Molecular Profiling of Antibiotic Resistant Bacteria and Genes in Raw and Drinking Water

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ABSTRACT

Antibiotic resistant bacteria and antibiotic resistant determinants are regarded as emerging public health threats in drinking water systems. Heterotrophic Plate Count bacteria in distribution pipes are used as direct parameters to assess the conditions of water in distribution systems. Moreover, the presence of pathogenic features in the organisms may also indicate the potential public health risks they may pose to consumers when present in the drinking water. The current study investigated the pathogenic potential of Heterotrophic Plate Count bacteria present in two drinking water distribution systems. A total of 40 samples were collected over four seasons from August 2016 to May 2017. Physiochemical analysis of the bulk water samples revealed that the pH levels were mostly alkaline and ranged between 7.37 and 9.62 for both the Mmabatho and Mafikeng Water Treatment Plants throughout the sampling period. Electric conductivity values for water from both plants exceeded the SANS 241 specified limits and ranged between 239 - 846 µs/cm per 100ml of bulk water sample. A total of 202 isolates were detected based on differences in pigmentation of colonies on chromogenic R2A medium. Isolates were screened against a panel of 11 antimicrobial agents using Kirby-Bauer agar disc diffusion method. A total of 68 multi-drug resistant isolates defined as those showing resistance to 3 or more antibiotics belonging to different classes were selected and further screened for pathogenic determinants such as Haemolysin, Proteinase, DNase, Oxidase and Lipase production. A large proportion 62% (42) of these isolates were β haemolytic, while only 27% (18) were α haemolytic. In addition, only a small proportion 19% (12) of the isolates produced the DNase enzyme. When screened for the presence of antibiotic resistant genes, by use of PCR assay a large proportion 39 (55.8%) of the isolates possessed the *strA* gene. On the contrary, the *strB* gene, *aadA* gene, *dfrb1, dfrb2* gene, *tetA* gene and *blacTX-M* were detected in 30.8% (21), 16.2% (11), 19.1% (13), 19.1% (13), 4.4% (3), and 7.4% (5) of the isolates respectively. The identities of the isolates was determined by bacterial 16S rRNA sequencing and results revealed that isolates belonged to the families *Enterobacteriaceae, Paenibacillaceae, Bacillaceae, Yersiniaceae, Xanthomonadaceae* and

Flavobacteriaceae that of which some members are of clinical importance. Data obtained in this study suggested that some members of the HPC isolates identified in the water bodies may pose severe public health complications to consumers as well as enormous therapeutic challenges to both the medical and veterinary professions.

Keywords: Antibiotic resistance, Heterotrophic Plate Count Bacteria, water distribution systems, drinking water quality

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CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 INTRODUCTION

Water is a fluid that contains no organic nutrients, however it is essential to all living organisms (Willey *et al.*, 2008). 'Drinking water' is defined as water suitable for human consumption and for use in domestic activities (WHO, 2002). In South Africa access to safe drinking water is a basic human right (Constitution of the Republic of South Africa, 1996), and this mandate has been intrusted to the Department of Water and Sanitation, which is the custodian of South Africa's water resources. In order to fulfil its mandate, the Department of Water and Sanitation's vision is to be "a dynamic, people centred department, leading the effective management of the nation's water resources, to meet the needs of current and future generations". This is achieved by striving to ensure that all South Africans gain access to clean water and dignified sanitation, thereby promoting effective and efficient water resources management to ensure sustainable economic and social development (Mothetha *et al.*, 2013).

Despite this, adequate supply of portable water remains a great socio-economic problem in most developing countries including South Africa (Riley *et al.*, 2011), and the situation is even worse in rural areas where there are still issues of backlog in portable water supply (Mothetha *et al.*, 2013). In the year 2012 the Organization for Economic Cooperation and Development (OECD) revealed with great concern that global water demand is expected to increase by an estimate of 55% by 2050 (World Water Assessment Programme, 2015). This was evident by the fact that by 2015 the North West Province had a population of 3,689,320 inhabitants with 57% (2,102,912) living in rural areas and a relatively large

proportion (25.5%; 535,493) of these households did not have access to basic water services (Bluedrop Report, 2014/5). Against this backdrop, individuals in these rural communities revert to raw or untreated water from alternative sources, which do not meet safe drinking water standards as well as the CDC guidelines for household water quality (WHO, 2003).

The socio-economic impacts associated with the consumption water that is usually of low quality from unprotected sources are very severe due to the potential of causing waterborne illnesses associated with millions of deaths especially among infants worldwide (Riley *et al.*, 2011). This therefore implies that the provision for safe drinking water is of great importance for consumers and water supplied to communities meet the minimum requirements indicated in the South African National Standard (SANS), 241 Drinking Water Specification. The SANS 241 regulatory document outlines that water intended for consumption should not pose any significant health risk over a lifetime for which it is consumed and should also not produce any hyper-sensitive reactions despite the immune status of the consumer. However, in a province like the North West in which 501 066 individuals are living with HIV and AIDS (Nicolay and Kotze, 2009; (National Department of Health SA, 2013), there is need to constantly monitor the quality of water supplied to consumers.

Quality drinking water is not only a requirement for promoting and maintaining public health, it also has a direct influence in food security and economic development (Annual National State of Water Resources Report, 2012). South Africa is classified as water stressed country with very few surface water bodies and therefore developmental activities that require water supply is largely dependent in groundwater sources (Basson,

1998). Irrigation alone accounts for 62 % of the national available water budget (SSA, 2010). In the North West province, 80% of water used for domestic and agricultural purposes is irrigated from underground sources (NWPEO, 2008). Due to high industrial activities in the province particularly mining and farming, there are always severe challenges resulting from the deterioration of water quality as well as its availability (NWPEO, 2008; IPD-review, 2015). Furthermore, technical difficulties resulting from poorly maintained water treatment infrastructures as well as service delivery backlogs compromise the production of adequate potable water.

To ensure compliance, water production facilities should be evaluated constantly for their purification efficiencies. However, the current water treatment infrastructures or facilities have proven to be inadequate to meet the challenges of water contamination (Pruden, 2014). Waste water is generated from both industrial processes and domestic household activities and therefore a variety of compounds including gasoline additives, surfactants, endocrine disruptors, and pharmaceuticals and personal care products (PPCP) have been detected in waste water (Ashton *et al.*, 2008; Picó and Barceló, 2015; Cizmas *et al*, 2015). In addition, the frequent use of antibiotics in human and veterinary medicine even in more advanced countries (Silbergeld *et al.*, 2008; Brunning, 2014; Rosi-Marshall and Kelly, 2015) has resulted to the detection of antimicrobial metabolites in environmental water systems which may lead to undesirable effects on consumers (Luo *et al.*, 2014; Cizmas *et al,* 2015; Evgenidou *et al.*, 2015) as well as the development of multiple antibiotic resistant strains.

In South Africa, waste water treatment plants are required to adhere to a set of basic minimum standard methods designed to remove both chemical and microbial

contaminants in waste water (DPW, 2011). Although the methods can be improved to enhance quality of the finished product, standard protocols require filtration/screening, analysis in an anaerobic reactor, flocculation, sedimentation, disinfection and disposal (DPW, 2011).The occurrence of droughts coupled with the increasing incidence of water scarcity has amplified the need for water re-use (Lapworth *et al.,* 2012). Aquifer recharge aids in increasing freshwater resources for drinking water production, and this mimics the natural groundwater formation processes. Surface water is typically used for the artificial recharge and this therefore indicates that, the presence of potential long-term contaminants in groundwater might occur due to short- circuit natural attenuation mechanisms in the soil and subsurface (Lapworth *et al.,* 2012). This therefore implies that constant evaluation of current water treatment processes particularly their potential to remove Pharmaceuticals and Protective Care Products (PPCP) and microbial contaminants is a great priority. Bergeron *et al.,* 2015 highlighted that chlorination during water treatment does effectively remove most bacteria and may also promote the release of free bacterial DNA.

Water treatment facilities receive waste water from various sources for treatment and may serve a bridge for the movement of these contaminants between the environment and animals and to consumers (Cizmas *et al,* 2015). Given that the presence of microbial contaminants in drinking water is not only limited to faecal contamination but may include organisms that may colonise distribution pipes and be present in the finished product, it is important to understand threats in drinking water systems from catchments to consumers and back to the natural water systems, which may compromise water quality. This may significantly improve treatment strategies thus effectively meeting public health requirements of consumers (Guidance to drinking water quality, 2011). Despite the fact

that previous studies in the study area indicated the presence of specific target pathogenic microorganisms in water that is intended for human consumption or in waste water to the best of our knowledge, there is no report on the occurrence of multiple antibiotic resistant organisms in the water treatment and distribution systems. This study is therefore designed to determine molecular profiles of antibiotic resistant bacteria and their associated resistance genes in raw and treated drinking water sources in the Mafikeng area, North West Province, South Africa. The findings of the study may provide identification of resistant organisms may assist in understanding agent causing infectious diseases (Woese *et al.*, 2000). Because 16S rRNA gene universal to all bacteria, it is not identical for all organisms as it differs in taxonomic groups. Through sequencing of the 16S rRNA gene in bacterial isolates sequence to be obtained, data may assist in distinguish phenotypic resistance to antimicrobial agents, which may expand on poorly previously described organism (Clarridge, 2004). The study will further give an indication of the efficiency of current water treatment processes as well as an assessment of finished product and hence indirectly indicate the potential health complications on consumers relying on the potable water.

1.2 PROBLEM STATEMENT

In the northern, inland provinces of South Africa many Waste Water Treatment Plants (WWTPs) are either not working efficiently or they are not fully operational, thus creating opportunities for wastewater to pollute environmental water bodies (DWA: Green Drop Report 2012). South Africa is classified as water stressed country that is situated in a semi-arid region and due to these challenges some municipal regions employ the water re-use scenario in providing potable water to consumers. The water re-use strategy involves the use of treated wastewater for beneficial purposes, which increases a

community's available water supply and makes it more reliable, especially in times of drought. However, due to population growth as well as increased urbanisation, contamination of water bodies has been on the increase and this therefore presents severe challenges to water treatment processes.

Many of the water provision systems in these northern inland provinces in South Africa are designed as open systems where water is used only once before being discarded. In such scenarios communities that are situated downstream from neighbouring towns, agricultural production systems and industrial/mining areas become indirect re-users. This is, however, not the case for a town such as Mafikeng. The drinking water provision system in Mafikeng is based on source water from both surface and groundwater sources (Mulamattathil *et al.*, 2014). Surface water is abstracted from the Modimola dam at a point that is downstream the WWTP, thus this is water that has been released from the WWTP upstream is then pumped in the dam, providing a semi-closed water use system, thus providing opportunities for pathogenic microorganisms to be transmitted to the finished product.

Recent studies in the North West Province have focused on the generation of data sets on the occurrence of antibiotic resistance bacteria (ARB) and their associated antibiotic resistance genes (ARG) on specifically isolated cultures. Data generated from such studies are likely to be biased since they usually fail to consider the total bacteria population (Carraro *et al.,* 2011). It is also challenging to evaluate the impact of data generated from an epidemiological point of view. This study is designed to monitor the dynamics of physiological parameters, antibiotic resistant bacteria and genes in raw and drinking water in selected water production facilities and associated drinking water

distribution systems in Mafikeng. Data generated will be evaluated to assess the public health implications it may have for current water production practices and the relationship that exist between source water and the targeted facilities. It is therefore envisaged that data obtained may provide suggestions for future water quality monitoring practices and may also be useful for future decisions regarding available water re-use strategies.

1.3 AIM AND OBJECTIVES

1.3.1 Aim

The aim of this study was to isolate and gather profiles of Antibiotic Resistant Bacteria and antimicrobial resistance gene determinants from two water distribution systems in the Mafikeng Local Municipality.

1.3.2 Objectives

The objectives of the study were to:

- determine the physio-chemical parameters of the water samples
- assess the microbiological quality of water samples by determining indicator bacteria counts
- isolate different antibiotic resistant bacteria present in the samples
- confirm the identities of ARB using gene specific PCR and bacterial 16S rRNA gene sequence analysis
- determine the virulence gene profiles of the isolates using phenotypic and genotypic techniques

CHAPTER 2

LITERATURE REVIEW

2.1 General overview

The contamination of water bodies with waterborne pathogens has been reported to have a direct link with waterborne-related diseases and which has been a major water quality concern throughout the world (EPA, 2012). In fact, contamination of water bodies with pathogenic microbes especially bacterial strains is a serious issue which makes it important to not only recognise and understand the modes of contamination but also determine their public health implications (EPA, 2012). Against this background, incidents of disease occurrences as a result of the consumption of contaminated water has been widely documented (Riley *et al.*, 2011).

Despite the fact that in the past only pathogens that are associated with the occurrence of diseases such as cholera and typhoid fever were the main concern in water contamination, more recently, newly recognised microbial contaminants pose greater challenges to water safety (WHO, 2003). Various studies have shown the presence of multiple antibiotic resistant opportunistic and pathogenic bacteria species in water catchments that are unprotected sources but used for drinking and household activities as well as treated drinking water in the North West Province, South Africa (Pavlov *et al.*, 2004; Ateba and Mbewe, 2011; Mulamattathil *et al.*, 2000; Ateba and Mbewe, 2013; Bezuidenhout *et al.*, 2013; Ateba and Mbewe, 2014; Cartens *et al.*, 2014; Mulamattathil *et al.*, 2014). The findings of some of these studies revealed that the isolated bacterial species also harboured virulence determinants such as production of extracellular

enzymes associated with cytotoxicity (Pavlov *et al.*, 2004); resistance to amoeba (Carstens *et al.*, 2014) coupled with antibiotic resistant genes.

Bacterial strains that harbour antibiotic resistance determinants as well as virulence genes present a serious health risk to consumers (WHO, 2011). Particularly in a country like South Africa with large number of immune-compromised individuals due to the high HIV/AIDS incidence (SSA, 2016; WHO, 2011). By the year 2016, the South African midyear population estimates indicated that 7.03 million individuals in the country live with HIV, which accounts for 12.7% of the country's population (SSA, 2016). In addition to this, it is also reported that in developing countries, particularly those in Africa, and not excluding South Africa, where water-borne diseases infect millions of consumers yearly (Fenwick, 2006). This therefore explains why each year 3.4 million people, mostly children, die from water-related diseases worldwide (WHO, 2014). These waterborne complications are due to the fact that reports from the World Health Organisation reveal that over 2.6 billion people lack access to clean water (WHO, 2010) and this accounts for the high mortality particularly among children. It is therefore important to improve the quality of water that is intended for human consumption since this can significantly reduce the global disease burden on consumers (WHO, 2010).

2.2 Antibiotics

2.2.1 General overview

The term antibiotics denote any class of organic molecule that inhibits or kills microbes by directly interacting with specific bacterial target(s) (Dougherty and Pucci, 2012). Initially discovered from environmental microorganisms, where they are believed to serve as an ecological advantage to inhibit competitors (Martínez, 2008), the discovery of antibiotics

has been of great benefit in the treatment of human and animal bacterial infections (Brunning, 2014). Antibiotics are also used in agriculture, specifically in veterinary medicine as biocides as well as feed additives for livestock and poultry (Silbergeld *et al.*, 2008). This therefore indicates that antibiotic resistance affects various sectors including human medicine, veterinary medicine, animal husbandry, trade, agriculture and the environment (WHO, 2011).

There are studies which suggest that some antibiotics might be involved in intercellular signalling at the low concentrations in most natural environments (Martinez and Baquero, 2009; Baquero *et al.*, 2008). Microbial analyses of rock surfaces dating back 4 million years revealed the presence of multidrug resistant bacteria that were stable in the presence of diverse antibiotics currently used in human medicine (Keen and Patrick, 2013). R-factors also known as resistance plasmids have also been documented in pristine environments in the first studies of antibiotic resistance in the environment by Gardner *et al.*, 1969 as cited by Martínez, 2012. Furthermore, the quinolone resistance determinant *Qnr* was found to be present in the chromosomal gene components of waterborne bacteria prior the introduction of the antibiotic, and again in areas where quinolones have not been utilized (Martinez and Baquero, 2009; Poirel *et al.*, 2005). This therefore indicates that the occurrence or absence of resistant genes and resistant bacteria in a given water body cannot be concluded without proper surveillance studies.

There are various classes of antibiotics, grouped according to the mechanisms in which they inhibit bacterial growth (Kohanski *et al.*, 2010). They target specific essential cellular functional sites thereby inhibiting cell wall, protein and nucleic acid synthesis which directly inhibit cell functions as well as other metabolic processes (Brunning, 2014). The

overall impact of the mechanisms of action of antibiotics to the cell results in two outcomes (Kohanski *et al.*, 2010). The first is bacteriostatic that inhibits bacterial growth and reproduction. The second outcome is bactericidal resulting in cell death (Kohanski *et al.*, 2010). The class of bactericidal agents includes antibiotics such as β-lactamase, named as such due to the presence of beta-lactam ring which functions by inhibiting bacterial cell wall biosynthesis (Kohanski *et al.*, 2010). Penicillins are the most prescribed and utilized bactericidal antibiotics while cephalosporins such as carbapenems, and monobactams are examples of beta-lactam antibiotics. Aminoglycosides inhibit bacterial protein synthesis by inducing inaccurate mRNA translation and these include streptomycin, kanamycin, tobramycin, gentamicin, and neomycin (Brunning, 2014).

Tetracyclines and Chloramphenicol are bacteriostatic agents that are considered to be broad-spectrum antibiotics due to the fact that they are highly effective and possess high efficacy against both gram negative and positive bacteria (Brunning, 2014). Tetracyclines are widely used for both human clinical complications as well as in animal feeds as a growth promoter (Chopra and Roberts, 2001). Other classes of antimicrobial agents include Macrolides, Glycopeptides, Oxazolidinones, Ansamycins, Quinolones, Streptogramins and Lipopeptides (Willey *et al.*, 2008; Brunning, 2014).

2.2.2 Mechanisms of antibiotic resistance

The occurrence of infectious diseases in humans and animals is part of their lives and therefore may be unavoidable. Fortunately, there are many strategies available to help protect humans and animals from an infection or to treat diseases once clinical signs and symptoms have developed in a patient (Rossolini *et al.*, 2014). Antibiotics are a range of

powerful drugs that destroy or slow down the growth of bacteria and therefore provide treatment options for diseases caused by bacteria species (Willey *et al.,* 2008).

Antibiotics were discovered in the 1940's, approximately a decade later in 1953, a *Shigella* outbreak caused by multiple drug resistant *Shigella dysenteriae* strains occurred in Japan (Brunning, 2014). The causative organism exhibited high levels of resistance to chloramphenicol, tetracycline, streptomycin and sulphonamide (Brunning, 2014). This was one of the first documented confirmation of previously susceptible microbial species displaying an evolutionary trend of bacterial resistance towards antibiotics in medical settings. Given that antibiotic resistance is known to be rising to dangerously high levels worldwide, new resistance determinants are constantly being identified and are spreading globally, other clinically important multiple drug resistant bacteria that have risen since the introduction of antibiotic include methicillin/oxacillin-resistant *Staphylococcus aureus* (MRSA) (WHO, 2014); vancomycin-resistant enterococci (VRE) (Rossolini *et al*, 2014); extended-spectrum beta-lactamases (ESBLs) and penicillin-resistant *Streptococcus pneumoniae* (PRSP) (Todar, 2012; Keen and Patrick, 2013). The development of resistance by bacterial species to antibiotics in a clinical setting does not only compromise the treatment of infectious diseases but also affects therapeutic procedures as transplants as well as anticancer therapies that involve immune-comprised individuals (Martínez, 2012).

Antibiotic resistance by a bacterial cell is achieved when the target molecule is modified or the concentration at which sensitivity is reached for sensitive organisms is not reached (Martinez and Baquero, 2009). Genetic alterations by punctual mutations, insertions or deletions are another form through which bacteria adapt and evade destruction by an

antimicrobial agent (Džidic *et al.*, 2008, Bergeron *et al.*, 2015). Spontaneous mutation frequencies for antibiotic resistance determinants among bacteria range from 10^{-8} to 10^{-9} (Martinez and Baquero, 2009) thus indicating their potential to spread widely once developed. Given that only hyper-mutant phenotypes of bacterial strains able to adapt to stress (Martinez and Baquero, 2009), under persistent selective pressure, once a resistant determinant develops the trait will dominant in the specific habitat.

Among bacterial species, mutation induced resistance can occur through a modification of merely a few bases (Džidic *et al.*, 2008). In *Escherichia coli* analysis of genomic data has revealed that alterations in at least seven amino acids accounted for the expression of the *gyrA* genes and three *parC* genes that were linked to for quinolones resistance (Džidic *et al.*, 2008). In addition, the *mecA* gene that is carried in the staphylococcal cassette chromosome results in an altered penicillin binding protein PBP2a, thus resulting in the development of methicillin-resistant *S. aureus* (Katayama and Hiramatsu, 2000). On the contrary, the *mecC* gene which possess 70% nucleotide identity to *mecA*, expresses the same phenotypic properties as *mecA* gene in isolates with the exception that isolates harbouring the former determinants are likely to be resistant to oxacillin (Stegger *et al.*, 2012). The *mecC* homologue poses significant diagnostic problems with the potential of host cells to be misidentified as methicillin-sensitive *S. aureus* and this may have severe health consequences for individual patients and for the surveillance of MRSA (Kim *et al.*, 2013).

Genetic mutation resulting in protein modification on target molecules within a cell is evidently displayed against some medically important antibiotics (Katayama and Hiramatsu, 2000). Triclosan is used as a broad-spectrum ingredient that is added to many

consumer products to help reduce or inhibit contamination with both Gram negative and Gram-positive bacteria, with the exception of those belonging to the genus *Pseudomonas* (Zhu *et al.*, 2010). Resistance to triclosan results from the presence of the *fabI* allele that encodes an additional enoyl-ACP reductase enzyme on the desired triclosan target of triclosan sensitive organisms, rendering isolates harbouring the genetic determinant insensitive (Zhu *et al.*, 2010). β-lactamases that hydrolyse penicillins, cephalosporins, monobactams, and Carbapenems as well as aminoglycoside-modifying enzymes and chloramphenicol acetyltransferases are three enzymes known to facilitate deactivation of antibiotics belonging to different classes (Zhu *et al.*, 2010; Santajit and Indrawattana, 2016). These resistant determinants are mainly transmitted between bacterial species through mobile genetic elements known as transposons and are known to exhibit resistance by inducing protein modifications or inactivation of antimicrobial agents designed to eliminate target organisms (Bergeron *et al.*, 2015).

Resistance may also occur through inherent properties, that results from either the lack of the transport system or target of the antimicrobial agent in the specific organism (Willey *et al.,* 2008; Martinez and Baquero, 2009). In some situations, antibiotic resistance may occur as a result of the fact that outer membrane of the organism prohibits the uptake of the antibiotic into the cell mainly due to the active efflux of the antibiotic out of the cell (Schmieder and Edwards, 2012). Bacterial efflux systems are located at the cytoplasmic membrane of bacterial cells and allows for the exclusion of either a specific drug or class of antimicrobial agents from the bacterial cells (Dougherty and Pucci, 2012). Genes that code for efflux mechanisms are mostly localized on transposons, integrons and plasmids while chromosomal efflux genes are most often expressed during certain growth characteristics such as in biofilms (Dougherty and Pucci, 2012). Biofilms are a collective

of one or more types of microorganisms that can grow on many different surfaces and display much greater resistance to antibiotics than their free-living counterparts.

Other bacterial cells achieve resistance through bypassing the inactivation of a target enzyme in a mechanism that is termed target bypass and this is often common among trimethoprim and sulphonamide resistant bacteria (Dzidic *et al.*, 2008). The mechanism involves bypassing the inhibition of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) enzymes that are involved in tetrahydrofolate biosynthesis and are reliable target sites by trimethoprim and sulfonamides, respectively. This results in the production of another enzyme that has a low affinity for the antibiotics thus resulting in resistance (Dzidic *et al.*, 2008). The need to constantly conduct surveillance studies for these resistance determinants and phenotypes may have significant improvements on public health.

In addition to these mechanisms, genetic determinants that confer bacterial resistance may also be transferred between bacterial cells with or without any direct contact (Willey *et al.*, 2008). Some resistance determinants are transferred through horizontal gene transfer by transduction (transmission of DNA through a bacteriophage vector), transformation (acquisition of naked DNA from the environment), or conjugation (DNA transfer through cell to cell contact) (Salyers *et al.*, 2004; Willey *et al.*, 2008; Martínez, 2012). Given that horizontally transferrable genes encode for many adaptive traits including virulence factors, antibiotic resistance, detoxifying agents and enzymes for secondary metabolism that are beneficial to the bacterial strains, they may present severe therapeutic challenges to patients (Martínez, 2008). However, as these acquired genetic determinants require specific but varied selective pressures for them to be expressed the

implementation of strategies that significantly reduce or limit these selective forces in the environment may have great epidemiological implications (Diaz *et al.,* 2000).

Amongst bacterial species, antibiotic resistance is most often associated with the acquisition of genetic determinants by previously susceptible bacteria (Martínez, 2012; Zhang and Feng, 2016). However, in addition to acquired resistance, bacterial species may display intrinsic resistance to different classes of antibiotics which does not depend on previous exposure to antibiotics and therefore has no association with horizontal gene transfer (Martínez, 2012). This explains why antimicrobial resistant organisms and antibiotic resistant determinants have been isolated in areas where there has been no evidence of human activities as well as the use of antimicrobial agents (Gardner *et al.*, 1969; Martínez, 2012; Keen and Patrick, 2013). A typical example is the discovery of the *qnrA* resistant gene that codes for resistance to the synthetic quinolone antibiotics that are prevalent among plasmids in human pathogens and has been reported to be part of the chromosomal makeup of a non-antibiotic producing environmental *Shewanella algae* (Poirel *et al.*, 2005). However, it is important not to exclude the transfer of these genes to other host strains that originally lack the biochemical and genetic traits associated with the genes as well as the possibility of modularity effects (Martinez and Baquero, 2009; Baquero *et al.*, 2008; Gould and Vrba, 1982). Modularity is an attribute of a system that can be decomposed into a set of repeated, conserved cohesive entities that are loosely coupled (Martinez and Baquero, 2009; Gould and Vrba, 1982).

Given that transferable elements are also not present at the same frequencies in different environments, the insertion of acquired resistant determinants may also differ significantly among bacterial species within regions (Martinez and Baquero, 2009). In addition, the

transformation efficiency is defined as the efficiency by which cells can take up extracellular DNA and express genes encoded by it very significantly among different species and genus, which indicates that evolution of antibiotic resistant strains may also vary from one region to another (Baquero *et al.*, 2008). This phenomenon explains the reasons for the expression of resistant phenotypes as well as genotypes by previously susceptible human pathogens. However, the continuous presence of antibiotics even at low concentrations in the environment may act as contributors for gene transfer (Jeters *et al.*, 2012).

The persistence of these elements not only describes the magnitude of environmental pollution but also their presence even in the absence of selection pressures (Martínez, 2012). Against this background, it has been reported that the natural resistome of bacteria possess an extensive array of potential resistance genes which may express different resistant potentials in different microbial populations residing in different environmental stress conditions (Poirel *et al.*, 2005; Martínez, 2012; Keen and Patrick, 2013). The need to constantly screen for resistant determinants coupled with investigations designed to assess the concentrations of antimicrobial metabolites in environmental samples are of great epidemiological importance.

2.2.3 Pathogenicity of Antibiotic Resistant Bacteria

Global antibiotic consumption trends were monitored for a decade between the years 2000 and 2010 and findings indicated a sharp increase of 36% of antibiotic consumption within that period (Van Boeckel *et al.*, 2015). Given that the BRICS countries including Brazil, Russia, India, China, and South Africa accounted for 76% of this overall global increase in antimicrobial consumption (Van Boeckel *et al.*, 2015). These reports may

indicate that despite the intention to use antibiotics to drastically reduce deaths and complications caused by bacterial infections, this increase in the usage of antimicrobial agents often promotes the emergence and spread of resistant strains (Almagor *et al.*, 2018). This further leads to the exhaustion of this limited resource with severe implications to public health even in South Africa. Antibiotic usage extends to agriculture and aquaculture industries, where they are used as additives to animal feed as well as for prophylaxis (Dzidic *et al.*, 2008). Half of the antimicrobial agents produced globally are utilized within these two industries (Dzidic *et al.*, 2008). Despite the fact that continuous utilization of antibiotics in distinct industries may seem isolated, the problem of antimicrobial resistance knows no boundaries. In addition, drug-resistant microbes are capable of moving among people and animals, and from one country to another without any notice resulting in the unintentional cross introduction of antimicrobial agents or their metabolites to other territories (Jacob *et al.*, 2008).

In the fermentation of corn starch to ethanol, a lucrative industrial process in which antibiotics such as penicillin, erythromycin, virginiamycin and tylosin are commonly used to optimize the process and also to eliminate bacterial population that may compete with the yeasts during the fermentation process (Jacob *et al.*, 2008). The by-product of this process is a nutrient-rich corn mash termed distillers' grains, which is regularly used in livestock or poultry feed. The macrolide antibiotics in dried distillers' grains have been found to remain active throughout the fermentation even following incorporation into livestock feeds (Basaraba *et al.*, 1999; Jacob *et al.*, 2008). Furthermore, antibiotic administration in animal farming is a common practice for growth promotion (Silbergeld *et al.*, 2008). As such, it is no surprise that resistance in organisms associated with animals have been reported in food supply where they may further be introduced to

humans through consumption (Dzidic *et al.*, 2008; Byaruganba *et al.*, 2011). The interaction of these micro-population with those that reside in human gut may later add to an already problematic antimicrobial resistance (Keen and Patrick, 2013).

The human gut microbial population is estimated to comprise approximately 1000 bacterial species that are reported to be crucial for life (Bäckhed, *et al.*, 2005). The ecological and physiological properties of opportunistic organisms such as *Pseudomonas*, *Acinetobacter, and Enterococcus* facilitates their dominance in the human microbiome (Vaz-Moreira *et al.*, 2014). In two separate analysis, Sommer *et al.* (2009) determined the occurrence of antibiotic resistance reservoirs in the microflora of two unrelated individuals who had not been exposed to antimicrobial agents for at least a year using metagenomics. A total of 27 unique beta-lactamase sequences comprising the previously detected *CblA, CfxA, CTX-M, TEM*, and *AmpC* as well as 10 unidentified beta-lactam gene families possessing 35% to 61% amino acid similarities to sequences previously deposited in GenBank (Sommer *et al.*, 2009). Overall 95 unique inserts containing functional antibiotic resistant genes were identified.

In a separate study, Koenig *et al.* (2001) also assessed the gut micro-flora of 3 days' old babies and detected methicillin and flouroquinolone resistance genes. In addition, streptomycin resistant *E. coli* were detected in human urine samples and a large proportion (48%) of these isolates possessed multiple antibiotic (ampicillin, streptomycin, and tetracycline) resistant traits that were transmissible (Walia *et al.* 2004).

In countries with more advance public health facilities and health care policies such as the United States of America (USA), approximately two million serious illnesses resulting from bacterial infection are reported annually, and thus over 250 million antibiotic prescriptions are written to patients each year (Sharma *et al.*, 2016). In the USA, bacterial infections caused by resistant strains such as Methicillin resistant *Staph. aureus* (MRSA) is implicated in the deaths of more Americans each year when compared to HIV/AIDS, Parkinson's disease, emphysema, and homicide combined thus accounting for approximately 11,285 deaths annually (Ventola, 2014). Statistics in South-East Asia indicate that antibiotic resistant bacterial infections claim the life of one child every 5 minutes (National Department of Health, 2015).

A surveillance carried out as part of a national program in understanding the state of antibiotic resistance in South Africa indicated a very high prevalence in drug resistant bacteria isolated from the bloodstream of patients in public hospitals (Bamford *et al.*, 2011). A thorough analysis of the antimicrobial resistance profiles of important clinically relevant pathogens such as *Escherichia coli, Klebsiella pneumoniae, Enterobacter* spp., *Pseudomonas aeruginosa, Acinetobacter baumannii* and *Staphylococcus aureus* revealed that *K. pneumoniae* and *Enterobacter* species displayed increased resistance to carbapenems (Bamford et al., 2011). Outbreaks of Vancomycin-resistant Enterococci (VRE) in South African public and private hospitals in 2012 resulted in efforts to control the overuse and misuse of antibiotics (Mendelson and Matsoso, 2015). These statistics indicate that if control measures are not put in place about 10 million people will lose their lives annually due to drug-resistant bacterial infections (Mendelson and Matsoso, 2015).

It has been reported that about 30% to 90% of antibiotics ingested by animals and humans is usually excreted unchanged (Sarmah *et al.*, 2006) and this resulted in the detection of β-lactams and methicillin resistant genes in both phage DNA extracted from a wastewater treatments (WWTP) as well as the receiving river water bodies (Colomer-Lluch *et al.*, 2011). This therefore implies that water bodies may receive these antimicrobial agents as well as their metabolites through rainfall runoffs, sewage waste and effluents from WWTP if proper sanitary and waste disposal mechanisms are not implemented. In a previous study approximately 10 and 1000 ng per litre of antimicrobial agents were detected in secondary effluents from a WWTP (Le-Minh *et al.*, 2010). Aquatic environments are now known to be a reservoir for ARG, thus facilitating their transmission to humans most often through plasmids, integrons and insertion sequences (Shi *et al*, 2012).

2.2.4 Antibiotic Resistance Monitoring

Antimicrobial resistance surveillance or monitoring is designed to track changes in microbial populations with the aim of facilitating the early detection of resistant strains of public health importance, and thus provide opportunities for the prompt notification and investigation of outbreaks (Johnson, 2015). The findings from surveillance are expected to inform clinical therapy decisions; to guide policy recommendations, as well as assess the impact of resistance containment interventions (Johnson, 2015; Mellon *et al.*, 2001Ref). WHO has categorised a number of strategies that are considered appropriate for the surveillance or monitoring of antimicrobial resistance that comprise i) *alert organism tracking* involving the identification, confirmation, and communication of specific organisms of great public health importance, such as vancomycin-resistant *Staphylococcus aureus* and extensively drug-resistant *Mycobacterium tuberculosis* (XDR-TB); ii) *enhanced routine surveillance* involving the active review,

interpretation, confirmation, and investigation of results generated in the course of routine clinical care and iii) targeted surveys that involve one-time or periodic study protocols to address specific scientific or public policy needs not adequately addressed by routine diagnostic test results.

In South Africa there are two main laws governing the usage of antimicrobial agents and these include the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act (Act 36 of 1947) and the Medicines and Related Substances Control Act (Act 101 of 1965). Both acts are designed to provide a list of antibiotics that should be acquired over the counter as well as the antibiotics that are primarily administered to humans respectively. It is important to mention that these policies may be contradictory in eradicating the problems associated with the misuse of antibiotics.

Data on antibiotic consumption trends is scarce in South Africa (Mellon *et al.*, 2001). As a result of lack in record keeping of stock remedy dispersion as well as remedies obtained without a prescription. Furthermore, antibiotics used for animal feeds administered by farmers who may not abide to regulations and guidelines (Henton *et al.*, 2011). The documentation of identical antibiotic resistant genes and antibiotic resistant mobile elements in bacterial organisms that colonises both animals and humans (Van et al., 2007) has led to the suggestion that the use of antibiotics as prophylaxis to be identified as a significant factor in the emergence of antibiotic resistance species common in animals and human beings (Acar and Röstel, 2001), as well as resistant bacteria which may be introduced to humans through food stuff (Byaruganba *et al.*, 2011). In 2000 WHO recommended that antimicrobial agents of different classes should be used in animal growth promoters, then to those in clinical therapy for human medicine (Milić *et al*, 2013)

in efforts to create variation in antibiotics used for animals and humans. Antimicrobial resistance affects various sectors medicine, veterinary medicine, animal husbandry, trade, agriculture and the environment (WHO, 2011).

Following the release on global antibiotic consumption trends for the years 2000 and 2010 period, data showed an increase in antibiotic consumption by 36% (Van Boeckel *et al.*, 2015). A number of programs have been established in South Africa in efforts to combat antibiotic resistance crisis by promoting rational use of antibiotics namely; the Global Antibiotic Resistance Partnership in South Africa (GARP-SA). The South African Antibiotic Stewardship Programme (SAASP) aims at educating and promoting appropriate antibiotic prescription in both public and private health sectors in SA as well as collection of Antimicrobial resistance activities.

2.3 Water Distribution systems

2.3.1 Constituents of distribution systems

Distribution systems include all water utility components for the distribution of finished or potable water by means of gravity storage feed or pumps though distribution pumping networks to customers or other users, including distribution equalizing storage (NRC, 2006). The sole purpose of water distribution system is to deliver water from treatment plant to end user, without deterioration of the water quality. There are four basic types of distribution system layouts namely grid, Ring/loop, Radial and Dead-End System, and each is dependent on road layouts of an area (Adeosun, 2014). The branch and grid/loop configurations are the two mostly used. A branch system is most commonly used in rural areas whereas grid/looped system is used in large municipal areas, however most

systems are a combination of both configurations (Adeosun, 2014). To enable water to be constantly looped, which reduces water stagnation and increases fire-fighting ability.

The integrity of a distribution system is determined by three main components that are physical, hydraulic and water quality integrity (van Zyl, 2014). Physical integrity is defined as the ability of the system components to withstand external and internal stress without failure. Physical integrity is compromised mainly by aging and corrosion of pipe material. Pipe material in water supply and distribution are generally of three generic types and these may be cementitious, metallic or plastic in nature (Momba *et al.*, 2000). Corrosion in water distribution systems is defined as the degradation of concrete linings and pipe material, as well as the deterioration of metallic pipe and valves via redox reactions (NRC, 2006). Corrosion may also be microbial mediated (Berry *et al.*, 2006). This may occur internally or outside the pipes. External corrosion may also be a result of low soil redox potential, low soil pH, stray currents, and dissimilar metals or galvanic corrosion. Whereas internal corrosion may be an influence of pH, disinfectant type as well as dose, genera of bacteria present in biofilms, water use patterns, use of inhibitors and more (NRC, 2006). Physical integrity loss may also imply a break in the barrier system from external environment, making the enclosed distribution system exposed to contamination (van Zyl, 2014).

Hydraulic integrity is on the other hand the ability of the systems to meet consumer demand at adequate pressures, velocities and water age in the system (van Zyl, 2014). It is however also mainly dependent on the systems physical integrity. Water quality integrity describes the ability of the system to deliver water of acceptable quality to its users. Various factors intrinsic and/or external factors are involved in deterioration of

drinking water quality in distributions. Contamination in drinking water poses great health concerns, and the origin may be from various sources including human or animal fecal contamination (e.g., noroviruses, *E. coli* O157:H7, *Cryptosporidium*), aquatic microorganisms (e.g., nontuberculous mycobacteria, *Legionella*), toxins from aquatic microorganisms (such as Cyanotoxins), and chemical contaminants (organic chemicals: benzene, polychlorinated biphenyls and pesticides; inorganic chemicals: arsenic and nitrates; metals: lead and copper and disinfection by- products)(van Zyl, 2014; NRC, 2006), discussed further in water quality parameters section in this document.

2.3.2 Biological

Distribution systems are vital in determining the final water quality as following purification the treated water is then exposed to various conditions which may alter quality status of the water (van Zyl, 2014). Another aspect of concern in distribution systems which does not involve mechanical failure is the development of biofilms (Momba *et al.*, 2000). Biofilms describe a layer of a consortium of microorganisms in an aquatic environment adhering to a substratum by a polymetric matrix (Willey *et al.*, 2008). Biofilm development is a result of initial accumulation of organic matter onto a surface then colonisation by bacteria. In distribution systems the organisms which go on to form biofilms are a result of ineffective disinfectant, pipe material (hydrophobic-hydrophilic potential), microbial resistance to disinfectants, availability of an organic carbon source and breakage in pipelines (Momba *et al.*, 2000).

Concern grows due to the bacterial population in biofilms that has been isolated from distribution systems which include secondary opportunistic pathogens in humans such as *Pseudomonas, Mycobacter, Klebsiella, Aeromonas*, *Legionella* spp., *Yersinia*

enterocolitica, *Salmonella typhimurium* and enterotoxigenic *E. coli* (Momba *et al.*, 2000; Mahapatra *et al.*, 2015). Biofilms have also been suggested to possible contribution to antibiotic resistance, based on the high cell density, close proximity, and accumulation of mobile genetic elements (Schwartz *et al.*, 2003). The abundance of biofilms in distribution pipes is greatly driven by the advantages which come from belonging to a community which harbour as intensified resistance to chlorine and other biocides (Berry *et al.*, 2006). Persister cells are believed to be the main driver explaining biofilm resistance to antimicrobial agents (Roberts and Stewart, 2005).

2.3.3 Removal of pharmaceuticals in water production facilities

Conventional water treatment plants employ coagulation and flocculation and sedimentation, filtrations and disinfection to improve water quality. From literature, it is evident that these processes are ineffective in completely removing many dissolved organic and/or inorganic contaminants (Azzouz and Ballesteros, 2013; Vieno *et al.*, 2005; Picó and Barceló, 2015). Gasoline additives, surfactants, endocrine disruptors, and pharmaceuticals and personal care products (PPCP) have been detected in water catchment used for the production of potable water (Ashton *et al.*, 2008; Cizmas *et al*, 2015; Picó and Barceló, 2015).

Azzouz and Ballesteros, (2013) screened for antibiotics in a treatment plant from each point in the treatment process from the reviving effluent to released water over three seasons. The number pharmaceuticals contaminants reported were not only large in terms of numbers but at higher concentrations than those collected in the warmer periods. These results agreed with publication in a previous report that also assessed seasonal variation in the occurrence of pharmaceuticals (Vieno *et al.*, 2005). Ibuprofen (46, 160

and 314 ng L-1), carbamazepine (127, 145 and 186 ng L-1) and diclofenac (58, 150 and 259 ng L-1) were reported to occur at the highest concentrations among the other compounds detected in summer, autumn and winter respectively. Following treatment during the cold period trace amounts (0.09–0.5 ng L-1) of ibuprofen and carbamazepine were still detected in water samples (Azzouz and Ballesteros, 2013).

Although Vieno *et al.,* 2005 showed the removal carbamazepine of up to 75.5%, a research by Zhang et al., (2013) shows that the removal of carbamazepine to be only about 2-3% in another treatment plant. Vieno *et al.,* 2006 also carried out a study about the drawbacks in removal of pharmaceuticals (Diclofenac, Ibuprofen, Bezafibrate, Carbamazepine, Sulfamethoxazole) where the coagulation process used in water treatment plant improved with high-molecular-weight DOM concentrations in ferric sulphate (5, 15 or 30 mg l-1 of DOC) for the removal of diclofenac, ibuprofen and bezafibrate being ionic compounds. In contrast the water treatment was ineffective for neutral pharmaceuticals such as carbamazepine and Sulfamethoxazole.

The South African Drinking Water Quality Framework has adapted a preventative risk management approach as recommendations of the World Health Organization (DWQ Framework SA, 2009). The entire water supply system from source to tap and back to water source is evaluated in efforts of identification present and possible risks which may be encountered as well as placing measures in how these risks can be managed. Therefore, it is relevant to discuss waste water treatment plants and their efficiency in eliminating biological and pharmaceutical compounds. A lab-scale re-creation experiment of an activated sludge as of that in waste treatment plants, showed effectively

removal of ibuprofen, and benzafibrate compounds, however poorly so towards carbamazepine and sulphamethoxazole (Clara *et al.*, 2005).

Though some antibiotics can be removed in treatment processes, concentration of antibiotics in waste water treatment plant effluent have been shown to be generally high (Le-Minh *et al.*, 2010; Martínez, 2012; Al Aukidy *et al.*, 2012). Hence waste water treatments are considered a point source of pharmaceutical contamination to the environment (Lapworth *et al.*, 2012). Sludge obtained from waste water treatment plants contains nutrients and organic matter which can improve soil quality. It is used as a natural fertiliser in farming; and this practice may create an indirect introduction of immobilized PPCP in the sludge to the environment (Radjenovi´c *et al.*, 2009). Concentrations of Thiabendazole have been documented to be as high as 5000 mg/kg in biosolids (Lapworth *et al.*, 2012). Point source contamination may also occur in landfill sites where household or industrial waste containing these products are dumped (Kaplan, 2013).

2.4 Water quality parameters

2.4.1 Physical parameters

2.4.1.1 Conductivity

Electrical conductivity is a measure to the ability of water to conduct electrical current. It also has a direct relationship to the concentration of salts (inorganic materials such as alkalis, chlorides, sulfides and carbonate compounds) dissolved in water. Therefore, the presence of inorganic material which dissolves to create ions in water results in higher conductivity in the water (EPA[a], 2012). It is a good measure of flowing (stream) water

quality, water bodies possess varying conductivity as an influence of properties of the geological area in which the water exists (EPA[a], 2012).

Temperature has a direct influence on conductivity, that is for every 1°C increase, conductivity values simultaneously increase by 2-4% hence conductivity is reported at 25 °C. Conductivity may also be used as an indicator for changes in a water system, when the average range of conductivity for the water bodies is known (Rose *et al.*, 2016). Other aspects which may influence conductivity measure include highly mineralized groundwater inflows, Agricultural runoff, and heavy rainfall (EPA[a], 2012).

2.4.1.2 Turbidity

Turbidity is a measure of water clarity which can be observed with the optical eye, and it is an expression of the amount of light scattered by particles in water (Rose *et al.*, 2016). It affects the physical transparency of water by appearing cloudy or murky. This property is often used as an optical indicator for water quality based on its clarity. Turbidity is an influence of suspended solids and dissolved coloured matter in water (inorganic, or organic matter such as algae, plankton and decaying material (EPA[a], 2012). It does not measure the direct quantity of the total suspended materials but may be used to indicate changes in the concentration of total dissolved solids in water (Rose *et al.*, 2016).

Water bodies with high amounts of decomposing vegetation are more turbid whereas moving water does not allow for sedimentation, instead keeps matter in suspension (EPA[a], 2012). Turbid water quality will therefore have reduced oxygen and a higher temperature due to the suspended solids in the water. Furthermore, turbidity provides

conditions for microorganisms, which may aid bacterial regrowth in distribution systems (Rose *et al.*, 2016).

2.4.1.3 pH

Potential of hydrogen (pH) is a numeric scale used to measure free hydrogen and hydroxyl ions in a solution, which indicates the acidity/ alkalinity of the solution (Willey *et al.*, 2008). It's a major determinant of water quality, influences various aspects of the water and activities which may occur in the water body such as solubility, biological inhabitants and nutrients availability (van Zyl, 2014; EPA[a], 2012). Levels which are too high or low may have negative effects not only on aquatic species but humans as well. Human consumable pH levels with none - to minimal health effects range from 4-11. SANS 241– Drinking water stipulates a pH range between ≥ 5 and ≤ 9,7 for potable water (van Zyl, 2014; SANS 241, 2011). pH level below 4 can cause skin and eye irritations whereas a value below 2.5 will cause irreversible damage to skin and organ linings (WHO, 2003).

It has also been reported the pH levels above 8.0 inhibit the effectiveness of disinfection with chlorine; pH levels of 6.5 - 9.5 in water can damage and corrode pipes in distribution systems while pH values above 11.0 can cause skin and eye irritations (WHO, 2003). In addition, water with high pH levels has a characteristic bitter taste (EPA[b], 2012).

During chlorination in water purification, pH affects the distribution of chlorinated by-products being that there is a presence of organic and/inorganic compounds in the water. A lower pH in this case will lower the concentration of THM produced, in contrast it increases formation of HAAs and vice versa (WHO, 2011). Even in ozonation, pH plays

a crucial role in the formation of bromite when bromide is present in the water (WHO, 2011).

2.4.1.4 Temperature

Water temperature is the measure of thermal energy of a substance (Willey *et al.*, 2008). This physical water property can be influenced by various aspects in the water column such as; Conductivity and salinity, suspended solids etc. (Rose *et al.*, 2016). It is crucial when determining other aspects of water quality. These include; Metabolic rates and photosynthesis production, Compound toxicity, Dissolved oxygen and other dissolved gas concentrations, Conductivity and salinity, Oxidation reduction potential (ORP), pH, and Water Density (Rose *et al.*, 2016).

Temperature has been shown to affect metabolic rates and biological activity of aquatic inhabitants (Wetzel, 2001). It is a selective parameter for aquatic life which may flourish at that condition. The effects of high temperature in water may increase the solubility of toxic compounds (cadmium, zinc, lead and ammonia). In contrast, reduce the availability of oxygen and other gases and as affect organism's tolerance limit (Bhadja and Vaghela, 2013). Water temperature is determined by a number of factors that is, the type of water body, depth, season, latitude and other components of the surrounding environment (Wetzel, 2001).

2.4.2 Chemical Parameters

2.4.2.1 Inorganic chemicals

Inorganic chemicals are present in water as dissolved salts such as carbonates and chlorides (van Zyl, 2014). They may be deposited in source water as a result of natural

leaching from mineral deposits into source waters, land-use activities, corrosion and leaching of pipes and fittings. Inorganic compounds with significant health concerns that may be present in water include Arsenic, Barium, Boron, Chromium, Fluoride, Selenium and Uranium (WHO, 2011). Water from lakes and reservoirs has been reported to contain higher concentration of DOC than groundwater (Momba *et al.*, 2000).

Some pharmaceutical vaccines used in therapeutic applications have also been documented to be contaminated with inorganic chemicals which include aluminium, Iron(II) Sulfide, Iron, Chromium nickel (Gatti and Montanari, 2016). Through the movement of water bodies from domestic and industrial discharge a variety of the compounds have been detected in water catchment used for the production of potable water (Ashton *et al.*, 2008; Picó and Barceló)

2.4.2.2 Organic chemicals

Organic compounds present in water are a consequence of either natural or human activity, however they are usually present at very low concentrations (Brettar and Hofle, 2008). Decomposition of animal and plant matter produces organic compounds, that is humic and fulvic acids which may enter natural sources. Humic and fulvic acids have been documented to produces secondary toxic organics when in contact with common disinfects which are of health risk to consumers (van Zyl, 2014; Templeton *et al.*, 2009). PPCP and endocrine disrupting chemicals are also emerging organic contaminants of concern in drinking water, and their introduction and concertation levels of these chemicals are an influence of social, cultural, technological and agricultural factors (Azzouz and Ballesteros, 2013; WHO, 2011; Silbergeld *et al.*, 2008; Brunning, 2014).

2.4.2.3 Disinfectants

Disinfection is a human activity introduced intentional in water to disrupt bacterial cell growth and prevent regrowth in distribution systems (Berry *et al.*, 2008). Following this purification process, residual disinfectants remain in water as it moves through the distribution system in order to regulate microbial population re-growth in water following purification at treatment facility till end consumer use (Berry *et al.*, 2008). Chlorine is one of the most common used disinfection chemical during water purification (van Zyl, 2014).

Although the use of chlorination is of benefit for safe water production, currently a number of publications have shown that the orthodox water purification techniques may not always be effective at removing bacterial cells (Pruden, 2014; Ashton *et al.*, 2008; Cizmas *et al.*, 2015). Chlorination may further promote the release of free bacterial DNA into the environment (Bergeron *et al.,* 2015). Literature on the effects of chlorination on the abundance of ARB can be traced back to as early as the 1970s (Grabow and van Zyl, 1976). A number of publications evaluating ARB in potable water demonstrate that ARB are more resistant to chlorination (Templeton *et al.*, 2009; Huang *et al.*, 2013; Sun *et al*, 2013). This therefore indicates the need to constantly monitor the occurrence of ARB in water treatment facilities and their respective distribution systems especially in areas that are most often faced with inadequate water quality management practices.

Sun *et al*, 2013 experiment demonstrated that TS001 strain of the genus *Sphingomonas* inoculated in 4 mg L^{-1} concentration of chlorine incubated for 24 h had no significant inactivation of only 5.5% of the cellular cultivation (Enumerated from HPC selective media). Huang *et al.*, experiment results also showed that tetracycline-resistant *E. coli*

had higher tolerance to chlorine than those that are sensitive to antibiotics which agrees with previous literature (Sun *et al.*, 2013; Grabow and van Zyl, 1976; Huang *et al.*, 2013).

Disinfectants may also act as a selective condition for bacterial species as it may decrease sensitive competitors. A study be Norton and LeChevallier, (2000) demonstrated effects of chlorination on bacterial population based on their cell wall composition and identified that the microbial population shifted from predominately Gram-negative bacteria (97%) in the raw water to Gram-positive organisms (98%) in post disinfected water (NRC, 2006).

The use of disinfectants such as chlorine in the presence of organic matter produces secondary toxic compounds (WHO, 2011; Templeton *et al.*, 2009). These include THMs, HAAs, haloacetonitriles, haloketones, monobromoacetic acid, hydrogen peroxide, ketones etc. depending on the source of organic contaminant (WHO, 2011). Ozonation is another disinfecting treatment process in water treatment known to produce formaldehyde and other aldehydes in the presence of organic compounds (van Zyl, 2014).In conditions where organic compounds are presence , bacterial growth phase and extracellular polymeric matrix, the efficiency of disinfectant in distribution pipes may be affected, leading to biofilm formation, microbial mediated corrosion (Khan *et al.*, 2016).

2.4.3 Microbial properties

Viruses, protozoa and bacteria are natural inhabitants of various habitats and may be present in water distribution systems (van Zyl, 2014). Drinking water distribution system may be considered as a niche itself as it houses conditions and parameters which influences microbial growth and water quality state (Inomata *et al.*, 2009). In the past

orthodox pathogens such as *Vibrio cholerae* and *Salmonella Typhi* were the main concern in water contamination, other recognised contaminants impose greater challenges in water safety (WHO, 2000).

The occurrence of culturable microorganisms in water bodies can act as a vehicle for transmission of virulent agents among resident microbes in the habitat (Walia *et al.*, 2004). The focus is on microbiological indicators which show evidence of contamination and possible pathogenic inhabitants. Organisms common in intestinal tracts of humans and animals are tested for in water post production using indicator organism *E. coli.* The presence of such organisms termed faecal coliforms; give an indication for micro-organisms that can cause gastro-intestinal and other diseases (van Zyl, 2014). The general microbial population in water is examined with the enumeration of heterotrophic bacteria (Allen *et al.*, 2004).

Heterotrophic bacteria belonging to the genus *Pseudomonas*, *Acinetobacter*, or family *Sphingomonadaceae* are commonly found in tap water (Vaz-Moreira *et al.*, 2014). The ecology and physiology of these organisms facilitates their inhabitation in waste, surface, or drinking water bodies. They also have the potential to integrate in the human microbiome and survive (Vaz-Moreira *et al.*, 2014). In addition to these other bacteria belonging to the coliform group that naturally occur as normal flora in the gastrointestinal tract on humans and warm-blooded animals and may thus be present in drinking water systems include *Klebsiella pneumoniae*, *Enterobacter aerogenes, Enterobacter cloacae* and *Citrobacter freundii* (NRC, 2006). Given that these organisms have been reported to harbour a wide variety of antibiotic resistant determinants (ARDs), ARBs and their associated ARDs are major emerging public health threats in drinking water systems

especially in countries with poor public health facilities (WHO, 2011). The frequent detection of ARG as well as ARB and their association with waterborne disease outbreaks based on genotypic typing data coupled with their potential to rapidly accumulate in the aquatic environments is of great public health concern.

Thus the development, implementation and constant assessment of the effectiveness of national and international guidelines for risk assessment in drinking water supply systems should be given great priority by increasing detection of multi-drug resistant bacteria strains (Pruden, 2014; Bergeron *et al.*, 2015). In South Africa, SANS 241 has set minimum concentration levels for microbiological water quality parameters for Total coliform counts (≤ 10/100 mL), *E. coli* or faecal coliform counts (Not Detected), Heterotrophic plate counts (≤ 1 000/mL), Cytopathogenic viruses (0/10 L), Somatic coliphages (0/10 mL) and, Protozoan parasites (*Cryptosporidium* and *Giardia* species) (0/10 L) (SANS 241: 2011). These are indicator organisms that are used for routine water quality screening, but it is also suggested that their presence may indicate the presence of other pathogenic bacteria. This therefore implies that the public health implications of water intended for human consumption should not be limited entirely to these organisms.

2.5 Heterotrophic Plate Count

2.5.1 General Overview

Strategies designed to prevent the occurrence of microbial populations in drinking water should not only be limited to faecal coliform contaminants but also to organisms which may colonise distribution pipes or which may be present in source water (Guidance to drinking water quality, 2011). Heterotrophic bacteria denote bacteria that require organic nutrient for growth and survival. Heterotrophic plate count (HPC) represents however only

a fraction of the bacteria population in any source (Allen *et al.*, 2004). They are obtained through an enumeration technique and the data generated may vary significantly depending on the temperature, incubation period, culture media that is used for isolation as well as the source of the water samples. Against this background HPC analysis have revealed that bacteria belonging to the genera *Citrobacter*, *Flavobacterium*, *Acinetobacter*, *Micrococcus* even the most frequently detected *Escherichia coli* as well as opportunistic pathogens belonging to the genus *Aeromonas*, *Klebsiella*, and *Pseudomonas* are most frequently associated with bacteria contamination in water sources (Inomata *et al.*, 2009).

2.5.2 Heterotrophic Plate Count in drinking water

Various methods are employed to assess the efficiency of water purification process for elimination of microbial populations and these include quality assessment techniques designed to determine the *E. coli* counts, Coliform counts as well as Heterotrophic Plate Count also known as Standard Plate Count (van Zyl, 2014). HPC in distribution pipes is assumed to be a direct implication of the condition(s) in the distribution system and have been directly associated with stagnation, loss of residual disinfectant, elevated Assimilable Organic Carbon (AOC) levels, higher water temperature, and availability of organic nutrients (Allen *et al.*, 2004). These parameters therefore need to be monitored since they facilitate bacteria growth and persistence hence negatively affecting the quality of the finished product.

Against this background, the HPC technique is used to as an indicator for determining the efficiency of water treatment process and may also be expanded to the effectiveness of the disinfection processes. Given that HPC analysis is a measure of the microbial state

in water distribution system conditions, where regrowth is assumed to be a result in the loss of residual disinfectants, availability of nutrients or high temperature (WHO, 2003; Wetzel, 2001; Inomata *et al.*, 2009), it therefore constitutes a very valuable analytical tool for water quality assessment to provide indication of potential pathogens that may present public health complications on consumers. It is based on these reasons that HPC analysis has been included in other industrial applications such as beverage vending machines, food processing, medical devices (WHO, 2003) to also provide data on the potential presence of pathogens.

In conclusion, HPC are primarily used as indicators rather than as a public health assessment parameter. This is based on the premise that HPC are believed to have no direct health implications on consumers when ingested (Hellard *et al.*, 2001; Allen *et al.*, 2004) but their presence at high levels provide an indication of the presence of pathogenic bacteria requiring proper screening and assessment of their virulence potentials. However, since some emerging pathogens such as *E. coli* strains belonging to the serotype O157 are known to have a very low infectious dose coupled with very high virulence potentials, HPC may in this light be considered a direct assessment of the public health implications of the water that is supplied to consumers. Unfortunately, for this conclusion to be made, the identities of isolates need to be determined and this procedure may be time consuming depending on the availability of resources. Notwithstanding, the presence of these emerging pathogens as well as other opportunistic pathogens in water that is supplied to communities especially in countries such as South Africa where the proportion of immune-compromised individuals is very high cannot be under-estimated (Colford *et al.*, 2002; WHO, 2003). It is against this background that the South African

Bureau of Standards (SABS), specification states that drinking water may not contain more than 1000 CFU of HPC per millilitre of drinking water (SANS 241: 2011).

2.5.3 Heterotrophic Plate Count health aspects

HPC is believed to have no direct significance in health effects when ingested (Allen *et al.*, 2004) with exception when the immune status of consumers are compromised (Colford *et al.* 2002; WHO, 2003). As is the case with any natural habitat, drinking water distribution systems provide conditions which may not favour the growth of microorganisms such as extreme pH, high salinity, reduced nutrient content and exposure to high chlorine content which may indirectly induce stress response mechanisms in the organisms thus promoting the selection of bacterial populations with greater or enhance resistance potentials (Bessa *et al.*, 2014).

A number of studies have evaluated the presence of microbial populations in post-purification processes and Inomata *et al.*, (2009) evaluated the virulence potentials of HPC isolates from tap water intended for human consumption using a haemolytic assay. Findings revealed that a large proportion (87%) of the isolates displayed haemolytic activity and thus was associated with the residual chlorine from the source. In addition, an analysis of the identities of the isolates to species level based on bacterial 16S rDNA sequence analysis revealed varied results characterised mainly by bacteria belonging to three distinct genera, namely *Sphyngomonas*, *Staphylococcus* and *Mycobacterium* but with three strains identified as *Micrococcus luteus*, *Bacillus* species as well as *Cupiaviadus metallidurans* identified. Moreover, a large proportion (88%) of the potentially pathogenic isolates have been previously reported as opportunistic pathogens that are associated with food poisoning in humans (Inomata *et al.*, 2009).

The frequent and misuse of antibiotics presents a serious public health concern to the society resulting from the accumulation of antimicrobial metabolites in various ecosystems including environmental water systems (Cizmas *et al.*, 2015). This is even amplified by the fact that following consumption of antimicrobial agents, the body unavoidably excretes excess of the drug. These antimicrobial agents or metabolites if excreted onto the environmental sources via urine, faeces, household waste or industrial emissions (Cizmas *et al.,* 2015) contribute significantly to the development of resistant bacteria strains that have a negative effect on therapeutic processes.

Although there are a number of processes that are frequently utilized to remove these antimicrobial contaminants in water bodies with the aim of also reducing their concentrations in aquatic environments (Luo *et al.*, 2014; Evgenidou *et al.*, 2015) given their undesirable consequences on aquatic organisms (Bessa *et al.*, 2014; Luo *et al.*, 2014), it has been reported that inactivation of tetracycline-susceptible *E. coli* was greater at a low concentrations of chlorine (1.5 mg Cl_2 L^{-1} and below). Overall, bacteria cells that display drug resistant phenotypes showed higher tolerance to chlorine when compared to their sensitive counterparts and this indicates the need to constantly evaluate the resistance profiles of bacteria contaminants in a given water reticulation system (Grabow and van Zyl, 1976; Sun *et al*, 2013). Data generated may be very useful in the modification of national guidelines for water treatment purposes.

2.6 Water quality frame work

Water quality describes the physical, chemical, biological and aesthetic properties of water which determine its fitness for a variety of uses and for protecting the health and

integrity of aquatic ecosystems (WHO, 2011). Water production facilities are responsible for providing safe drinking water which does not present any significant risks to the health of consumers over a lifetime, including the development of hyper-sensitive reactions to consumers (SANS; 241) and this is a strict requirement for Drinking Water Specifications (WHO, 2011).

To achieve this goal the Water Services Authority that comprise a variety of integrated stakeholders including the Department of Health, the Department of Water Affairs and Forestry Affairs and the National treasury have the responsibility of ensuring that water supplied to consumers meet the SANS 241 standards. Whilst the Department of Water Affairs and Forestry is the principal institution for the regulation of drinking water quality (DWQ Framework SA, 2009), in 2009 the department published a document titled 'A Drinking Water Quality Framework' aiming at promoting effective management of drinking water quality with regards to protecting public health in the country. To ensure efficiency the Stockholm Framework (WHO, 2011) was also included in the document which has now been incorporated by The National Water Act (No. 36 of 1998) and the National Water Resource Strategy (2004). The frame work follows a holistic approach for risk assessment and risk management within drinking-water supply system.

Prior to the incorporation of this framework Drinking Water Quality Management in South Africa primarily focused on compliance monitoring, which had a narrow approach to quality control. Given that the document overlooked the possibility of contamination from sources that could compromise drinking water quality to only be recognized following quality monitoring at treatment plants, this had severe implications on consumers since complication were the only signal of poor water quality after they have been exposed to

pathogens (DWQ Framework SA, 2009). Based on these limitations, the current Drinking Water Quality Framework for South Africa, now follows a preventative risk management approach evaluating the entire water supply system from source water to tap and back to source water (DWQ Framework SA, 2009). To enforce this constant water quality assessment requirement, the Department of Water Affairs and Forestry has entrusted its mandate to municipalities who are expected not only to provide water to consumers but ensure that the water is of "good quality" and hence safe for drinking.

The effectiveness of operational procedures in any water treatment plant depends largely on the management processes that are utilized as well as the human resources capacity. In the North West Province in particular, a previous report indicated that lack of appropriate capacity is the most important challenge that negatively affects the delivery of services to communities and this includes but not limited to the provision of safe drinking water. Given that the principal function of every water treatment plant is to provide safe drinking water to communities and households (Smith, 2009) it is important to constantly monitor both the quality as well as the efficiency of the treatment protocols. An assessment of adherence to standard treatment protocols and the implementation of proper management practices will therefore improve the quality of the finished product.

It is generally known that three main drinking water supply key areas are most often used to determine health risks as well as the socio-economic consequences of water supplied to consumers and these include process control (classification of works and operational capacity and process control competency in terms of the draft Regulation 813), drinking water quality assessments (consisting of compliance to the WSA monitoring programmes, and microbiological and chemical compliance to SANS standards) and risk

management (which is based on continued water safety planning, conducting of a full SANS analyses and use of monitoring programmes that are risk informed) (SANS 2011: 1). This study focuses mainly on the drinking water quality assessments as a way of indirectly determining the compliance of water treatment protocols to the WSA monitoring programmes and SANS standards.

CHAPTER 3

METHODS AND MATERIALS

3.1 Clearance for the study

Permission to conduct the study was obtained from the Mafikeng Local municipality and Sedibeng Water (Appendix 1). Ethical Clearance for the study was also approved by the Health Ethics Committee of the North West University, South Africa prior to sample collection.

3.2 Study area

Water samples were collected from five sampling points in two water distribution systems in the Ngaka Modiri Molema Municipality in Mafikeng. The water distribution systems were the Mafikeng Water Treatment Works and Mmabatho Water Treatment Works situated in Unit 15 and Danville respectively. These water treatment plants provided both raw and treated (drinking) water samples in the study. Raw water samples were also collected from Molopo Eye while treated (drinking) water samples was obtained from the Modimola dam. Mixed water that had undergone treatment from both sources are mixed at the Signal hill reservoir and distributed to some areas in the city. Samples were also collected from this reservoir. During this study, the choice of these sampling points was motivated by the fact that water from Molopo eye and Modimola dam are purified and used for human consumption as well as for recreational, agricultural, and industrial purposes in the area.

3.3 Sampling

Raw and treated water samples were collected in February, April, July, and October 2016, to cover the four different seasons. A total of forty water samples that comprised ten per season were collected using 500 mL bottles. The sampling points where chosen to reflect the different stages within the water production cycle from catchment area to end user. After collection the samples were properly labelled and immediately transported on ice to the laboratory for analysis. Upon arrival in the Laboratory, the samples were analysed immediately based on standard procedures as outlined in the South African National Standard (SANS) 241. The number of samples and the locations from which they were collected are shown in Table 3.1.

Table 3.1: Type and quantity of water samples that was collected from the different sampling points in the water distribution system per season.

Sampling site	**Quality of Water**	**No of samples collected per season**
Molopo Eye	raw untreated water	1
Treatment plants	Inlet water abstracted from catchment area prior to treatment	2
Treatment plants	potable water prior to release to reservoir	2
Randomly selected household taps	treated water	4
Modimola Dam	Mixed water	1
Total Number collected per season		**10**
Total Number of samples collected during the study		**40**

3.4 Determination of the physio-chemical parameters of water samples

Physio-chemical parameters of the water samples that include temperature, pH, turbidity and electrical conductivity were determined immediately after collection on site based on standard methods (APHA, 1992). The samples were analysed in triplicates in order to obtain an average value and standard error means per sample.

3.4.1 Temperature

The temperature and pH conditions of the water samples ware measured using digital pH meter (CRISON, model SensION+ MM40+), based on a standard protocol (APHA, 1992).

3.4.2 pH Measurement

The pH of the water samples was measured using a digital pH meter (CRISON, model SensION+ MM40+), based on a standard protocol (APHA, 1992).

3.4.3 Determination of Electrical Conductivity

The electrical conductivity of the water samples was measured using a digital conductivity meter (CRISON, model SensION+ MM40+).

3.4.4 Turbidity Measurement

The turbidity of the water samples was measured using a digital turbidity meter (CRISON, model SensION+ MM40+) based on a standard protocol (NRCC 2011).

3.5 Bacterial enumeration

3.5.1 Screening of water samples for proportions of indicator organisms

Aliquots of 100 mL from each water sample were aseptically filtered using 0.45 mm sterile membrane filters (Pall Corporation, USA) on a water pump machine (Model Sartorius 16824). Filters were placed on mFC agar (Biolab, South Africa) for selective enumeration of faecal coliforms and Membrane Lactose Glucuronide agar (MLGA) (pH= 7.2 ± 0.2) (Oxoid Ltd, UK) to assay for *E. coli* counts. The MLGA contains a chromogenic substrate X-glucuronide that is broken down by the β-glucuronidase enzyme which is highly specific for *E. coli*. mFC and MLGA plates were incubated aerobically at 37 °C for 24 hours. After incubation, the isolates with blue colonial morphologies on mFC agar represented faecal coliform bacteria while green coloured colonies on MLGA represented *E. coli*. These indicator organisms on both agar plates were counted and results were recorded.

3.5.2 Isolation of heterotrophic bacteria from water samples

Aliquots of 100 mL of the water sample were aseptically filtered using 0.45 mm sterile membrane filters (Pall Corporation, USA) on a water pump machine (Model Sartorius 16824). Filters were placed aseptically on R2A media (pH= 7.2 ± 0.2) (Lab M, UK) and plates incubated aerobically at 35 °C for up to 3 days. Isolates displaying different colonial morphologies were picked in triplicates, purified on R2A agar and plates incubated aerobically at 35 °C for up to 3 days. Pure isolates were stored at 4 °C and used for further bacterial identification and characterisation assays.

3.6 Primary biochemical identification tests

Presumptive heterotrophic isolates were identified using the following criteria;

3.6.1 Cellular morphology

Heterotrophic isolates were subjected to Gram-staining using a standard technique that is designed to place isolates into the two groups based on their cellular morphologies (Cruikshank *et al.*, 1975). A smear of each pure isolate was prepared on a microscope slide and was fixed by passing briefly over a flame. Slides were stained with 1% (w/v) crystal violet for 1 minute and later rinsed with sterile water. Slides were later flooded with iodine for 1 minute and rinsed with sterile water. Excess crystal violet stains on the slide was removed by briefly washing with 70% (v/v) ethanol and rinsed with sterile water. Slides were finally counter stained with Safranin-O for 1 minute and rinsed with sterile water. Slides were viewed using a light microscope under oil immersion. Gram-positive and Gram-negative organisms were subjected to antimicrobial susceptibility tests in order to determine their antimicrobial resistance profiles.

3.6.2 Determination of isolates possessing antibiotic resistant phenotypes

The antimicrobial resistant profiles of the isolates were determined using the Kirby-Bauer agar disc diffusion technique (Bauer *et al.,* 1966). Results were interpreted using Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2007), for known microbial inhibition disk diffusion standards. For antibiotic standards which were not available on the CLSI guidelines, manufactures suggestions were followed for MIC results interpretation. A panel of eleven different antimicrobial agents that appear in Table 3.2. Isolates were revived on R2A media and 0.5 McFarland's bacteria suspensions with a desired a desired absorbance reading of 0.4 to 0.6 at 600nm wavelength. Suspensions were prepared using sterile saline solution. Aliquots of 100 µL of the bacteria suspensions were spread-plated on Mueller Hinton Agar (BioLab, South Africa) in order to prepare a bacteria lawn after aerobic incubation. Eleven antimicrobial discs obtained from Davies

Diagnostics (Pty) Ltd and impregnated with different standard concentrations of the drugs were placed at equitable distances from each other onto the inoculated Mueller Hinton agar plates using sterile forceps. Plates were incubated aerobically at 37 °C for 24 hours. Bacterial growth inhibition zone diameter data were measured in millimetres (mm) and values were used to classify isolates as sensitive, intermediate resistance or resistant to a particular antibiotic based on standard reference values (CLSI, 2007). Isolates showing resistance to 3 or more antibiotics belonging to different classes were defined as multiple antibiotic resistant strains (Rota *et al.*, 1996) and were selected for further analysis. Multiple antibiotic resistant (MAR) phenotypes for the isolates were generated using abbreviations that appear on the discs.

Table 3.2: List of antibiotics used and their specifications

Group	**Antibiotic**	**Disc conc. (µg)**	**Abbr.**	**Zone Diameter Nearest whole mm**			**Equivalent MIC Breaking Point µg/mL**	
Penicillin	Ampicillin	**10**	AP	**R**	I	**S**	**R**	**S**
	Penicillin-G	10	PG	≤13	14-16	≥17	≥32	≤8
Macrolide	Erythromycin	15	E	≤15	-	-	-	-
Phenicol	Chloramphenicol	30	CHL	≤ 13	14-22	≥23	≥8	≤0.5
Cephalosporin	Cephalosporin	30	CIP	≤12	13-17	≥18	≥32	≤8
Aminoglycoside	Streptomycin	300	S	-	-	-	-	-
	Kanamycin	30	KAN	≤6	7-9	≥10	-	-
	Neomycin	30	NE	≤13	14-17	≥18	≥25	≤6
Glycopeptide	Vancomycin	30	VA	≤ 17-	-	≥23	-	-
Tetracycline	Oxy-tetracycline	30	OT	≤ 12	10-11	≥9	-	-
DHFR Inhibitor	Trimethoprim	2.5	TMP	≤18-	-	≥25	-	-
				≤10	11-15	≥16	≥16	≤4

3.7 Secondary biochemical identification tests

Confirmed MAR isolates were subjected to assays designed to determine their abilities to produce extracellular enzymes. Fresh isolates were spot inoculated onto the appropriate plates and were incubated aerobically at 37 °C for 24 hours. Specific assays were used for screening the potential of isolates to produce haemolysin, proteinase, DNase and lipase using standard methods (Venter and Bezuidenhout, 2015).

3.7.1 Extracellular enzyme production tests

3.7.1.1 Haemolysin production

Haemolysin production is a virulent trait of bacteria species and haemolysin is a cytolytic protein that is capable of lysing red blood cells (Willey *et al.*, 2008). Single pure bacteria colonies were sub-cultured on 5% (w/v) sheep blood agar to determine their haemolytic activity. Plates were incubated aerobically at 37 °C for 24 hours. The detection of clear zones around the bacterial colonies indicated beta (β)-haemolysin production while a greenish colouration around bacterial colonies represented alpha (α)-haemolysin production and the absence of haemolytic patterns around colonies was interpreted as gamma (γ)-haemolysis (Pavlov *et al.*, 2004).

3.7.1.2 Proteinases

Isolates were screened for their potential to produce proteinases which are enzymes that facilitate the catabolism of peptide bonds on long protein chains which bind amino acids (Willey *et al.*, 2008). Single pure colonies of the test isolates were spot-inoculated on Brain Heart Infusion Broth (Biolab, SA) (pH 7.4 ± 0.2) supplemented with 3% (w/v) skim milk solution (Oxoid, UK). Plates were incubated aerobically at 37 °C for 24 hours and

isolates that produced clear zones around the colony were recorded as positive for proteinases and vice versa.

3.7.1.3 DNase

DNase enzyme degrades nucleic acids (Willey *et al.*, 2008). In order to assay for the DNase enzyme a single pure colony of test isolates was sub-cultured on DNase agar (pH 7.3 ± 0.2) (Fluka Analytical, Switzerland) supplemented with 0.01% toluidine blue O solution (Sigma Aldrich, US). Plates were incubated aerobically at 37 °C for 24 hours. Following incubation, the plates were flooded with a 0.1% (v/v) 1M HCl (Merck, US) solution, and isolates that produced a zone of clearing or a pink halo around the bacterial colony indicated a positive reaction for DNase enzyme and vice versa.

3.7.1.4 Lipase

Lipase is an enzyme that facilitates the catabolism of triacylglycerols into simpler compounds that include monoacylglycerols, diacylglycerols, free fatty acids and glycerol (Willey *et al.,* 2008). Single pure colonies of the test isolates were sub-cultured on Tryptone soy agar (Merck, US) supplemented with 1% (v/v) Tween 80 (Sigma Aldrich, US) which served as the substrate for the lipase enzyme. Plates were incubated aerobically at 37°C for 24 hours. The production of a turbid halo around the bacteria colonies was indicative of a positive reaction for Lipase enzyme and results were recorded.

3.8 Bacterial Storage

Multiple antibiotic resistant isolates were suspended in 1 mL of 20% (v/v) glycerol contained in 1.5 µL sterile Eppendorf tubes. The contents in the tubes were homogenised by vortexing and tubes were then stored at -80 °C for future identification tests.

3.9 Extraction of Chromosomal DNA from MAR isolates

All presumptive heterotrophic MAR isolates were revived on R2A media and used for extraction of chromosomal DNA. Following aerobic incubation single colonies of pure fresh cultures were inoculated in 5 mL of pre-autoclaved (121 °C for 15 minutes) nutrient broth and suspended by vortexing. The suspension was incubated aerobically for 24 hours at 37 °C. Genomic DNA was extracted from bacteria cells using the Zymo Research Genomic DNA™ Tissue MiniPrep Kit (The Epigenetics Company, USA), supplied by Biotechnical Industries (Pty) Ltd Sunnyside Pretoria, South Africa following the manufacturer's instructions. Genomic DNA extracted from the isolates was quantified using a Nanodrop Lite spectrophotometer (Model 1558) obtained from Thermo Scientific, USA. The DNA samples were stored at -20°C and used for molecular characterisation assays.

3.10 Bacterial 16S rRNA gene PCR analysis

Bacterial 16S rRNA gene fragments were amplified using chromosomal DNA obtained from MAR isolates and primer sequences listed in Table 3.3 (Korzeniewska and Harnez, 2013). PCR reaction mixtures comprised standard 25 µL reaction volumes consisting of 12.5 µL of a 2X DreamTag Green Master Mix (0.4 mM dATP, 0.4 mM dCTP 0.4 mM dGTP and 0.4 mM dTTP, 4Mm $MgCl_2$ and loading buffer), 11 µL of nuclease free water, 0.25 µL of each oligonucleotide primer and 1 µL of template DNA. A DNA thermal cycler (Bio-Rad C1000 Touch™ Thermal Cycler) was used in the PCR amplification reactions. All

PCR reagents were Fermentas, USA products obtained from Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa.

3.11 PCR amplification of antibiotic resistance genes

A total of 68 antibiotic resistant isolates were subjected to specific PCR assays to amplify antibiotic resistant genes using a DNA Thermal cycler (Bio-Rad C1000 Touch™ Thermal Cycler). PCR reaction mixtures comprised standard 25 µL reaction volumes consisting of 12.5 µL of a 2X DreamTag Green Master Mix (0.4 mM dATP, 0.4 mM dCTP 0.4 mM dGTP and 0.4 mM dTTP, 4Mm MgCl2 and loading buffer), 11 µL of nuclease free water, 0.25 µL of each oligonucleotide primer and 1 µL of template DNA. All PCR reagents were Fermentas, USA products obtained from Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. Oligonucleotide primer sequences used as well as cycling conditions are listed in Table 3.3.

3.12 Bacterial 16S rRNA gene sequence analysis

Bacterial 16S rRNA gene fragments were sequenced at the Department of Microbiology, Potchefstroom Campus, South Africa and the sequence data were subjected to a blast search using the Blast Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cg) in order to confirm the identities of the isolates.

Table 3.3: Oligonucleotide primer sequences that were used to amplify antibiotic resistance genes by PCR analysis

Target Gene	Primer sequence (5'-3')	Amplicon Size (bp)	PCR Conditions	References
erm(B)	F: GAT ACC GTT TAC GAA ATT GG	364	95°C for 1 minute; 40 cycles of 95°C 15 seconds, 58°C for 1 minute, 72°C for 1 minute and a final elongation of 72°C for 1 minute.	Burch *et al.*, 2013
	R: GAA TCG AGA CTT GAG TGT GC			
sul1	F: CCG TTG GCC TTC CTG TAA AG	67	94°C for 5 minutes; 30 cycles of 94°C 30 seconds,58°C for 1 minute, 72°C for 1 minute and a final elongation of 72°C for 10 minutes.	Burch *et al.*, 2013
	R: TTG CCG ATC GCG TGA AGT			
tet(A)[a]	F: GCT ACA TCC TGC TTG CCT TC	210	95°C 1 minute; 40 cycles of 95°C 15 seconds, 62°C for 1 minute, 72 °C for 1 minute and a final elongation of 72 °C for 10 minutes.	Bergeron *et al.*, 2015
	R: CAT AGA TCG CCG TGA AGA GG			
tet(W)[a]	F: GAG AGC CTG CTA TAT GCC AGC	168		
	R: GGG CGT ATC CAC AAT GTT AAC			
tet(X)[a]	F: AGC CTT ACC AAT GGG TGT AAA	278		
	R: TTC TTA CCT TGG ACA TCC CG			
mec(A)[a]	F: ATGCGCTATAGATTGAAAGGAT	163	95 °C 1 minute; 40 cycles of 95°C for 15 seconds, 60 1 minute for 1 minute, 72 °C for 1 minute and a final elongation of 72 °C for 10 minutes.	Bergeron *et al.*, 2015
	R: TACGCGATATCTAACTTTCCTA			
ampC	F: TTCTATCAAMACTGGCARCC	1048	95°C for 1 minute; 35 cycles of 94°C 30 seconds, 49 °C for 30 seconds, 72°C for 1 minute and a final elongation of 72°C for 10 minutes.	Poirel *et al.*, 1999
	R: CCYTTTTATGTACCCAYGA			
strA	F: CTTGGTGATAACGGCAATTC	548	94 °C for 1 minute; 30 cycles of 94 °C for 45 seconds58 °C for 45 seconds,	Gebreyes and Altier, 2002
	R: CCAATCGCAGATAGAAGGC			
strB	F: ATCGTCAAGGGATTGAAACC	509		

	R: GGATCGTAGAACATATTGGC		72 °C for 45 seconds and a final elongation of 72 °C for 7 minutes	Gebreyes and Altier, 2002
aadA	F: GTGGATGGCGGCCTGAAGCC R: AATGCCCAGTCGGCAGCG	525	95°C for 4 minutes, 30 cycles of 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 45 seconds and a final elongation of 72°C for 10 minutes	Lanz *et al.*, 2003
cmlA	F: CCGCCACGGTGTTGTTGTTATC R: CACCTTGCCTGCCCATCATTAG	698	95°C for 2 minutes; 30 cycles of 94°C for 1 second, 40 °C for 1 second, 72°C for 15 seconds and a final elongation of 72°C for 10 minutes.	Gebreyes and Altier, 2002
vanA	F: TTG GGG GTT GCT CAG AGG AG R: CTT CGT TCA GTA CAA TGC GG	732	94°C for 3 minutes; 32 cycles of 94 °C for 30 seconds, 60 °C for 45 seconds, 72 °C for 2 minutes and (v) a final elongation of 72 °C for 10 minutes	Dutka-Malen, 1995
dfrB1, *dfrB2*	F: CAAAGTAGCGATGAAGCCA R: CAGGATAAATTTGCACTGAGC	205	95°C 10 minutes; 30 cycles of 95◦C for 15 seconds, 50 for 1 minute, (iv) 72 ◦C for 1 minute (v) and a final elongation of 72°C for 10 minutes	Kadlec *et al.*, 2005
blaCTX-M	F: ATGTGCAGYACCAGTAA R: CCGCTGCCGGTYTTATC	536	94°C for 5 minutes; 40 cycles of 94 °C for 1 minute, 57 °C for 1 minute, 72 °C for 1 minute and a final elongation of 72 °C for 10 minutes	Novais *et al.*, 2010

3.13 Agarose gel electrophoresis of PCR products

PCR products and bacterial DNA were separated by electrophoresis on a 1.3 % (w/v) agarose gel in 1 x TAE buffer [20mM Acetic acid (Merck, US), 40mM Tris (Sigma Aldrich, US) and 1mM EDTA (Merck, US) at pH 8.0]. A 100 bp DNA molecular weight marker was included in the gel and used to confirm the sizes amplicons (Bergeron *et al.*, 2015). Fragments were separated at 70 V, 250 mA for 60 minutes and gels were stained in ethidium bromide (0.001µg/mL). Amplicons were visualized on ChemiDoc Imaging System (Bio-RAD ChemiDoc™ MP Imaging System, Gene snap version 6.00.22. UK) under UV light at 420nm. Gel images were captured using Gene Snap (version 6.00.22).

CHAPTER 4

RESULTS AND INTERPRETATION

4.1 Physio-chemical parameters of water samples

The quality of the water samples was measured on site using a multi-probe analyser for physiochemical properties. These included; temperature, pH, electrical conductivity (EC) and total dissolved solids (TDS) and detailed results for the different sampling times are shown in Tables 4.1 and 4.2. The values obtained during sampling were equated to the drinking water parameters stipulated by SANS 241 (2011).

Table 4.1: Physical results for Plant NW- Mmabatho Water Treatment Plant 2016 - 2017

Sampling date	Sampling site	Temperature (°C)	pH	EC (µS/cm)	TDS (mg/L)
	SANS 241:2011	**-**	**≥ 5 to ≤ 9.7**	**≤ 170**	**≤ 1200**
August 2016	**Raw**	18.7	9.53	800	508
	Inlet	16.5	9.40	776	489
	AF	18.5	8.94	846	533
	D1	21.0	9.17	750	473
	D2	19.6	9.62	656	413
November 2016	**Raw**	25.7	9.94	763	481
	Inlet	23.9	9.93	774	487
	AF	24.8	9.47	773	487
	D1	27.0	9.17	703	443
	D2	28.3	8.37	677	426
March 2017	**Raw**	28.5	7.42	283	180.9
	Inlet	22.5	7.37	239	152.3
	AF	22.3	7.47	256	164.1
	D1	27.8	7.34	287	183.2
	D2	26.4	7.37	876	559
May 2017	**Raw**	23.5	8.86	375	765
	Inlet	20.9	8.58	374	764
	AF	21.1	8.28	398	811
	D1	22.4	9.03	400	816
	D2	21.8	8.66	387	789

Raw- Modimola Dam, Inlet- Abstracted water from catchment prior treatment, AT- Water After treatment, D1: Random Household tap water from treatment plant distribution system, D2- Second Random Household tap water from treatment plant distribution system.

Table: 4.2 Physical results for Plant NW- Mafikeng Water Treatment Plant (MK) 2016 - 2017

Sampling date	Sampling site	Temperature (°C)	pH	EC (µS/cm)	TDS (mg/L)
	SANS 241:2011	**-**	**≥ 5 to ≤ 9.7**	**≤ 170**	**≤ 1200**
August 2016	**Raw**	10.4	7.76	405	827
	Inlet	21.0	8.55	289	182
	AF	21.2	8.52	309	195
	D1	22.2	7.73	805	507
	D2	27.0	8.17	360	192.9
November 2016	**Raw**	24.6	8.96	390	246
	Inlet	26.5	8.79	305	192
	AF	24.5	8.85	321	202
	D1	31.1	8.56	321	202
	D2	26.5	8.78	305	192.2
March 2017	**Raw**	24.5	7.84	262	167.3
	Inlet	25.2	7.64	270	169.2
	AF	24.8	7.6	271	172.9
	D1	26.8	7.64	280	179.6
	D2	29.2	7.61	284	182.5
May 2017	**Raw**	17.6	9.56	366	747
	Inlet	20.0	8.65	357	717
	AF	21.0	8.43	355	727
	D1	24.6	8.88	370	760
	D2	25.6	8.62	338	690

Raw- Molopo Eye river, Inlet- Abstracted water from catchment prior treatment, AT- Water After treatment, D1: Random Household tap water from treatment plant distribution system, D2- Second Random Household tap water from treatment plant distribution system.

4.1.1 Temperature

For the cold periods August and May 2017 the temperature was lower than that of the warm periods November 2016 and March 2017. The temperature in the distribution system following extraction from catchment was higher than that in raw water. The temperature also increased following the purification process through to the distribution systems. The temperature in the distribution system ranged from 21°C to 31°C, with exception to the distribution system (D2) from Mmabatho Water Treatment Plant in August 2016 (cold period).

4.1.2 pH

The pH values were above 7.0 in all sampling points for both the treatment plants although these values varied throughout the systems ranging from 7.3 to 9.9. The values fell within the limits stipulated by SANS 241 (2011) (≥ 5 to ≤ 9.7) with exception of the raw and inlet water samples for the November 2016 collected from the Mmabatho Water Treatment Plant which were above the standard limits at 9.94 and 9.93 respectively. The pH values for the March 2017 were more neutral (7.34 - 7.47).

4.1.3 Electrical conductivity (EC)

All the EC values obtained for both the treatment plants were above the standard limits SANS 241 (2011) of ≤ 170 µS/cm and thus ranged from 239 µS/cm to as high as four times the limit (846 µS/cm). Although the EC values for March 2017 was lowest ranging between 239 to 287 µS/cm, these values were also above the reference standard values (SANS 241; 2011). Despite this, the EC value of water collected from a sampling point that was intended for direct human consumption was the highest at 876 µS/cm.

4.1.4 Total dissolved solids (TDS)

The total dissolved solids in all the sampling points for both the treatment plants fell below the maximum standard set by the South African National Standards of ≤ 1200 mg/L throughout the sampling periods. However, the TDS value was highest in the May 2017 (717 to 816 mg/L) sampling period when compared to the March 2017 sampling period that had the lowest TDS values (152.3 to 183.2 mg/L). On the contrary water samples collected from a second consumption point (D2) had the highest TDS of 559 mg/L.

4.2 Detection of Indicator organisms

The microbial quality of the water samples was also evaluated through the detection of indicator organisms, *E. coli* and Coliform bacteria as indicated in Materials and Methods

(Section 3.5). Results were also compared with drinking water parameters stipulated by SANS 241 (2011). Data in Tables 4.3 to 4.6 reveal the bacterial loads in the samples for the different sampling periods.

Table 4.3: Indicator organisms detected in water samples for the period August 2016 by plate count

Sampling Site	*E. coli*	Coliform	Faecal Coliform
Standard limits SANS 241 (2011)	**Not detected/100ml**	**< 10/100ml**	**Not detected/100ml**
		MM	
Raw	-	5	2
Inlet	2	3	4
AT	-	-	5
D1	-	4	3
D2	-	-	-
		MK	
Raw	1	1	7
Inlet	2	5	7
AT	-	-	1
D1	-	21	9
D2	-	2	-

Raw- Modimola Dam, Raw(MK)- Molopo Eye river, Inlet- Abstracted water from catchment prior treatment, AT- Water After treatment, D1: Random Household tap water from treatment plant distribution system, D2- Second Random Household tap water from treatment plant distribution system.

Table 4.4:

Indicator organisms detected in water samples for the period November 2016 by plate count

Sampling Site	*E. coli*	Coliform	Faecal Coliform
Standard limits SANS 241 (2011)	**Not detected/100ml**	**< 10/100ml**	**Not detected/100ml**
		MM	
Raw	24	17	7
Inlet	5	1	1
AT	-	-	-
D1	-	-	-
D2	-	1	1
		MK	
Raw	62	52	12
Inlet	-	-	6
AT	-	-	-
D1	-	-	-
D2	-	-	-

Raw (MM)- Modimola Dam, Raw(MK)- Molopo Eye river, Inlet- Abstracted water from catchment prior treatment, AT- Water After treatment, D1: Random Household tap water from treatment plant distribution system, D2- Second Random Household tap water from treatment plant distribution system.

Table 4.5: Indicator organisms detected in water samples for the period March 2017 by plate count

Sampling Site	*E. coli*	Coliform	Faecal Coliform
Standard limits SANS 241 (2011)	**Not detected/100ml**	**< 10/100ml**	**Not detected/100ml**
MM			
Raw	-	5	2
Inlet	2	3	4
AT	-	-	5
D1	-	4	3
D2	-	-	-
MK			
Raw	1	1	7
Inlet	2	5	6
AT	-	-	1
D1	-	21	9
D2	-	-	-

Raw (MM)- Modimola Dam, Raw(MK)- Molopo Eye river, Inlet- Abstracted water from catchment prior treatment, AT- Water After treatment, D1: Random Household tap water from treatment plant distribution system, D2- Second Random Household tap water from treatment plant distribution system.

Table 4.6: Indicator organisms detected in water samples for the period May 2017 by plate count

Sampling Site	*E. coli*	Coliform	Faecal Coliform
Standard limits SANS 241 (2011)	**Not detected/100ml**	**< 10/100ml**	**Not detected/100ml**
MM			
Raw	17	1	3
Inlet	7	-	-
AT	-	-	-
D1	-	-	-
D2	-	-	-
MK			
Raw	5	21	1
Inlet	1	3	5
AT	-	-	-
D1	-	-	-
D2	-	-	-

Raw (MM) - Modimola Dam, Raw(MK)- Molopo Eye river, Inlet- Abstracted water from catchment prior treatment, AT- Water After treatment, D1: Random Household tap water from treatment plant distribution system, D2- Second Random Household tap water from treatment plant distribution system.

Generally, the number of indicator organisms detected in water samples were higher in raw water, as would be expected. For the sampling periods from August 2016 to May 2017 water from both treatment plants in the two districts produced relatively similar bacteria contamination results. The Mmabatho Treatment Plant indicated the presence of *E. coli* (2 – 24/100 mL) in both the raw (catchment source) as well as in their intake water. On the contrary, water from the Mafikeng Treatment Plant revealed *E. coli* contamination of 1 - 62/100 mL only in the untreated water samples. Interestingly, after the purification process no *E. coli* cells were detected from consumers tap throughout the sampling periods from August 2016 to May 2017.

In the Mmabatho Treatment Plant, both Total and faecal coliforms were detected at 1-17/100 mL and 1-7/100 mL respectively. Water from the Mafikeng Treatment Plant revealed Coliform and faecal coliform counts of 1-52/100 mL and faecal coliform at 1-12/100 mL, respectively. Higher numbers of coliforms and faecal coliforms were detected in raw (range 24/100 mL and 52/100 mL respectively) than in consumers tap, with exception to the first distribution (D1) from the Mafikeng Treatment plant distribution system where the coliform and faecal coliform counts were 21/100 mL and 9/100 mL for both the March and August sampling periods respectively. These values are above those that are stipulated by the South African National Standards requiring that no faecal coliform bacteria should be detected in 100 mL of water sample for it to be considered safe by public health standards.

4.1 Detection of Heterotrophic bacteria

In all the 40 samples that were collected throughout the 4 seasons and analysed for heterotrophic bacteria, 202 isolates where enumerated using chromogenic R2A medium.

Bacteria contaminants from the different sampling points displayed variations in microbial growth counts as well as colonial morphologies with the colour of isolates ranging from yellow, pink, brown, orange and white or cream. Generally, the colonial morphologies of isolates obtained throughout the different sample collection periods was most often consistent and similar with exception of the detection of brown colonies in samples collected during the summer (November 2016 and March 2017) periods from the Mafikeng Treatment plant. Pure colonies of the heterotrophic bacteria contaminants were subjected to bacteria identification tests to species level using genetic techniques and results are summarised in Table 4.13.

4.2 Cellular morphologies of isolates

Following isolation HPC isolates were subjected to gram staining and isolates displayed varied gram results throughout the sampling period. For the second cold period May 2017 the majority of enumerated isolates were gram positive.

4.3 Antimicrobial resistance profiles of isolates

Antibiotics tested on each isolate was dependent strictly on its Gram status. Gram positive isolates were screened using all the antibiotics that appear in Table 3.2 while for Gram negative bacteria the antibiotics Ampicillin, Penicillin G, as well as Vancomycin were not tested due to possible intrinsic resistance (Kristich *et al.,* 2014). Isolates which displayed resistance to 3 or more antibiotics of different classes where analysed further for extracellular enzyme production assays. Detailed data of the proportion of isolates from the different sampling sites that were resistant to the antibiotics tested are shown in Tables 4.7 to 4.10. Results indicated that large proportion (50% - 100%) of the isolates from Molopo Eye river were most often resistant to Ampicillin, Penicillin G, Streptomycin, Kanamycin, Neomycin, Vancomycin, Cephalosporin and Trimethoprim. Even though a

similar trend was observed for isolates from water obtained in the Modimola Dam, the proportion of isolates that were resistant to these antibiotics were much lower (12.5% - 66.7%) except for isolates from water after treatment. None of the isolates obtained in water from both treatment plants in August 2016 were resistant to Chloramphenicol.

Similarly, large proportions (50% - 100%) of the isolates from water collected in November 2016 from the Modimola Dam were resistant to Ampicillin, Penicillin G and Trimethoprim when compared to those that were resistant to Erythromycin (16.7%-33.3%), Chloramphenicol (33.3% - 66.7%) except for isolates from raw water and water after treatment in the Molopo Eye river that were all resistant to this drug. A similar pattern of resistance to Chloramphenicol was obtained when isolates were screened against Streptomycin. Only a small proportion (33.3%) of the isolates collected from the raw and inlet water from the Modimola Dam and Molopo Eye river were resistant to Vancomycin. Interestingly, none of the isolates from water that was randomly collected household taps from Molopo Eye river treatment plant distribution system during the November 2016 sampling period was resistant the antibiotics. A large proportion (33.3% - 100%) of isolates obtained from water for both the March and May 2017 sampling periods were most often resistant to Ampicillin, Penicillin G and Trimethoprim.

Table: 4.7 Antibiotic resistance percentages for Plants NW August 2016

Site	Antibiotic											
	AP	**PG**	**E**	**CHL**	**C**	**S**	**K**	**NE**	**VA**	**OT**	**CIP**	**TMP**
		MM										
Raw	33.3	-	-	-	-	-	-	33.3	33.3	-	33.3	-
Inlet	28.6	-	-	-	-	42.9	28.6	-	-	28.6	-	57.1
AT	100	33.3	-	-	-	50	66.7	16.7	50	66.7	-	50
D1	16.7	-	16.7	-	-	-	-	-	16.7	16.7	-	50
D2	12.5	50	-	-	-	25	62.5	-	12.5	62.5	12.5	50
		MK										
Raw	100	33.3	-	-	-	66.6	100	16.6	66.6	83.3	-	83.3
Inlet	85.7	71.4	-	-	14.3	-	57.1	-	57.1	85.7	14.3	71.4

AT	100	33.3	-	-	0	33.3	16.7	50	66.7	50	16.7	100
D1	50	-	-	-	-	66.7	50	50	66.7	66.7	-	100
D2	33.3	-	-	-	-	-	-	-	-	33.3	-	100

Raw (MM)- Modimola Dam, Raw(MK)- Molopo Eye river, Inlet- Abstracted water from catchment prior treatment, AT- Water After treatment, D1: Random Household tap water from treatment plant distribution system, D2- Second Random Household tap water from treatment plant distribution system.

Table: 4.8 Antibiotic resistance percentages for Plants NW November 2016

Site	Antibiotic											
	AP	**PG**	**E**	**CHL**	**C**	**S**	**K**	**NE**	**VA**	**OT**	**CIP**	**TMP**
						MM						
Raw	50	50	16.7	33.3	-	33.3	-	-	33.3	16.7	-	100
Inlet	75	75	-	50	-	-	-	-	-	25	-	100
AT	100	100	-	50	-	-	-	-	-	-	-	100
D1	100	100	33.3	66.7	-	-	66.7	-	-	33.3	-	66.7
D2	-	-	16.7	66.7	16.7	88.3	33.3	16.7	-	66.7	-	66.7
						MK						
Raw	-	-	-	100	100	100	-	66.7	-	66.7	-	100
Inlet	50	50	-	33.3	-	33.3	-	-	33.3	66.7	-	100
AT	-	-	33.3	100	-	100	100	33.3	-	100	-	100
D1	-	-	-	-	-	-	-	-	-	-	-	-
D2	-	-	-	-	-	-	-	-	-	-	-	25

Raw (MM)- Modimola Dam, Raw (MK)- Molopo Eye river, Inlet- Abstracted water from catchment prior treatment, AT- Water After treatment, D1: Random Household tap water from treatment plant distribution system, D2- Second Random Household tap water from treatment plant distribution system.

Table: 4.9 Antibiotic resistance percentages for Plants NW March 2017

Site	Antibiotic											
	AP	**PG**	**E**	**CHL**	**C**	**S**	**K**	**NE**	**VA**	**OT**	**CIP**	**TMP**
							MM					
Raw	33.3	33.3	-	33.3	-	-	-	-	-	-	-	33.3
Inlet	-	-	-	20	-	-	-	-	-	-	-	40
AT	-	33.3	-	-	-	-	-	-	-	-	-	-
D1	16.7	16.7	-	33.3	-	-	-	-	-	-	-	83.3
D2	33.3	50	16.7	33.3	-	-	-	-	33.3	-	-	50
							MK					
Raw	33.3	33.3	33.3	33.3	-	33.3	-	-	-	-	-	-
Inlet	-	-	-	100	-	-	-	-	-	-	-	33.3
AT	33.3	33.3	-	33.3	66.7	-	-	-	-	-	-	16.7
D1	30	30	-	30	30	10	-	10	10	-	-	40
D2	22.2	11.1	-	22.2	-	-	-	-	-	-	-	33.3

Raw (MM)- Modimola Dam, Raw(MK)- Molopo Eye river, Inlet- Abstracted water from catchment prior treatment, AT- Water After treatment, D1: Random Household tap water from treatment plant distribution system, D2- Second Random Household tap water from treatment plant distribution system.

Table: 4.10 Antibiotic resistance percentages for Plants NW May 2017

Site	Antibiotic											
	AP	**PG**	**E**	**CHL**	**C**	**S**	**K**	**NE**	**VA**	**OT**	**CIP**	**TMP**
		MM										
Raw	40	40	-	0	0	0	0	0	0	0	0	60
Inlet	42.9	42.9	-	0	0	0	42.9	0	0	0	0	0
AT	40	40	-	0	0	0	60	0	0	20	0	0
D1	66.7	66.7	-	0	0	16.7	0	16.7	0	0	0	16.7
D2	100	80	-	0	0	0	60	0	0	0	0	20
		MK										
Raw	100	100	-	-	-	-	-	-	-	-	-	100
Inlet	100	83.3	16.7	-	-	-	-	-	16.7	-	-	66.7
AT	60	80	-	-	-	-	60	-	-	-	-	-
D1	60	80	-	-	-	-	20	-	-	-	-	20
D2	50	50	-	-	-	16.7	-	-	-	-	-	33.3

Raw (MM) - Modimola Dam, Raw(MK)- Molopo Eye river, Inlet- Abstracted water from catchment prior treatment, AT- Water After treatment, D1: Random Household tap water from treatment plant distribution system, D2- Second Random Household tap water from treatment plant distribution system.

4.4 Extracellular enzyme production

4.4.1 Haemolytic patterns on blood agar

A total of 68 multi-drug resistant isolates were screened to determine their haemolytic patterns and results obtained are shown in Table: 4.11. Generally, a majority (78%) of the isolates displayed haemolytic patterns on blood agar and these comprised thirty-seven (70%) that were β haemolytic and 16 (30%) that produced greenish colourations around bacterial colonies thus exhibiting α-haemolytic patterns. On the contrary, 15 isolates displayed no haemolytic patterns on blood agar and were interpreted as γ-haemolysis (Table 4.11).

Table 4.11: Haemolysis patterns of isolates from the different sampling periods

Collection period	**Number tested**	**β Haemolysis**	**α Haemolysis**	**γ Haemolysis**
August 2016	22	13	5	4
November 2016	15	9	4	2
March 2017	21	10	6	5
May 2017	11	4	1	6
	68	37	16	15

Figure: 4.1 illustrates a summary of extracellular productivity of all MAR heterotrophic

plate count isolates. 4 extracellular enzyme production were tested. Only 3 isolates displayed the capability of nucleic acids degradation. For the proteinase and lipase activity, isolates displayed varied results however majority of the isolates were positive for the testes respectively. 76% of the isolates were lipase positive. While 52% showed protein catabolism activity. All the isolates tested positive for the oxidase test, indicated by a dark blue colour reaction. With note 16S rRNA reveiled identities of isolates in the Enterobacteriaceae family which are known to be oxidase negative. Results observed obatines for oxidase activity may be of instrumental contamination during results interpretation.

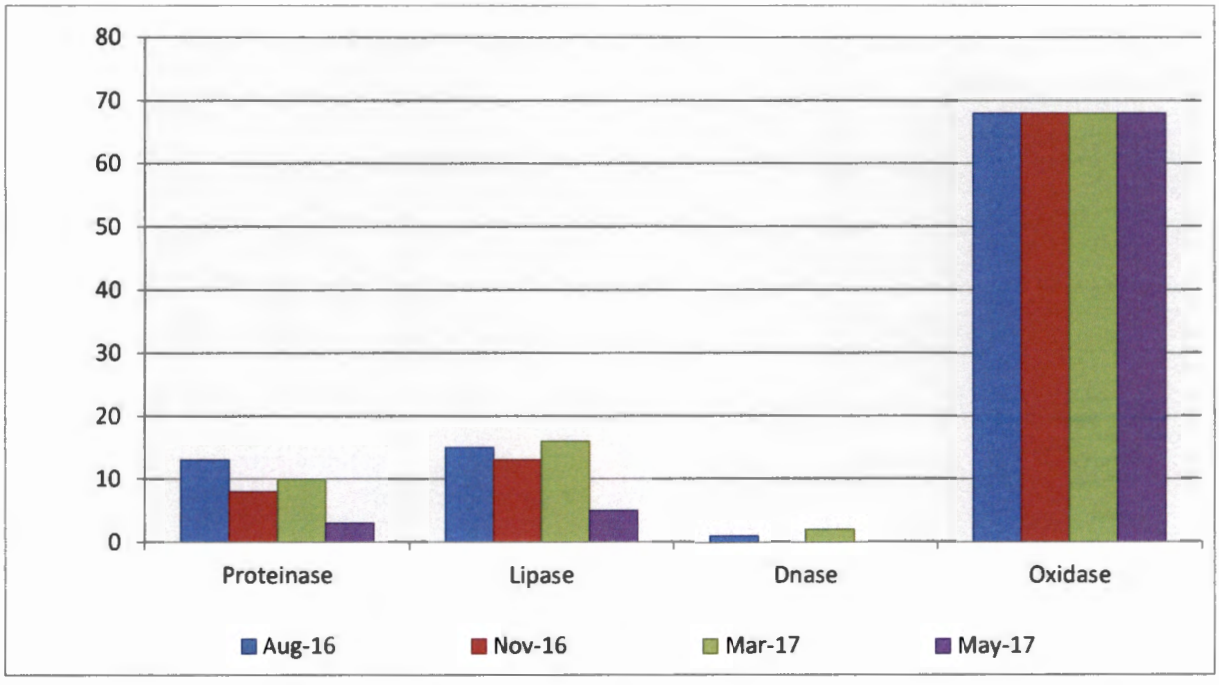

Figure 4.1: The number of isolates per samping period positive for extracellular enzyme investigated.

4.5 Chromosomal DNA extracted from MAR HPC isolates

Genomic DNA was extracted from all the 68 pure heterotrophic bacteria isolates. The DNA extracted was of good quantity with no RNA or fragmentation observed.

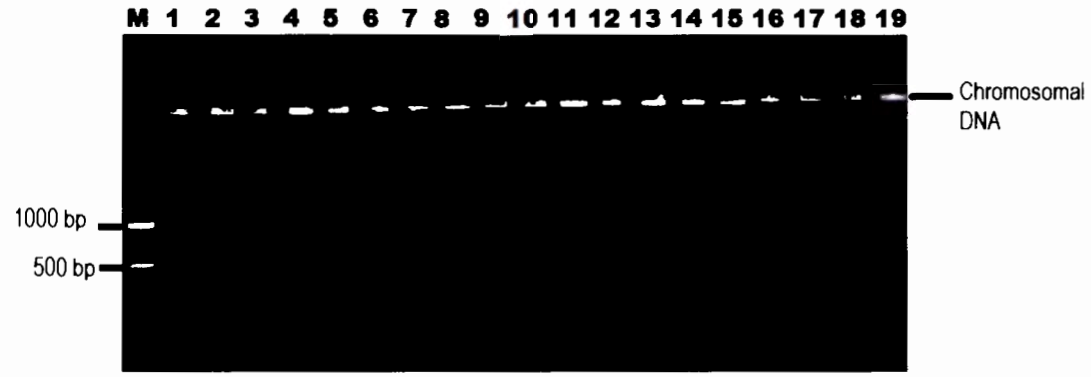

Figure 4.2: An image depicting a 1.3% (w/v) agarose gel of chromosomal DNA extracted from isolates. Lane M= 100 base pairs DNA ladder; Lanes 1-19: DNA extracted from representative isolates.

4.6 16S rRNA gene PCR analysis

Bacterial 16S rRNA gene was amplified for all 68 MAR heterotopic plate count bacteria using conditions stated on Table 3.3. Amplification produced the expected 16S rRNA universal bacteria gene fragments with the size of 1420 bp. Bacteria 16S rRNA gene fragments of representative isolates are shown in Figure 4.3.

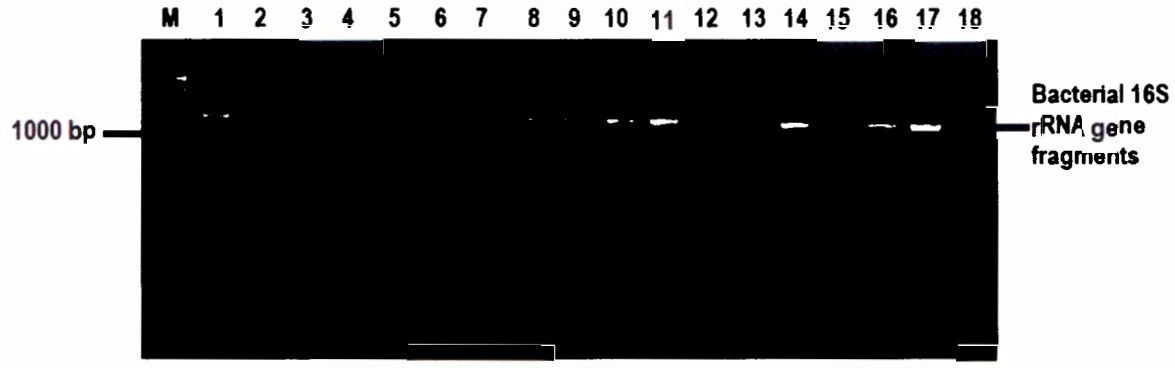

Figure 4.3: An image depicting a 1.3% (w/v) agarose gel of bacterial 16S rRNA gene fragments amplified from isolates. Lane M= 100 base pairs DNA ladder; Lanes 1-17: bacterial 16S rRNA gene fragments of representative isolates and Lane 18: No template DNA negative control reaction.

4.7 PCR amplification of antibiotic resistance genes

4.7.1 Detection of streptomycin resistant *strA* and *strB* gene fragments

All the 68 isolates that displayed phenotypic resistance to 3 or more antibiotics from different classes (Table 3.2) were subjected to specific PCR assays designed to screen for the presence of resistant determinants. Results indicated that a large proportion (55.8%) of these isolates harbored the *strA* gene that codes for resistance to streptomycin (Gebreyes and Altier, 2002). In addition, 30.8% of these isolates were positive for the *strB* gene fragments. Figures 4.4 and 4.5 indicate 548 bp and 509 bp gene fragments representing the *strA* and *strB* genes respectively from representative isolates in the study.

Figure 4.4: An image depicting a 1.3% (w/v) agarose gel of the *strA* gene fragments amplified from isolates. Lane M= 100 base pairs DNA ladder; Lanes 1-11 and 13-14: *strA* gene fragments of representative isolates and Lane 12: No template DNA negative control reaction.

Figure 4.5: An image depicting a 1.3% (w/v) agarose gel of the *strA* and *strB* gene fragments amplified from isolates. Lane M= 100 base pairs DNA ladder; Lanes 1-10: *strB* gene fragments of representative isolates and Lane 11: No template DNA negative control reaction.

4.7.2 Detection of aminoglycoside antibiotic resistant *aadA* gene fragments

All the isolates were screened for the presence of the Streptomycin 3"-adenylyltransferase (*aadA*) gene encoding for resistance to Aminoglycoside antibiotics (Pinto-Alphandary *et al.*, 1990) and hence mediates bacterial resistance to the antibiotics streptomycin and spectomycin. Of the 68 multi-drug resistant heterotrophic isolates only a small proportion (16.2%) harbored the *aadA* gene and amplifications produced

fragments with the expected amplicons sizes of 525 bp. Figure 4.6 indicates the *aadA* genes amplified from representative isolates in the study.

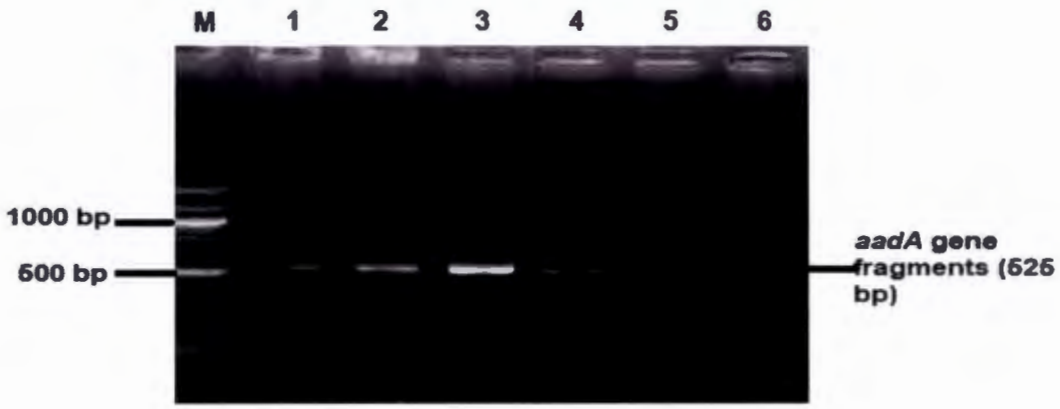

Figure 4.6: An image depicting a 1.3% (w/v) agarose gel of the *aadA* gene fragments amplified from isolates. Lane M= 100 base pairs DNA ladder; Lanes 1-5: *aadA* gene fragments of representative isolates and Lane 6: No template DNA negative control reaction.

4.7.3 Detection of Trimethoprim antibiotic resistant *dfrB1, dfrB2* gene fragments

All the 68 heterotrophic isolates that displayed phenotypic resistance to 3 or more antibiotic discs of different antibiotic classes were screened for the presence of Trimethoprim resistance genes *dfrB1* and *dfrB2*. Results indicated that 19.1% of the isolates harboured the *dfrB1* and *dfrB2* resistant gene segments in their chromosomes and fragments were of the expected size (205 base pairs). Figure 4.7 indicates the *dfrB1 and dfrB2* genes amplified from representative isolates in the study.

Figure 4.7: An image depicting a 1.3% (w/v) agarose gel of the *dfrB1* and *dfrB2* gene fragments amplified from isolates. Lane M = 100 base pairs DNA ladder; Lanes 1-15: *dfrB1* and *dfrB2* gene fragments of representative isolates.

4.7.4 Detection of b-lactamase resistance *blaCTX-M* gene fragments

All the 68 Isolates were also screened for the presence of the *blaCTX-M* beta-lactamase gene that encodes for resistance to beta-lactam antibiotics (Novais *et al.*, 2010). From these only a small proportion (7.4%) of the isolates were positive for this gene and amplifications produced fragments with the expected size of 536 bp (Figure 4.8).

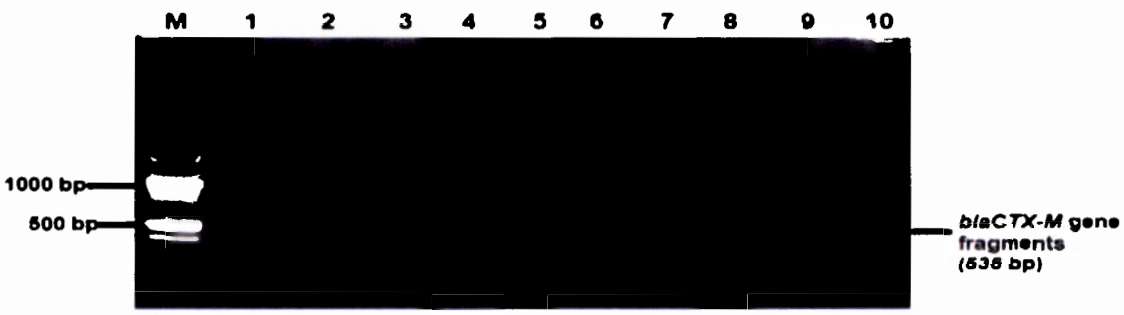

Figure 4.8: An image depicting a 1.3% (w/v) agarose gel of the *blaCTX-M* gene

fragments amplified from isolates. Lane M = 100 base pairs DNA ladder; Lanes 1-9: *blaCTX-M* gene fragments of representative isolates and Lane 10: No template DNA negative control reaction.

4.7.5 Detection of Tetracycline resistance *tetA* gene fragments

Tetracycline antibiotics have a broad-spectrum activity against a wide range of microorganisms' protozoa and chlamydiae (Chopra and Roberts, 2001). All the 68 isolates were screened for the presence of the *tetA* tetracycline resistance gene. Despite the very high phenotypic resistance against this drug, only a small proportion (4.4%) of these isolates harbored the *tetA* resistance gene. The *tetA* gene fragments amplified in this study were of the expected size (210 bp) and Figure 4.9 indicates the *tetA* gene fragments amplified from representative isolates.

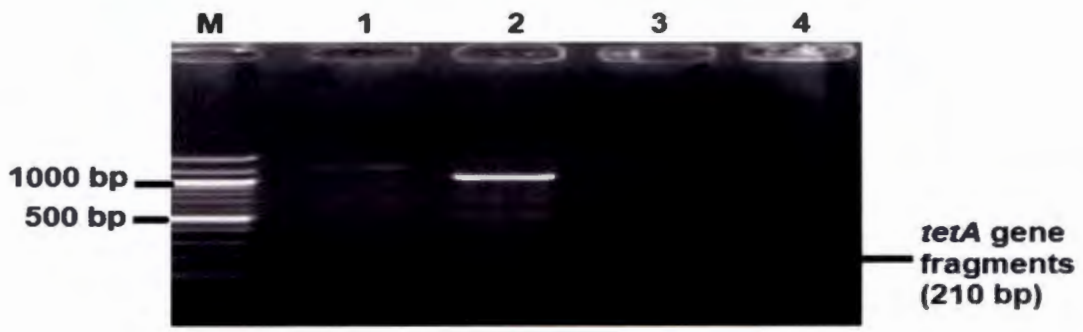

Figure 4.9: An image depicting a 1.3% (w/v) agarose gel of the *tetA* gene fragments amplified from isolates. Lane M = 100 base pairs DNA ladder; Lanes 1-3: *tetA* gene fragments of representative isolates and Lane 4: No template DNA negative control reaction.

4.7.6 Summary of antibiotic resistant determinants in isolates

The proportions of the different antibiotic resistant genes amplified successfully from heterotrophic bacterial isolates from the different sample sites and seasons were computed and summarised. Detailed data is shown in Table 4.12. As shown in Table 4.12 only 6 resistant genes were successfully amplified among the isolates. The *StrA* and *StrB* genes were the frequently detected resistant determinants among the isolates in 38 and 21 isolates respectively. Despite the fact that an appreciable proportion of the isolates possessed the *dfrB1* and *dfrB2* genes (13 isolates) as well as the *aadA* gene (11 isolates), only very small proportions possessed the *tetA* (3 isolates) and the *blaCTX-M* gene (5 isolates).

Table: 4.12 Summary of the proportion of resistant determinants harboured by MAR isolates from the different sampling sites in the study.

		MAR heterotrophic plate count isolates positive for Resistant genes tested					
Sample Period	**Site**	***StrA***	***StrB***	***aadA***	***tetA***	***blaCTX-M***	***dfrB1, dfrB2***
August (MM)	**Raw**		1	1		1	1
	Inlet	2	1				1
	AT	1	1	1			
	D2	2	1				1
(MK)	**Raw**	2					
	Inlet	3	2				2
	AT	2					
November (MM)	**Raw**	3			1	1	
	AT	1					
	D1	1	1				1
	D2	1	1				1
(MK)	**Raw**	1	2		1	1	
	Inlet	2					1
	AT						1
	D1				1		
March (MM)	**Raw**			1		1	
	Inlet	1		2		1	1
	AT	1					
	D1	2	2	1			1
	D2	2	1				
(MK)	**Raw**	2	2				
	AT	1	1				

	D1	3	2	2			
	D2	2					
May (MM)	**Raw**	1					
	Inlet	1					
	AT		1				
	D2		1	1			2
(MK)	**Raw**			1			
	AT	1	1	1			
Total Isolates positive of 68 tested		**38**	**21**	**11**	**3**	**5**	**13**

4.8 Identification of MAR isolates based on bacterial 16S rRNA gene sequence data

Bacterial 16S rRNA sequencing data of the HPC isolates revealed that isolates possessed more than 90 percent similarities to previously characterised species, from seven different families that comprised *Enterobacteriaceae*, *Paenibacillaceae*, *Bacillaceae*, *Yersiniaceae*, *Xanthomonadaceae* and *Flavobacteriaceae*. With interest MKOW2 which had 64.32% similarities to a *Pseudoflavonifractor* species had never been named previously. The sequence data of four isolates did not match any strain in the data base. Isolates MKOW2 and MKOB1 possessed sequence data with very high percentage similarities to *Serratia marcescens* subsp. Marcescens. Interestingly, these isolates presented with brown colonial morphologies on agar and could not have been considered to belong the genus *Serratia*. It is therefore suggested that the preliminary identification characteristics of bacteria isolates must be complemented with more reliable genetic identification assays to ensure correct classification.

Table 4.13: Identities of MAR isolates based on bacterial 16S rRNA gene sequences.

Name	Top-hit taxon	Top-hit strain	Similarities (%)	Top-hit taxonomy	Completeness (%)
1-2-_907-R_E08_14	*Escherichia coli*	NCTC9001(T)	99.33	Bacteria;Proteobacteria;Gammaproteobacter	59.2

MPW2				ia;Enterobacterales;Enterobacteriaceae;*Escherichia*	
4_907-R_A07_01 MKIW2	*Citrobacter pasteurii*	CIP 55.13(T)	99.65	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Enterobacteriaceae;*Citrobacter*	59.2
4-2-_907-R_F08_17 MMH2P1	*Bacillus paramycoides*	NH24A2(T)	92.75	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;*Bacillus*	60.0
5-2-_907-R_G08_20 MMH2P3	*No hits found*	No hits found	0		0
6_907-R_B07_04 MPO3	*Stenotrophomonas maltophilia*	MTCC 434(T)	99.41	Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;*Stenotrophomonas*	59.1
7-2-_907-R_H08_23 MDW2	*Klebsiella variicola*	DSM 15968(T)	99.76	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Enterobacteriaceae;*Klebsiella*	59.0
8_907-R_C07_07 MKIW2	*Citrobacter amalonaticus*	CECT 863(T)	99.33	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Enterobacteriaceae;*Citrobacter*	59.5
10-2-_907-R_A09_03 MDO3	*Delftia lacustris*	LMG 24775(T)	96.75	Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;*Delftia*	59.2
13-3-_907-R_B09_06 MKH2B3	*Blastomonas natatorial*	DSM 3183(T)	98.24	Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae;*Blastomonas*	59.4
16_907-R_D07_10 MKIW2	*Stenotrophomonas maltophilia*	MTCC 434(T)	99.53	Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;Stenotrophomonas	59.2
19_907-R_E07_13 MKOO3	*Stenotrophomonas maltophilia*	MTCC 434(T)	99.42	Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;*Stenotrophomonas*	59.5
19-2-_907-R_C09_09 MPW1	*Pseudoxanthomonas mexicana*	AMX 26B(T)	97.02	Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;*Pseudoxanthomonas*	59.2
19-3-_907-R_D09_12 MMH1Y3	*Bacillus wiedmannii*	FSL W8-0169(T)	84.74	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;*Bacillus*	59.7

20-4-_907-R_E09_15 MMH1W3	*No hits found*	No hits found	0		0
22-2-_907-R_F09_18 MKIY2	*Stenotrophomonas maltophilia*	MTCC 434(T)	99.52	Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;*Stenotrophomonas*	59.3
23-2-_907-R_G09_21 MKOY2	*Stenotrophomonas maltophilia*	MTCC 434(T)	99.65	Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;*Stenotrophomonas*	59.2
23-3-_907-R_H09_24 MMH2W1	*Stenotrophomonas pavanii*	DSM 25135(T)	99.49	Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;*Stenotrophomonas*	59.4
24-4-_907-R_A10_01 MMH2O2	*Pseudomonas indoloxydans*	IPL-1(T)	95.03	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;*Pseudomonas*	59.2
25-3-_907-R_B10_04 MMH2W3	*Klebsiella singaporensis*	LX3(T)	80.51	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Enterobacteriaceae;*Klebsiella*	59.4
27-3-_907-R_C10_07 MMH1Y1	*JHEE_s*	250J	98.17	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;*Pseudomonas*	59.3
28_907-R_F07_16 MMH2O1	*Stenotrophomonas maltophilia*	MTCC 434(T)	99.76	Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;*Stenotrophomonas*	59.1
30_907-R_G07_19 MKOW2	*JQ084175_s*	WT_dss_B1_D07	64.32	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;*Pseudoflavonifractor*	45.9
32-3-_907-R_D10_10 MKH1W2	*Enterobacter ludwigii*	EN-119(T)	99.63	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Enterobacteriaceae;*Enterobacter*	59.2
34-3-_907-R_E10_13 MPW2	*Serratia marcescens subsp. Marcescens*	ATCC 13880(T)	98.19	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Yersiniaceae;Serratia;*Serratia marcescens*	59.0
35_907-R_H07_22 MKIP	*Klebsiella singaporensis*	LX3(T)	96.96	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Enterobacteriaceae;*Klebsiella*	58.8

38-2-_907-R_F10_16 MMIY2	*Paenibacillus typhae*	CGMCC 1.11012(T)	99.37	Bacteria;Firmicutes;Bacilli;Bacillales;Paenibacillaceae;*Paenibacillus*	59.0
41-4-_907-R_G10_19 MMH2W1	*Chryseobacterium gambrini*	DSM 18014(T)	84.77	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;*Chryseobacterium*	59.8
43_907-R_A08_02 MKIY2	*Stenotrophomonas maltophilia*	MTCC 434(T)	100.00	Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;*Stenotrophomonas*	58.4
44_907-R_B08_05 MPP2	*Stenotrophomonas maltophilia*	MTCC 434(T)	99.53	Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;*Stenotrophomonas*	59.4
44-3-_907-R_H10_22 MDW1	*No hits found*	No hits found	0		0
44-4-_907-R_A11_02 MMH2W3	*Klebsiella pneumoniae subsp. Ozaenae*	ATCC 11296(T)	94.11	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Enterobacteriaceae;Klebsiella;*Klebsiella pneumoniae*	59.5
46-4-_907-R_B11_05 MKOW2	*Serratia marcescens subsp. Marcescens*	ATCC 13880(T)	99.50	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Yersiniaceae;*Serratia*;*Serratia marcescens*	59.5
50-3-_907-R_C11_08 MKOB1	*Serratia marcescens subsp. Marcescens*	ATCC 13880(T)	98.66	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Yersiniaceae;*Serratia*;Serratia marcescens	59.2
51_907-R_C08_08 MKH1W3	*Citrobacter europaeus*	97/79(T)	99.07	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Enterobacteriaceae;*Citrobacter*	59.2
52-4-_907-R_D11_11 MMOW2	*Serratia nematodiphila*	DSM 21420(T)	99.61	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Yersiniaceae;*Serratia*	59.2
54-4-_907-R_B11_05 MMOW3	*Bacillus paramycoides*	NH24A2(T)	99.88	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;*Bacillus*	59.6
58_907-R_D08_11 MDW1	*No hits found*	No hits found	0		0

CHAPTER 5

DISCUSSION

5.1 General Discussion

The main objective of this study was to isolate different types of antibiotic resistance bacteria in raw and drinking water samples from two water distribution systems in the Mafikeng Local Municipality. To avoid bias the study focused on heterotrophic plate count bacteria since these organisms are capable of proliferating in controlled water distribution systems and thus provide an indication of the microbial status of the water (Allen *et al.*, 2004).

In addition, heterotrophic bacteria counts also provide an indication of the efficiency and adherence of the water management procedures as well as protocols to standard operational procedures that are designed to eliminate or reduce microbial growth (WHO, 2003; Wetzel, 2001; Inomata *et al.*, 2009). The main motivation of this is based on previous reports in the North West Province that revealed the presence of multi-drug resistant opportunistic bacteria harbouring a variety of virulence determinants in water from river catchments that are unfortunately used for drinking by individuals in communities that do not have access to potable water(Pavlov *et al.*, 2004; Mulamattathil *et al.*, 2000; Ateba and Mbewe, 2011; 2013; 2014; Bezuidenhout *et al.*, 2013; Cartens *et al.*, 2014; Mulamattathil *et al.*, 2014;).

Moreover, this study was also motivated by the fact that the presence of antibiotic resistant organisms in drinking water may pose challenges to water production facilities resulting from the deterioration of water quality. This also goes a long way to pose severe health challenges on consumers who rely on water from municipal treatment facilities.

These concerns are even aggravated in a country like South Africa were the incidence of HIV/AIDS is significantly high and therefore the importance of studies designed to assess water quality cannot be over-emphasized. Data generated from this study will provide an overview of the survival of micro-organisms within the different stages within two water distribution systems from raw water to household taps were the finished product is provided to consumers.

In South Africa, the Service Charter is designed to "uphold the Constitutional responsibility of the State clearly articulated in the Bill of Rights to deliver services to the citizenry" (PSCBC Resolution 1 of 2013). Article 4 of the Service Charter actually outlines some of the services that the State must provide to the citizens and among others the provision of Water and sanitation. In order to achieve this, the Department of Water and Sanitation that is the custodian of South Africa's water resources has been given the mandate to ensure that all South Africans gain access to clean water and dignified sanitation. The department also promotes effective and efficient water resources management to ensure sustainable economic and social development.

Given that the main objective of any organisation is to ensure that the finished product that is supplied to consumers is of great quality, there must be systems in place to constantly determine the proportion of indicator organisms whose concentrations do not only provide an indication of the presence of other pathogenic but may serve as an indirect assessment of the public health risks on consumers.

An analysis of indicator organisms in water from both the districts treatment plants indicated relatively similar results. Though the presence of *E. coli* organisms in catchment areas as well as inlet water for both plants was evident (1-62/100mL), results showed

effective purification measures as none were enumerated following treatment. With note the raw and inlet sampling points, the inlet water in each plant displayed a lower quantity of indicator organisms to that in raw water. Because the water is of the same source, the lower level of indicator organisms observed in the inlet sampling point may be a result of natural purification which occurred in the raw water. Coliforms and Faecal Coliforms on the other hand were detected throughout the system from catchment source to the taps of consumers for both plants.

The proportion of total coliform bacteria cells detected in water from both plants following treatment ranged from 1 – 4/100 mL of water and was within the water quality safety limits of less than 10 coliform per 100 mL in water as recommended by the SANS 241 regulations. However, the proportion of faecal Coliforms in water from the Mmabatho and Mafikeng Treatment plants after treatment were 1 - 5/100 mL and 1 - 9/ 100 mL of water respectively and this violated the SANS 241 recommendations that requires no faecal coliform bacteria to be detected in every 100 mL of water intended for human consumption. Despite the fact that these results showed the presence of significantly higher numbers of coliforms and faecal coliform bacteria in water from both treatment plants, the proportion of these indicator organisms were also higher in raw water than in water collected from the tap of consumers. These findings indicate that water from both treatment plants do not meet safe drinking water recommendations as stipulated in SANS 241 and may serve as a potential source for the transmission of pathogens to consumers.

In addition, USEPA highlights that a number of organisms may survive current treatment processes thus rendering the finished product unsafe for drinking (EPA[c], 2002). Resistance to treatment protocols and chemicals may even be worsened by the ability of bacteria contaminants to form biofilms in distribution systems (EPA[c], 2002). Moreover, it has also been identified that opportunistic pathogens isolated from bacterial biofilms in

water distribution systems have been associated with secondary infections in immunocompromised individuals (Momba *et al.*, 2000; Mahapatra *et al.*, 2015). This therefore amplifies the need to constantly assess the effectiveness of water purifications systems as well as the quality of water produced from the plants in order to indirectly determine the public health implications on consumers. Given that the persistence of waterborne pathogens in water is not ideal, WHO also highlights that most of these pathogens do not grow or proliferate outside a host (WHO, 2006). Against this background, pathogens are easily transmitted through inadequate personal hygiene than by consuming contaminated water (WHO, 2006) and this outlines the need for the implementation of proper hygiene practices.

In this study, R2A selective media was used to successfully isolate planktonic HPC bacteria in bulk water samples from the Mmabatho and Mafikeng Treatment plants as well as the drinking water distribution systems. Despite the fact that pigmented HPC colonies varied slightly among the different sampling sites with colours ranging from yellow, orange, pink to brown some colonies did not produce any -pigments and appeared as either white or clear colonies. However, the detection of these representative pigmented and non-pigmented colonies in samples from the different sites throughout the study period indicates regularity great similarity in the species distribution throughout both the distribution systems.

Another objective of the study was to determine the physiochemical properties of water in both water distribution systems. This is based on the motivation that the physiochemical properties of water greatly influence bacterial re-growth and persistence therefore requiring the implementation of control strategies. Moreover, adherence to the SANS 241 specified guidelines minimises or eliminates the potential of undesired bacteria contaminants to persist in water and present public health challenges to consumers. The

pH values for water from both the Mmabatho and Mafikeng Treatment Plants for the sampling period August 2016 to May 2017 was mostly alkaline and ranged between 7.37 and 9.62. It is hereby suggested that the pH values of water samples might have contributed significantly to the Electric conductivity and Total Dissolved Solids parameters detected in the bulk water samples.

The Electric conductivity for both plants exceeded the SANS 241 specified limits and between 239 - 846 µS/cm per 100 mL of bulk water sample. EC and pH values showed a pattern in which the EC decreased when the pH was neutral (pH 7.34-7.47) and this was common among samples analysed in March 2017 and increased when the pH was alkaline. Total Dissolved Solids in the samples were within the stipulated limits even in bulk samples from untreated water. The values for Total Dissolved Solids were also lower when the pH of the water was neutral (pH 7.34-7.47).

During the analysis of water samples in this study, samples collected during the warm periods (November 2016 and March 2017) brought on the enumeration of brown pigmented colonies for the Mafikeng Treatment plant following 16S rRNA universal gene analysis using Blast Search Tool which gave identities to different bacterial strains *Blastomonas natatorial* and *Serratia marcescens subsp. Marcescens.* And clear colourless colonies observed on both two the plants November 2016 sampling period as well as for the May 2017 sampling period for Mafikeng Treatment plant distribution system. Temperature is known to influence metabolic rates and biological activity of aquatic inhabitants (Wetzel, 2001). The observation of these pigmented colonies may be as a result of the change in temperature in the water source due to season. It may also be an implication of availability of nutrients which dissolve easier at higher temperature (WHO, 2003; Wetzel, 2001; Inomata *et al.*, 2009). Furthermore, the variety of HPC obtained in this study is limited as incubation was at 37°C with interest to bacterial cells

which may potentially survive in a human host furthermore used of HPC could only determine the presence of active and culturable bacteria and do not support the growth of all bacteria that can inhabit chlorination distribution systems.

A further objective of the study was to isolate different antibiotic resistance bacteria present in the water samples as well as their associated resistant genes. Large proportion (50% - 100%) of the isolates from Molopo Eye river were most often resistant to Ampicillin, Penicillin G, Streptomycin, Kanamycin, Neomycin, Vancomycin, Cephalosporin and Trimethoprim. Even though a similar trend was observed for isolates from water obtained in the Modimola Dam, the proportion of isolates that were resistant to these antibiotics were much lower (12.5% - 66.7%) except for isolates from water after treatment. None of the isolates obtained in water from both treatment plants in August 2016 were resistant to Chloramphenicol.

From a total of 68 MAR heterotrophic isolates that were screened for the *strA* gene, a significant proportion 38 (55.8%) harbored this gene. A similar observation was reported by Gebreyes and Altier, (2002) in which 30.8% of planktonic bacteria were positive for the *strA* gene that codes for resistance to streptomycin. In addition to the *strA* gene fragment, the *strB* gene is also associated with resistance to streptomycin (Sundin and Bender, 1996). Moreover, both the *strA* and *strB* have also been linked to resistance of isolates to sulphonamide. However, none of the isolates in this study haboured the *sul1* gene that is primarily responsible for sulfonamide resistance.

Streptomycin resistance that was initially documented in 1945 (Sundin and Bender, 1996), is mediated by chromosomal mutations or the expression of streptomycin-inactivating enzymes with the latter accounting for a large proportion of treatment failures among infections caused by clinically relevant Gram-negative and gram-positive bacteria

(Walia *et al.,* 2004; Brunning, 2014). In addition, the *strA* and *strB* have been identified in a variety of broad host range non-conjugative plasmids within bacteria species isolated from animals and on the *Tn*3-type *Tn*5393 transposon that are present in conjugative plasmids among bacterial isolates (Sundin and Bender, 1996). The presence of these resistance determinants on mobile genetic elements facilitates the transmission to other susceptible isolates thus resulting in a modification of both their phenotypic and genotypic characteristics (Sundin, 2000). This might account for the recent detection of the *Tn*5393 resistant determinant in clinically relevant organisms such as *Campylobacter jejuni* and *Pseudomonas aeruginosa* (Sundin, 2000). The findings of this study also indicate that these isolates may serve as a potential source for the transmission of resistant determinants among bacteria species in the area.

The antimicrobial agent Streptomycin has been reported to be an important drug for synergistic treatment of serious and life-threatening infections caused by *Enterococcus* species. In addition, the *aadA* gene, which encodes resistance to streptomycin and spectinomycin via an adenylyltransferase enzyme and these are commonly harboured by Gram-negative organisms (Courvalin *et al.*, 1986; Tait *et al.*, 1985; Chinault *et al.*, 1986) and *Staphylococcus aureus* isolates (Courvalin and Fiandt, 1980). Two main mechanisms have been reported to facilitate the expression of resistance to streptomycin and these include ribosomal mutation as well as enzymatic modification of the drug. Despite the fact that the present study was not designed to determine the mechanisms of resistance to this drug an assessment of the occurrence of the *aadA* gene in isolates was performed. A significantly large proportion (16.2%) of the heterotrophic isolates harbored the *aadA* gene and displayed phenotypic resistant profiles to streptomycin. Moreover, the detection of both the *aadA* and *strA* genes in three isolates was a cause for concern. Despite the fact that two of these isolates did not possess extracellular

enzymatic traits that may initiate disease in their hosts, an MKH1Y3 that was identified as a *Bacillus wiedmannii* was isolated from the water distribution system after purification and has been reported to be pathogenic to humans. In summary, these strains possessed resistance genes *strA* and *strB* as well as *aadA* and also displayed potential to produce extracellular enzymes Proteinase, Lipase and β-haemolysin. The pathogenesis of *Bacillus wiedmannii* is highly associated with the potential to produce potent toxins. Despite the fact that pasteurization is capable of eliminating the pathogen in food products especially milk thus rendering it safe for consumption, the spores are known to survive in refrigeration and are capable of producing cellular pores that result in diarrhoea.

The antibiotic Trimethoprim is commonly used in the treatment of urinary tract infections in all parts of the world (Huovinen et al., 1995) and resistance to this drug has been reported among several bacterial species (Skold, 2001) thus increasing the global burden of antibiotic resistance to humans. Trimethoprim resistance was detected in 19.1% of the isolates in the present study through amplification of the *dfrB1, dfrB2* gene when compared to the much larger proportion (54%) of the isolates that displayed phenotypic resistance to this drug. Previous studies that were designed to evaluate the occurrence of metabolites to Trimethoprim in waste water treatment plants revealed that there was very little elimination of the drug in water samples collected during the different stages of purification (Carballa *et al.*, 2004; Perez *et al.*, 2005; Lindberg *et al.*, 2005). These observations could account for the high levels of resistance among the isolates from this study. However, the findings of this study do not agree with those of a previous report in which large proportions (96% and 69%) of *E. coli* and *K. pneumoniae* harboured 13 of the *dfr*-genes analysed (Brolund et al., 2010). Despite the fact that, phenotypic antibiotic resistance data may not be relied on to conclusively indicate Trimethoprim resistance,

they may serve as baseline data to inform and assist in the development and implementation of strategic control measures.

Amplification of Tetracycline resistance gene (*tetA*) was detected in a very small proportion (4.4%) of heterotrophic isolates in this study. The amplification was also of poor quality as illustrated on Figure 4.9 as amplification of desired gene also generated nonspecific binding amplicons. This finding contradicts a previous report in the area were marked multiple antibiotic resistances (over 70%) were observed for tetracycline amongst *E. coli* isolated from wastewater samples, Modimola Dam and tap water from homes (Wose Kinge.et al., 2010). Tetracycline antibiotics are frequently used as broad-spectrum drug against a wide range of microorganisms (Chopra and Roberts, 2001), and the drug is easily accessible over the counter. This therefore explains why large proportions of isolates are most often resistant to this antibiotic. However, the detection low levels of tetracycline resistance determinants among environmental isolates indicate that the proportion of isolates that are resistant to a specific drug at any given time should not be based on previous reported but on data obtained through experimental analysis.

The last objective of the study was to determine the identities of the heterotrophic isolates obtained in the study using bacteria 16S rRNA sequence analysis I. Isolates belonged to families *Enterobacteriaceae, Paenibacillaceae, Bacillaceae, Yersiniaceae, Xanthomonadaceae* and *Flavobacteriaceae*. Despite the fact that some of the isolates such as *Klebsiella pneumoniae* obtained in this study have not been extensively studied when compared to their more pathogenic counterparts due to the fact that they are often considered to be opportunistic pathogens. However, these bacterial species have been associated with nosocomial infections in clinical settings and within households. *Klebsiella pneumoniae subsp. Ozaenae* has been implicated as the main causative agent

of rhinopharynx chronic inflammatory disease as well as tracheo bronchopathia (Magro *et al.*, 2007). These isolates may pose public health complications to humans especially in a country like South Africa where the incidence of HIV/AIDS is very high.

Another *Klebsiella* species isolated in this study was *K. Variicola,* which accounts for 10% of the clinical *Klebsiella* infections (Alves *et al.*, 2006). It is however usually misidentified as *K. pneumoniae* when using routine methods as it is genetically related to *K. pneumoni*ae and the two can only be differentiated using the adonitol fermentation test (Seki *et al.*, 2013). This genetic relatedness largely accounts for a large proportion of misdiagnosis within the clinical sector and this has been reported to pose life threatening complications to patients (Seki *et al.*, 2013)). In addition, Maatallah *et al.*, (2014) reported that *Klebsiella variicola* than *K. pneumoniae* have been associated with higher mortality rates in humans suffering from bloodstream infections. Thus the detection of this bacteria strain in water that is utilized by individuals in rural communities was a great cause for concern.

CHAPTER 6

CONCLUSIONS

6.1 Conclusion

In the present study, physico-chemical parameters of water samples were successfully determined and compared against standard guideline values as recommended by SANS 241. Bulk water from both municipal distribution systems had alkaline pH values. The alkaline pH has a potential to affect the effectiveness of the disinfection process with chlorine (pH 8), damage and corrode distribution pipes (pH6 6.5-9.5) (WHO, 2003). Electric conductivity for both plants exceeded the specified limits while Total Dissolved Solids in all the sampling points for both the treatment plants meet the standards set by the South African National Standards.

Indicator organisms were also detected in the water samples, but *E. coli* was only detected in raw water based on phenotypic agar detection procedures. On the contrary, faecal coliform bacteria was detected in water samples collected at the different sampling points in both water distribution systems and values violated specified limits.

Heterotrophic bacteria were also successfully enumerated. Bacteria contamination was higher in raw water as would be expected. The proportion of gram-positive bacteria was higher among HPC bacterial population isolated throughout the different seasons. The identities of the HPC isolates were successfully determined using bacterial 16S rRNA sequence analysis and isolates predominantly belonged to the families *Enterobacteriaceae, Paenibacillaceae, Bacillaceae, Yersiniaceae, Xanthomonadaceae* and *Flavobacteriaceae*. These families comprise species that are known to be of great clinical importance.

Isolates obtained in the study possessed multi-drug resistant profiles and the *strA* gene was dominant among the isolates and this was followed by the *strB* gene.

6.2 Recommendations

- To further, future studies aiming at validation of the results and methods used in this study should include known and characterised bacterial strains with virulent characteristics representing HPC group. To be used as positive controls for the genetic resistant determinants tested.

- The HPC method used in this study only enumerated culturable HPC bacteria 37°C for 72 hours. As previously studied conditions of enumeration greatly influence bacteria culturable. On that note the pathogenic potential unculturable bacteria at the specified conditions number were no assessed. Therefore further studies should examine genetic resistant determinants from bacteria enumerated at various conditions to get a greater at view the prevalence of resistant determinants in the water bodies examined.

- Because this study aimed at examining culturable organisms at 37°C the incubation temperature extended to faecal coliforms detection, though bacterial cells we observed at this temperature, it is of important to note that cells observed may not be a true reflection of the total culturable organisms in the water samples as optimum temperature for coliforms is at 44.5 ± 0.5°C and therefore would be a limitation on the coliform bacterial cells enumerated.

- Though 16S rRNA sequences were generated in this study, no phylogenetic relationships were determined with other known strains, for future studies it would be beneficial to shed light on these relations.

- Because hygiene greatly contributes to bacterial load in tap water, is would be more accurate to use the same sampling point repeatedly following distribution of water post treatment throughout the study period.

BIBLIOGRAPHY

A Drinking Water Quality Framework for South Africa (DWQ Framework). The Department: Water Affairs and Forestry retrieved from http://www.dwaf.gov.za/Documents/Other/DWQM/DWQMFrameworkDec05.pdf [accessed, November 2016].

Acar, J., Röstel, B., 2001. Antimicrobial resistance: An overview. Revue scientifique et technique. International Office of Epizootics 20(3), 797-810.

Adeosun, O.O., 2014. Water Distribution System Challenges and Solutions. Water Online Retrieved from https://www.wateronline.com/doc/water-distribution-system-challenges-and-solutions-0001

Ajithkumar, B., Ajithkumar, V.P, Iriye, R., Doi, Y., Sakai, T., 2003. Spore-forming Serratia marcescens subsp. sakuensis subsp. nov., isolated from a domestic wastewater treatment tank. International Journal of Systematic and Evolutionary Microbiology 53, 253–258.

Al Aukidy, M., Verlicchi, P., Jelic, A., Petrovic, M., Barcelò, D., 2012. Monitoring release of pharmaceutical compounds: occurrence and environmental risk assessment of two WWTP effluents and their receiving bodies in the Po Valley. Italy Science of the Total Environment 438, 15-25.

Allen, M.J., Edberg, S.C., Reasoner, D. J., 2004. Heterotrophic plate count bacteria—what is their significance in drinking water?. International Journal of Food Microbiology 92, 265– 274.

Almagor, J., Temkin, E., Benenson, I., Fallach, N., Carmeli, Y., On behalf of the DRIVE-AB consortium (2018) The impact of antibiotic use on transmission of resistant bacteria in hospitals: Insights from an agent-based model. PLoS ONE 13(5),197111.

Alves, M.S., Dias, R.C., de Castro, A.C., Riley, L.W., Moreira, B.M., 2006. Identification of clinical isolates of indole-positive and indolenegative Klebsiella spp. Journal of Clinical Microbiology 44, 3640–3646.

American Public Health Association (APHA), 1992. Standard Methods for Examination of Water and Waste 18th Ed., American Public Health Association, Washington, D.C.

Ashton, D., Hiltonb, M., Thomas, K.V., 2004. Investigating the environmental transport of human pharmaceuticals to streams in the United Kingdom. Science of the Total Environment 333, 167–184.

Ateba, C.N., Mbewe, M., 2011. Detection of *Escherichia coli* O157:H7 virulence genes in isolates from beef, pork, water, human and animal species in the northwest province, South Africa: public health implications. Research in Microbiology 162(3), 240-248

Ateba, C.N., Mbewe, M., 2013. Determination of the genetic similarities of fingerprints from *Escherichia coli* O157:H7 isolated from different sources in the North West Province, South Africa using ISR, BOXAIR and REP-PCR analysis. Microbiology Research 168(7), 438-46

Ateba, C.N., Mbewe, M., 2014. Genotypic characterization of *Escherichia coli* O157:H7 isolates from different sources in the North-West Province, South Africa, Using Enterobacterial Repetitive Intergenic Consensus PCR Analysis. International Journal of Molecular Science 15(6), 9735-9747

Azzouz, A., Ballesteros, E., 2013. Influence of seasonal climate differences on the pharmaceutical, hormone and personal care product removal efficiency of a drinking water treatment plant. Chemosphere 93, 2046–2054.

Bäckhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A. and Gordon, J.I. (2005). Host-bacterial mutualism in the human intestine. Science 307, 1915–1920.

Bamford, C., Bonorchis, K., Ryan, A., Simpson, J., Elliott, E., Hoffmann, R., Naicker, P., Ismail, N., Mbelle,N., Nchabeleng, M., Nana, T., Sriruttan, C., Seetharam, S., Wadula, J., 2011. Antimicrobial susceptibility patterns of selected bacteraemic isolates from South African public sector hospitals, 2010. South African Journal of Epidemiology and Infect: (Part II) 26(4), 243-250.

Baquero, F., Martinez, J.L., Canton, R., 2008. Antibiotics and antibiotic resistance in water environments. Current Opinions in Biotechnology 19, 260-265.

Basaraba, R.J.; Oehme, F.W.; Vorhies, M.W.; Stokka, G.L., 1999. Toxicosis in cattle from concurrent feeding of monensin and dried distillers grains contaminated with macrolide antibiotics. International Journal of Vaccines and Vaccination 11, 79–86.

Basson, M. S., Van Rooven, J. A., 1998. Tunneling into the Future: The Role of Water Resources Development in South Africa. Tunnelling and Underground Space Technology 13(1), 35-38.

Bergeron, S., Boopathy, R., Nathaniel, R., Corbin, A., LaFleur G., 2015. Presence of antibiotic resistant bacteria and antibiotic resistance genes in raw source water and treated drinking water. International Biodeterioration and Biodegradation 102, 370-374.

Berry, D., Xi, C., Raskin, L., 2006. Microbial ecology of drinking-water distribution systems. Current Opinion in Biotechnology. 17, 297-302.

Bessa, L.J., Dias, V.F., Mendes, A., Martins-Costa, P., Ramos, H., da Costa, Paulo, M. C., 2014. How Growth Ability of Multidrug-Resistant Escherichia coli Is Affected by Abiotic Stress Factors. Open Journal of Preventive Medicine 4, 250-256.

Bhadja, P., & Vaghela, A. 2013. Effect of temperature on the toxicity of some metals to Labeo bata. International Journal of Advanced Life Sciences 6(3).

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O., Piddock, L. J. V., 2015. Molecular mechanisms of antibiotic resistance. Nature Reviews Microbiology 13, 42–51.

Blue Drop report, Department of water and Sanitation, 2014. Available at http://www.ewisa.co.za/misc/BLUE_GREENDROPREPORT/GREEN%20BLUE%20DROP/2014BlueDropReportExecutiveSummary_ToPDF.pdf. [Date of access: November 2016]

Boucher, Y., Labbate, M., Koenig, J.E., Stokes, H.W., 2007. Integrons: mobilizable platforms that promote genetic diversity in bacteria. Trends in Microbiology 15, 301–309.

Brettar, I., Hofle, M.G., 2008. Molecular assessment of bacterial pathogens - a contribution to drinking water safety. Current Opinion in Biotechnology 19:274–280

Brolund A, Sundqvist M, Kahlmeter G, Grape M. 2010. Molecular Characterisation of Trimethoprim Resistance in Escherichia coli and Klebsiella pneumoniae during a Two-Year Intervention on Trimethoprim Use. PLoS ONE 5(2): e9233. doi:10.1371/journal.pone.0009233

Brunning, A., 2014. An Overview of Antibiotics. Retrieved from: https://longitudeprize.org/blog-post/overview-antibiotics [Date of access: April 2016].

Byarugaba, D.K., Kisame, R., Olet, S., 2011. Multi-drug resistance in commensal bacteria of food from animal origin in Uganda. African Journal of Microbiology Research 5(12), 1539-1548.

Carballa, M., Omil, F., Lema, J.M., Llompart, M., Garcia-Jares, C., Rodriguez, I., 2004. Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. Water Resources 38, 2918–2926.

Carraro, L., Maifreni, M., Bartolomeoli, I., Martino, M. E., Novelli, E., Frigo, F., Marino, M., Cardazzo, B., 2011. Comparison of culture-dependent and -independent methods for

bacterial community monitoring during Montasio cheese manufacturing. Research in Microbiology 162, 231-239.

Carstens, A., Bartie, C., Dennis, R., Bezuidenhout, C.C., 2014. Antibiotic resistant heterotrophic plate count bacteria and amoeba resistant bacteria in aquifers of the Mooi River, North West province, South Africa. Journal of Water and Health 12(4), 835-845.

Chinault A C, Blakesley V A, Roessler E, Willis D G, Smith C A, Cook R G, Fenwick R G., Jr 1986. Characterization of transferable plasmids from *Shigella flexneri* 2a that confer resistance to trimethoprim, streptomycin, and sulfonamides. Plasmid (15), 119–131

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

Cizmas, L., Sharma, V.K., Gray, C., McDonald, T.J., 2015. Pharmaceuticals and personal care products in waters: occurrence, toxicity, and risk. Environmental Chemistry Letters 13(4), 381-394.

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

Clarridge, J.E., 2004. Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases. Clinical Microbiology Reviews 17 (4), 840–862.

Clinical and Laboratory Standards Institute (CLSI), 2007. M100-S17. Performance standards for antimicrobial susceptibility testing; 17th informational supplement, Clinical and Laboratory Standards Institute, Wayne, PA

Colomer-Lluch, M., Jofre, J., Muniesa, M., 2011. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. Public Library of Science ONE 6 (3), e17549.

Courvalin P, Carlier C, Collatz E. Evolutionary relationships between plasmid-mediated resistance to aminocyclitol antibiotics in group D streptococci. Journal of Bacteriology 143 (2), 541–551

Courvalin, P, Fiandt, M., 1980. Aminoglycoside-modifying enzymes of *Staphylococcus aureus*;

Courvalin, P., Carlier, C., Collatz, E., 1981. Evolutionary relationships between plasmid-mediated aminoglycoside-modifying enzymes from gram-positive and gram-negative bacteria. In: Grassi G, Sabath L D, editors. New trends in antibiotics: research and therapy. New York, N.Y: Elsevier/North-Holland Biomedical Press , 95–109.

Cruikshank, R., Duguid, J. P., Marmoin, B. P., Swain, R. H., 1975. Medical Microbiology, 12th Ed. Longman, New York. 2, 3-4.

Department of Environmental Affairs (DEA), 2016. 2nd South Africa Environment Outlook. A report on the state of the environment. Executive Summary. Department of Environmental Affairs, Pretoria. Chapter 8, 132- 153.

Department of Public Works (DPW), 2011. Small Waste Water Treatment Works DPW Design Guidelines, Retrieved from http://www.publicworks.gov.za/PDFs/consultants_docs/Design_guidline_for_Small_Waste_Water_Treatment_Works.pdf [Date of access June 2016]

DeZuane, J., 1996. Handbook of Drinking Water Quality, 2nd Ed. Wiley and Sons. ISBN: 978-0-471-28789-6

Diaz Ricci, J.C., Hernandez, M.E., 2000. Plasmid effects on Escherichia coli metabolism. Critical Reviews in Biotechnology 20, 79–108.

Dougherty, T.J. and Pucci, M.J., 2012. Antibiotic Discovery and Development. Springer New York Dordrecht Heidelberg London chapter 1 and 3.

Dutka-Malen,S., Evers, S., Courvalin, P., 1995. Detection of Glycopeptide Resistance Genotypes and Identification to the Species Level of Clinically Relevant Enterococci by PCR. Journal of Clinical Microbiology 33(1), 24–27.

DWA Department of Water affairs: Green Drop Report, 2012 available at http://www.idc.co.za/images/Content/Tenders/Chapter12-IntroBackground30042012.pdf. [Date of access: April 2016].

Dzidic, S., Suskovic, J., Kos, B., 2008. Antibiotic Resistance Mechanisms in Bacteria: Biochemical and Genetic aspects. Food Technology and Biotechnology 46(1), 11-21.

Environmental Protection Agency United States, EPA[a] (2012). 5.9 Conductivity. In Water: Monitoring and Assessment. Retrieved from http://water.epa.gov/type/rsl/monitoring/vms59.cfm [Date of access June 2017]

Environmental Protection Agency United States, EPA[b] (2012). pH. In Water: Monitoring and Assessment. Retrieved from http://water.epa.gov/type/rsl/monitoring/vms54.cfm [Date of access June 2017]

Environmental Protection Agency United States, EPA[c]. 2002. Health risks from microbial growth and biofilms in drinking water distribution systems. Washington: United States Environmental Protection Agency, 52 .

Evgenidou, E.N., Konstantinou, I.K., Lambropoulou, D.A., 2015. Occurrence and removal of transformation products of PPCPs and illicit drugs in wastewaters: a review. Science of the total Environment 505, 905-926.

Galán, J.C., González-Candelas, F., Rolain, J.M., Cantón, R., 2013. Antibiotics as selectors and accelerators of diversity in the mechanisms of resistance: from the resistome to genetic plasticity in the β-lactamases world. Frontiers of Microbiology 4(9), 1-17.

Gatti, A.M., Montanari, S., 2016. New Quality-Control Investigations on Vaccines: Micro- and Nanocontamination. International Journal of Vaccines and Vaccination 4(1), 1-13.

Gillings, M.R., 2014. Integrons: Past, present and future. Microbiology and Molecular Biology Reviews 78, 257–277.

Gould, S. J., and Vrba, S. (1982). Exaptation: a missing term in the science of form. Paleobiology 8, 4–15.

Grabow, W.O.K., van Zyl, M., 1976. Behaviour in conventional sewage purification processes of coliform bacteria with transferable or non-transferable drug resistance. Water Research Journal 10, 717–723.

Guidance to drinking water quality 4th Edition. World Health Organization. 2011. WHO Press, Switzerland. Available at http://apps.who.int/iris/bitstream/10665/44584/1/9789241548151_eng.pdf. [Date of access: April 2016].

Hellard, M.E., Stewart, M.I., Forbes, A.B., Fairley, C.K., 2001. A randomized, blinded, controlled trial investigation of the gastrointestinal health effects of drinking water quality. Environmental Health Perspectives 109(8), 773-778.

Henton, M.M., Eagar, H.A., Swan, G.E., van Vuuren, M., 2011. Part VI. Antibiotic management and resistance in livestock production. South African Medical Journal 101(8), 583-586.

Hoefel, D., Monis, P.T., Grooby, W.L., Andrews, S., Saint, C.P., 2005. Profiling bacterial survival through a water treatment process and subsequent distribution system. Journal of Applied Microbiology 99,175–186.

Huang, J.J., Hu, H.Y., Wu, Y.H., Wei, B., Lu, Y., 2013. Effect of chlorination and ultraviolet disinfection on tetA-mediated tetracycline resistance of Escherichia coli. Chemosphere 90, 2247–2253.

Huovinen P, Sundstrom L, Swedberg G, Skold O. 1995. Trimethoprim and sulfonamide resistance. Antimicrobial Agents Chemotherapy 39, 279–289.

Inomata, A., Chiba, T., Hosaka, M., 2009. Identification of Heterotrophic Plate Count Isolated from drinking water in Japan by DNA Sequencing Analysis. Biocontrol Science 14(4), 139-145.

Integrated Development Plan (IPD)-review. 2015. Ngaka Modiri Molema District Municipality available at http://www.nmmdm.gov.za/Documents/IPD-review-2014-2015.pdf . [Date of access: January 2017].

Jacob, M.E.; Fox, J.T.; Narayanan, S.K.; Drouillard, J.S.; Renter, D.G.; Nagaraja, T.G., 2008. Effects of feeding wet corn distillers grains with solubles with or without monensin and tylosin on the prevalence and antimicrobial susceptibilities of fecal foodborne pathogenic and commensal bacteria in feedlot cattle. Journal of Animal 86, 1182–1190.

Jacoby, G. A., 2009. AmpC β-lactamases. Clinical Microbiology Reviews 22(1), 161–182.

Jeters, R.T., Wang, G.R., Moon, K., Shoemaker, N.B., Salyers, A.A., 2009. Tetracycline-associated transcriptional regulation of transfer genes of the Bacteroides conjugative transposon. Journal of Bacteriology 191, 6374–6382.

Johnson, A.P., 2015. Surveillance of antibiotic resistance. Philosophical Transmissions Royal Society B 370. 1-12.

Kaplan, S., 2013. Review: Pharmacological Pollution in Water. Critical Reviews in Environmental Science and Technology 43(10), 1074-1116.

Katayama, Y., Ito, T., Hiramatsu K., 2000. A new class of genetic element, staphylococcal cassette chromosome mec, encodes methicillin resistance in Staphylococcus aureus. Antimicrobial Agents Chemotherapy 44(6), 1549-1555

Keen, P.L., Patrick, D.M., 2013. Tracking Change: A Look at the Ecological Footprint of Antibiotics and Antimicrobial Resistance. Antibiotics 2, 191-205.

Khan, S., Beattie, T.K., Knapp, C.W., 2016. Relationship between antibiotic- and disinfectant-resistance profiles in bacteria harvested from tap water. Chemosphere 152, 132-14.

Khan, U., Nicell, J. (2015). Human health relevance of pharmaceutically active compounds in drinking water. American Association of Pharmaceutical Scientists Journal 17, 558–585.

Kim, P.S., Shin, N.R., Kim, J.Y., Yun, J.H., Hyun, D.W., Bae, J.W., (2013). Gibbsiella papilionis sp. nov., isolated from the intestinal tract of the butterfly Mycalesis gotama, and emended description of the genus Gibbsiella. International Journal of Systematic and Evolutionary Microbiology 63, 2607–2611.

Koenig, J.E., Spor, A., Scalfone, N., 2011. Succession of microbial consortia in the developing infant gut microbiome. Proceedings of the National Academy of Sciences of the United States108, 4578–4585.

Kohanski, M.A., Dwyer, D.J., Collins, J.J., 2010. How antibiotics kill bacteria: from targets to networks. Nature Reviews Microbiology 8(6), 423–435.

Korzeniewska, E., Harnisz, M. 2013. Beta-lactamase-producing Enterobacteriaceae in hospital effluents. Journal of Environmental Management 123, 1-7.

Kristich, C.J., Rice, L.B., Arias, C.A., 2014. Enterococcal Infection—Treatment and Antibiotic Resistance. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK190420/pdf/Bookshelf_NBK190420.pdf [Date of access: April 2016].

Lapworth, D.J., Baran, N., Stuart, M.E., Ward, R.S., 2012. Emerging organic contaminants in groundwater: a review of sources, fate and occurrence. Environmental Pollution 163, 287-303.

LeChevallier, M. W., Seidler, R. J., Evans, T. M., 1980. Enumeration and characterization of standard plate count bacteria in chlorinated and raw water supplies. Applied Environmental Microbiology 40, 922–930.

Lehtola, M.J., Miettinen, I.T., Keinanen, M.M., Kekki, T.K., Laine, O., Hirvonen, A., Vartiainen, T., Martikainen, P.J., 2004[a]. Microbiology,chemistry and biofilm development in a pilot drinking water distribution system with copper and plastic pipes. Water Resource 38, 3769-3779.

Lehtola, M.J., Nissinen, T.K., Miettinen, I.T., Martikainen, P.J., Vartiainen, T., 2004[b]. Removal of soft deposits from the distribution system improves the drinking water quality. Water Resources 38, 601-610.

Le-Minh, N., Khan, S.J., Drewes, J.E., Stuetz, R.M., 2010. Fate of antibiotics during municipal water recycling treatment processes. Water Resources 44, 4295-4323

Lindberg, R.H., Wennberg, P., Johansson, M.I., Tysklind, M., Andersson, B.A.V., 2005. Screening of human antibiotic substances and determination of weekly mass flows in five sewage treatment plants in Sweden. Environmental Science Technology 39, 3421–3429.

Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I., Zhang, J., Liang, S., Wang, X.C., 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Science of the Total Environment (473-474), 6190-641.

Maatallah, M., Vading, M., Kabir, M.H., Bakhrouf, A., Kalin, M., 2014. Klebsiella variicola Is a Frequent Cause of Bloodstream Infection in the Stockholm Area, and Associated with Higher Mortality Compared to K. pneumoniae. PLoS ONE 9(11), 1135-1139.

Magro, P., Garand, G., Cattier, B., Renjard, L., Marquette, C.H., Diot, P., 2007. Association of tracheobronchopathia osteochondroplastica and ozene. Revue Des Maladies Respiratoires journal 24, 883-887.

Mahapatra, A., Padhi, N., Mahapatra, D., Bhatt, M., Sahoo, D. Jena, S., Dash, D., Chayani, N., 2015. Study of Biofilm in Bacteria from Water Pipelines. Journal of Clinical and Diagnostic Research 9(3), 9-11.

Martínez, J.L., 2008. Antibiotics and antibiotic resistance genes in natural environments. Science 321: 365–367.

Martínez, J.L., 2012, Natural antibiotic resistance and contamination by antibiotic resistant determinants: the two ages in the evolution of resistance to antimicrobials. Frontiers in Microbiology 3(1), 1-3.

Martinez, J.L., Fernando, B., 2009. Antibiotics and the Evolution of Antibiotic Resistance. n: Encyclopedia of Life Sciences. John Wiley & Sons, Ltd: Chichester.

Martins, A., 2014. How growth ability of multidrug-resistant *Escherichia coli* is affected by abiotic stress Factors. Open Journal of Preventive Medicine 4, 250-256.

Mellon M, Benbrook C, Benbrook KL 2001. Hogging It! Estimates of antimicrobial abuse in livestock. Cambridge: Union of Concerned Scientists Publications. http://www.ucsusa.org/assets/documents/food_and_agriculture/hog_chaps.pdf [Date of access August 2012]

Mendelson, M., Matsoso, M.P., 2015. The South African Antimicrobial RESISTANCE Strategy Framework. Monitoring, Surveillance and national Plans. Available at http://www.globalhealthdynamics.co.uk/wp-content/uploads/2015/06/08_Mendelson-Matsotso.pdf [Date of access August 2017]

Milić, N., Milanović, M., Letić , N.G., Sekulić, M.T., Radonić, J., Mihajlović, I., Miloradov, M.V., 2013. Occurrence of antibiotics as emerging contaminant substances in aquatic environment. International Journal of Environmental Health Research 23(4), 296-310.

Miller, G. (2006). Integrated concepts in water reuse: managing global water needs. Desalination. Vol. 187, pp 65–75.

Miller, R.A., Beno, S.M., Kent, D.J., Carroll, L.M., Martin, N.H.,Boor, K.J., Kovac, J., 2016. Bacillus wiedmannii sp. nov., a psychrotolerant and cytotoxic Bacillus cereus group species isolated from dairy foods and dairy environments International Journal of Systematic and Evolutionary Microbiology 66, 4744–4753.

Momba, M.N.B., Kfir, R., Venter, S.N., Cloete, T.E. 2000. An overview of biofilm formation in distribution systems and its impact on the deterioration of water quality. Water South Africa 26 (1), 59-66.

Mothetha, M., Z. Nkuna, Z., Mema, V., 2013. The challenges of rural water supply: a case study of rural areas in Limpopo Province. Council for Scientific and Industrial Research Retreved fromhttp://researchspace.csir.co.za/dspace/bitstream/handle/10204/7593/Mothetha_2013.pdf;jsessionid=5D7239AA2F96A42B6FCF4E7245751ADD?sequence=1

Mulamattathil, S.G., Bezuidenhout, C., Mbewe, M., 2014.Biofilm formation in surface and drinking water distribution systems in Mafikeng, South Africa. South African Journal of Science 110 (11/12), 1-9.

Nada, T., Baba, H., Kawamura, K., Ohkura, T., Torii, K., and Ohta, M., 2004. A small outbreak of third generation cephem-resistant Citrobacter freundii infection on a surgical ward. The Japanese Journal of Medical Science and Biology 57,181–182.

National Research Council (NRC), 2006. Drinking Water Distribution Systems; Assessing and Reducing risks. The National Academies Press Washington, D.C

National State of Water Resources Report 2012/13 Retrieved from http://www.dwa.gov.za/Groundwater/documents/Annual%20National%20State%20Water%20Report%20for%20Hudrological%20Year%202012-13_Final.pdf. [Date of access: March 2017].

Natura Research Council of Canada (NRCC), 2011. Effects of Sodium and Potassium in the Canadian Environment No. 150154. Associate Committee on Scientific Criteria for Environmental Quaity Otttawa. from Retrieved from http://water.epa.gov/type/rsl/monitoring/vms59.cfm [Date of access June 2017]

Nicolay, N. and Kotzé, J. 2009. ASSA2003 (Provincial) AIDS and Demographic Model. Retrieved from:http://www.callawayleadership.com/downloads/CLI_LE_episode18_summary_HIV_stats_SA.pdf . [Date of access: March 2017].

Norman, A., Hansen, L.H., Sorensen, S.J., 2009. Conjugative plasmids: vessels of the communal gene pool. Philosophical Transactions of the Royal Society B: Biological Sciences 364, 2275–2289.

North West Province Environment Outlook (NWPEO), 2008. Report on the state of the environment, North West Department of Agriculture, Conservation and Environment, 132 – 138.

Novais, A., Comas, I., Baquero, F., Canto´n, R., Coque, T.M., Moya,A., Gonza´ lez-Candelas, F., Gala´n, J.C., 2010. Evolutionary Trajectories of Beta-Lactamase CTX-M-1 Cluster Enzymes: Predicting Antibiotic Resistance. Public Library of Science Pathology 6(1), e1000735.

Parolini, M., Pedriali, A., Binelli, A. (2013). Application of a biomarker response index for ranking the toxicity of five pharmaceutical and personal care products (PPCP) to the bivalve Dreissena polymorpha. Environmental Contamination and Toxicology 64, 439–447.

Pavlov, D., De Wet, M.E., Grabow, W.O.K., Ehlers, M.M. 2004. Potentially pathogenic features of heterotrophic plate count bacteria isolated from treated and untreated drinking water. International Journal of Food Microbiology 92, 275–287.

Payment, P., Coffin, E., Paquette, G., 1994. Blood Agar To Detect Virulence Factors in Tap Water Heterotrophic Bacteria. Applied and Environmental Microbiology, 1179-1183.

Perez, S., Eichhorn, P., Aga, D.S., 2005. Evaluating the biodegradability of sulfamethazine, sulfamethoxazole, sulfathiazole, and trimethoprim at different stages of sewage treatment. Environmental Toxicology Chemicals 24, 1361–1367.

Picó, Y., Barceló, D., 2015. Transformation products of emerging contaminants in the environment and high-resolution mass spectrometry: a new horizon. Analytical and Bioanalytical Chemistry 407, 6257-6273.

Pinto-Alphandary, H.,Mabilat, C., Courvalint, P., 1990. Emergence of Aminoglycoside Resistance Genes *aadA* and *aadE* in the Genus Campylobacter. Antimicrobial Agents and Chemotherapy 34(6), 1294-1296.

Poirel, L., Rodriguez-Martinez, J. M., Mammeri, H., Liard, A., Nordmann, P., 2005. Origin of plasmid- mediated quinolone resistance determinant QnrA. Antimicrobial Agents Chemotherapy 49, 3523–3525.

Pruden, A., 2014. Balancing water sustainability and public health goals in the face of growing concerns about antibiotic resistance. Environmental Science and Technology 48, 5-14.

Radjenovi´c, J., Petrovi´c, M., Barceló, D., 2009. Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. Water Research 43, 831–841.

Republic of South Africa (RSA), 1996, Constitution of the Republic of South Africa, Act No. 108 of 1996, chapter 8, Cape Town.

Riley, M.R., Gerba, C.P., Elimelech, M. 2011. Biological approaches for addressing the grand challenge of providing access to clean drinking water. Journal of Biological Engeneering 5 (2), 1754-1611.

Roberts, M.E., Stewart, P.S., 2005. Modelling protection from antimicrobial agents in biofilms through the formation of persister cells. Microbiology 151, 75–80

Rose, K., Kelly, D., Kemker, C., Fitch, K., Card, A., Fondriest Environmental, Inc., 2014. Conductivity, Salinity and Total Dissolved Solids. Fundamentals of Environmental Measurements. Retrieved from http://www.fondriest.com/environmental-measurements/parameters/water-quality/ [Date of access: March 2016]

Rosewarne, C.P., Pettigrove, V., Stokes, H.W., Parsons, Y.M., 2010. Class 1 integrons in benthic bacterial communities: abundance, association with Tn402-like transposition modules and evidence for coselection with heavy-metal resistance. Federation of European Microbiological Societies Microbiology Ecology 72, 35–46.

Rosi-Marshall, E.J., Kelly, J.J., 2015. Antibiotic stewardship should consider environmental fate of antibiotics. Environmental Science and Technology 49, 5257-5258.

Rossolini, G.M., Arena, F., Pecile, P. and Pollini, S., 2014. Update on the antibiotic resistance crisis. Current Opinion in Pharmacology 18, 56–60.

Rota, J.S., Heath, J.L., Rota, P.A., King, G.E., Celma, M.L., Carabana, J., Fernandez-Munoz, R., Brown, D., Jin, L., Bellini, W.J., 1996. Molecular epidemiology of measles virus: identification of pathways of transmission and implications for measles elimination. Journal of Infectious Diseases 173, 32-37.

Salyers, A.A., Gupta, A., Wang, Y., 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends in Microbiology 12(9), 412-416.

Santajit, S., Indrawattana, N., 2016. Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. BioMed Research International,1-8.

Sarmah, A.K., Meyer, M.T., Boxall, A.B.A., 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65, 725–759.

Schmieder, R., Edwards, R., 2012. Insights into antibiotic resistance through metagenomic approaches. Future Microbiology 7(1), 73-89.

Schwartz, T., Kohnen, W., Jansen, B., Obst, U., 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. Federation of European Microbiological Societies Microbiology Ecology 43, 325–335

Seki, M., Gotoh, K., Nakamura, S., Akeda, Y., Yoshii, T., Miyaguch, S., Inohara, H., Horii, T., Oishi, K., Iida, T., Tomono, K., 2013. Fatal sepsis caused by an unusual Klebsiella species that was misidentified by an automated identification system. Journal of Medical Microbiology 62, 801–803.

Service Charter, Republic of South Africa, PSCBC Resolution 1 of 2013, Retrieved from http://www.dpsa.gov.za/documents/PUBLIC%20SERVICE%20CHARTER%202013.pdf

Silbergeld, E.K., Davis, M., Leibler, J.H., Peterson, A.E., 2008. One reservoir: redefining the community origins of antimicrobial resistant infections. Medical Clinics of North America 92(6), 1391–1407.

Skold O. 2001. Resistance to trimethoprim and sulfonamides. Veterinary Research 32: 261–273.

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

South Afrian National Standards (SANS), 2011. Specifications: Drinking water (SANS 241:2011). South African National Standards.

Statistics South Africa (SSA), 2010. Water Management Areas in South Africa. Discussion document: D0405.8. Statistics South Africa, Pretoria, Retrieved from https://www.statssa.gov.za/publications/D04058/D04058.pdf [Date of access: March 2016]

Stegger, M., Andersen, P.S., Kearns, A., Pichon, B., Holmes, M.A., Edwards, G., Laurent, F., Teale, C., Skov, R., Larsen, A.R., 2012. Rapid detection, differentiation and typing of methicillin-resistant Staphylococcus aureus harbouring either mecA or the new mecA homologue mecA(LGA251). Clinical Microbiology and Infection 18(4), 395-400.

Sun, W., Liu, W., Cui. L., Zhang, M., Wang, B., 2013. Characterization and identification of a chlorine-resistant bacterium, Sphingomonas TS001, from a model drinking water distribution system. Science of the Total Environment 458(460), 169-175.

Sundin, G. W., Bender, C. L., 1996. Dissemination of the strA-strB streptomycin-resistance genes among commensal and pathogenic bacteria from humans, animals, and plants. Molecular Ecology 5, 133-143.

Sundin, G.W., 2000. Examination of base pair variants of the *strA-strB* streptomycin resistance genes from bacterial pathogens of humans, animals, and plants. Journal of Antimicrobial Chemothererapy 46, 848 – 849.

Tait R.C, Rempel H, Rodriguez R.L, Kado CI. 1985. The aminoglycoside-resistance operon of the plasmid pSa: nucleotide sequence of the streptomycin-spectinomycin resistance gene. Gene. 36, 97–104

Tait R.C, Rempel H, Rodriguez R.L, Kado CI. 1985. The aminoglycoside-resistance operon of the plasmid pSa: nucleotide sequence of the streptomycin-spectinomycin resistance gene. Gene. 36: 97–104

Templeton, M.R., Oddy, F., Leung, W.-k, Rogers, M., 2009. Chlorine and UV disinfection of ampicillin-resistant and trimetoprim-resistant Escherichia coli. Can. Journal of Civil Engineering 36, 889-894.

Todar, K. 2009. The mechanisms of bacterial pathogenicity. In: Todar's online Textbook of Bacteriology. [Web: http://www.textbookofbacteriology.net/pathogenesis.html] . [Date of access: March 2017]

Todar, K. 2012. Todar's Online Textbook of Bacteriology. "The Good, the Bad, and the Deadly" available at [Retrieved from: http://textbookofbacteriology.net/resantimicrobial.html] [Date of access: March 2017]

Van Boecke,l T.P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B.T., Levin, S.A., Laxminarayan, R., 2010. Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. Lancet Infectious Disease 14(8), 742-50.

Van der Merwe-Botha, M. 2009. Water quality: A vital dimension of water security. Development Planning Division. Working Paper Series No.14, DBSA: Midrand.

Van Essen-Zandbergen, A., Smith, h., Veldman, k., Mevius, D., 2007. Occurrence and characteristics of class 1, 2 and 3 integrons in *Escherichia coli, Salmonella* and *Campylobacter* spp. in the Netherlands. Journal of Antimicrobial Chemotherapy 59, 746-750.

Van Zyl, J.E., 2014. Introduction to Operation and Maintenance of Water Distribution Systems 1st Ed. *Practical Guidelines for Operation and Maintenance of Water Distribution Systems in South Africa* (WRC Project No. K5/2135). Retrieved from http://www.wrc.org.za/Knowledge%20Hub%20Documents/Research%20Reports/TT600-14.pdf

Van, T.T.H., Moutafis, G., Tran, L.T., Coloe, P.J., Antibiotic Resistance in Food-Borne Bacterial Contaminants in Vietnam. Applied and Environmental Microbiology, 7906–7911.

Vaz-Moreira, I., Nunes, O.C. and Manaia, C.M., 2014. Bacterial diversity and antibiotic resistance in water habitats: searching the links withthe human microbiome. Federation of European Microbiological Societies Microbiology Reviews 38(4),761-778.

Venter, L., Bezuidenhout, C. C., 2015. Antibiotic resistant opportunistic pathogenic bacteria from drinking water biofilms, FEMS Microbiology (Special Edition). http://www.metam.co.za/documents_v2/File/RedRibbon_2009/ASSA2003%20AIDS%20and%20Demographics%20Projections%20for%202009.doc [Date of access: April 2017]

Ventola, C.L. (2014). The Antibiotic Resistance Crisis, Part 1: Causes and Threats. Pharmacy and Therapeutics 40(4), 277–283.

Vieno, N., Tuhkanen, T., Kronberg, L., 2006. Removal of Pharmaceuticals in Drinking Water Treatment: Effect of Chemical Coagulation. Environmental Technology 27(2), 183-192.

Vieno, N.M., Tuhkanen, T., Kronberg, L., 2005. Seasonal variation in the occurrence of pharmaceuticals in effluents from a sewage treatment plant and in the recipient water. Environmental Sciences and Technology 39, 8220–8226.

Walia, S.K., Kaiser, A., Parkash, M. Chaudhry G.R., 2004 Self-Transmissible Antibiotic Resistance to Ampicillin, Streptomycin, and Tetracyclin Found in Escherichia coli Isolates from Contaminated Drinking Water. Journal of Environmental Science and Health Part A- Toxic/Hazardous Substances & Environmental Engineering 39(3), 651–662.

Water South Africa, 26(1), 59-66.Water Wise, RandWater South Africa, 2017. Water Situation in South Africa. Retrieved from http://www.waterwise.co.za/site/water/environment/situation.html

Wetzel, R. G. (2001). Limnology: Lake and River Ecosystems (3rd ed.). San Diego, CA: Academic Press.

Willey, J.M., Sherwood, L.M. & Woolverton, C.J., 2008. Prescott, Harley, and Klein's Microbiology. 7th ed. New York: McGraw Hill.

Woese, C. R., G. J. Olsen, M. Ibba, and D. Soll. 2000. Comparisons of complete genome sequences allow the most objective and comprehensive descriptions possible of a lineage's evolution. Microbiology and Molecular Biology Review 64, 202–236.

World Health Organitzation WHO, 2002. Expert consensus. In: Bartram J.C.J, Exner, M., Fricker, C.R., Glasmacher. Heterotrophic plate count measurement in drinking water safety management. Geneva: World Health Organization.

World Health Organization (WHO), 2003. A Heterotrophic Plate Count and Drinking Water Safety. The Significance of HPCs for Water Quality and Human Health IWA Publishing, London.

World Health Organization (WHO), 2003. Emerging Issues in water and Infectious Diseases, World Health Organization, Geneve Switserland available at: www.who.int/water_sanitation_health/emerging/emergingissues/en/. [Retrieved from: April 2016]

World Health Organization (WHO). 2006. Guidelines for drinking-water quality - incorporating first addendum to third edition -volume 1: Recommendations. Geneva: World Health Organization. page 595.

World Health Organization . (2003). pH in Drinking-water. In Guidelines for drinking-water quality. Retrieved from http://www.who.int/water_sanitation_health/dwq/chemicals/en/ph.pdf

World Health Organization, 2011. Guidance to drinking water quality 4th Edition. WHO Press, Switzerland. Available at http://apps.who.int/iris/bitstream/10665/44584/1/9789241548151_eng.pdf. [Date of access: April 2016].

Wose Kinge C.N, Ateba C.N, Kawadza D.T. 2010. Antibiotic resistance profiles of *Escherichia coli* isolated from different water sources in the Mmabatho locality, North-West Province, South Africa. South African Journal of Science 106 (1-2), 44-49

WWAP (United Nations, World Water Assessment Programme). United Nations 2015. The United Nations World Water Development Report 2015: Water for a Sustainable World. Paris, UNESCO.

Yuk, H-G., MARSHALL, D.L., 2004. Adaptation of *Escherichia coli* O157:H7 to pH alters fluid acid. Applied and Environmental Microbiology 70, 3500–3505.

Zhang, W., DiGiano, F.A., 2002. Comparison of bacterial regrowth in distribution systems using free chlorine and chloramine: a statistical study of causative factors. Water Resources 36, 1469-1482.

Zhu, L., Lin, J., Ma, J., Cronan, J. E., Wang, H., 2010.Triclosan resistance of *Pseudomonas aeruginosa* PAO1 is due to FabV, a triclosan-resistant enoyl-acyl carrier protein reductase. Antimicrobial Agents and Chemotherapy 54, 689–698.

APPENDICES

APPENDIX 1

APPENDIX 1A: Antibiotic susceptibility patterns for Heterotopic plate count isolates obtained from Plant NW (MM) (August 2016)

AP- Ampicillin; KF – Cephalothin; CHL – Chloramphenicol; CIP- Ciprofloxacin; E- Erythromycin; K – Kanamycin; NE- Neomycin; OT – Oxy-tetracycline; PG - Penicillin G; S- Streptomycin; TM – Trimethoprim; VA- Vancomycin; R- Resistant; S- Sensitive

Site	Isolate	Gram reaction	AP	PG	E	CHL	C	S	K	NE	VA	OT	CIP	TMP
Raw	MDW1	+	R	S	S	S	S	S	S	R	S	S	R	S
	MDW2	+	S	S	S	S	S	S	S	S	R	S	S	S
	MDW3	+	S	S	S	S	S	S	S	S	S	S	S	S
Inlet	MMIW1	+	S	S	S	S	S	S	S	S	S	S	S	S
	MMIW2	+	R	S	S	S	S	R	S	S	S	S	S	R
	MMIW3	-			S	S	S	S	S	S		S	S	R
	MMIY1	+	S	S	S	S	S	S	S	S	S	S	S	S
	MMIY2	+	S	S	S	S	S	S	S	S	S	S	S	S
	MMIY3	+	R	S	S	S	S	R	R	S	S	R	S	R
	MMIP1	+	S	S	S	S	S	R	R	S	S	R	S	R
AT	MMOW1	+	R	R	S	S	S	S	R	S	S	R	S	R
	MMOW2	+	R	R	S	S	S	S	S	S	S	S	S	R
	MMOW3	+	R	S	S	S	S	S	S	S	S	S	S	R
	MMOO1	+	R	S	S	S	S	R	R	S	R	R	S	R
	MMOO2	+	R	S	S	S	S	R	R	S	R	R	S	R
	MMOO3	+	R	S	S	S	S	R	R	R	R	R	S	R
D1	MMH1W1	+	S	S	S	S	S	S	S	S	S	R	S	R
	MMH1W2	+	S	S	S	S	S	S	S	S	S	S	S	R
	MMH1W3	+	R	S	S	S	S	S	S	S	S	S	S	R
	MMH1O1	-			S	S	S	S	S	S		S	S	S
	MMH1O2	-			S	S	S	S	S	S		S	S	S
	MMH1O3	+	S	S	S	S	S	S	S	S	R	S	S	S
D2	MMH2W1	+	S	S	S	S	S	S	S	S	S	S	S	S
	MMH2W2	+	S	S	S	S	S	S	S	S	S	S	S	S
	MMH2W3	+	S	S	S	S	S	S	S	S	S	S	S	S
	MMH2O1	+	S	S	S	S	S	R	R	S	S	R	S	R
	MMH2O2	+	S	R	S	S	S	R	R	S	S	R	S	S
	MMH2P1	+	S	R	S	S	S	S	R	S	R	R	S	R
	MMH2P2	+	S	R	S	S	S	S	R	S	S	R	R	R
	MMH2P3	+	R	R	S	S	S	S	R	S	S	R	S	R

APPENDIX 1B: Antibiotic susceptibility patterns for Heterotopic plate count isolates obtained from Plant NW (MK) August 2016

Site	Isolate	Gram reaction	AP	PG	E	CHL	C	S	K	NE	VA	OT	CIP	TMP
Raw	MPW1	+	R	R	S	S	S	S	R	S	R	R	S	R
	MPW3	+	R	S	S	S	S	R	R	S	S	S	S	R
	MPW2	+	R	S	S	S	S	R	R	S	R	R	S	S
	MPP1	+	R	S	S	S	S	S	R	R	R	R	S	R
	MPP2	+	R	R	S	S	S	R	R	S	S	R	S	R
	MPP3	+	R	S	S	S	S	R	R	S	R	R	S	R
Inlet	MKIW1	+	R	S	S	S	S	S	S	S	S	S	S	R
	MKIW2	+	R	R	S	S	S	S	S	S	R	R	S	R
	MKIW3	+	R	R	S	S	S	S	R	S	R	R	R	S
	MKIY1	+	R	S	S	S	S	S	R	S	S	R	S	R
	MKIY2	+	R	R	S	S	S	S	R	S	R	R	S	R
	MKIY3	+	S	R	S	S	R	S	S	S	R	R	S	S
	MKIP	+	R	R	S	S	S	S	R	S	S	R	S	R
AT	MKOW1	+	R	R	S	S	S	S	S	S	S	S	S	R
	MKOW2	+	R	R	S	S	S	S	S	S	R	R	S	R
	MKOW3	+	R	S	S	S	S	S	S	S	S	S	S	R
	MKOO1	+	R	S	S	S	S	R	S	R	R	R	R	R
	MKOO2	+	R	S	S	S	S	S	R	R	R	R	S	R
	MKOO3	+	R	S	S	S	S	R	S	R	R	S	S	R
D1	MKH1Y1	+	R	S	S	S	S	S	S	S	R	S	S	R
	MKH1Y2	+	R	S	S	S	S	S	S	S	R	S	S	R
	MKH1Y3	+	R	S	S	S	S	R	S	R	R	R	S	R
	MKH1W1	+	S	S	S	S	S	R	R	S	S	R	S	R
	MKH1W2	+	S	S	S	S	S	R	R	R	S	R	S	R
	MKH1W3	+	S	S	R	S	S	R	R	R	R	R	S	R
D2	MKH2W1	+	S	S	S	S	S	S	S	S	S	S	S	R
	MKH2W2	+	R	S	S	S	S	S	S	S	S	R	S	R
	MKH2W3	+	S	S	S	S	S	S	S	S	S	S	S	R

AP- Ampicillin; KF – Cephalothin; CHL – Chloramphenicol; CIP- Ciprofloxacin; E- Erythromycin; K – Kanamycin; NE- Neomycin; OT – Oxy-tetracycline; PG - Penicillin G; S- Streptomycin; TM – Trimethoprim; VA- Vancomycin; R- Resistant; S- Sensitive

APPENDIX: 1C Antibiotic susceptibility patterns for Heterotopic plate count isolates obtained from Plant NW (MM) November 2016

Site	Isolate	Gram reaction	AP	PG	E	CHL	C	S	K	NE	VA	OT	CIP	TMP
Raw	MDW1	-			S	S	S	S	S	S		S	S	R
	MDW2	-			S	S	S	R	S	S		S	S	R
	MDW3	-			S	S	S	S	S	S		S	S	R
	MDO1	+	R	R	S	R	S	S	S	S	R	S	S	R
	MDO2	+	R	R	S	S	S	S	S	S	S	S	S	R
	MDO3	+	R	R	R	R	S	R		S	R	R		R
Inlet	MMIC1	+	R	R	S	S	S	S	S	S	S	S	S	R
	MMIY1	+	R	R	S	R	S	S	S	S	S	S	S	R
	MMIY2	+	R	R	S	R	S	S	S	S	S	R	S	R
	MMIY3	+	S	S	S	S	S	S	S	S	S	S	S	R
AT	MMOW1	+	R	R	S	S	S	S	S	S	S	S	S	R
	MMOW2	+	R	R	S	R	S	S	S	S	S	S	S	R
D1	MMH1C1	+	R	R	R	R	S	S	S	S	S	S	S	R
	MMH1P1	+	R	R	S	R	S	S	R	S	S	S	S	S
	MMH1P2	+	R	R	S	S	S	S	R	S	S	R	S	R
D2	MMH2Y1	-			S	R	S	S	S	S	S	S	S	S
	MMH2Y2	-			S	R	S	R	R	S	S	S	S	S
	MMH2C1	-			R	R	R	R	S	S		R	S	R
	MMH2C2	-			S	S	S	R	S	S		R	S	R
	MMH2C3	-			S	S	S	R	S	S		R	S	R
	MMH2P1	-			S	R	S	R	R	R		R	S	R

AP- Ampicillin; KF – Cephalothin; CHL – Chloramphenicol; CIP- Ciprofloxacin; E- Erythromycin; K – Kanamycin; NE- Neomycin; OT – Oxy-tetracycline; PG - Penicillin G; S- Streptomycin; TM – Trimethoprim; VA- Vancomycin; R- Resistant; S- Sensitive

APPENDIX: 1D Antibiotic susceptibility patterns for Heterotopic plate count isolates obtained from Plant NW (MK) November 2016

Site	Isolate	Gram reaction	AP	PG	E	CHL	C	S	K	NE	VA	OT	CIP	TMP
Raw	MPW1	-			S	R	R	R	S	R		S	S	R
	MPW2	-			S	R	R	R	S	S		R	S	R
	MPW3	-			S	R	R	R	S	R		R	S	R
Inlet	MKIY1	+	R	R	S	S	S	S	S	S	S	R	S	R
	MKIY2	-			S	R	S	R	S	S	R	R	S	R
	MKIY3	+	S	S	S	S	S	S	S	S	S	S	S	R
AT	MKOY1	-			S	R	S	R	R	S		R	S	R
	MKOY2	-			S	R	S	R	R	S		R	S	R
	MKOY3	-			R	R	S	R	R	R		R	S	R
D1	MKH1Y1	+	S	S	S	S	S	S	S	S	S	S	S	S
D2	MKH2B1	-			S	S	S	S	S	S		S	S	S
	MKH2B2	-			S	S	S	S	S	S		S	S	S
	MKH2C1	+	S	S	S	S	S	S	S	S	S	S	S	S
	MKH2C2	+	S	S	S	S	S	S	S	S	S	S	S	R

AP- Ampicillin; KF – Cephalothin; CHL – Chloramphenicol; CIP- Ciprofloxacin; E- Erythromycin; K – Kanamycin; NE- Neomycin; OT – Oxy-tetracycline; PG - Penicillin G; S- Streptomycin; TM – Trimethoprim; VA- Vancomycin; R- Resistant; S- Sensitive

APPENDIX: 1E Antibiotic susceptibility patterns for Heterotopic plate count isolates obtained from Plant NW (MM) March 2017

Site	Isolate	Gram reaction	AP	PG	E	CHL	C	S	K	NE	VA	OT	CIP	TMP
Raw	MDO1	+	R	R	S	R	S	S	S	S	S	S	S	R
	MDW1	-			S	S	S	S	S	S		S	S	S
	MDW2	-			S	S	S	S	S	S		S	S	S
Inlet	MMIY1	-			S	S	S	S	S	S		S	S	S
	MMIY2	-			S	S	S	S	S	S		S	S	R
	MMIP1	-			S	S	S	S	S	S		S	S	S
	MMIW1	-			S	R	S	S	S	S		S	S	S
	MMIW2	-			S	S	S	S	S	S		S	S	R
AT	MMOW1	+	S	R	S	S	S	S	S	S	S	S	S	S
	MMOW2	-			S	S	S	S	S	S		S	S	S
	MMOW3	-			S	S	S	S	S	S		S	S	S
D1	MMHIW1	-			S	S	S	S	S	S		S	S	R
	MMH1W2	-			S	R	S	S	S	S		S	S	R
	MMH1W3	-			S	S	S	S	S	S		S	S	R
	MMH1Y1	-			S	S	S	S	S	S		S	S	S
	MMH1Y2	+	R	R	S	R	S	S	S	S	S	S	S	R
	MMH1Y3	+	S	S	S	S	S	S	S	S	S	S	S	R
D2	MMH2W1	+	R	R	S	R	S	S	S	S	R	S	S	R
	MMH2W2	+	R	R	S	R	S	S	S	S	R	S	S	R
	MMH2W3	-			S	S	S	S	S	S		S	S	R
	MMH2Y1	-			S	S	S	S	S	S		S	S	S
	MMH2Y2	+	S	R	R	S	S	S	S	S	S	S	S	S
	MMH2Y3	-			S	S	S	S	S	S		S	S	S

AP- Ampicillin; KF – Cephalothin; CHL – Chloramphenicol; CIP- Ciprofloxacin; E- Erythromycin; K – Kanamycin; NE- Neomycin; OT – Oxy-tetracycline; PG - Penicillin G; S- Streptomycin; TM – Trimethoprim; VA- Vancomycin; R- Resistant; S- Sensitive

APPENDIX: 1F Antibiotic susceptibility patterns for Heterotopic plate count isolates obtained from Plant NW (MK) March 2017

Site	Isolate	Gram reaction	AP	PG	E	CHL	C	S	K	NE	VA	OT	CIP	TMP
Raw	MPP1	-			R	S	S	S	S	S		S	S	S
	MPW1	-			S	S	S	S	S	S		S	S	S
	MPW2	+	R	R	S	R	S	R	S	S	S	S	S	S
Inlet	MKIW1	-			S	R	S	S	S	S		S	S	S
	MKIW2	-			S	R	S	S	S	S		S	S	S
	MKIW3	-			S	R	S	S	S	S		S	S	R
AT	MKOB1	+	R	R	S	R	R	S	S	S	S	S	S	S
	MKOY1	+	S	S	S	S	R	S	S	S	S	S	S	R
	MKOY2	-			S	S	S	S	S	S		S	S	S
	MKOW1	+	R	R	S	R	R	S	S	S	S	S	S	S
	MKOW2	+	S	S	S	S	R	S	S	S	S	S	S	S
	MKOW3	-			S	S	S	S	S	S		S	S	S
D2	MKH1W1	-			S	S	S	S	S	S		S	S	S
	MKH1W2	+	R	R	S	R	S	S	S	S	S	S	S	R
	MKH2W3	+	R	R	S	R	S	S	S	S	S	S	S	R
	MKH1P1	+	R	R	S	S	R	S	S	S	S	S	S	R
	MKH1B1	-			S	S	S	S	S	S		S	S	S
	MKH1B2	-			S	S	S	R	S	S		S	S	S
	MKH1Y1	-			S	S	R	S	S	R		S	S	R
	MKH1Y2	+	S	S	S	S	S	S	S	S	S	S	S	S
	MKH1Y3	+	S	S	S	R	R	S	S	S	R	S	S	S
	MKH1Y4	+	S	S	S	S	S	S	S	S	S	S	S	S
D2	MKH2B1	-			S	S	S	S	S	S		S	S	S
	MKH2B2	+	S	S	S	S	S	S	S	S	S	S	S	S
	MKH2B3	+	S	S	S	S	S	S	S	S	S	S	S	S
	MKH2Y1	-			S	S	S	S	S	S		S	S	R
	MKH2Y2	-			S	S	S	S	S	S		S	S	S
	MKH2Y3	+	R	S	S	S	S	S	S	S	S	S	S	S
	MKH2W1	-			S	S	S	S	S	S		S	S	R
	MKH2W2	+	R	R	S	R	S	S	S	S	S	S	S	R
	MKH2W3	-			S	R	S	S	S	S		S	S	S

AP- Ampicillin; KF – Cephalothin; CHL – Chloramphenicol; CIP- Ciprofloxacin; E- Erythromycin; K – Kanamycin; NE- Neomycin; OT – Oxy-tetracycline; PG - Penicillin G; S- Streptomycin; TM – Trimethoprim; VA- Vancomycin; R- Resistant; S- Sensitive

APPENDIX: 1G Antibiotic susceptibility patterns for Heterotopic plate count isolates obtained from Plant NW (MM) May 2017

Site	Isolate	Gram reaction	AP	PG	E	CHL	C	S	K	NE	VA	OT	CIP	TMP
Raw	MDO1	+	S	S	S	S	S	S	S	S	S	S	S	R
	MDO2	+	S	S	S	S	S	S	S	S	S	S	S	S
	MDO3	+	S	S	S	S	S	S	S	S	S	S	S	S
	MDW1	+	R	R	S	S	S	S	S	S	S	S	S	R
	MDW2	+	R	R	S	S	S	S	S	S	S	S	S	R
Inlet	MMIO1	+	S	R	S	S	S	S	S	S	S	S	S	S
	MMIO2	+	R	R	S	S	S	S	R	S	S	S	S	S
	MMIO3	-			S	S	S	S	R	S		S	S	S
	MMIW1	+	R	S	S	S	S	S	R	S	S	S	S	S
	MMIW2	+	S	S	S	S	S	S	S	S	S	S	S	S
	MMIW3	+	S	S	S	S	S	S	S	S	S	S	S	S
	MMIY1	+	R	R	S	S	S	S	S	S	S	S	S	S
AT	MMOW1	+	R	R	S	S	S	S	R	S	S	R	S	S
	MMOW2	+	S	S	S	S	S	S	R	S	S	S	S	S
	MMOW3	+	R	R	S	S	S	S	R	S	S	S	S	S
	MMO1	-			S	S	S	S	S	S		S	S	S
	MMO2	+	S	S	S	S	S	S	S	S	S	S	S	S
D1	MMH1O1	+	R	R	S	S	S	R	S	R	S	S	S	S
	MMH1O2	+	S	S	S	S	S	S	S	S	S	S	S	S
	MMH1O3	-			S	S	S	S	S	S		S	S	S
	MMH1W1	+	R	R	S	S	S	S	S	S	S	S	S	S
	MMH1W2	-			S	S	S	S	S	S		S	S	S
	MMH1W3	-			S	S	S	S	S	S		S	S	R
D2	MMH2O1	+	R	S	S	S	S	S	S	S	S	S	S	S
	MMH2O2	+	R	R	S	S	S	S	S	S	S	S	S	R
	MMH2W1	+	R	R	S	S	S	S	R	S	S	S	S	S
	MMH2W2	+	R	R	S	S	S	S	R	S	S	S	S	S
	MMH2W3	+	R	R	S	S	S	S	R	S	S	S	S	S

AP- Ampicillin; KF – Cephalothin; CHL – Chloramphenicol; CIP- Ciprofloxacin; E- Erythromycin; K – Kanamycin; NE- Neomycin; OT – Oxy-tetracycline; PG - Penicillin G; S- Streptomycin; TM – Trimethoprim; VA- Vancomycin; R- Resistant; S- Sensitive

APPENDIX: 1H Antibiotic susceptibility patterns for Heterotopic plate count isolates obtained from Plant NW (MK) May 2017

Site	Isolate	Gram reaction	AP	PG	E	CHL	C	S	K	NE	VA	OT	CIP	TMP
Raw	MPO1	+	R	R	S	S	S	S	S	S	S	S	S	R
	MPO2	+	R	R	S	S	S	S	S	S	S	S	S	R
	MPO3	+	R	R	S	S	S	S	S	S	S	S	S	R
	MPW1	+	R	R	S	S	S	S	S	S	S	S	S	R
	MPW2	+	R	R	S	S	S	S	S	S	S	S	S	R
	MPW3	+	R	R	S	S	S	S	S	S	S	S	S	R
Inlet	MKIO1	+	R	S	S	S	S	S	S	S	S	S	S	S
	MKIO2	+	R	R	R	S	S	S	S	S	R	S	S	R
	MKIO3	-			S	S	S	S	S	S		S	S	S
	MKIW1	+	R	R	S	S	S	S	S	S	S	S	S	R
	MKIW2	+	R	R	S	S	S	S	S	S	S	S	S	R
	MKIW3	+	R	R	S	S	S	S	S	S	S	S	S	R
AT	MKOW1	+	R	R	S	S	S	S	R	S	S	S	S	S
	MKOW2	+	R	R	S	S	S	S	R	S	S	S	S	S
	MKOW3	+	R	R	S	S	S	S	S	S	S	S	S	S
	MKOO1	+	S	S	S	S	S	S	S	S	S	S	S	S
	MKOO2	+	S	R	S	S	S	S	R	S	S	S	S	S
D1	MKH1W1	-			S	S	S	S	S	S		S	S	R
	MKH1W2	-			S	S	S	S	S	S		S	S	S
	MKH1W3	-	S	S	S	S	S	S	S	S	S	S	S	S
	MKH1C1	+	R	R	S	S	S	S	S	S	S	S	S	S
	MKH1C2	+	S	R	S	S	S	S	R	S	S	S	S	S
D2	MKH2O1	-			S	S	S	S	S	S		S	S	S
	MKH2O2	+	S	S	S	S	S	R	S	S	S	S	S	S
	MKH2O3	-			S	S	S	S	S	S		S	S	S
	MKH2W1	+	R	R	S	S	S	S	S	S	S	S	S	R
	MKH2W2	+	R	R	S	S	S	S	S	S	S	S	S	S
	MKH2W3	+	R	R	S	S	S	S	S	S	S	S	S	R

AP- Ampicillin; KF – Cephalothin; CHL – Chloramphenicol; CIP- Ciprofloxacin; E- Erythromycin; K – Kanamycin; NE- Neomycin; OT – Oxy-tetracycline; PG - Penicillin G; S- Streptomycin; TM – Trimethoprim; VA- Vancomycin; R- Resistant; S- Sensitive

APPENDIX 2

APPENDIX: 2A Extracellular enzyme production outcome on MAR heterotrophic bacteria August 2016

Isolate ID	Proteinase	Lipase	DNase	Haemolysis	Oxidase
MKH1W1	+	+	-	B	+
MMOO1	+	+	-	A	+
MMIW1	-	+	-	B	+
MKH1W2	-	-	-	B	+
MMH2O1	+	-	+	B	+
MKH1W3	+	+	-	B	+
MKIP	-	-	-	B	+
MKIY2	+	-	-	B	+
MMIP1	-	+	-	B	+
MKOW2	+	+	-	B	+
MMOW1	+	+	-	A	+
MPO3	-	+	-	B	+
MKIY1	+	+	-	B	+
MMH2P1	-	+	-	B	+
MKH1W3	-	+	-	A	+
MMIY1	-	-	-	A	+
MKOO2	+	-	-	A	+
MPW2	+	+	-	B	+
MKOO3	-	+	-	Y	+
MKIW2	+	+	-	Y	+
MPP2	+	+	-	Y	+
MDW1	+	-	-	Y	+

Y- Gramma, α- Alpha, β- Beta, (+) - positive,(-) - negative

APPENDIX: 2B Extracellular enzyme production outcome on MAR heterotrophic bacteria November 2016

Isolate ID	Proteinase	Lipase	DNase	Haemolysis	Oxidase
MDO3	-	+	-	A	+
MMOW2	+	-	-	B	+
MMH1P1	+	+	-	Y	+
MMH2C2	-	+	-	B	+
MMIY2	+	+	-	B	+
MKOY2	+	+	-	B	+
MMHIC2	+	+	-	B	+
MPW1	-	+	-	B	+
MPW3	-	+	-	B	+
MDW2	-	+	-	B	+
MPW2	-	+	-	A	+
MMH2P1	+	-	-	Y	+
MKIY2	-	+	-	A	+
MDO2	+	+	-	B	+
MKH1Y3	+	+	-	A	+

Y- Gramma, α- Alpha, β- Beta, (+) - positive,(-) - negative

APPENDIX: 2C Extracellular enzyme production outcome on MAR heterotrophic bacteria March 2017

Isolate ID	Proteinase	Lipase	DNase	Haemolysis	Oxidase
MMIY2	+	+	-	B	+
MKH2B3	+	-	-	A	+
MMH1Y3	-	-	-	A	+
MMH2W2	+	+	-	A	+
MKH2W2	-	-	-	B	+
MMH2Y1	+	-	+	B	+
MMH2W3	-	+	-	B	+
MKH1O1	-	+	-	A	+
MKH1Y4	+	+	-	B	+
MKH2W1	-	+	-	B	+
MMH1W3	-	+	-	B	+
MKOB1	+	+	-	Y	+
MKH1Y1	-	+	-	Y	+
MMH2W1	+	+	-	A	+
MPW1	+	+	+	A	+
MMOW2	-	+	-	B	+
MPP1	-	+	-	B	+
MDW1	-	+	-	B	+
MPW2	+	+	-	Y	+
MKH1W3	+	+	-	Y	+
MMH1Y1	-	-	-	Y	+

Y- Gramma, α- Alpha, β- Beta, (+) - positive,(-) - negative

APPENDIX: 2D Extracellular enzyme production outcome on MAR heterotrophic bacteria May 2017

Isolate ID	Proteinase	Lipase	DNase	Haemolysis	Oxidase
MMOW3	-	+	-	β	+
MMOW2	+	+	-	β	+
MKOW1	+	+	-	α	+
MMH2O2	-	-	-	β	+
MMH2W1	+	-	-	γ	+
MKIO2	-	+	-	β	+
MPW3	-	+	-	γ	+
MPO1	-	-	-	γ	+
MKIW2	+	+	+	γ	+
MKH2W3	-	-	+	γ	+

Y- Gramma, α- Alpha, β- Beta, (+) - positive,(-) - negative

APPENDIX: 3 Summaries of resistant genes detected from MAR heterotrophic plate count isolates obtained and their Extracellular Protein results

Sample Period	Site	Isolate ID	Resistant genes detected from MAR heterotrophic plate count isolates						Extracellular Protein Production				
			StrA	*StrB*	*aadA*	tetA	*blaCTX-M*	*dfrB1, dfrB2*	Proteinase	Lipase	DNase	Haemolysis	Oxidase
August (MM)	**Raw**	MDW1		X	X		X	X	+	-	-	γ	
	Inlet	MKIW2						X	+	+	+	γ	
		MMIY1	X						-	-	-	α	
		MMIP1	X	X					-	+	-	β	
	AT	MMOO1			X				+	+	-	α	
		MMOW1	X	X					+	+	-	α	
	D2	MMH2P1	X					X	-	+	-	β	
		MMH2O1	X	X					+	-	+	β	
(MK)	**Raw**	MPO3	X						-	+	-	β	
		MPP2	X						+	+	-	β	
	Inlet	MKIP	X	X				X	-	-	-	β	
		MKIY2						X	+	-	-	β	
		MKIW2	X						+	+	-	γ	
		MKIY1	X	X					+	+	-	β	
	AT	MKOO3	X						-	+	-	γ	
		MKOO2	X						+	-	-	α	
November (MM)	**Raw**	MDO3	X			X	X		-	+	-	α	
		MDO2	X						+	+	-	β	
		MDW2	X						-	+	-	β	
	AT	MMOW2	X						+	-	-	β	
	D1	MMH1P1	X	X				X	+	+	-	γ	
		MMH1C2							+	+	-	β	
	D2	MMH2P1	X	X				X	+	-	-	γ	
(MK)	**Raw**	MPW2		X		X	X		-	+	-	α	
		MPW1	X	X					-	+	-	β	
	Inlet	MKIY2	X					X	-	+	-	α	
		MKIY1	X						-	+	-	β	

	AT	MKOY2						X	+	+	-	β	
	D1	MKH1W1				X			-	+	-	β	+
March (MM)	**Raw**	MDW1			X		X		-	+	-	β	+
	Inlet	MMIY2			X		X	X	+	+	-	β	+
	AT	MMOW2	X						-	+	-	β	+
	D1	MMH1Y2	X		X			X	-	-	-	α	
		MMH1Y3		X				X	-	-	-	α	+
		MMH1Y1	X	X					-	-	-	γ	+
	D2	MMH2W1	X						-	-	-	β	+
		MMH2W2		X					+	+	-	α	+
		MMH2Y2	X						+	-	+	β	+
	(MK)	**Raw**	MPW1	X	X					+	+	+	α	+
			MPW2	X	X					+	+	-	γ	+
	AT	MKOB1	X	X					+	+	-	Y	+
	D1	MKH1Y3	X	X	X				+	+	-	β	+
		MKH1W3	X	X					+	+	-	γ	+
		MKH1Y1			X				-	+	-	γ	+
		MKH1W2	X						+	+	-	α	+
	D2	MKH2B1	X						-	-	-	α	
		MKH2B3	X						+	-	-	α	+
May (MM)	**Raw**	MDW2	X						-	+	-	β	
	Inlet	MMIY1	X						+	+	-	β	
	AT	MMOW2		X					+	+	-	β	
		Mmow3	X		X				-	+	-	β	
	D2	MMH2W3			X			X	-	+	-	β	+
		MMH2W1						X	+	-	-	γ	
		MMH2O2		X					-	-	-	β	
	(MK)	**Raw**	MPW2			X				+	+	-	β	
	AT	MKOW2			X				+	+	-	β	
		MKOW1	X	X					+	+	-	α	