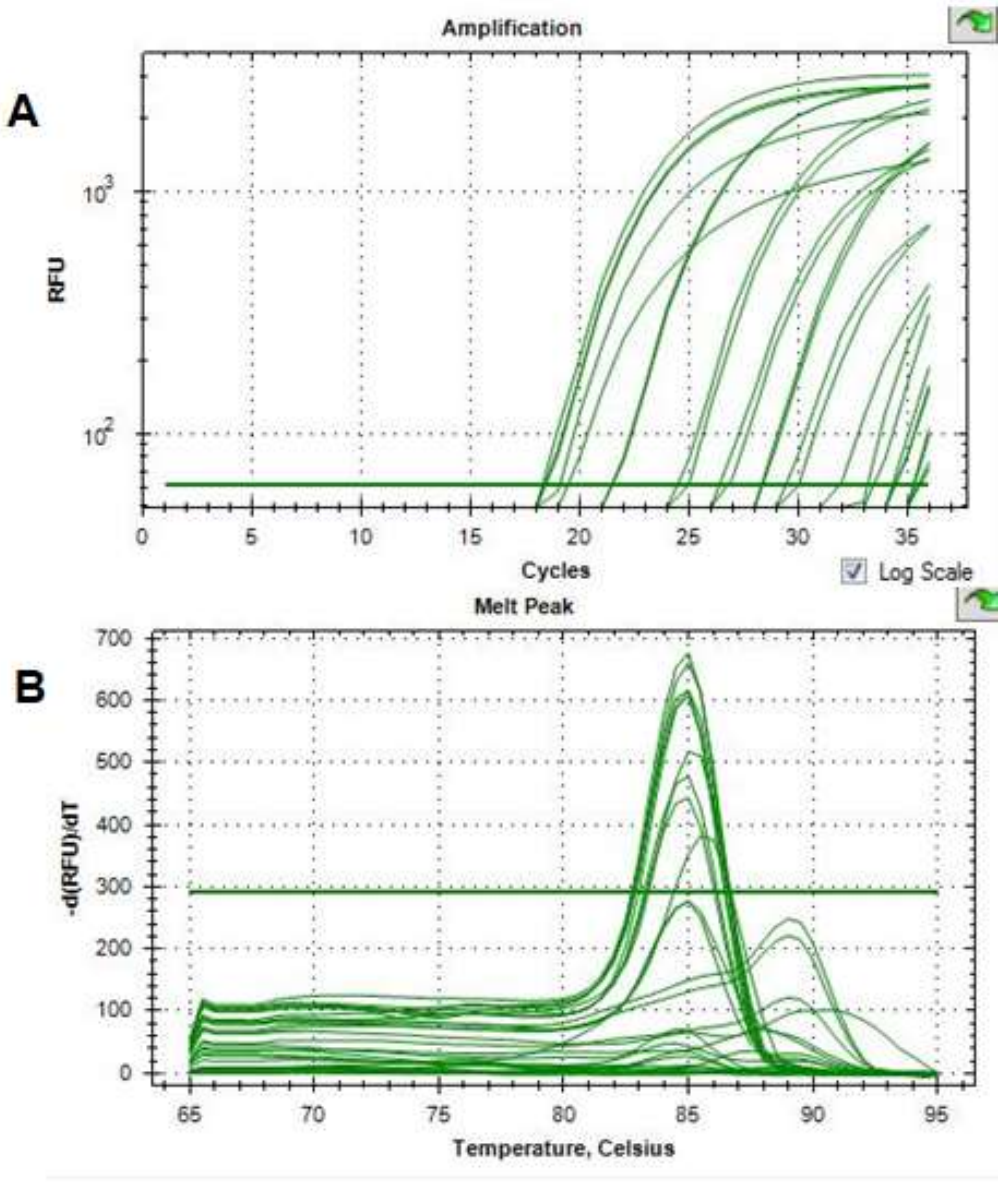

Figure 10: Image of quantification curves (A) and melt curves (B) of *nptII* quantification on plasmid and genomic DNA during qPCR.

Some of the melt curves (figure 10 B) were not exactly the same as the standard melt curve peaks. The standards all had a melting temperature of 85°C. The samples with melt curves at about 89°C are those of samples C7 (genomic DNA) and M3 (plasmid DNA enriched with kanamycin). These are also the only two samples that were quantifiable even though their melt curves are under the threshold for the lowest dilution of the positive control. The dissociation curves show no primer dimer formation.

Table 8: Summary of the average copies of *nptII* detected in genomic and plasmid DNA per nanogram of DNA using qPCR and ddPCR.

Sample	Real-Time PCR (average copies/ng DNA)	ddPCR (average copies/ng DNA)
GC1+	Negative	3.70×10^{-5}
GC3+	Negative	Negative
GC7	1.58×10^{4}	Negative
PC7+	Negative	Negative
PM3+	2.12×10^{3}	1.46×10^{-6}

[G-Genomic DNA, P-Plasmid DNA, (+) kanamycin]

Results presented in Table 8 indicate that genomic DNA of enriched samples from C1 and C3 (kanamycin enriched) were negative when tested for *nptII* genes using qPCR. Plasmid DNA from C7 (kanamycin enriched), was negative for the *nptII* gene using both quantification methods. However, ddPCR was able to detect copies of the *nptII* gene in enriched genomic DNA from C1 (kanamycin enriched) that was not quantifiable using qPCR. In the case of plasmid DNA of M3, enriched in the presence of kanamycin, both qPCR and ddPCR were able to quantify the *nptII* gene. The average copy number for ddPCR was considerably lower compared to the qPCR copies detected.

(ii) Detection of the β-lactam *ampC* resistance gene

(a) End-point PCR

Plasmid DNA samples from site C1 to C7 and M1 to M7 that were enriched in media, but did not contain kanamycin, were all positive for the *ampC* gene. Samples of sites M5 and M6 that were supplemented with kanamycin (50 µg/ml) were positive for the gene, since they were in line with the 550 bp marker.

Results therefore indicate a high occurrence of plasmid DNA samples containing the *ampC* gene. An illustration of *ampC* amplification on an agarose gel (2%) stained with EtBr is shown in figure 11.

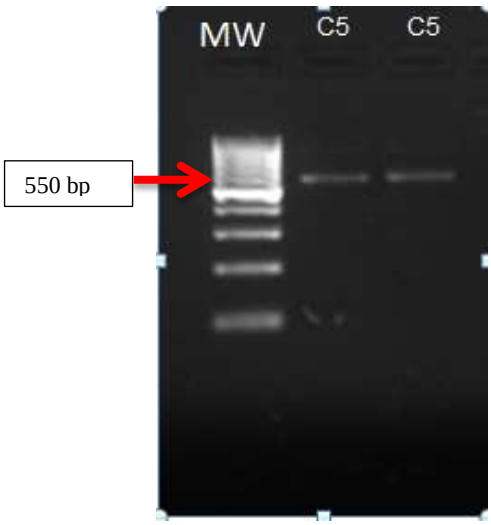

Figure 11: Image of an agarose gel (2%) stained with EtBr of the *ampC* resistance gene products from total plasmid DNA extractions.

(b) Real-time PCR and ddPCR

Molecular quantification using qPCR and ddPCR was performed for plasmid DNA samples that tested positive for *ampC* during end-point PCR analysis. Using the standard curves (figure 12) created for the *ampC* gene, the copy number of the target genes could be calculated.

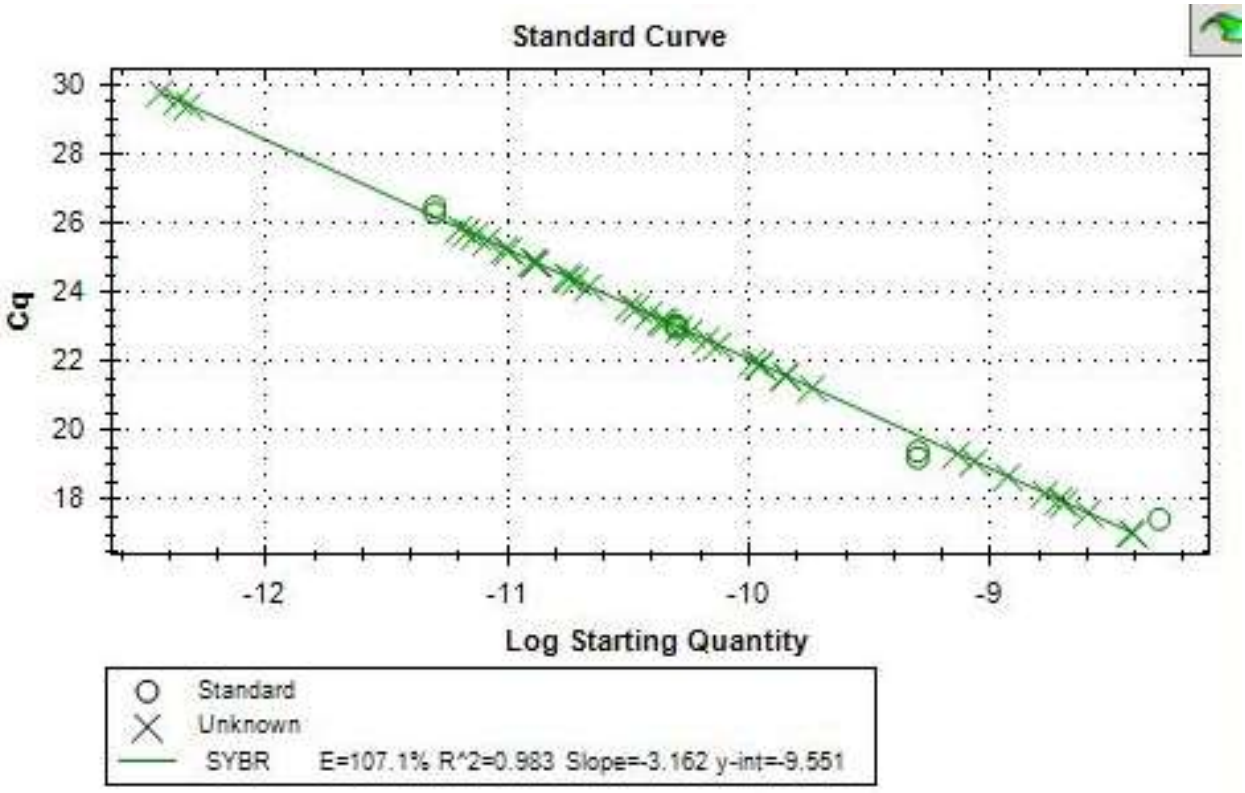

Figure 12: Standard curve of *ampC* gene for plasmid DNA samples.

Reactions were performed in triplicate and the PCR efficiency for this approach was 107.11%, with a slope of -3.162 and R^2 value of 0.983 as can be seen on the standard curve (figure 12). To normalise the data, all starting quantities, as determined by the standard curve, were inserted into a copy number calculator (http://cels.uri.edu/gsc/cndna.html). The absolute quantity of copies detected according to the amplicon size, could thus be determined. Specificity of the qPCR products were determined by analysing the melt curves (figure 13 B). For the ddPCR a dilution of the positive control determined the threshold for positive and negative droplet detection. The threshold was consistently applied for each sample to quantify the amount of gene detected. The average copy number determined per sample is summarised in Table 9.

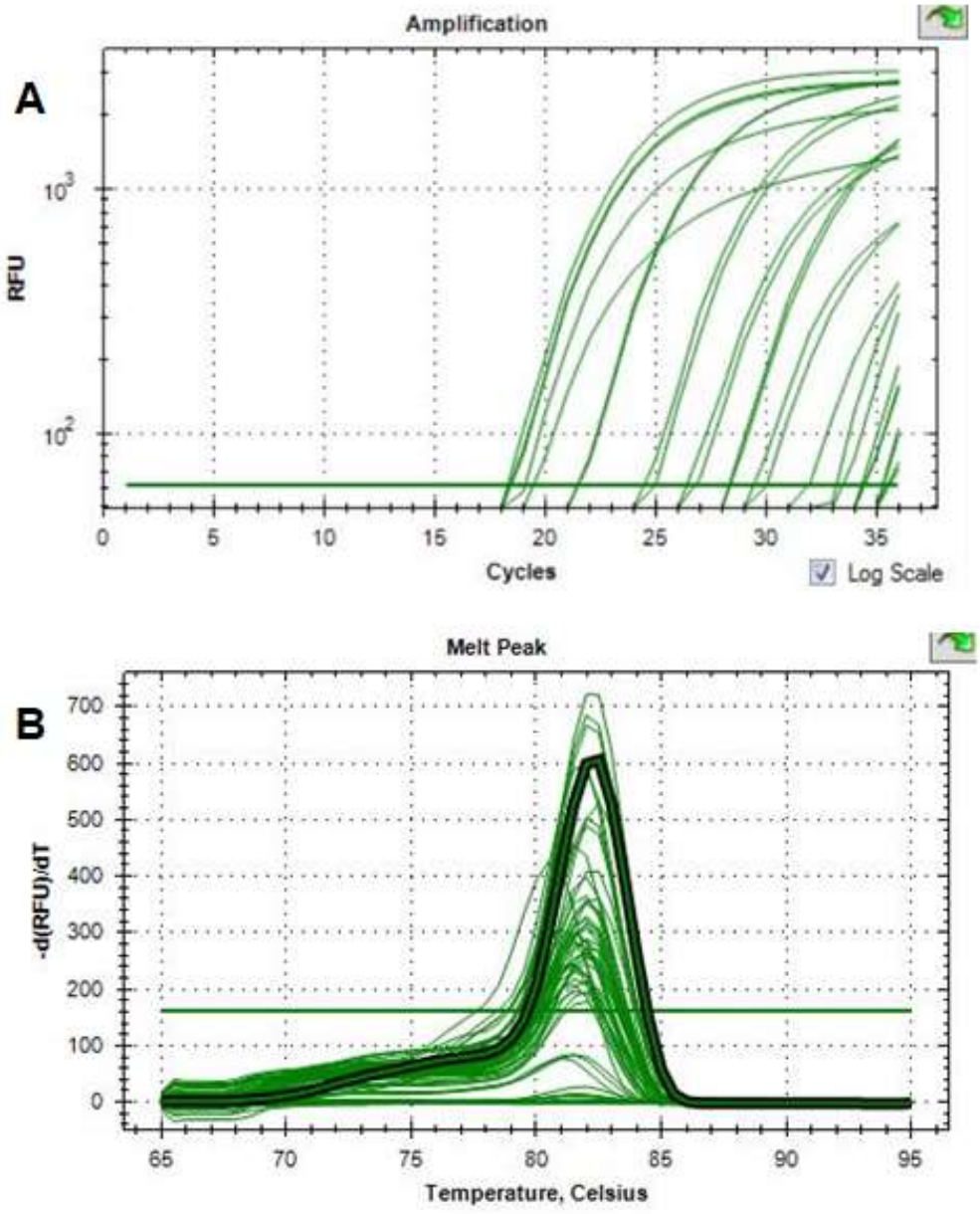

Figure 13: Image of quantification curves (A) and melt curves (B) of *ampC* quantification on plasmid DNA during qPCR.

The melt curve results for the *ampC* gene show most samples with approximately the same melting temperature (82°C) as the standard melt curves (figure 13 B). Some samples show melting temperatures of approximately 81°C, which are possibly mutations or variation in the gene, picked up by plasmid DNA from the total environmental bacterial sample. There were no primer dimers, since the no template control yielded no peaks and there were no peaks at the characteristic lower temperatures of the dissociation curves.

Figure 14 depicts an illustration of droplets that amplified for the *ampC* gene of plasmid DNA samples. The threshold was consequently set at 14,000 droplets for all samples, according to the positive control.

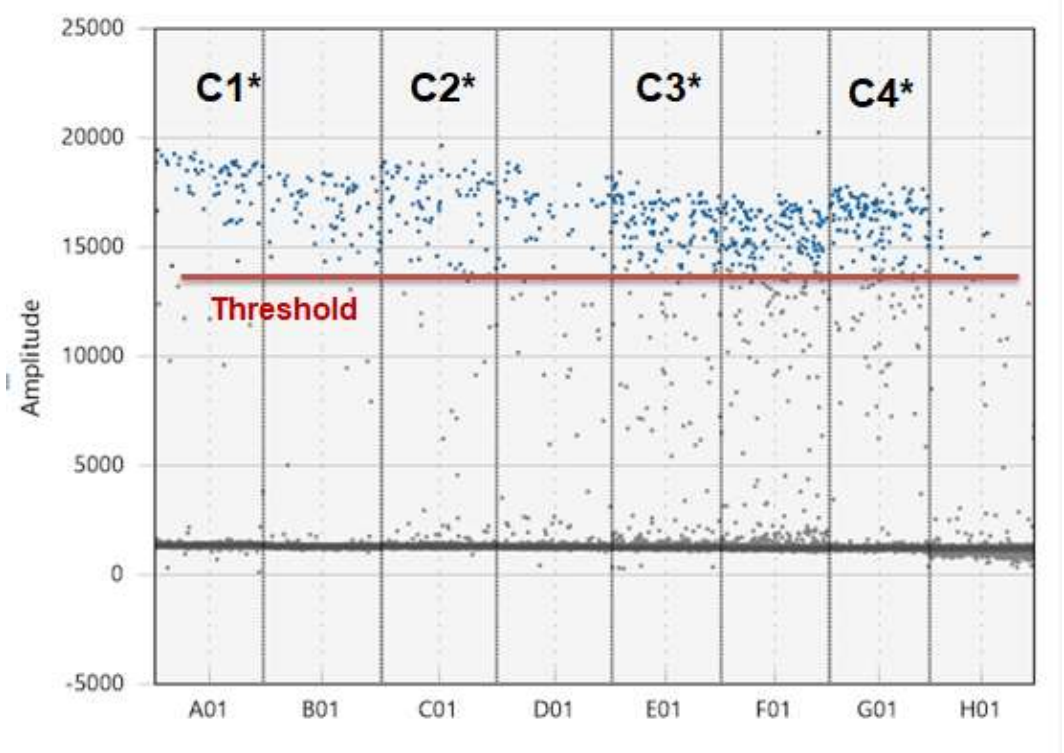

Figure 14: Image of ddPCR results of *ampC* gene for plasmid DNA samples C1 to C4 (enriched with kanamycin).

Results in figure 14, show clear amplification of the *ampC* gene and condensed grouping of droplets can be seen above the set threshold, indicating positive results. The blue droplets are positive and the grey droplets are negative results.

Results presented in Table 9 indicate that a greater copy number of the *ampC* gene was quantified compared to the *nptII* gene for both qPCR and ddPCR. However, qPCR results show higher *ampC* gene average copies (11-13 logs more) per nanogram of DNA compared to the ddPCR. The ddPCR was, however, able to detect copies of the *ampC* gene from plasmid DNA

of enriched samples M1 and M7, as well as M5 and M6 (kanamycin enriched), where the qPCR could not.

Table 9: Summary of the average copies of *ampC* detected in plasmid DNA per nanogram using qPCR and ddPCR.

Sample	Real-Time PCR (average copies/ng DNA)	ddPCR (average copies/ng DNA)
C1	2.60×10^{7}	1.67×10^{-4}
C2	7.50×10^{7}	1.47×10^{-4}
C3	7.02×10^{7}	3.34×10^{-4}
C4	4.54×10^{7}	1.93×10^{-4}
C5	2.50×10^{8}	1.01×10^{-3}
C6	1.58×10^{7}	1.65×10^{-4}
C7	1.33×10^{8}	5.80×10^{-4}
M1	Negative	2.87×10^{-5}
M2	1.34×10^{8}	6.63×10^{-3}
M3	2.06×10^{8}	1.39×10^{-4}
M4	1.10×10^{7}	6.77×10^{-5}
M5	4.90×10^{9}	3.13×10^{-3}
M5+	Negative	6.55×10^{-3}
M6	2.86×10^{9}	4.78×10^{-3}
M6+	6.58×10^{5}	1.23×10^{-5}
M7	Negative	4.03×10^{-3}

[(+) kanamycin]

(iii) Detection of *intI 1* class one integrase gene

(a) End-point PCR

Genomic DNA from sites C4 and M2 that were enriched with media and contained kanamycin (50 µg/ml) amplified the *intI 1* gene. Sites M2, M3, M5, M6 and C5 that were enriched in media, but did not contain kanamycin, showed positive amplification of the *intI* 1 gene.

The *intI* 1 gene was amplified in plasmid DNA for sites M5, M6, C5 and C6 that were isolated from samples that were enriched in media and also contained kanamycin (50 µg/ml). Plasmid DNA from sites M1, C1, C2, C5, C6 and C7, which were enriched in media that did not contain kanamycin also showed positive amplification for the *intI* 1 gene.

Results therefore indicate a high occurrence of samples containing the *intI 1* gene. An illustration of *intI 1* amplification at exactly 500 bp, according to the 100 bp molecular weight marker, on an agarose gel (2%) stained with EtBr is shown in figure 15.

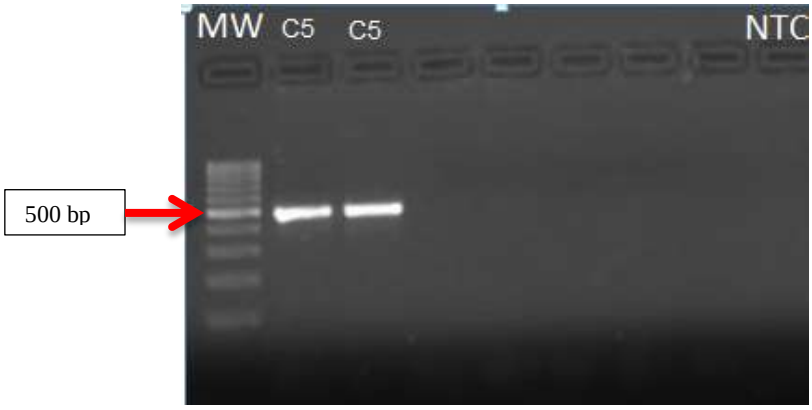

Figure 15: Image of an agarose gel (2%) stained with EtBr of the *intI 1* gene products from genomic DNA extractions.

(iv) Detection of efflux pump gene *msrA/B*

(a) End-point PCR

Genomic DNA showed positive amplification of the efflux pump gene at sites C1, C2, C3, C7, M5, M6 and M7, which were enriched in media that contained kanamycin (50 µg/ml). Sites C2, C6 and M4, which were enriched in media, but did not contain kanamycin were also positive for the gene. Plasmid DNA from site M5 was enriched in media containing kanamycin and also amplified the gene. Plasmid DNA from sites C2, C6 and M6, which were enriched in media, but

did not contain kanamycin were positive for the *msrA/B* efflux pump. An illustration of the *msrA/B* efflux pump gene amplification at exactly 400 bp, according to the 100 bp molecular weight marker, on an agarose gel (2%) stained with EtBr is shown in figure 16.

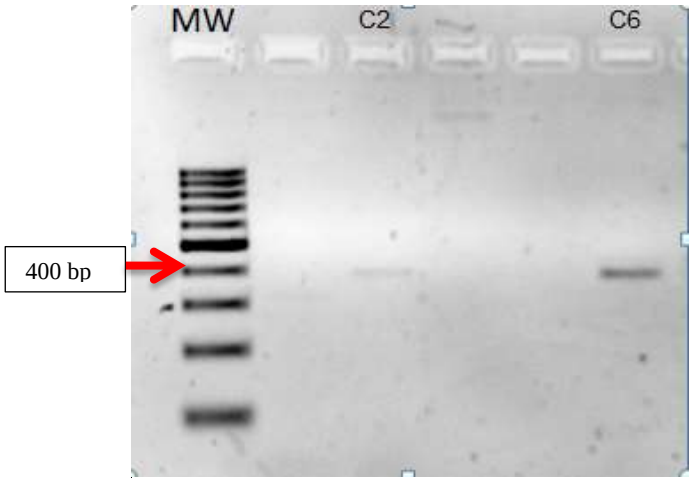

Figure 16: Inverted image of an agarose gel (2%) stained with EtBr of the *msrA/B* efflux pump gene products from genomic DNA extractions.

The results show a higher frequency of the efflux pump gene in the genomic DNA than in the plasmid DNA, particularly in samples exposed to kanamycin as a selective pressure.

4.3 Culture-independent method 2017

(i) Detection of *nptII* aminoglycoside resistance gene

(a) End-point PCR

Environmental DNA extracted directly from filtered membranes tested positive for the *nptII* resistance gene at sites C6 and C7.

(b) Real-time PCR and ddPCR

Molecular quantification using qPCR and ddPCR was performed for eDNA from all sites of the two river systems. Reactions were performed in triplicate and the PCR efficiency for this approach was approximately 91%, with a slope of -3.345 and R^2 value of 0.999 for both Rivers, as can be seen in figure 17.

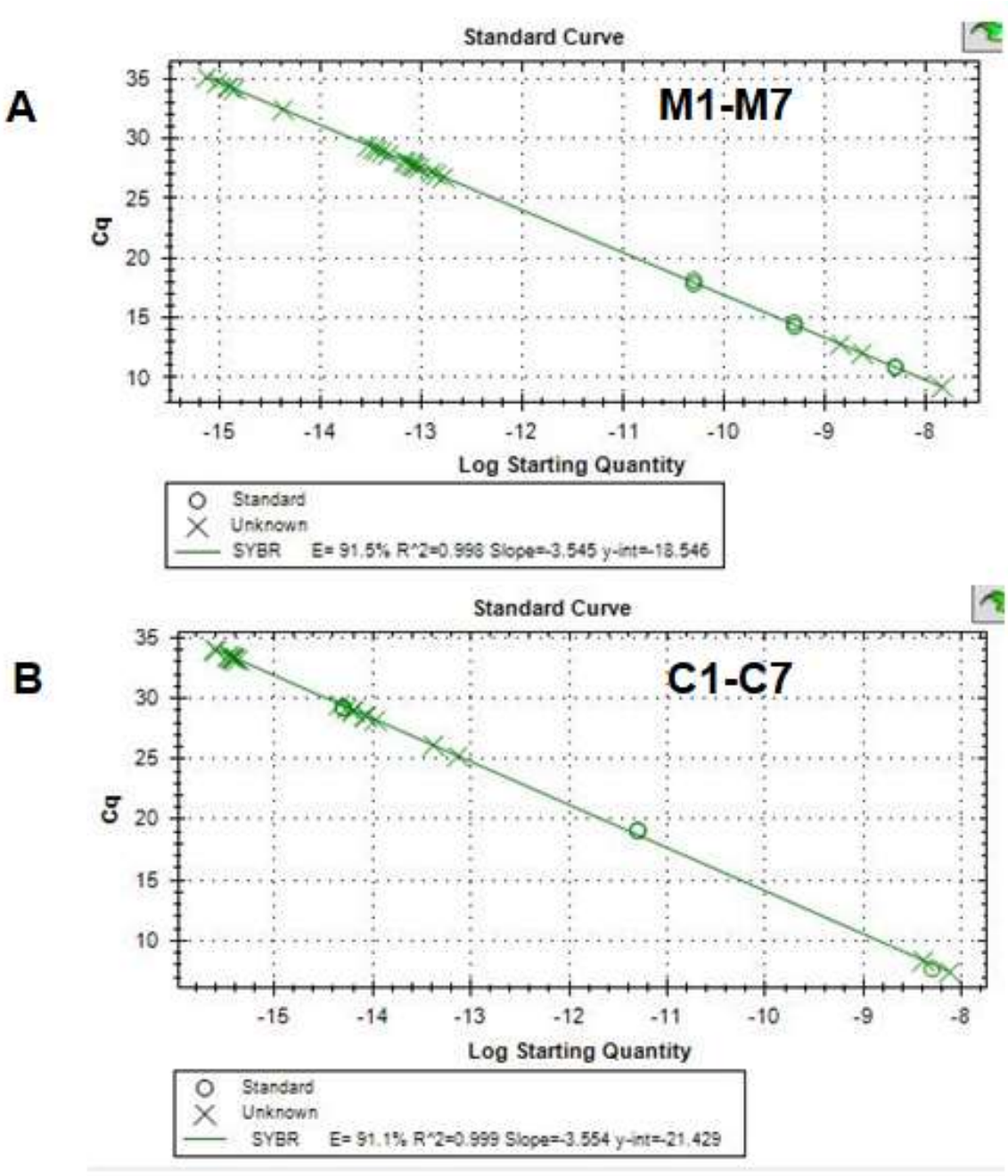

Figure 17: Standard curves of *nptII* gene on eDNA from Marico River sites M1 to M7 (A) and Crocodile River C1 to C7 (B).

Normalisation of data and copy number calculation was done as in 4.2 (b). The specificity of the qPCR products were determined by analysing the melt curves (figure 18 B and 19 B). For the ddPCR, a dilution of the positive control determined the threshold for positive and negative droplet detection. The threshold was consistently applied for each sample to quantify the amount of gene detected. The average copy number determined per sample is summarised in Table 10.

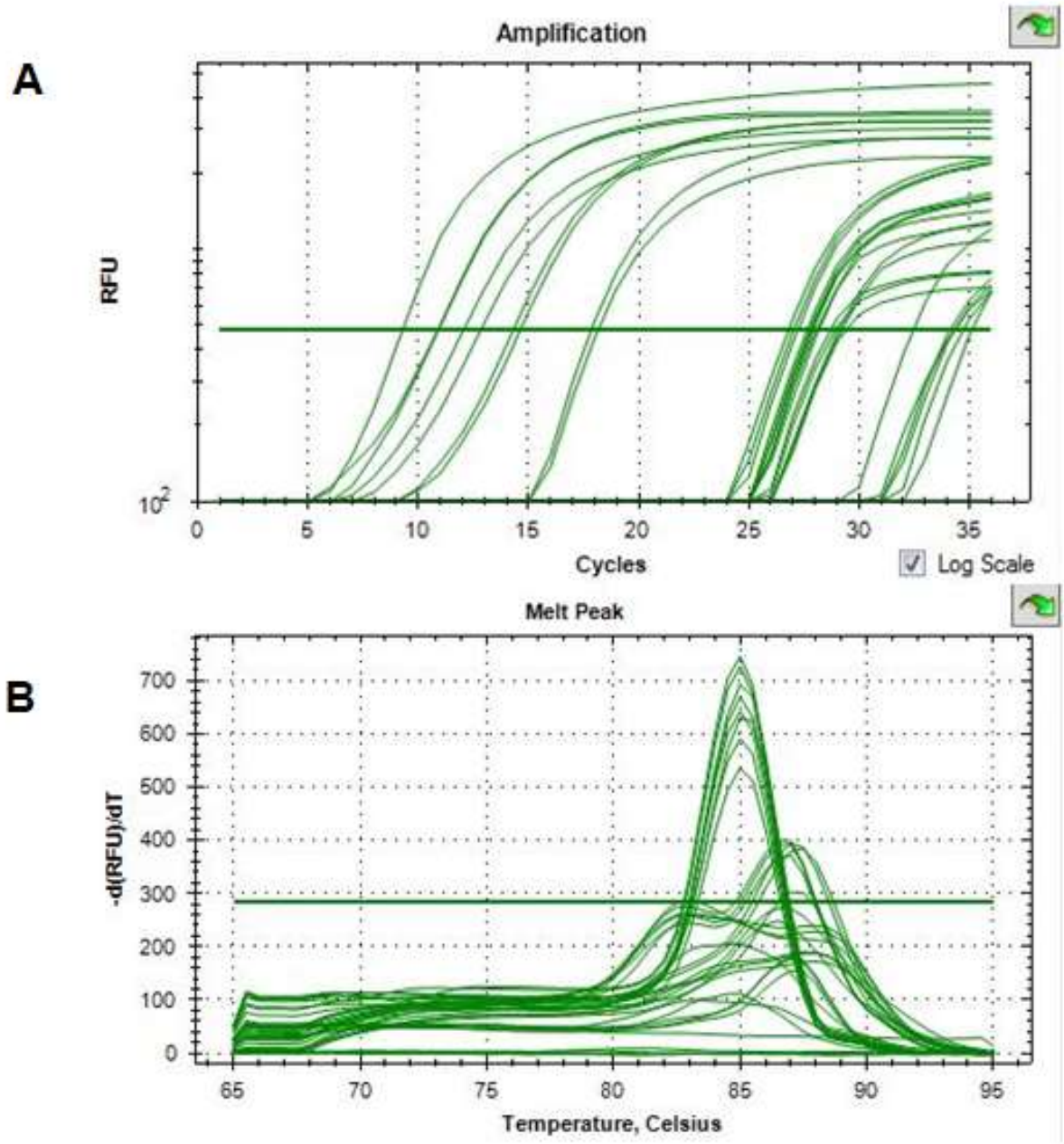

Figure 18: Image of quantification curves (A) and melt curves (B) of *nptII* quantification on eDNA extracted from sites M1 to M7 during qPCR.

The melt curve results in figures 10 (B), 18 (B) and 19 (B) show samples amplifying that are not consistent as the standard curve melting temperature, which is expected at approximately 85°C. In figure 10, the samples with melting curves at about 89°C are those of samples C7 (genomic DNA) and M3 (plasmid DNA enriched with kanamycin). The same pattern is observed in figures 18 and 19 where the melting curves are at around 87°C and 88°C. Figure 18 shows two melt curve peaks; one at 82°C and one at 88°C. This could possibly indicate the presence of heterozygous bacteria in the mixed eDNA from the Marico River samples. The samples showing this particular dual melt curve pattern are M3 and M6.

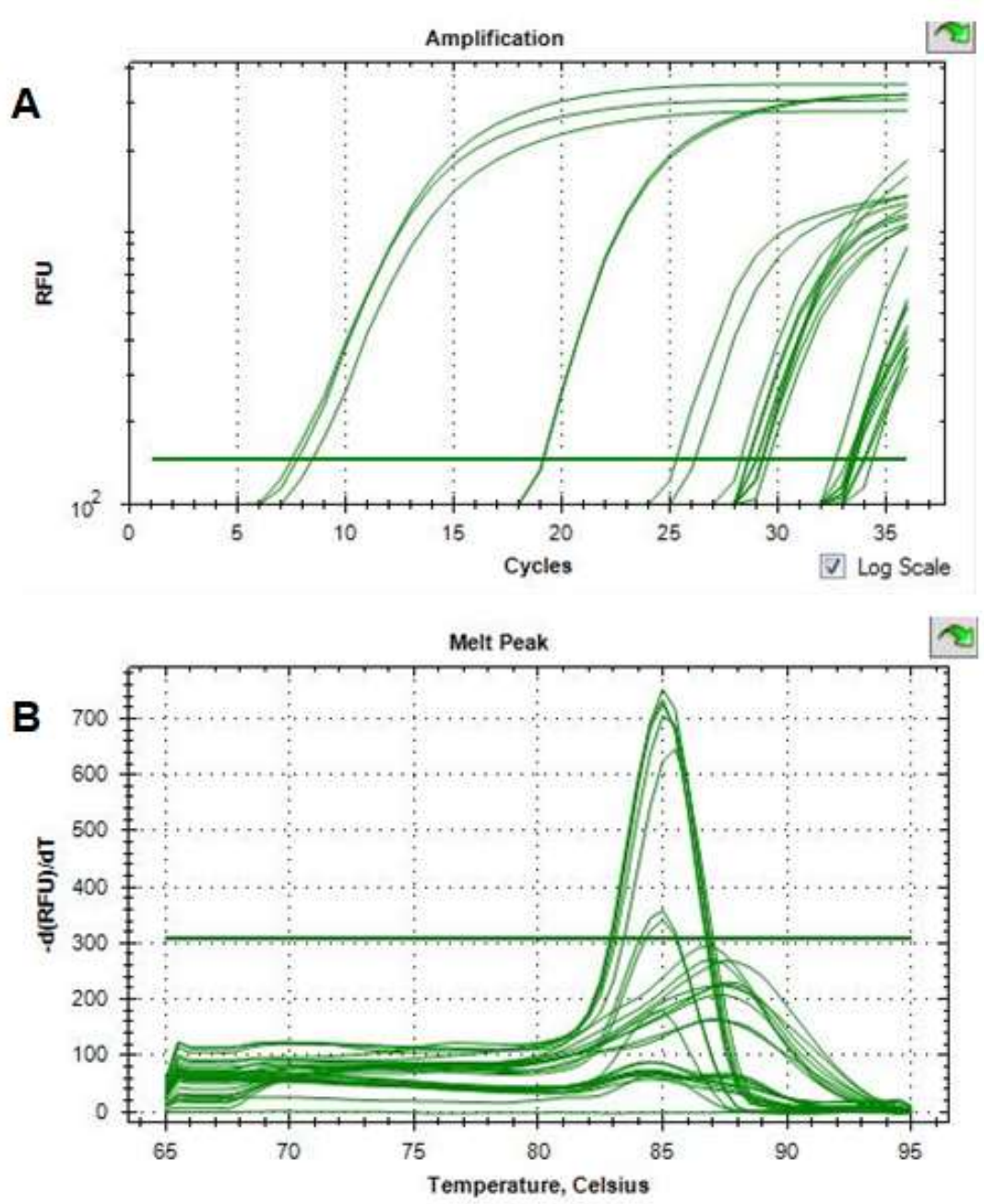

Figure 19: Image of quantification curves and melt curves of *nptII* quantification on eDNA extracted from sites C1 to C7 during qPCR.

The higher melt curve temperature observed for *nptII* quantification, compared to the standard curves, in figures 10 (B), 18 (B) and 19 (B) could be caused by gene variation in the bacteria, since the samples are of mixed environmental DNA. The possibility of mutations existing in the gene occurring in the environmental DNA is also feasible. The possibility of primer dimers is not probable, since the no peaks were detected at lower temperatures of the dissociation curves.

Table 10 shows that the qPCR was more successful in quantifying the *nptII* gene from the culture-independent extracted eDNA. The qPCR results were not available for samples C5, C6, C7, M2 and M5, since the copy numbers were too low.

Table 10: Summary of average copies of *nptII* gene detected per nanogram of eDNA using qPCR and ddPCR.

Sample	Real-Time PCR (average copies/ng DNA)	ddPCR (average copies/ng DNA)
C1	1.22×10^4	Negative
C2	1.24×10^4	Negative
C3	8.30×10^4	Negative
C4	7.94×10^3	Negative
C5	Negative	Negative
C6	Negative	Negative
C7	Negative	Negative
M1	5.66×10^4	Negative
M2	Negative	Negative
M3	1.81×10^5	Negative
M4	1.23×10^5	Negative
M5	Negative	Negative
M6	1.11×10^5	Negative
M7	5.10×10^4	Negative

(iii) Detection of *intI 1* class one integrase gene

There was a higher occurrence of samples containing the *intI 1* gene for environmental DNA directly extracted from membrane filters. All sites were positive and amplified at the same size (500 bp) as the positive control. An illustration of the *intI 1* gene amplification at exactly 500 bp, according to the 100 bp molecular weight marker, on an agarose gel (2%) stained with EtBr is shown in figure 20.

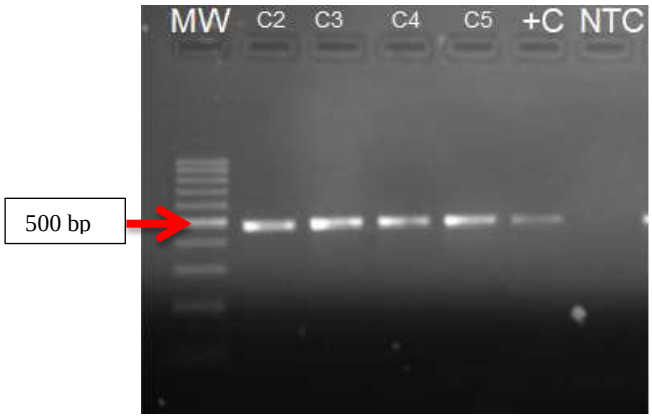

Figure 20: Image of an agarose gel (2%) stained with EtBr of the *intI 1* gene products from culture-independent eDNA extractions.

(iv) Detection of efflux pump gene *msrA/B*

Of the eDNA from membrane extractions, sites M2, M5, M6 and C1 were positive for the efflux pump gene.

4.4 Summary of genes that amplified for each approach during end-point PCR

Table 11 provides a summary of the number of isolates, as well as which gene amplified at what site during end-point PCR by testing extracted DNA from culture-dependent and culture-independent approaches applied in 2015 and 2017 respectively. These results give an indication of which method is more supportive in providing a snapshot of the overall appearance of an environment, when screening for genes of interest.

Table 11: Summary of samples that amplified selected genes using various approaches from 2015 and 2017.

Genes investigated	2015 culture-dependent samples		2017 enriched samples					2017 culture-independent samples	
	Total	Sample (sites)	Total	Plasmid DNA sites		Genomic DNA sites		Total	Sites
				+	-	+	-		
nptII	**NEG**	-	**5**	C7, M3	NEG	C1, C3	C7	**2**	C6, C7
ampC	**1**	CW26 (C2)	**16**	M5, M6	C1 - C7, M1 - M7	NA	NA	**NA**	NA
intI 1	**2**	CW26 (C2), MS37(M6)	**17**	M5, M6, C5, C6	M1, C1, C2, C5, C6, C7	C4, M2	M2, M3, M5, M6, C5	**14**	C1 - C7, M1 - M7
msrA/B	**6**	MW38(M6), MS17(M3), MS30(M6), MS33(M6), MS37(M6), MS38(M6)	**14**	M5	C2, C6, M6	C1, C2, C3, C7, M5, M6, M7	C2, C6, M4	**4**	M2, M5, M6, C1

[NA ~ Not applicable, since only plasmid DNA was relevant for *ampC* detection. (+) ~kanamycin selection present, (-) ~enriched broth with no kanamycin, NEG ~negative for amplification]

Results presented in Table 11 indicate that most genes identified with the culturing approach, applied in 2015, were detected in multi-aminoglycoside resistant isolates from sites C2, M3 and M6. The enrichment approach of 2017 indicates that more genes are detected in the plasmid DNA when no kanamycin selective pressure is involved. Sites that were positive were from the Crocodile River, particularly sites C2 and C6. However, when the kanamycin selective pressure was present, the plasmid DNA of sites M5 and M6, which were from Marico River, were more frequently positive. Genomic DNA contained more genes when kanamycin selective pressure was involved (n = 11), particularly in samples from sites C1 and C3. Integrase 1 (*intI 1*) was detected at all sites using eDNA from the culture-independent approach. This gene was also frequently detected when screening DNA from the other approaches, demonstrating the potential for ARGs to be transferred not only to plasmids within the same isolates, but also to other bacteria within the same or other species.

CHAPTER 5 – DISCUSSION

The aim of this study was to quantify and characterise aminoglycoside resistant bacteria and screen for relevant antibiotic resistance genes from aquatic environments using culture-dependent and culture independent methods. In this chapter, the results obtained from each culturing method are described to meet this aim.

5.1 Levels of aminoglycoside resistant bacteria

In this part of the study the interest was to focus on the levels of kanamycin resistant HPC bacteria when compared to the levels of the general population of HPC. Results indicated that aminoglycoside resistant bacteria were present in relatively high numbers, when compared to the heterotrophic bacterial level of the same sample from the corresponding sites of the Crocodile and Marico Rivers respectively. During the wet season high levels of kanamycin resistant bacteria were detected in the Marico River at sites M4, M6 (surface water and sediment) and C5 (surface water) of the Crocodile River. Site C1 showed the highest levels of kanamycin resistant bacteria in the sediment of the wet season, whereas site C5 had the highest levels in the dry season. In the dry season, site M6 again showed the highest levels of kanamycin resistant bacteria (surface water and sediment) and the heterotrophic bacterial level was also the highest for this site in the wet and dry seasons.

The selection of the high levels of kanamycin bacteria in these environments could potentially be due to anthropogenic activities and pollution at or upstream from the various sampling sites. For example, at sites M4, M6, C1 and C5 (Tables 1 and 2) there are agricultural activities. Animals and irrigation runoff could potentially be part of return flows (Bezuidenhout *et al.,* 2017). Furthermore, various physical conditions and chemicals could also contribute towards the observed results at the sites.

Physical parameters such as temperature, conductivity, salinity, pH, water density, gas concentrations (Murdoch *et al.,* 2000), as well as chemicals such as organic carbon, nitrates and phosphates are required for bacterial growth. Combinations of these could have either stimulatory or inhibitory effects. Additionally, chemical substances such as metals and antimicrobial substances have definite inhibitory effects. For example, toxicity of metals in aquatic ecosystems is enhanced by increased water temperature (Murdoch *et al.,* 2000). In a study by Degerman *et al.* (2013), it was demonstrated that an increase in temperature and nutrients, as expected with global warming, will alter bacterial community composition in terms of growth rates and shortened response times. Bacterial communities have various

adaptations that they use throughout yearly cycles to respond to fluctuations in temperature, nutrients and possibly to unfavourable substances like antibiotics (Degerman *et al.,* 2013). Physico-chemical results from Bezuidenhout *et al*. (2017) indicated that the physico-chemical parameters of these two river systems (Marico and Crocodile Rivers) were generally within the target water quality range for a number of applications such as irrigation and other agricultural use. Levels of various parameters in the Crocodile River were elevated indicating potential effects of pollution events occurring. These pollution events could thus be sources of the kanamycin resistant bacteria and the conditions created could thus have resulted in the maintenance of the HPC population with this resistance phenotype.

5.1.1 Kanamycin resistant bacteria level trends at various sites

A closer scrutiny of the results revealed that the Crocodile River sediment had higher levels of kanamycin resistant bacteria during the wet season, with the highest detected at site C1. Surface water samples had higher levels of kanamycin resistant bacteria, with the highest levels at sites C3 and C5 of the Crocodile River and sites M4 and M6 of the Marico River. According to Table 2, the sub-catchment of C1 is influenced by industrial activities and urbanization (Bezuidenhout *et al.,* 2017). There are also a large number of wastewater treatment plants (WWTP) impacting site C1, as well as some of the other sites where the kanamycin resistant bacteria were considerably high (Bezuidenhout *et al.,* 2017). There were also agricultural activities at sites C1, C5, M4 and M6, where some irrigation activities are adjacent to the rivers. Antibiotics may thus enter these aquatic environments through various sources. These could be directly from poorly or untreated sewage, or from grazing of pasture-reared animals (Smalla *et al.,* 1993), or from feed lots or chicken production (Kemper, 2008; Gothwal & Sashidhar, 2015; Singer *et al.,* 2016). These are all activities associated with the river systems under investigation.

Wastewater has been recognised as a major contributor to antibiotics and antibiotic resistant bacteria (Pruden *et al.,* 2013; Grenni *et al.,* 2017) in surface water resources. A non-metabolized fraction of active antibiotics are present in wastewater, since it is not completely broken down in the human body (Kümmerer, 2009). Furthermore, WWTPs are not equipped to remove these compounds (Carvalho & Santos, 2016; Grenni *et al.,* 2017). Bacterial transformation is also common in WWTPs, because bacteria are exposed to selective pressures from various antibiotics (Pruden *et al.,* 2013; Grenni *et al.,* 2017). These active substances, together with the transformed bacteria, are therefore discharged with the effluent into environmental waters (Gothwal & Shashidhar, 2015; Carvalho & Santos, 2016; Grenni *et al.,* 2017).

The Green Drop score is awarded to WWTPs based on the ability to deliver wastewater treatment services (collect, treat and discharge sewage) (DWA, 2013a). A score above 90% is awarded the Green Drop Certification status, whereas a score below 30% means the wastewater system is in a critical state (DWA, 2013a). The Green Drop progress report of 2014 stated that certain WWTPs are in critical and high risk conditions (DWS, 2014). Many plants in the Gauteng and North-West Provence were in high to critical risk conditions. The Zeerust WWTP upstream from site M6 was one of the WWTPs in critical condition that requires immediate intervention (DWS, 2014). If one considers that WWTPs in South Africa discharge approximately 2.1 billion m^3 effluent per annum (DWA, 2011) and that a large proportion of this is untreated, then the contribution to antibiotic resistance becomes enormous.

Environmental water is mainly used as is in the agricultural sector in South Africa. This is a water scarce country and agriculture requires 60% of all available water (DWA, 2011). The quality of the environmental water is impacted by upstream users and return flows from these activities. Water quality may thus have an impact on the production system and could consequently directly contribute towards antibiotic resistance. Furthermore, beef and milk production animals are frequently treated with antibiotics to prevent and/or treat diseases such as mastitis in cattle (Ateba *et al.,* 2010), gastro intestinal disorders in pigs (Kemper, 2008) and other infectious diseases. At sub-therapeutic levels, aminoglycosides and other antibiotics are applied in feedlots for growth promotion (Smalla *et al.,* 1993; Summers, 2006, Kümmerer, 2009; Zhang *et al*., 2009; Kwon-Rae *et al.,* 2011; Moyane *et al.,* 2013). These factors contribute further towards levels of antibiotic resistant bacteria and antibiotic in adjacent aquatic systems.

Higher levels of kanamycin resistant bacteria were generally detected in sediments. These environments possess various ecological niches, where bacteria are in constant competition for space and nutrients (Nesme & Simonet, 2015). Metals are known to precipitate when the pH is high and tend to accumulate in the sediment. The pH of both river systems were neutral to alkaline in range, with the Crocodile River showing elevated levels in the dry season (Bezuidenhout *et al.,* 2017). This could be due to the lack of rain in the dry season causing the parameters in the water to become more concentrated. Nutrients, antibiotics, micro pollutants and bacteria precipitate into the sediment over time. This combined with the metal precipitation from the increased pH create the ideal environment for co-selection and bacterial transformation (Bezuidenhout *et al.,* 2017). This is accomplished through the uptake of beneficial genetic material including ARGs (Smalla *et al.,* 1993; Nesme & Simonet,

2015) and potentially efflux pumps. It is thus feasible that the kanamycin resistant levels from the sediments were higher, because they may have been exposed to a greater concentration of metals and antibiotics compared to those from the water column.

Physico-chemical properties combined with a selective pressure from constant antibiotic release into the environment, may explain the presence of the multi-aminoglycoside resistant bacteria isolated from the various sampling sites. Table 6 indicates that most of the multi-aminoglycoside resistant isolates were originally from the Marico River sediment (n = 21). These results could mean that a substantial fraction of the kanamycin resistant bacteria in Table 4 and 5, detected at the various sites during the wet season, are potentially multi-aminoglycoside resistant.

5.2 Identification and characterisation of multi-aminoglycoside resistant bacteria

Of the multi-aminoglycoside resistant bacteria, 22 sequences could be obtained to confirm their identity. The identity of the isolates, along with their accession numbers are provided in Table 7. Considering Table 7, it seems that sites C1, C4 and C7 of the Crocodile River and sites M3, M6, M7 and M8 from the Marico River were potential hot spots for multi-aminoglycoside resistant bacteria during the wet season. Most of the identified organisms were, however, non-pathogenic environmental bacteria commonly found in similar aquatic environments that they were isolated from. However, there were some organisms that possessed potential pathogenic capabilities.

Oerskovia turbata, isolated from sediment of site C1, is a Gram positive pleomorphic (can alter shape or size), motile organism, which may cause endocarditis and septicaemia in patients that have compromised immunity (McNeil *et al.*, 2004). Cases of infection were reported in patients with cancer and AIDS when implants of catheters and homograph valves were involved (McNeil e*t al.,* 2004). The organism is commonly found in soil environments. Even though few cases have reported the presence of this organism in clinical settings, it is alarming that this organism was isolated from environmental river sediment and possesses multi-aminoglycoside resistant capabilities. It is possible that the organisms could have been discharged from wastewater, since there is a WWTP present in this sub-catchment, or it is feasible that the organism could have landed in the sediment due to runoff from adjacent soil.

The genus, *Chryseobacterium* is widely distributed in aquatic and soil environments and they are described as Gram-negative, non-spore-forming and non-motile bacilli (Kirby *et al.,* 2004; Tai *et al.,* 2006; Hantsis-Zacharov & Halpern, 2007; Pires *et al.,* 2010). Some of the species in this genus cause defects in food products such as meat, fish and dairy products (Hantsis-Zacharov & Halpern, 2007). Others are pathogenic to humans and animals (Hantsis-Zacharov & Halpern, 2007). According to Kirby *et al.* (2004), these organisms are capable of surviving chlorine-treatment water purification processes and are known to colonize in sink basins and taps. These organisms are a threat to human health, since they create potential reservoirs for nosocomial infections (Kirby *et al.,* 2004). Chryseobacteria have been described as the etiological agents of infections from hospital equipment, surgically implanted devices, bacteraemia, endocarditis, ocular and other bodily infections (Kirby *et al.,* 2004).

Several species of this genus were resistant to all aminoglycosides used in this study. The *Chryseobacterium* taiwanese strain (sediment of site C4) and *Chryseobacterium haifense* (sediment of site M6), were originally isolated from raw milk (Hantsis-Zaccharov *et al.,* 2007). It is possible that the isolate could have come from dairy farms in the surrounding area or through wastewater. *Chryseobacterium humi* was isolated from sediment of site M6 and is chemoheterotrophic (Pires *et al.,* 2010). According to Pires *et al*. (2010), this organism is resistant to a variety of antibiotics including gentamicin (10 µg), polymixin B (300 µg) and penicillin G (10 µg). The isolates were susceptible to streptomycin discs at 10 µg (Pires *et al.,* 2010). In the present study, this species was resistant to four aminoglycosides, which included gentamicin and streptomycin, at concentrations of 50 µg/ml. The findings indicate that this organism could have acquired improved fitness from being exposed to metals and antibiotics in the sediment of the Marico River. If this hypothesis is correct, it is cause for concern. Although the organisms isolated from this genus are mostly non-pathogenic environmental bacteria, the isolates in the present study were resistant to all aminoglycosides used and may possess the potential to exchange genetic material to create potential reservoirs for ARGs.

The genus *Sphingobacterium* is described as Gram-negative, non-spore-forming, non-motile rods (Schmidt *et al.,* 2012; Xiao *et al.,* 2013). *Sphingobacterium* strains are usually isolated from a variety of environments including clinical environments, compost of cow dung, farm soil and activated sludge. (Xiao *et al.*, 2013).The multi-aminoglycoside resistant sphingobacteria, which were detected multiple times using the single isolate culturing approach, were mostly isolated from site M6 and one at site C7. *Sphingobacterium alimentarium* isolated from the sediment of site M6, is usually isolated from dairy products

(Schmidt *et al.,* 2012). *Sphingobacterium hotanense,* was isolated from water and sediment of site M6. According to Xiao *et al.* (2013), *S. hotanense* could be resistant to various antibiotics including, but not limited to, ampicillin (10 µg), kanamycin (30 µg), neomycin (30 µg) and streptomycin (10 µg). In the present study, this species was resistant to kanamycin, neomycin and streptomycin at 50 µg/ml. The multi-aminoglycoside resistant isolate *S. hotanense,* identified in the present study was isolated from both water and sediment environments of site M6. This site is influenced by a WWTP, as well as urban and agricultural activities (Bezuidenhout *et al.,* 2017).

The organism *Ensifer adhaerens*, isolated from sediment at site M7, is described as a Gram-negative non-obligative predator of other bacteria in soil environments (Casida, 1982). It can attach to other bacteria and cause cell lysis when nutrients are limited (Casida, 1982; Rogel *et al.,* 2001). A study by Rogel *et al.* (2001) found that this organism had the potential to nodulate when they contain symbiotic plasmids from *Rhizobium tropici.* The latter species is abundant in soil and assist with nitrogen fixation (Rogel *et al.,* 2001). If the multi-aminoglycoside resistant *E. adhaerens* were to possess ARGs, it could be possible for this organism to influence soil plant relationships via plasmid conjugation. A study done by Rathore *et al.* (2015) confirmed the ability of *E. adhaerens* to allow horizontal gene transfer (HGT) into plant genomes under natural conditions.

The genus *Microbacterium* is described as Gram-positive and non-spore-forming rods (Ohta *et al*., 2013). Some of the species are motile, while others are not. Species from this genus are widely distributed in various environments, ranging from fermented food, cheese, human blood, soil, insects and plant roots (Ohta *et al.,* 2013). According to Funke *et al*. (1995), most strains are usually isolated from environmental settings, however, occurrences of isolates from clinical samples are becoming more recurrent. The organism *Microbacterium arborescens* was isolated from the Crocodile River sediment (site C4). A study done by Funke *et al*. (1995) demonstrated that *M. arborescens* is a motile strain that is susceptible to gentamicin, however, the present study found that the organism is multi-aminoglycoside resistant. *Microbacterium saccharophilum* was isolated from the Marico River sediment (site M3). *Microbacterium fluvii* was isolated from the Marico River sediment (site M6). *Microbacterium* species are generally detected in the environment, demonstrating that environmental organisms, seemingly harmless, have the potential to develop resistance to relevant aminoglycosides commonly applied in human and veterinary medicine. The three species of *Microbacterium* were isolated from different sites. This demonstrates the widespread occurrence of these multi-aminoglycoside resistant isolates, that could

potentially share resistance determinants, since *M. saccharophilum* possesses the efflux pump gene (Table 7).

Pontibacter rhizosphera was isolated from the Crocodile River sediment (site C7). This species is described as Gram-negative, motile and straight to curved rod shaped, with pink pigmentation (Raichand *et al.,* 2011). The genus *Pontibacter* belongs to the phylum Bacteroidetes and members of this genus were previously detected in a vast array of environmental sources, such as marine, fresh water and desert habitats (Roiko *et al.,* 2017). A recent study by Roiko *et al*. (2017) isolated and characterized an organism from human blood in a clinical setting. The organism was isolated from a patient with apparent sepsis and antimicrobial susceptibility testing conveyed that the organism was highly resistant to gentamicin (Roiko *et al.,* 2017). As previously mentioned, aminoglycosides are commonly used in human medicine to treat Gram-negative infections (Gad *et al.,* 2011). The fact that a member of this genus, mainly detected in environmental sources, could cause illness in a clinical environment and resist antibiotics associated with the treatment thereof, is alarming.

Bacillus subtilis was isolated from the Marico River sediment (site M6 and M8). *B. subtilis* is one of the most well studied organisms and are populous in soil environments containing *Streptomyces* (Prozorov*,* 2003). This group of bacteria is used for laboratory and biotechnology purposes, since it possesses the potential to secrete microbial inhibiting substances such as bacitracin (Prozorov, 2003). Studies on the transfer of mobile elements between *B. subtilis* and more harmful organisms have been conducted (Bernhard e*t al.,* 1978; Prozorov, 2003; Dai *et al.,* 2012; Gueimonde *et al.,* 2013). The present study found this isolate to be multi-aminoglycoside resistant. This environmental organism is capable of creating a selection pressure on the surrounding bacterial species by secreting inhibitory substances, as well as transferring antibiotic resistant determinants to human pathogens in the same genus. The *B. subtilis* isolated from site M6 possessed an efflux pump gene (Table 7). Although the *B. subtilis* strain isolated is a non-pathogenic environmental strain, the aforementioned studies showed that this organism is capable of horizontal gene transfer. When using plasmids as mobile vehicles for gene transfer, the possibility of gene transfer between pathogenic species such as *B. cereus* and *B. subtilis* is of concern.

Algoriphagus jejuensis was isolated from the Marico River sediment (site M6). This species is described as a Gram-negative, pink pigmented, non-motile, rod shaped and strict aerobic organism, which was originally isolated from seawater (Lee *et al.,* 2012). This organism has the ability to resist gentamicin, kanamycin, neomycin, streptomycin and polymyxin B (Lee *et al.,* 2012). The present study also confirmed this affluence of resistance to aminoglycosides.

Bacteria are known to thrive in waste, surface and drinking water sources (Vaz-Moreira *et al.,* 2014). The fact that *A. jejuensis* possessed multi-aminoglycoside resistant capabilities is a possible risk for potential transformation and exchange of resistance determinants to other bacteria present in the aquatic environment. Site M6, from which the organism was isolated, is considered to be a hot spot for antibiotic resistance bacteria and is influenced by various anthropogenic activities (Table 1).

Pseudoxanthomonas wuyuanensis was isolated from the Marico River sediment (site M6). This organism is described as Gam-negative, aerobic and motile rods with a polar flagellum, originally isolated from saline-alkaline soil environment (Li *et al.,* 2014). According to Li *et al.* (2014), the organism is susceptible to various antibiotics including gentamicin, streptomycin, tetracycline and neomycin to name a few. However, the present study demonstrated that the organism was multi-aminoglycoside resistant. The presence of an organism, with potential multi-aminoglycoside resistant capabilities in the sediment is of concern. Selective pressures from anthropogenic activities at site M6 could aid in the non-species specific transfer of mobile genetic elements, containing resistance determinants.

Sanguibacter soli was isolated from the Marico River sediment at site M6. This organism is described as Gram-positive, non-spore-forming, motile rods that were originally isolated from soil (Kim *et al.,* 2008). *S. soli* may have experienced selective pressure from the anthropogenic activities influencing site M6, which aided in the development of multi-aminoglycoside resistant capabilities shown in the present study.

The multi-aminoglycoside resistant isolates identified in this study are considered to be non-pathogenic, but all have the potential to resist all the aminoglycoside antibiotics, which are relevant for human and veterinary medicine. Anthropogenic activities, especially the discharge of treated or untreated effluent from WWTPs, pose a risk for antibiotic resistance and pathogenic propagation in receiving water environments (Rodriguez-Mozaz *et al.,* 2015; Ju *et al.,* 2016).

Tables 1 and 2 give a description of the activities influencing the water quality in the Crocodile and Marico catchments. WWTPs are widely distributed and urbanisation has an influence, especially in the Crocodile River catchments. In the Marico and Crocodile catchments, natural land cover exists in the form of open bush, grassland and low shrub land area. The Marico catchment has a total of approximately 84% of this land cover type, whereas the Crocodile catchment possesses nearly 69% (Bezuidenhout *et al.,* 2017). These open areas are ideal for livestock grazing and farm activities. Along the Marico River natural

land cover, agriculture, farming and irrigation activities take up much of the land cover type. The influence that agriculture, livestock and crops, as well as wastewater have on the natural aqueous environment, and the bi-directional route between humans, are displayed in figure 1.

Antibiotic resistance is increasing in clinical environments (Nesme & Simonet, 2015). Kanamycin is an important antibiotic, especially considering its role in the treatment of XDR-TB (Bardien *et al.,* 2009). Therefore, monitoring the presence of the kanamycin resistance is extremely important, considering the potential for dissemination of genes involved in antibiotic resistance. According to Bennet (2008), antibiotic modification is possible, because a large pool of resistance genes exists in the environment. The exchange between bacterial replicons and their host is crucial to be able to respond to the selective pressure caused by anthropogenic antibiotic use. According to Vaz-Moreira *et al.* (2014), antibiotic resistant determinants will persist in the environment and could spread, not only to other ubiquitous bacteria, but also human pathogens.

5.3 Molecular detection of ARGs

5.3.1 End-point PCR detection of relevant ARGs from DNA of various culturing methods

In order to understand how anthropogenic activities are influencing bacterial kanamycin resistance, it is important to know what genes are present in the resistance gene pool, as well as the transferability of the genes present, in the Crocodile and Marico Rivers. Results presented in Table 11, suggest that the single isolate culturing approach, performed in 2015, generally generated the least amount of the genes that were screened for in the present study. One *ampC* gene, two *intI 1* and six samples containing efflux pump genes were detected, among the various isolates. This approach is the most time consuming and does not give a clear representation of the state of the environment regarding resistance genes screened for. Results indicate that from the multi-aminoglycoside resistant isolates, the most genes detected, were from sites C2, M3 and M6. Sites M3 and M6 were also identified as potential hot spots for multi-aminoglycoside resistant bacteria and M6 also had the highest levels of kanamycin resistant bacteria in the water and sediment.

All the multi-aminoglycoside resistant bacteria were negative for the *nptII* gene. This gene has been associated with conferring resistance to both kanamycin and neomycin (Ramirez & Tolmasky, 2010). According to Smalla *et al.* (1993), few kanamycin resistant bacteria contain

the *nptII* gene and the phenotypic resistance is often caused by several other resistance mechanisms. Fernández-Martínez *et al.* (2015) demonstrated that aminoglycoside resistance phenotype was not an accurate prediction of the aminoglycoside modifying enzymes conferring the resistance. Frequent occurrence of kanamycin resistant phenotype could be attributed to the consistent application of aminoglycosides in veterinary medicine (Smalla *et al.*, 1993). Cross resistance is a possible explanation for the absence of the *nptII* gene. In the present study, various efflux pumps were detected in the multi-aminoglycoside resistant isolates. MDR efflux pumps can decrease the concentration of aminoglycosides in the cell, affecting susceptibility to the entire class of aminoglycoside compounds (Jana & Deb, 2006). This phenomenon also contributes to phenotypic resistance (Blanko *et al.,* 2016).

The 2017 culture enrichment technique showed a greater frequency of all the genes screened for. This approach provided a better representation of the general state of the environment under investigation. Both genomic DNA and plasmid DNA of bacteria from each sample could be investigated. Presence or absence of genes could be determined when there was nutrient enrichment with or without the presence of kanamycin. The latter combination served as a selective pressure on the bacterial population present in the given sample, selecting for the kanamycin resistant population. Results in Table 11 indicated that more genes were present in the plasmid DNA, when no kanamycin enrichment was involved. Plasmid DNA from sites C2 and C6 (Crocodile River) were more frequently positive during PCR amplification of the resistance genes screened for. When enrichment in the presence of kanamycin was conducted, plasmid DNA from sites M5 and M6 (Marico River) showed frequent amplification for all genes. The opposite effect was observed in the genomic DNA. When kanamycin enrichment was added to the broth, more frequent amplification of the genes occurred. This was especially the case with genomic DNA samples from sites C1 and C3 (Crocodile River). The highest levels of kanamycin resistant bacteria (Table 5) were also detected at these sites. Overall, five *nptII*, sixteen *ampC*, seventeen *intI 1* and fourteen efflux pump genes were detected using the enrichment approach.

The results indicate that efflux pumps and *intI 1* genes may have played an important part in the resistance to kanamycin, since efflux pump genes were found in the genomic DNA more frequently than the plasmid DNA. This result supports the possibility that the bacteria incorporated the efflux pump gene from plasmid DNA to the genomic DNA as means of surviving the selective pressure, supporting the findings from Hall *et al.* (2017). In the study of Hall *et al.* (2017), soil populations were exposed to mercury as a selective pressure,

resulting in frequent incorporation of the mercury resistance transposable element from plasmid to chromosomal DNA.

Considering the results in Table 11, it appears that the *intI 1* gene may also play an important role in plasmid mediated *ampC* when nutrient enrichment is involved. Mobile integrons are known to co-select with plasmid associated ARGs (Stalder *et al.,* 2012). Class one integrase genes are ubiquitous in anthropogenic impacted environments and their role in acquiring and disseminating antibiotic resistance determinants cannot be denied (Stalder e*t al.,* 2012; Gillings *et al.,* 2015). A variety of resistance genes were detected when enrichment was evolved, whether by nutrient or kanamycin enrichment is not relevant. What is important, is that bacterial genomes have high plasticity (Stalder *et al.,* 2012). Class one integrons possess the ability to rapidly adapt to environmental pressure and can readily transfer between bacteria (Gillings *et al.,* 2015).

Antibiotics are considered to be the main force behind the emergence of antibiotic resistance. A study by Knöppel *et al.* (2017) demonstrated that selective forces other than antibiotics could also contribute to the evolution of antibiotic resistance. In that study it was demonstrated that growth medium caused adaptation mutations for growth rates that could decrease susceptibility to various antibiotic classes (Knöppel *et al.,* 2017). Selective pressures caused by anthropogenic activities therefore play an important part in bacterial populations, whether it is fluctuating physico-chemical parameters, antibiotic levels or other antimicrobials in the aquatic environment. Bacteria genetically adapt to various growth conditions and antibiotic selective pressures could utilise these mutations to drive resistance evolution (Knöppel *et al.,* 2017). Constant effluent discharge from poorly maintained WWTPs and improper use of antibiotics in human and veterinary medicine, may all contribute to the proliferation of antibiotic resistance determinants (Manaia *et al.,* 2016). These could be ARGs, efflux pump regulators or mutagenic substances.

Environmental DNA (eDNA) isolated directly from membrane filters did not provide much more information compared to the single isolate approach. Overall this approach yielded two *nptII,* fourteen *intI* 1 and four efflux pump genes. The culture-independent approach provided relevant evidence on the abundance of the *intI 1* gene. The integrase 1 (*intI 1*) gene was detected at all sites using eDNA and is frequently associated with other resistance determinants, as well as mobile genetic elements such as plasmids and transposons (Sundsfjord *et al.,* 2004). Gillings *et al.* (2015) suggested using the *intI 1* gene as a proxy for anthropogenic pollution, because it is linked to genes that confer antibiotic and metal resistance. The *intI 1* gene is also abundant in pathogenic and non-pathogenic bacteria

(Gillings *et al.,* 2015). Class 1 integrons are frequently circulating in human-dominated ecosystems and are present in up to 80% of enteric bacteria present in humans and farm animals (Gillings *et al.,* 2015). It has been suggested that abundance of genes such as the *intI 1* gene, increases during wastewater treatment (LaPara *et al.,* 2011; Gillings *et al.,* 2015).

The *intI 1* gene was detected at all sites using the culture-independent method. This illustrates that the sites sampled are influenced by human activities and the possession of this gene creates diverse advantages to bacteria that possess it. This gene was also frequently detected when screening DNA from the other approaches, demonstrating the potential for ARGs to be transferred not only to plasmids within the same isolates, but also to other bacteria within the same or other species (Rowe-Magnus & Mazel, 2002; Sundsfjord *et al.,* 2004; Hall *et al.,* 2017). This is relevant, because an elusive group of pathogens, collectively known as ESKAPE, owe their adaptive capabilities to plasmids (Ramirez *et al.,* 2014). Plasmids represent a significant threat to human health, since they contribute to the dissemination of relevant resistant determinants. Acquisition of these determinants enables the host to adapt and counteract current antimicrobial therapies, thus contributing to the antibiotic resistance crisis (Carattoli, 2013).

The results are relevant, because integrons are widely distributed in various bacterial species (Stalder *et al.,* 2012). The fact that this gene was detected at every site sampled in the present study, confirms the potential for bacterial adaptation and proliferation. The environmental bacteria in the 14 sites under investigation could therefore become reservoirs of ARGs for pathogenic bacteria.

5.3.2 Quantification of aminoglycoside resistance gene *nptII* and β-lactam gene *ampC*

Occurrence of the *nptII* gene, associated with kanamycin and neomycin resistance in aquatic environments, is suggested to be connected to sewage, animal manure and municipal wastewater discharges (Smalla *et al*., 1993; Zhu, 2007). Aminoglycosides are important in the treatment of Gram-negative infections and are used in combination with β-lactams, because they have a synergistic effect (Sundsfjord *et al.,* 2004; Jana & Deb, 2006; Ramirez & Tolmasky 2010). Furthermore, β-lactams are some of the most commonly used antibiotics in human medicine (Nesme & Simonet, 2015). Investigating the levels of these genes is important in understanding how anthropogenic activities influence the Crocodile and Marico Rivers.

The qPCR assay used in the present study was able to quantify the abundance of *nptII* and *ampC* genes. The average copies per nanogram of DNA were compared to the results from ddPCR quantification. In all the results, the average copies per nanogram DNA was higher when using qPCR than ddPCR for both genes. The two methods usually produce comparable results, but environmental DNA may contain PCR inhibitors. In the present study, the two methods were used to supplement one another.

The culture enumeration method only had two samples that were quantifiable for the *nptII* gene using qPCR. Sample C7 (genomic DNA) contained 1.58×10^4 copies/ng DNA and sample M3 (plasmid DNA enriched with kanamycin) contained 2.12×10^3 copies/ng DNA. Results from Table 10 indicate that the qPCR technique was more successful in quantifying the *nptII* gene from the culture-independent extracted eDNA. Average copies per nanogram DNA ranged from 7.94×10^3 to 1.23×10^5. Sites M4 and M6 yielded the highest average copies per nanogram input of DNA. The qPCR results from the present study are comparable to a study by Zhu (2007) where qPCR results indicated that *nptII* was present in environmental river water from undetectable to 4.36×10^6 copies/L of water sample. The eDNA extracted in the present study was also from one litre of water and is comparable to the study of Zhu (2007).

The melt curves of the *nptII* gene, detected in the eDNA showed variations, that could be a result of sequence variation or mutation in the gene. This could perhaps explain the low copy number of the ddPCR when compared to the qPCR results. It is feasible that the qPCR process was still able to quantify the genes, but recognized the variation in the melting temperatures, whereas ddPCR process possibly observed the mutation as a negative result. Samples were still assumed to be positive in qPCR even if they were below the lowest 5 femtogram standard Cq value.

Quantification of the *ampC* gene yielded higher average copies per nanogram DNA input, ranging from undetectable to 1.58×10^7 copies per nanogram. The highest quantity of the gene was detected from samples M5 (4.90×10^9 copies per nanogram) and M6 (2.86×10^9 copies per nanogram). qPCR results again yielded higher copy numbers of the *ampC* gene than the ddPCR. The ddPCR was, however, able to detect copies of the *ampC* gene in samples that were below the detection limit for qPCR. It is feasible that inhibitors in the DNA were causing the lower copy number in ddPCR or possible mutations in the gene, since the melt curves of some of the samples were also not at precisely 82°C, as the standard was. It has been argued that ddPCR is more sensitive and tolerant to PCR inhibitors and sequence variations compared to qPCR (Sanders *et al.,* 2011). However, in the present study, the

opposite result was demonstrated. According to the findings of Pavšič *et al.* (2016), qPCR can produce 50% higher or lower copy number variations when compared to ddPCR. Pavšič *et al.* (2016) also found qPCR to be more sensitive when compared to two ddPCR platforms. The fact that the copy numbers vary so dramatically could be caused by a variety of factors. These include, but are not limited to, human error in calculation, the PCR process itself, sample load restrictions of ddPCR compared to qPCR, primer specificity, the lack of probes when testing mixed environmental DNA or error in setup of assay during the experiment.

In the treatment of human infections, β-lactam based antibiotics are frequently used (Nesme & Simonet, 2015) and the high abundance of these genes in plasmids suggests they are easily transferable, enabling them to spread from the environment to human pathogens. Plasmid mediated AmpC enzymes have been isolated in patients from clinical settings and risk factors, especially for immune compromised patients, include bloodstream infections from *K. pneumonia,* increased hospitalization and prior treatment with broad-spectrum β-lactamase inhibitors (Jacoby, 2009).

A study by Ju *et al.* (2016) found similar prevalence patterns between β-lactam and aminoglycoside ARG types. This study suggests that the same antibiotic can select for different ARG types and that co-resistance, where various ARGs are located within the same genetic element, such as class 1 integrons, drives this resistance (Ju *et al.,* 2016). This same pattern was observed in a study where the class 1 integrons, containing resistance determinants to aminoglycosides, β-lactam and trimethoprim, were detected in Vibrio strains from aquatic environments in Mozambique (Taviani *et al.,* 2008). These results support the findings in the present study. Although quantification of *nptII* yielded low copy numbers, clinically relevant plasmid mediated *ampC* was abundant. The fact that these resistance determinants are associated with class 1 integrons, suggests that they might be easily transferred, especially since the *intI 1* gene was detected at all sites in the Crocodile and Marico River using the culture-independent extracted eDNA.

5.4 Evaluation of overall results from methods used and existing trends

The results presented in this study showed that aminoglycoside resistant bacteria and relevant ARGs were present in high levels in the Crocodile and Marico Rivers. Culturing methods were used to isolate, identify and characterise kanamycin resistant bacteria, detect relevant ARGs, as well as quantify the *nptII* and *ampC* genes.

It is important to consider that the culturing technique used to characterise the aminoglycoside resistant bacteria does not compensate for the 99% of viable-but-not-cultivable bacteria present in the samples (Smalla *et al.,* 1993; Foughy *et al.,* 2014). The culture based, single isolate screening approach of 2015 could only confirm the presence of multi-aminoglycoside resistant bacteria that were selected from kanamycin supplemented nutrient agar. This means that potentially other multi-aminoglycoside resistant bacteria were discounted, adding some level of bias to this method.

The enrichment broth enumeration approach also has some level of bias. Nutrients in broth can select for certain bacterial species to grow, whereas other organisms that cannot be cultivated under the selected conditions, and may be carrying potential ARGs, are discounted (Hilton *et al.,* 2016). Addition of kanamycin to the culturing broth adds another level of selection for bacteria containing genes that could aid in the survival of the selective pressure.

Results in the present study indicate that the presence of a selection pressure causes greater variation, when the gene detection results are considered. When using culture enumeration, certain bacterial species are selected in a sample and other bacteria are suppressed, adding a level of bias to the method. This does not, however, change the fact that bacteria possessing various relevant ARGs were still detected in the samples. This method is thus useful to investigate the effect that selection from anthropogenic activities may have on the detection of ARGs in aquatic environments.

The direct eDNA extraction from filtered water samples is an example of a method where bias is reduced, since no selection process takes place. DNA from total bacteria in a given sample are extracted, thus the entire population (potential ARGs) are present in the final eluted DNA. Culture-independent DNA extraction complemented the culture enrichment method in assessing the occurrence of relevant genes such as the *intI 1* gene that was detected in every extraction of each site under investigation.

Using eDNA from membrane filtered samples to detect ARGs and pathogenic bacteria in aquatic environments is a widely used approach in various studies (Zhu, 2007; Silva de Faria *et al.,* 2016). Culture-independent techniques are sensitive when detecting low level resistance (Sundsfjord *et al.,* 2004), because they focus on genetic profiles, instead of phenotypic attributes (Hilton *et al.,* 2016). Therefore, the combination of eDNA with PCR assays offer relatively quick and specific results (Hilton *et al.,* 2016).

Results of this study, regarding *nptII* detection and quantification, supports previous studies on cultured kanamycin resistant bacteria that also found few isolates that contain the *nptII* gene (Smalla *et al.,* 1993; Leff *et al.,* 1993; Zhu, 2007). According to Manaia (2017), ARGs that are too low to be quantified, still pose a risk for human health. It is therefore crucial to recognise the potential risks associated with anthropogenic activities (Manaia, 2017). Considering the results in the present study, anthropogenic activities are causing certain trends to occur at various sites, creating 'hot spots' for antibiotic resistance. From all the culturing methods used and analyses performed, the sites that were most frequently positive were M6, followed by C1. The potential 'hot spots' could have been influenced by one or more point sources of pollution that exist in each catchment. Sample sites C1 and M6 are located in a sub-catchments dominated by urban and industrial development, especially in the Gauteng Province for site C1 (DWAF, 2004). There are also agricultural and irrigation activities, adjacent to the Rivers (Bezuidenhout *et al.,* 2017). Surface runoff from streets and inadequate WWTP processes also occur in each the aforementioned sub-catchments (Bezuidenhout *et al.,* 2017). Antibiotic, metal and micro pollutant contamination from these sources, which come into contact with bacteria in aquatic environments can select for antibiotic resistance.

The multi-aminoglycoside resistant organisms identified in this study were mostly non-pathogenic; however, the ability of environmental bacteria to exchange ARGs to and from clinically relevant bacteria is a reality (Ju *et al.,* 2016). *S. hotanense,* identified in the present study, was isolated from both water and sediment environments of site M6. This site is influenced by a WWTP, urban and agricultural activities (Bezuidenhout *et al.,* 2017). It is plausible that the organism has acquired increased fitness from being exposed to the sources that impact the Marico River in this sub-catchment. A study by Rathore *et al.* (2015) confirmed the ability of *E. adhaerens* to allow HGT into plant genomes under optimal conditions. The presence of efflux pumps, and various ARGs identified in this transformed organism provided resistance to kanamycin, streptomycin and adaptive capabilities to neomycin (Rathore *et al.,* 2015). The ARGs screened for in the present study were not detected in the two organisms mentioned above. This may indicate that their resistance capabilities could be provided from other relevant ARGs or mechanisms that were not screened for in the present study. Aminoglycoside resistance is mostly caused by aminoglycoside modifying enzymes (AMEs) (Ramirez & Tolmasky, 2010). AMEs are usually located on mobile elements such as integrons and plasmids, increasing their transferability (Jana & Deb, 2006; Zhang *et al.,* 2009).

Since aminoglycosides are often applied in human medicine, especially in illnesses such as septicaemia and MDR-TB, multi-aminoglycoside resistant infections could be catastrophic (Peterson & Rogers, 2015). In a country such as South Africa, where the public health system has many cases of immune compromised patients, it is important to consider the impact aminoglycoside resistance could have (Bardien *et al.,* 2009). The environmental reservoirs of bacterial populations are a potential source and reservoir of ARGs that could spread to pathogens (Forsberg *et al.,* 2012; Ju *et al.,* 2016).

Dissemination of ARGs among bacteria is a threat to human and environmental health (Zhang *et al.,* 2009). Environmental settings, such as the urban water cycle, where anthropogenic activities occur and water is reused, are important factors in the cycling of resistance (Vaz-Moreira *et al.,* 2014; Manaia *et al.,* 2016). Evidence suggests that ARGs can be exchanged via HGT between bacteria from farm animal or soils and clinical isolates, creating multidrug resistant pathogens (Forsberg *et al*., 2012; Li *et al*., 2015a). This emphasizes the importance of studying environmental bacteria and the influence anthropogenic activities may have on the transferability of ARGs to and from these environments.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

The present study investigated the presence of aminoglycoside resistant bacteria and other relevant resistance genes in the Crocodile and Marico Rivers, using various culturing and culture-independent approaches.

6.1 Kanamycin resistant bacterial levels in the surface water and sediment

Using the standard culturing technique, it was possible to determine the levels of kanamycin resistant bacteria compared to heterotrophic bacteria of the same site. Results showed that the levels of kanamycin resistant bacteria were high, especially in the sediment. The results also show potential 'hot spots' of antibiotic resistant bacteria and how anthropogenic activities could be related to the aminoglycoside resistance proliferation.

6.2 Identification and characterisation of multi-aminoglycoside resistant bacteria

Kanamycin resistant bacteria from the culturing method were purified by means of streak plate and Gram staining. Pure isolates were tested for resistance to gentamicin, streptomycin and neomycin at 50 µg/ml concentrations. Most of the multi-aminoglycoside resistant bacteria were originally from the Marico River sediment, followed by the Crocodile River sediment. Multi-aminoglycoside resistant isolates were identified using 16S rRNA sequencing. Twenty-two isolates were identified and there were various genera present.

6.3 Molecular detection of *nptII* and other relevant resistance genes, using various culturing methods

The multi-aminoglycoside resistant isolates did not contain any *nptII,* but a few were positive for the other genes screened for. Therefore, the single isolate culturing method did not yield much information on the presence of an aminoglycoside resistance gene, even if kanamycin resistant bacteria were abundant. The culture enumeration approach was positive for *nptII* and yielded the greatest variety and abundance of ARGs screened for. This method allowed for selection of antibiotic resistance in plasmid and genomic DNA with nutrient broth, as well as nutrient broth in the presence of kanamycin. Culture enumeration therefore provided four variables to test and gave an estimation of what influence selection from anthropogenic activities could have on bacterial resistance proliferation. For the culture-independent approach, eDNA was positive for a variety of ARGs screened for, including *nptII*. This

approach gave a good overview of the state of the environment under investigation, regarding ARGs present. Depending on the aim of a study, the culture enumeration and culture-independent technique can be used separately or in combination, to investigate the overall state of an environment. These techniques, together with PCR assays, should provide insights on the influence antibiotic pollution sources may have in the aquatic environment.

6.4 Molecular quantification of *nptII* and *ampC* using qPCR and ddPCR

Quantification of *nptII* and a*mpC* was possible using qPCR and ddPCR. Less *nptII* was detected compared to *ampC*. Quantification using qPCR yielded higher average copies per nanogram of input DNA for both *nptII* and *ampC* detection. qPCR results indicated that possible mutations or sequence variations were present in the environmental samples.

The hypothesis for this study was correct that aminoglycoside resistance is present in the environment. However, enrichment enumeration and culture-independent methods provided better resolution regarding the overall presence of ARGs in the River systems.

6.5 Summary of the study

The data presented in this study demonstrated that aminoglycoside resistant bacteria are present in the Crocodile and Marico Rivers and anthropogenic activities may have an influence on antibiotic resistant bacteria proliferation and prevalence patterns in aquatic environments. It also demonstrated that ARGs, associated with mobile elements such as integrons and plasmids, are abundant in the rivers. This serves as a potential risk for horizontal gene transfer between environmental bacteria and human pathogens. It can therefore be concluded that the study was successful and all objectives were achieved. This study emphasizes the importance of examining antibiotic resistance in the environment. Aminoglycosides are frequently used in human and veterinary medicine and resistance thereof, by clinical pathogens, could be catastrophic.

6.6 Recommendations for further studies

Considering the experimental results, the following recommendations should be considered:

- The *nptII* gene as detected in eDNA, presented variations in the melting curves during qPCR analysis. In order to determine what the variations mean, next generation sequencing of the gene can be done.
- Antibiotic resistance genes detected, using the single isolate culturing method could be sequenced to determine if the gene present in the multi-aminoglycoside resistant isolate possessed any sequence variations or mutations.
- Other relevant antibiotic resistance genes, particularly associated with mobile genetic elements discharged from wastewater treatment plants, need to be investigated in the Crocodile and Marico Rivers. Special focus needs to be placed on cross-resistance genes, such as multi-drug resistant efflux pumps and integrons, since they could serve as a proxy for anthropogenic pollution. Quantification of these genes using primer specific probes should also be considered to determine the role that integrons have on the resistance prevalence at each site.
- Further study as to what are the main drivers of resistance in these river systems need to be addressed. This can be done by doing surveys of the sampling points and surrounding environment. Analysis of the water itself can also be done to determine which antibiotics, as well as at what levels they are present. It is also important to investigate the social aspects regarding the water use of these two rivers and to what extent humans come into contact with the bacteria possibly carrying antibiotic resistance genes. This should raise awareness of the impact factor antibiotic resistance genes in the water could have on immune-compromised individuals, particularly in rural communities.
- Metagenomic exploration of relevant ARGs present at each site over two years can also be done and compared to the anthropogenic activities influencing each site. This is relevant to determining the influence humans have on antibiotic resistance gene proliferation over time.

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APPENDIX A

Table 1: GPS coordinates of the Crocodile River sampling sites.

Site	Name and description of site	S	E
1	Before Brits (K1)	25° 40.9” 9.71’	27° 47” 33.66’
2	After Brits (K2)	25° 32” 58.05’	27° 42” 50.84’
3	Pienaars River (K3)	25° 06” 24.7’	27° 33” 55.2’
4	Koedoeskop bridge (K4)	25° 03” 59.6’	27° 31” 06.2’
5	Croc Thaba (K5)	24° 39” 53.63’	27° 22” 40.39’
6	Croc after Thabazimbi (K6)	24° 24” 5.35’	27° 05” 51.73’
7	Croc turn (K7)	24° 12” 57.30’	26° 53” 54.28’

Table 2: GPS coordinates of the Marico River sampling sites.

Site	Name and description of site	S	E
1	M1 - Marico Eye.	25°47’22.16 ’	26°21'59.85 ’
2	M2 – 20 km downstream of Eye.	25°39’34.64 ’	26°26’01.0 ’
3	M3 – Sterkstroom 5km before its confluence with Marico River.	25°38’50.82 ’	26°29’20.47 ’
4	M4 – 10 km above Marico Bosveld Dam.	25°35’19.28 ’	26°24’38.85 ’
5	M5 – Klein Marico River 5 km above Bospoort Dam.	25°32’31.80 ’	26°06’14.26 ’
6	M6 – Klein Marico River 1 km below Bospoort Dam.	25°30'59.47 ’	26° 9'30.72 ’
7	M7 – Marico River immediately below Marico Bosveld Dam.	25°27’42.51 ’	26°23’30.92 ’
8	M8 – Marico River at Derdepoort.	24°50’42.62 ’	26°29’11.08 ’