# Incidence and characteristics of antibiotic resistant bacteria in raw and drinking water from Western Cape water production facilities

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# DECLARATION

I declare that, this dissertation for the degree of Master in Science of Microbiology at North-West University, Potchefstroom Campus hereby submitted, has not been submitted by me for a degree at this or another University, that it is my own work in design and execution, and that all materials contained herein has been duly acknowledged

Karabo Tsholo Date

…… ……

# DEDICATION

Dithuto tsame ke di-lebisa kwa losikeng lotlhe lwa ga Tsholo, Mogale le Mokaila. Ke ka ntlha ya maele, lerato le kemonokeng ya bona ke kgonneng go tsamaya leoto le tshwana le le. Ba nthutile gore gona sepe se se thata mo botshelong, fela o baya tshepo ya gago yotlhe Modimomg. Thapelo ya me ya metlhe e tswa mo bukaneneg ya Dipesalema 23:1-6.

**MORENA KE MODISA WA ME**

**Pesalome ya ga Dafita.**

1 Morena ke modisa wa me,
ga nkitla ke tlhoka sepe.
2O mpothisa mo mafulong
a matalana,
o nkgogela kwa metsing a tapoloso.
3O lapolosa mowa wa me;
o ntsamaisa mo ditseleng
tsa tshiamo
ka ntlha ya leina la gagwe.
4Le fa ke tsamaya mo mogorogorong
wa moriti wa loso,
ga nkitla ke boifa bosula bope;
gonne o na le nna.
Tsamma ya gago
le seikokotlelo sa gago
di a nkgomotsa.
5O baakanya bojelo fa pele ga me
go lebagana le baba ba me;
o ntlotsa tlhogo ka lookwane;
senwelo sa me se tletse,
se a penologa.
6Ruri molemo le boitshwarelo
di tla ntshala morago
ka malatsi otlhe a bophelo jwa me;
ke tla nna mo Ntlong ya Morena
go ya bosakhutleng.

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# ABSTRACT

South Africa is one of the driest countries in the world. There are some parts in the country which are classified as water stressed region like Western Cape Province. This province had been reliant on surface and groundwater for drinking water production. A recent drought and in some cases prolonged drought has led to the establishment of a direct portal reuse (DPR) plant in one of the municipalities. It is known that traditional water sources and particularly wastewater is contaminated with agricultural, pharmaceutical personal care products such as antibiotic residues, antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs), besides other contaminants, including pathogens. It is well documented that conventional drinking water processes are not designed to remove antibiotic residues, ARB and ARGs. Their presence in drinking water alters water quality which is an emerging public concern that persists in developing and developed countries. Antibiotic resistance in water sources has up to now often been overlooked. The aim of the present study was to determine the incidence and characteristics of ARB from selected water production facilities in the Western Cape Province. In addition, results obtained were used to determine the effectiveness of two DWPFs in removal of heterotrophic ARB, associated antibiotic resistant genes (ARGs) as well as antibiotic residues. Samples were collected from two water production facilities in the Western Cape (i) direct potable reuse (DPR) facility WC-A linked to a conventional drinking water production facility (DWPF) WC-A and (ii) a separate conventional drinking water production facility WC-F. Various physico-chemical parameters were measured *in situ* and in the laboratory. Selective medium was used to isolate heterotrophic plate count (HPC) bacteria, faecal coliforms and *E. coli*. Only HPC bacteria were further characterized for antibiotic resistance and virulence characteristics as well as presence of ARGs. Virulence was based on production of various associated extracellular enzymes. Water samples were subjected to isolation of environmental DNA, which was used to detect ARGs. Antibiotic residues were extracted directly from water environments using the SPE-DEX system and quantified using ultra-high performance liquid chromatography (UHPLC). Redundancy analysis (RDA) was used to plot the correlation of physico-chemical parameters and microbiological agents.

Physico-chemical parameters of drinking water from WC-A and WC-F were within the limits set by South African National Standards (SANS 2015:241) of drinking water. Microbiological agents such as faecal coliforms, *E. coli* were not detected in drinking water. There was a reduction of HPC bacteria from raw to drinking water at WC-A and WC-F. Antibiotic resistance patterns among the isolates indicated that HPC bacteria were in general resistant

to trimethoprim, ampicillin, cephalothin and chloramphenicol. An interesting finding was that the DPR plant produced drinking water that had lowest counts of ARB compared to the conventional system. Another interesting observation was that water immediately after treatment had fewer HPC bacteria compared to the distribution networks. An antibiotic resistant index that was used demonstrated that the multiple antibiotic resistant (MAR) index of reclaimed water at WC-A was lower than 0.2, indicating lower number of bacteria resistant to the range of antibiotics. Using Gram-staining and 16S rRNA gene sequencing, *Pseudomonas* spp. and *Bacillus* spp. were the dominant HPC bacteria isolated from water samples. Other bacteria identified included *Massilia* spp.*, Undibacterium* spp.*, Acidovorax* spp.*, Chromobacterium* spp…Most HPC bacteria species were β and α haemolytic. Production of extracellular enzymes was as follows; 82.48% lecithinase, 77.99% DNase, 66.80% proteinase, 56.44% gelatinase and 41.67% lipase. Ciprofloxacin was detected in all water samples. Streptomycin was detected in most water samples, except for wastewater and drinking water from the DPR plant. As expected, most of the antibiotic residues were detected in wastewater samples. The *ermB* was the gene in most water samples. The *ermF, intI1* and *ampC* were the most prevalent genes in drinking water samples. The $bla_{TEM}$ and *tetM* were not detected in water samples. The *ermB* and *intI1* genes were only detected in eDNA from wastewater at WC-A and mixed raw water at WC-F. Statistical analysis showed that the growth of microbiological agents has a strong correlation with physico-chemical parameters of the water environments. This study shows that the DPR plant and DWPFs are effectively reducing physico-chemical parameters and microbiological agents. However, conventional treatment processes applied in the DWPFs are not designed to remove ARB, antibiotic residues and ARG in water. The advanced treatment processes that are used to treat wastewater and prepare it for potable use appear to be more effective.

**Keywords:** Physico-chemical parameters; antibiotic resistant bacteria (ARB); antibiotic resistant genes (ARGs); Environmental DNA (eDNA); HPC bacteria; Antibiotic residues; DPR plant; DWPFs.

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| | |
|---|---|
| ARB | Antibiotic Resistant Bacteria |
| ARG | Antibiotic Resistant Gene |
| DPR | Direct Potable Reuse |
| DWA | Department of Water Affairs |
| DWPF | Drinking Water Production Facility |
| DWS | Department of Water and Sanitation |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| GDP | Gross Domestic Product |
| HGT | Horizontal Gene Transfer |
| HPC | Heterotrophic plate count |
| NDP | National Development Plant |
| NO2- | Nitrite |
| NO3- | Nitrate |
| PO4-3 | Phosphate |
| SDG | Sustainable Development Goal |
| WCWSS | Western Cape Water Supply System |
| WWTP | Wastewater treatment plant |

# CHAPTER 1

# INTRODUCTION

## 1.1 General overview and problem statement

Water is essential for survival and growth of all living things. In higher animals and humans, it facilitates digestion, absorption of food, transportation of nutrients in the body and elimination of waste products via urine (Panneerselvam and Arumugan, 2012; Patil *et al*., 2012). However, access to sufficient and safe drinking water remains a major public health concern. More than one billion people worldwide do not have access to safe and clean drinking water (Sharma *et al.,* 2016; Fonyuy, 2014). South Africa is ranked as the 30$^{th}$ driest country in the world (GreenCape, 2017). One of the provinces in the country, Western Cape is classified as a water stressed region (GreenCape, 2017). Thus, Western Cape depends on borehole and surface water and lately also wastewater, for production of drinking water (Matthews, 2015). These water sources undergo various water treatment processes in the drinking water production facilities (DWPFs) to eliminate water pollutants (Matthews, 2015; Mokhosi and Dzwairo, 2015).

Water harbour both non-pathogenic and pathogenic microorganisms (Bedada *et al.,* 2018). Pathogenic bacteria are associated with waterborne infections and alteration of drinking water quality which is a public health concern (Pandey *et al.,* 2014). Microbiological quality of drinking water could be determined by assessment of the heterotrophic plate count (HPC) bacteria, faecal coliforms and *E. coli* (Ikonen *et al.,* 2013). HPC bacteria are used to assess the ability of DWPFs to eliminate microorganisms and their potential pathogenicity (Bedada *et al.,*2018; Figueras and Borrego, 2010). Faecal coliforms and *E. coli* are indicator microorganisms for faecal pollution in drinking water (Ellis *et al.,* 2017). Drinking water is safe and clean for human consumption when it contains 0 cells of faecal coliforms and *E. coli* in 100 ml water samples (Edokpayi *et al.,* 2018, SANS 241, 2015).

Drinking water quality could also be altered by the presence of high and unregulated amounts of physical and chemical agents (Rahmanian *et al.,* 2015). Water easily gets contaminated with physico-chemical parameters from the environment due to its solvent nature that dissolves organic and inorganic compounds (Qureshimatva and Solanki, 2015). Factors that are used to assess physico-chemical parameters of drinking water quality and suitability include taste, odour, colour and levels of organic and inorganic compounds (Rahmanian *et al.,* 2015). Therefore, appropriate drinking water production practices must be implemented to control physico-chemical and microbiological parameters of drinking water (Mokhosi and Dzwairo, 2015). The Department of Water Affairs implemented South

African National Standards (SANS) 241 for drinking water that monitors water institutes that produce drinking water (DWS, 2011). SANS ensures that the level of physico-chemical parameters, as well as microbiological agents present in drinking water are safe for human consumption (DWS, 2011).

The problem of water, however, stretches beyond the scope of availability and quality. The world also faces a major public health concern of antibiotic resistant bacteria (ARB; Devipriya and Kalaivani, 2012) and the potential that water could be a vehicle to spread this. The extensive and improper usage of antibiotics in agriculture, human medicine and veterinary medicine has led to the spread of antibiotic residues in the water environment (Pruden *et al.,* 2013; Yuan *et al.,* 2015). As a result, ARB and antibiotic resistant genes (ARGs) are frequently isolated from wastewater, soil and dams (Yuan *et al.,* 2015). DWPFs are not designed to eliminate ARB and their associated genes from the environment (Yang *et al.,* 2017; Ju *et al.,* 2016). A study by Yuan *et al*. (2015) shows that the most commonly used disinfection process, chlorination, selects for ARB and ARGs. Drinking water could be a reservoir and vector for ARB and ARGs (Vaz-Moreira *et al.,* 2014). ARB can transmit and incorporate their ARGs into other organisms via horizontal gene transfer (HGT; Hiltunen *et al.,* 2017; Chee-Sanford *et al.,* 2009).

There are limited studies in Western Cape that investigate drinking water quality in the province. Antibiotic resistance and their potential pathogenicity can pose public health threats to immunocompromised patients, children and elderly people (Horn *et al.,* 2016; Pavlov *et al.,* 2004; Yoshikawa, 2002).

## 1.2 Research aim and objectives

### 1.2.1 Aim

The aim of the study was to determine the incidence and characteristics of antibiotic resistant bacteria in raw and drinking water from two Western Cape water production facilities.

### 1.2.2 Objectives

Specific objectives of this study were to:

I. determine the physico-chemical and general microbiological quality of water
II. isolate and identify antibiotic resistant bacteria and to determine antibiotic resistant patterns and their associated genes

III. determine whether isolates are pathogenic based on patterns of extracellular enzyme production

IV. Determine if antibiotic residues are present in raw and drinking water

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Water availability and use in South Africa

Earth is composed of 71% of the hydrosphere (Genda, 2016). However, only less than 1% of earth's water is considered safe for human use (Zia *et al.,* 2013). One of the challenges that most parts of the world, including South Africa is availability of freshwater (Carden and Armitage, 2013). South Africa is a country with low rainfall and very low dam levels, thus resulting to shortage of freshwater (Edokpayi *et al.,* 2018; Mulamattathil *et al.,* 2014a). This shortage is enhanced by many factors that may include an increase in the population size, changing climate, deteriorating water quality, lack of water engineers and exploitation of water (DWS, 2018; IWA, 2016). South Africa has defined water sources that are inadequate to supply sufficient drinking water to all citizens (Edokpayi *et al.,* 2018). Despite having limited water sources, South Africa has water consumption of about 233 litres/capita/day (l/c/d) which is 53 l/c/d more than the international benchmark (GreenCape, 2018). The annual rainfall in South Africa is 465 mm, which is equivalent to half of the world's average (DWS, 2018). It is estimated that South Africa has 49 000 million $m^3$/a of water runoff (DWS, 2018). The annual rainfall in South Africa varies year to year (MacKellar *et al*., 2014). It also varies greatly between different parts of South Africa (Botai *et al.,* 2018; du Plessis and Schloms, 2017). The annual rainfall in the eastern Highveld is between 500 mm/a and 900 mm/a (occasionally exceeding 2000 mm/a), northwestern receives rainfall below 200 mm/a, central parts have an average rainfall of 400 mm/a and the rainfall in the areas closer to the coast varies (Botai *et al.,* 2018).

Approximately 70% of water runoff is stored in 252 largest dams in the country (DWS, 2018). Dams were built to prevent flooding and subsequently to store freshwater that is important in power generation, beverage production, agriculture and domestic uses (Blersch and du Plessis, 2017; Kusangaya *et al.* 2014; Paulse *et al*., 2009). DWS (2018) states that South Africa has available surface water of about 10 200 million $m^3$/a. Dam water is used as the main source for production of drinking water in South Africa (Collins and Herdien, 2013; Paulse *et al*., 2009). However, due to low rainfall, high evaporation rates and low conversion of rainfall to runoff, dams do not produce sufficient freshwater to meet the demand of the public (Palamuleni and Akoth, 2015).

Even though dams are recognised as the main source of drinking water, groundwater is excessively used due low water levels in dams across the globe (Mahon and Gill, 2018; Currell *et al.,* 2012). Previously, groundwater was used to provide rural areas, small towns,

villages and water scarce areas in South Africa (DWS, 2015). Currently, boreholes are sunk as a strategic plan implemented by the DWS to remedy water shortages in the country (DWS, 2015). South Africa has an estimated accessible groundwater of 4 500 million $m^3$/a DWS (2018). The country has already exhausted between 2 000 and 3 000 million $m^3$/a of the groundwater DWS (2018).

Freshwater is extensive and improperly used in the agricultural sector and urbanisation (Zia *et al.,* 2013; Currell *et al.,* 2012). The agricultural sector is important in ensuring the country produces sufficient food to compensate for the increase in the population size (Kachi *et al.,* 2016; Ojha *et al.,* 2015). Other beneficial impacts that agricultural sector have on South Africa are to improve food security and reducing poverty thus creating job and business opportunities (BFAP, 2018). However, this sector poses a negative impact on water quality and quantity (Kachi *et al.,* 2016). The agricultural sector in South Africa uses 392 670 356 $m^3$/a of freshwater. This quantity is allocated as follows; 387 650 971 $m^3$/a of water used for irrigation, 3 715 023 is used for livestock and 1 304 352 is reserved for farms (Cole *et al.,* 2018). According to DWS (2018), the agricultural sector is using 61% of the freshwater in South Africa.

Urbanisation uses 24% of the freshwater in South Africa (DWS, 2018). Urbanisation is a process in which large numbers of people migrate or develop rural areas into urban areas (Rashid *et al.,* 2018). With urbanisation comes a challenge that may pose a significant threat to natural dynamics, resource availability and environmental quality (McGrane *et al.,* 2015). These changes come as a result of improper regulation and lack of planning in urban developments (Turok and Borel-Saladin, 2014; Carden and Armitage, 2013). The rapid growth of urbanisation in South Africa has significantly impacted the availability and provision of freshwater (Turok and Borel-Saladin, 2014). According to the DWS (2018), other sectors in South Africa that use water includes afforestation (3%), rural development (3%), industries which are not part of the urban domestic (3%), livestock and watering and nature conservation (2%), mining (2%), and power generation (2%).

## 2.2 Water availability in Western Cape

Western Cape situated in the Southwest region of South Africa is characterised by a Mediterranean climate (Blersch and du Plessis; 2017; du Plessis and Schloms, 2017). The Southwest region has wide range of climatic and topographic heterogeneity (du Plessis and Schloms, 2017). The climate ranges from semiarid to relatively wet on the windward slopes of the mountains (Blamey *et al.,* 2018). The Southwest is the only region in South Africa that has winter rainfall (Blamey *et al.,* 2018; Reason and Rouault, 2005), caused by cold fronts,

associated extratropical cyclones, other westerly disturbance such as cut-on lows and ridging high pressure (Blamey *et al.,* 2018; Engelbrecht *et al.,* 2015). The rainfall in the Southwest region is associated with organized synoptic-scale weather, while the rainfall in the inland parts of the country such as Northern and Eastern regions rainfall is due to their convective and sometimes tropical nature (Engelbrecht *et al.,* 2015).

Western Cape has an estimated rainfall of 350 mm per annum (Western Cape Government, 2005). The rainfall in the province is below the country's average. Water crisis in Western Cape is set to escalate (DWS, 2012a). DWS (2012a) predicts that the rainfall in the province will decrease by 30% in 2050. Currently, the water level of major dams is calculated to be 22.8% in Western Cape and only 12.3% of the water is usable (Botai *et al.,* 2017). Western. As a result, South Africa is one of the first countries in the world to pass the law that regulates water usage to ensure that water systems have sufficient water to sustain life (WWF, 2016). Shortage of freshwater in Western Cape was triggered by a strong El Nino phase which occurred between 2014-2016 (Western Cape Government, 2017). Due to El Nino phase Western Cape is experiencing the worst droughts since 1904 (Botai *et al.,* 2017).

## 2.3 Effects of freshwater shortage in Western Cape

The Western Cape water supply system (WCSS) that supplies various municipalities in the Western Cape with freshwater form one of the main drivers of the economic growth in the province and the country at large. According to GreenCape (2018), the WCSS supplies industries that contribute 80% of the provincial Gross Domestic Product (GDP) and 11% of the national GDP. The economic growth of Western Cape between 2011 and 2016 was predicted to 4.2% per annum (GreenCape, 2014). However, due to lack of rainfall, harvesting has dropped and this has a negative impact of the economy (Baudoin *et al.,* 2017).

Western Cape is a province with the highest agricultural activities in the country. According to GreenCape (2017), Western Cape produces 55% to 60% of the country's agricultural exports. Approximately 11.5 million hectares in Western Cape is used for agricultural purposes (GreenCape 2017). Western Cape is well known for cultivation of grapes and production of wine. The production of wine in Western Cape yields more than 50% of the GDP and it is also estimated that wine making creates more than 8% jobs in the province (Araujo *et al.,* 2015). Thus it is evident that shortage of freshwater is a limiting factor for economic growth (Andersson *et al.,* 2009). In the WWF (2017) report it was shown that due to losses in maize exports during the fourth quarter of 2015, about 37 000 jobs were lost. This has led to an increase of unemployment and subsequently the price of food (WWF,

2017; Baudoin *et al.*, 2017). According to the World Economic Forum (WEF) (2017), shortage of freshwater in South Africa is ranked as the third highest risk why other nations would not invest in the country. This factor also persists in other parts of the world (GreenCape 2018).

In addition to economic growth, shortage of freshwater is one of the limiting factors of social development (Liefferink *et al.,* 2017). South Africa has drafted a National Development Plan (NDP), which is aimed to eradicate poverty, provide citizens with safe and clean drinking water, as well as proper sanitation by 2030 (Coetzee *et al.,* 2016; NPC, 2011; RSA, 1996). However, shortage of freshwater is one of the limiting factors for the country to reach its target goals on the NDP (WWF, 2016). The goals of NDP are in line with the Sustainable Development Goals (SDG) drafted by the United Nations (Geere *et al.,* 2018; WWF, 2016).

Western Cape is one of the fast growing provinces in South Africa, thus leading to rapid development and growth. According to the Western Cape Government (2006), approximately 90% of Western Cape population is urbanised. This is 40% higher than the average urbanisation population in South Africa. According to STATS SA (2018b), Western Cape is expected to have large inflow of migrants of approximately 311 004 between 2016 and 2021. One of the consequences of urbanisation is elevated consumption levels of water due to transformation (Karthiyayini and Sundaram, 2016); which then exerts far more pressure unto Western Cape to provide sufficient freshwater.

## 2.4 Alternative resources

Predictions made by Jurdi *et al*. (2002) were that an increase in urbanisation, agricultural activities, rapid economic growth and social development would increase the demand of freshwater and lead to limited water resources by 2010. Currell *et al*. (2012) state that above mentioned factors have indeed led to depletion of freshwater. Furthermore, these factors, including pollution, circumstances around bulk water and wastewater infrastructures may lead to alteration of water quality (Sershen *et al.,* 2016). Altered water quality makes freshwater less potable for humans and acceptable for other purposes as well.

To ensure that the provincial and nation GDP grows, Western Cape must continue to increase the agricultural exports. This would not only benefit the economic growth but social development as well. If the Western Cape could increase its export rates it will help create more jobs and eradicate poverty. According to Statistic South Africa (STATS SA) (2018a), the unemployment rate has increased by 153 000 or 0.4% in the first quarter of 2018 as compared to the fourth quarter of 2017. This takes the unemployment rate up to 59.3%.

Creating more jobs and eradicating poverty will be putting South Africa in a better position to achieve its goals in the NDP by 2030. However, in order for Western Cape to reach this target, whether directly or indirectly, the willingness to collaborate from different stakeholders would have to occur. Water experts are needed not only to come up with new water technologies but also to educate the public on how to conserve and protect natural resources. A research by Cosgrove and Loucks (2015) stated that the water crisis that is already a global issue and will still remain one of the main causes of societal problems. To prevent this from occurring alternative water technologies must be implemented to compensate for the high demand of freshwater. As shown, traditional water sources such as dams and borehole water do not produce sufficient freshwater (Rathnayaka *et al.,* 2016; Matthews, 2015).

Through scientific researches water experts have come up with new alternative water sources such as reclaimed water, grey water, desalinated water, and stormwater to compensate for the high demand of freshwater (Rathnayaka *et al.,* 2016). These water sources are subjected to various water treatment processes depending on what purpose the water will serve. However, advanced water technologies are costly and can only be afforded by developed countries to relieve the stress posed by shortage of freshwater, while this stress deteriorate the well-being of the least developed countries (Rodda *et al.,* 2016).

### 2.4.1 Greywater

The Western Cape uses grey waters as an alternative water source. According to the DWS (2017), greywater is the wastewater collected from homes and various commercial buildings. This excludes wastewater collected from kitchen sinks, dishwasher and toilet water (Brain *et al.,* 2015). This water is termed blackwater. Greywater is non-potable mainly used for agricultural and industrial purposes (Chukalla *et al.,* 2018; Chaabane *et al.,* 2017). Greywater may not be meant for human use but it is subjected to various water treatment processes due to high concentration of salts, sodium, boron, as well as extreme pH can negatively affect the plant's growth (WRC, 2017).

### 2.4.2 Desalination

Desalination is the process that converts salty seawater or brackish groundwater through advanced water treatment processes for water reuse purposes (Blersch and du Plessis, 2017). Desalination system may require high energy to operate however, they becoming widely used since they need less pretreatment of water and have been shown to be robust (Swartz *et al.,* 2006). The thermal desalination and membrane desalination are the most studied process of the desalination robust (Likhachev and Li, 2013; Swartz *et al.,* 2006). The

various processes involved in the thermal desalination include multiple effect distillation, multi-stage flash and vapour compression distillation, whereas membrane-based processes include reverse osmosis, nanofiltration and electrodialysis (Likhachev and Li, 2013). According to Blersch and du Plessis (2017) there are six distillation plants in South Africa and three of them are situated in the Southern Cape to combat shortage of water in Western Cape that occurred in 2009/2010.

### 2.4.3 Direct potable reuse (DPR) plant

The DPR plant depends on wastewater for production of drinking water (Matthews, 2015). The DPR plant recycles wastewater that is directly used for human consumption and it was established in Western Cape in January 2011 (Collins and Herdien, 2013). This plant was installed as new water technology that allows water to be reused and subsequently compensate for the high demand of drinking water as the province is experiencing shortage of freshwater (Matthews, 2015). Wastewater that enters the plant is associated with high level contaminants from agricultural and urban runoffs (Adefisoye and Okoh, 2017; Singh and Lin, 2015). Traditionally, treated effluent from the Western Cape wastewater treatment works (WWTW) was used for irrigation (Grimmer and Tuner, 2013). Now, treated effluents undergo ultra-filtration and reverse osmosis for treatment in the production of drinking water (Grimmer and Tuner, 2013; Matthews, 2015).

## 2.5 Water quality: Physico-chemical parameters

An increase in drinking water demand caused by industrialization, urbanization and agricultural activities has led to an increased rate of pollution in the water environment (Ayangunna *et al.,* 2016; Baghvand *et al.,* 2010). Water is composed of hydrogen and oxygen atoms ($H_2O$), which are highly reactive and can solubilize easily (Reda, 2016). Hence, water contains harmful particles from the environment such as minerals, organic compounds and gases (Saritha *et al.,* 2017). Usually these pollutants present in water are not easily biodegradable (Javid *et al*., 2015). The current study was focused on physico-chemical parameters such as phosphate, nitrate, nitrite, salinity, electrical conductivity, total dissolved solids, temperature, pH, turbidity and free chlorine.

### 2.5.1 Phosphate, nitrate and nitrite

Phosphate and nitrogen compounds such as nitrate and nitrite are important nutritional elements needed to stimulate human, animal and plant growth (Naylor *et al.,* 2018; Goody *et al.,* 2017). These compounds are the active ingredients of fertilizers (Naylor *et al.,* 2018; Goody *et al.,* 2017; Aydin, 2007, 2007). These compounds are spread into the water

environment through wastewater, faeces and agricultural runoff (Gupta *et al*., 2017; Goody *et al.,* 2017). The spread of nitrate and nitrite into the water environment depends on mineralization, nitrification and denitrification process (Charles and Ogoko, 2012). It is important to monitor the levels of nitrate in drinking water as it may increase risks of cancer, methemoglobinemia and organ failure (Aydin, 2007; Naylor *et al.,* 2018). Nitrate concentration that is greater than 1,000 µg/L creates favourable conditions for the growth and development of algae in surface water, resulting in eutrophication (Naylor *et al.,* 2018). Phosphate also causes eutrophication and human health risks such as muscle damage, breathing problems and kidney failure for humans (Gupta *et al*., 2017; Nduka *et al.,* 2008).

### 2.5.2 Salinity, total dissolved solids (TDS) and electrical conductivity (EC)

Salinity is the composition of sodium and chloride ion, which are the active elements of sodium chloride, commonly known as salt (Ivanković *et al.,* 2017). Intake of drinking water that contains high salt concentration poses health risks to humans (Schelbeek *et al.,* 2017). Prolonged effects of salt lead to hypertension and cardiovascular diseases (Nahian *et al.,* 2018; Schelbeek *et al.,* 2017). Individual who are more susceptible are immunocompromised patients, children and elderly people (Nahian *et al.,* 2018; Khan *et al.,* 2016; 2011). Salt contents in water are affected by electrical conductivity and total dissolved solids (Daud *et al.,* 2017).

TDS is the measure of the total concentration of solid particles which are present in water (Hubert and Wolkersdorfer, 2015). Solids are suspended and dissolved particles in water (Qureshimatva *et al.,* 2015). These solids give drinking water a peculiar taste when proper drinking water purifications are not followed (Buridi and Gedala, 2017). Solids particles may include organic and inorganic dissociated anions and cations as well as undissociated dissolved species (Hubert and Wolkersdorfer, 2015). Intake of drinking water that has high levels of TDS could cause abnormalities in the central nervous system, and paralysis of the tongue, lips, face, irritability and dizziness (Gupta *et al*., 2017).

TDS may be used to determine EC under controlled condition (Ewebiyi *et al.,* 2015). A factor of 0.65 is used for the convertion of TDS into EC (µmho/cm) at constant of temperature of 25°C (Abhineet and Dohare*,* 2014). EC is the ability of water to conduct electric current (Qureshimatva and solanki, 2015; Chandne, 2014). The conduction of electric current is strongly dependent on the total concentration of the dissolved electrolytes and gases (Hubert and Wolkersdorfer, 2015).

### 2.5.3 Temperature

Temperature is an important ecological and physical factor that plays a role in the regulation of physico-chemical and microbiological parameters in the environment (Anbarasu and Anubuselvan, 2017; Qureshimatva and Solanki, 2015). Water affects the function of both living and non-living organisms (Palamuleni and Akoth, 2015). Temperature regulates metabolic activities, growth, feeding, reproduction, distribution, migratory behaviour of microorganisms and functioning of the ecosystem (Elahi *et al.,* 2015; Palamuleni and Akoth, 2015; Chandne, 2014).

### 2.5.4 pH

pH is the negative logarithm of hydrogen ion concentration expressed as pH = -log $[H^+]$ (Qureshimatva *et al.,* 2015). pH values are determined by measuring the concentration of hydrogen and hydroxyl ion in water (Bisi-Johnson *et al.,* 2017). The measured values of pH are important in determining whether water samples are acidic, neutral or alkaline (Rahmanian *et al.,* 2015). Water samples with the pH value lower than 7.0 is acidic, 7.0 denotes neutral value and pH greater than 7.0 is considered alkaline (Jamdade and Gawade*,* 2017). Concentrated acid and alkaline are corrosive to pipes used to transport water to distribution networks (WHO, 2007). Acidic pH is corrosive to metal and plumbing systems thus releasing toxic metals such as lead, copper etc. (Rahmanian *et al.,* 2015; Buridi and Gedala, 2014; Patil *et al.,* 2012). There are no health implications that are directly associated with pH (WHO. 2007).

### 2.5.5 Turbidity

Turbidity is the measurement of scattering and absorption of light by suspended particles in water (Voichick *et al.,* 2018; Roos *et al.,* 2017). The effect of suspended particles is affected by size, shape, refraction index and colour of the silt, clay and organic matter (Voichick *et al.,* 2018; Roos *et al.,* 2017). Turbidity usually forms when light weight particles cannot be suspended and removed by coagulation-flocculation process (Baghvand *et al.,* 2010). High turbidity makes it difficult for filtration processes to remove pathogenic microorganisms from the drinking water production facilities (Gupta *et al*., 2017). Turbidity creates favourable conditions for bacteria to resist conventional water processes (Baghvand *et al.,* 2010; Gupta *et al*., 2017). However, there are no health implications that are directly associated with turbidity of drinking water (Roos *et al.,* 2017)

### 2.5.6 Free chlorine

Chlorination is a common and frequently used disinfection process used for the removal of waterborne pathogens (Gabelicha *et al.,* 2002). Some of the chlorine residues remain in excess in drinking water after a few minutes of chlorination (Dippong *et al.,* 2014). These residues are called free chlorine (Dippong *et al.,* 2014). Free chlorine occurs in a wide range of concentrations (Jamdade and Gawande, 2017). High concentration of free chlorine deteriorates polyamide membranes of the water treatment plant if not properly regulated (Gabelicha *et al.,* 2002). Regulated free chlorine present in drinking water systems prevents recontamination of water in a distribution network (Dippong *et al.,* 2014).

## 2.6 Water quality: Microbiological parameters

Microorganisms are the most common and deadly contaminants present in drinking water (Rajendra *et al.,* 2012). Therefore, access to drinking water free from microorganisms can reduce waterborne infections (Edokpayi *et al.,* 2018). Waterborne infections due to poor sanitation and contaminated water sources constitute up to 80% of the total numbers of health problems in the world (Bedada *et al.,* 2018; Abera *et al.,* 2011). Water that is aimed for human consumption should not harbour microorganisms (Dippong *et al.,* 2014). Microorganisms that are going to be investigated in this study include faecal coliform, *Escherichia coli (E. coli)* and heterotrophic plate count (HPC) bacteria.

### 2.6.1 Faecal coliforms

Faecal Coliforms belong to *Enterobacteriaceae* family which comprises of bacteria that are facultative anaerobic, Gram negative, non-spore forming, and rod shaped in nature (Divya and Solomon, 2016; Figueras and Borrego, 2010). This group includes *Escherichia coli, Enterobacter* spp. and *Klebsiella.* spp. (Mann, 2016). These bacteria inhabit large intestines and play a role in the digestive system of mammals including humans and other warm-blooded animals (Kinika *et al.,* 2017). These bacteria reach water sources through human waste, animals' manure, sewage discharge and water runoff (Divya and Solomon, 2016). They are are used as indicators of faecal contamination (Harwood *et al.,* 2005). Faecal coliforms can be detected with laboratory methods that are affordable, reliable and easy to interpret, thus making making them good indicators of pathogenic microorganism in water (Kinika *et al.,* 2017). Faecal indicators are essential for the evaluation of microbiological quality of drinking water (Jagals *et al.,* 2000; Kinika *et al.,* 2017; Harter *et al.,* 2014).

### 2.6.2 *E. coli*

Since the discovery of *E. coli,* scientists have a better understanding of phenotypic and genotypic virulence in the different environments, including water (Brandt and Albertsen, 2018; Grunert *et al.,* 2018). *E. coli* is a predominant member of faecal coliform present in mammal faeces and it is also used as faecal indicator for assessment of faecal contamination of both pathogenic and non-pathogenic bacteria present in drinking water (Clarke *et al.,* 2017). Non-pathogenic strains of *E. coli* occur as normal flora of the gut, thus contributing to the production of vitamin K2 in the host cell and further prevent the occurrence of other pathogenic strains in the intestines (Odonkor and Ampofo, 2013). However, there are five *E. coli* strains that are pathogenic and associated with diarrhoea that include Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enterohaemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC) and Entero-invasive *E. coli* (EIEC) (Stange *et al*., 2016; Traoré *et al.,* 2016). *E. coli* serotype O157:H7 present in drinking water is an opportunistic pathogen associated with health implications (heamorrhagic enteritis and haemolytic uremic syndrome) especially in children, elderly people or immunocompromised patients (Clarke *et al.,* 2017; Adzitey *et al.,* 2015; Figueras and Borrego, 2010). *E. coli* should not be detected in drinking water samples (Fadaei, 2014; Kumar *et al.,* 2013).

### 2.6.3 Heterotrophic plate count (HPC) bacteria

Heterotrophic plate count (HPC) bacteria are aerobic and facultative anaerobic microorganisms (Bedada *et al.,* 2018). HPC bacteria generate carbon source from organic carbon rather than carbon dioxide (Rusin *et al.,* 1997). Humans get in contact with the HPC bacteria from vegetation, air, soil, food and water environments (Allen *et al.,* 2004). Since there are no universal indicator microorganisms to assess the quality of drinking water (Bedada *et al.,* 2018), HPC bacteria are used to monitor microbiological quality of drinking water and evaluate the efficiency of the drinking water treatment plant processes (Mokhosi and Dzwairo, 2015; Bedada *et al.,* 2018). Drinking water production facilities are effective if the numbers of HPC bacteria are reduced from raw to treated water (Mokhosi and Dzwairo, 2015). HPC bacteria are culturable and viable microorganisms that can be enumerated and isolated from water samples using easy laboratory techniques (Allen *et al*., 2004; Defives *et al.,* 1999).

## 2.7 Pathogenicity

The ineffectiveness of the drinking water production facilities may lead to high and unregulated levels of HPC bacteria in drinking water (Chowdhury, 2011; Mokhosi and

Dzwairo, 2015). Some HPC bacteria are able to produce extracellular enzymes in unfavourable conditions for their survival (Tropeano *et al.,* 2013). Extracellular enzymes interact with the outside intracellular compartment by breaking down C-O and C-N bonds link monomers (Cunha *et al.,* 2010). These extracellular enzymes produced by bacteria are used in industries for various purposes and biotechnological processes (Tropeano *et al.,* 2013). However, the presence of extracellular enzymes in drinking water may pose health threats such as gastrointestinal illness characterised by fever, nausea, vomiting and diarrhoea (Horn *et al.,* 2016). Individuals who are mostly susceptible are children, immunocompromised patients and elderly people (Chowdhury, 2011; Pavlov *et al.,* 2004).

## 2.8 Water treatment

The world is faced with a public health concern of physical, chemical and microbiological pollutants in drinking water (Saritha *et al.,* 2017). This problem persists even in developed and developing countries with advanced public health facilities (Naylor *et al.,* 2018). It is then important to monitor and regulate physico-chemical parameters, as well as microbiological agents in drinking water (Reda, 2016). The regulation and monitoring of water pollutants is crucial in the production of safe and clean drinking water (Matthews, 2015).

Water quality in South Africa is monitored by the Department of Water Affairs (DWA), now known as Department of Water and Sanitation (DWS) (WWF, 2016). The DWS introduced national guidelines known as South African National Standards (SANS) of drinking water (DWS, 2011). SANS of drinking water are national standards used to determine whether drinking water produced across South African water production facilities contains safe levels of physical, chemical and microbiological parameters (Mokhosi and Dzwairo, 2015).

### 2.8.1 Coagulation-flocculation process

Raw water entering the water plant is subjected to coagulation-flocculation process (Matthews, 2015). Coagulation-flocculation process is primarily used for the removal of colloidal particles, suspended solids, COD, turbidity and metals present in the wastewater (Ayangunna *et al.,* 2016). This is achieved by destabilizing and forming flocculants (Pontuis, 2016, Aghapour *et al.,* 2016). Chemical coagulants are used to neutralize negatively charged colloidal particles by cationic hydrolysis products to form agglomeration of flocculants (Sher *et al.,* 2013; Baghvand *et al.,* 2010). Flocculants are removed from the drinking water treatment plant as sludge (Teh, 2016).

### 2.8.2 Rapid sand filtration

Water leaving the flocculation-coagulation process is passed through rapid sand filtration (Matthews, 2015). Rapid sand filtration is a purification process that has long been used in drinking water production facilities to ensure water is clean and safe for industrial and domestic use (Mwiinga *et al.,* 2004; Lombard and Haarhoff, 1995). This process is used for its supremacy to trap and remove coagulated and flocculated materials that may have not been removed in the coagulation and flocculation treatment processes (van der Walt and van der Walt*,* 2009). These flocculants are removed by a special feature of the rapid sand filtration known as backwash (Ceronio *et al.,* 2002). The porous size of these filters is 0.3 mm (Mwabi *et al.,* 2012)

### 2.8.3 Ultrafiltration membrane

Ultrafiltration is becoming more recognised in the water purification processes due to its low cost run and ability to enhance water quality (Fesenko *et al*., 2018). Ultrafiltration consists of membrane filters with the porous size that ranges from 0.01 µm to 0.01 µm water (Al-Sarkal and Arafat, 2013). These filters enable the ultrafiltration not only to remove colloidal substances but also to eliminate bacteria and protozoa (Al-Sarkal and Arafat, 2013).

However, the application of ultrafiltration is limited to medium and higher molecular weight components (Maddah *et al.,* 2017). This creates suitable condition for other microbiological agents (viruses) as well as natural organic matter to pass through the membrane (Winter *et al.,* 2017). The application of ultrafiltration membranes is essential for pretreatment and protection of reverse osmosis (Al-Sarkal and Arafat, 2013). Utlrafiltration membranes prevent against the occurrence of membrane fouling of reverse osmosis that may be caused by high levels of particulate matter (greater than 4 SDI) and aluminium-based chemical coagulants (greater than 100 µl; Vera *et al*., 2013). Ultrafiltation and reverse osmosis are becoming frequently used as technologies that treat wastewater, salty seawater and brackish ground water for domestic, industrial and human use (Fesenko *et al*., 2018; Blersch and du Plessis, 2017; Matthews, 2015).

### 2.8.4 Reverse osmosis

Reverse osmosis is an efficient water technology that requires low reagents for operation (Fesenko *et al.,* 2018). Reverse osmosis process is used to recycle water to compensate for the normal and high water demand of fresh and drinking water (Greenlee *et al*., 2009). Reverse osmosis treats water by passing it through semi permeable membrane under osmotic pressure from less to more concentrated solution (Blandin *et al*., 2016;

Wimalawansa, 2013). The porous size of membrane filter ranges between 0.0001 and 0.1 microns (Greenlee *et al.,* 2009). As a result, reverse osmosis are used to remove micro dissolved matter, petrochemical chemicals and pharmaceutical and personal care products (Wimalawansa, 2013). Reverse osmosis may also be used to remove non-hazardous pollutants to ensure that the odour, taste and colour of drinking water is enhanced (Greenlee *et al*., 2009).

### 2.8.5 Advanced oxidation

Advanced oxidation process generates highly reactive hydroxyl radicals (HO-) and other reactive oxygen species (Moreira *et al*. 2018; Zhang *et al.* 2016). Hydroxyl and other reactive oxygen species generated react and oxidize a wide range of non-biodegradable organic contaminants in water (Zhang *et al.* 2016; Micheal *et al*. 2012; Ferro *et al.* 2017). Advanced oxidation processes are essential in the water treatment facilities that recycle wastewater (Micheal *et al.* 2012). Advanced oxidation is used for its supremacy to inactivate antibiotic resistant bacteria and antibiotic resistant gene by lysis of the DNA (Zhang *et al.* 2016).

There are four types of advanced oxidation and these include: Fenton oxidation, photo-Fenton process, TiO2 photocatalysis and UV/H2O2 (Zhang *et al.* 2016). Of the four, photocatalysis is the most cost effective, environmental-friendly as it uses natural or artificial solar radiation (Karaolia *et al.* 2018). Furthermore, photocatalysis proves adventitious over the other listed processes since it is non-selective and has no disinfection by-products (Xiong and Hu. 2013).

### 2.8.6 Disinfection

The application of water disinfection is applied to remove waterborne pathogens that may cause health hazards to humans (Collivignarelli *et al.,* 2017). Disinfection process is used to eliminate both pathogenic and non-pathogenic organisms to ensure microbiological quality in drinking water production facilities (Lin *et al.,* 2016b). The most common disinfection process is chlorination which was firstly used in the 19$^{th}$ century because it is cost effective (Lin *et al.,* 2016b). The application of chlorine as a water disinfectant confers changes in the enzymatic activities, thus subsequently inhibits bacterial metabolism that eventually leads to lysis of bacterial cells (Collivignarelli *et al.,* 2017).

## 2.9 Disinfection selects for antibiotic resistance

The efficiency of drinking water production facility treatment processes has been for many years optimized by environmental engineers and scientists that have been focused on the

elimination of physical, chemical and microbiological pollutants (Ju *et al.,* 2016; Yang *et al.,* 2017). However, these experts have been turning a blind eye over the contaminants of emerging concern such as pharmaceutical products, antibiotic resistant bacteria and antibiotic resistant genes (Ju *et al.,* 2016; Yang *et al.,* 2017). Hence, the drinking water treatment processes can successfully eliminate suspended solids, organic matter, nitrogen and phosphate, but has a limited capacity to biodegrade contaminants of emerging concern, which are not initially included in the routine monitoring scheme of the drinking water treatment processes (Martínez, 2008; Li *et al.,* .2017a; Munir *et al.,* 2011; Sousa *et al.,* 2017).

The ineffectiveness of most commonly used disinfection processes was shown in a study by Guo at al. (2017). A similar study was done by Sullivan *et al*. (2017) where the concentration of antibiotic resistant bacteria and antibiotic resistant genes was compared before and after chlorination. The antibiotic resistant bacteria and antibiotic resistant gene concentrations were higher after chlorination as compared to the initial concentration. This is evident that disinfection processes do not always reduce, but can select for antibiotic resistant bacteria and their associated genes (Guo *et al.,* 2017). This makes drinking water a vector for antibiotic resistant bacteria and their associated genes (Zhang *et al.,* 2016).

## 2.10 Incidence of antibiotic resistance

The world is faced with a major public health issue of emergence of antibiotic resistant bacteria and their associated genes in drinking water (Adefisoye and Okoh, 2017; Lin *et al.,* 2016b). The discovery of antibiotics has caused a tremendous change in medicine by protecting lives again pathogenic microorganisms, thus increasing life expectancy of humans and animals (Davies and Davies, 2010; Chropra, 2012). The first antibiotic, penicillin was discovered by a Nobel prize winner Alexandra Fleming in 1928, but the drug became commercially available to the public in 1940 (Berglund, 2015; Davies and Davies, 2010; Brown and Wright, 2016). The era (1940-1962) in which penicillin, together with aminoglycoside, cephalosporins, macrolides, glyocopeptides, quinolones, streptomycin, chloramphenicol and tetracycline were discovered was termed the golden age (Penesyan *et al.,* 2015; Chropra, 2012). However, two years after the distribution of penicillin, the first antibiotic resistant bacteria emerged (Berglund, 2015).

The world has entered a post antibiotic age where antibiotic resistance is set to escalate, causing antibiotic therapy to be complex and expensive (Brown and Wright, 2016). In this era, the application of antibiotics is the main cause of therapy failure (Hancock, 2015). The

occurrence of antibiotic resistance has affected and threatens the prevention and treatment with antibiotics resulting in high morbidity and mortality rates (Singh *et al.,* 2016).

This is a life-threatening situation since clinical resistance is disseminated and spread into the water environment (Zhang *et al.,* 2009). The extensive and improper usage of antibiotics in agriculture, veterinary medicine, and prophylactic and continuous exposure to antibiotics in human medicine has led to the emergence, selection and spread of antibiotic resistance in surface water, groundwater and drinking water (Adefisoye and Okoh, 2017; Stange *et al.,* 2016).

### 2.10.1 Antibiotics in human and veterinary medicine

The application of antibiotics in human and veterinary medicine is essential in the inhibition of metabolic activities of the bacterial cell (Brown and Wright, 2016). The bacterial cell cannot grow, divide and reproduce when the metabolism is halted (Brown and Wright. 2016). Antibiotics are not limited to prevention and treatment of bacterial infections (Marti *et al.,* 2014). Application of antibiotics have a wide range of applications for patients undergoing surgery such as chemotherapy, bone marrow or organ transplants, joint replacements and care of premature infants (Marti *et al.,* 2014).

There is no governing body or organisation that regulates and monitors the application of antibiotics (Woon and Fisher, 2016). As a result, it is easy to access antibiotics since some pharmaceutical companies issue these drugs (over the counter medicine) without any prescription from the doctors (Woon and Fisher, 2016; Bergeron *et al.,* 2015). Some patients undergoing antibiotic therapy either overdose or do not finish their prescription (Bergeron *et al.,* 2015). Improper usage of antibiotics does not just lead to treatment failure but also enhances the virulence of antibiotic resistant bacteria (Danesh *et al.,* 2017; Saga and Yamaguichi, 2009). Antibiotics are not completely metabolized by the body (Roca *et al.,* 2015), 30-90% of the antibiotic residues are released into the water environment in their original form (Liu *et al.,* 2013). Antibiotics are spread in the environment through wastewater, sewage and animal manure runoff (Yang *et al.,* 2017; Chen *et al.,* 2015a; Reinthaler *et al.,* 2003). Bacteria are then in contact with these antibiotics present in the water environment at sub-inhibitory concentrations (Akegoke *et al*., 2017). This contact creates a favourable condition for bacteria to acquire antibiotic resistant characteristics (Vaz-Moreira *et al.,* 2014; Timi and Adeniyi, 2013).

### 2.10.2 Antibiotics in agriculture

Antibiotics are not only useful in human medicine as they are used as growth stimulator of livestock to compensate for the high demand on food (Roca *et al.,* 2015). Animals are fed with antibiotics to stimulate growth and subsequently to yield more meat within a short period of time (Zishiri *et al.,* 2016). Woon and Fisher (2016) study indicated that penicillin and tetracycline can be used to enhance the relative body mass of animals by 15-20%, but the mechanism of growth remains unknown (Nakayama *et al.,* 2017). Water runoff is often a vehicle that spreads antibiotic resistant bacteria from the environment to the aquatic systems and it impacts badly on the quality of water (de Faria *et al.,* 2016).

## 2.11 Antibiotic resistant genes in water

Environments where there are high anthropogenic activities serve as suitable habitats for microorganisms to acquire antibiotic resistance determinants (Rizzo *et al.,* 2013). The extensive usage of antibiotics in agriculture, veterinary and human medicine has led to the occurrence of antibiotic resistant bacteria and antibiotic resistant genes in water environments (Vaz-Moreira *et al.,* 2014; Timi and Adeniyi, 2013). The detection of antibiotic resistant bacteria and antibiotic resistant genes in drinking water is a public health concern worldwide (Xi *et al.,* 2009). Consumption of drinking water that harbors antibiotic resistant bacteria and antibiotic resistant genes may pose health risks to immunocompromised patients, children and elderly people (Xi *et al.,* 2009).

### 2.11.1 Antibiotic resistant genes related to erythromycin

Erythromycin antibiotics belonging to the macrolides were derived from *Saccharopolyspora erythroaeae* found in soil environmental samples in 1949 but were made commercially available in 1952 (Jelić and Antolović, 2016; Procópio *et al.,* 2012). Macrolides antibiotics are synthesized from *Steptomyces* characterized by 14-, 15- or 16-member lactose ring (Hawkyard and Koerner, 2007). Erythromycin antibiotics consist of 14-member lactose ring (Jelić and Antolović, 2016; Hawkyard and Koerner, 2007). Macrolides inhibit bacterial protein synthesis of 23S subunit of the bacterial ribosome, leading to premature release of peptides during translation (Etebu and Arikekpar, 2016; Choi *et al.,* 2018). Erythromycin are effective in protection against Gram-positive cocci and bacilli and some Gram-negative bacteria (Choi *et al.,* 2018). Erythromycin has a wide range of applications as it is active against respiratory, gastrointestinal, and genital tract infections, streptococcal tonsillopharyngitis, otitis media, acute bronchitis, primary atypical pneumonia, as well as skin and soft tissue infections (Jelić and Antolović, 2016; Welling, 1979).

The first antibiotic resistance emergence of erythromycin was reported in the 1950s when 70% of *S. aureus* strains conferred resistance to erythromycin (Davies and Davies, 2010). The *erm* genes encode resistance to macrolides (Choi *et al.,* 2018). The *erm* genes can also confer resistance to other classes of antibiotics, namely chloramphenicol and vancomycin (Zhang *et al.,* 2009). The resistance mechanisms that are associated with *erm* genes include rRNA methylation and efflux pumps (Zhang *et al.,* 2009). The *erm* genes are carried in the genetic elements, such as plasmids and transposons, thus making it easy for the genes to be shared between bacterial species (Dzyubak and Yap; 2016; Zhang *et al.,* 2009).

### 2.11.2 Antibiotic resistant genes related to ampicillin

Ampicillin antibiotics inhibit the membrane-bound enzymes that are involved in the synthesis of the bacterial cell wall (Chudobova *et al.,* 2014; Ashnagar and Naseri, 2007). According to Lachmayr *et al*. (2009) β-lactams make up around two-thirds, by weight, of all the antibiotics used in human medicine. Ampicillins are effective against both Gram-positive and Gram-negative bacteria (Kaushik *et al.,* 2014). Ampicillins are commonly used for treatment against enteric fever, respiratory infections, urinary tract infections, skin and soft tissue infections (Kaushik *et al.,* 2014).

The extensive usage of ampicillins has led to the synthesis of β-lactamase enzymes (Chudobova *et al.,* 2014). These enzymes catalyse the hydrolysis of amide bond of four membered β-lactam ring and confer resistance to destroy cell wall (Shahid *et al.,* 2011; Lachmayr *et al.,* 2009; Singh *et al.,* 2016). There are four classes of β-lactamases, namely A, B, C and D (Shahid *et al.,* 2011). Classes A, C and D are serine enzymes, whereas class B is Zn-metalloenzyme (Shahid *et al.,* 2011). Class A is the most common mechanism of resistance displayed by most bacterial species (Yin *et al.,* 2013).

The $bla_{TEM}$ is the most studied gene from extended spectrum β-lactamases (ESBLs) in class A (Shahid *et al.,* 2011; Lachmayr *et al.,* 2009). The $bla_{TEM}$ gene consists of more than 220 different distinct alleles, which encode different amino acid polymorphisms that extended their substrate range (Adesoji and Ogunjobi, 2016). The $bla_{TEM}$ gene is associated with mobile genetic elements and enhances the chance of multidrug resistance (Zhang *et al.,* 2009). According to Overdevest *et al*. (2011) in the 1990 $bla_{TEM/SHV}$ genes were involved in the cross infection in hospitals but these genes are now associated with non-clinical environments. The spread of the $bla_{TEM}$ gene in the water environment is facilitated by transposons and integrons (Lachmayr *et al.,* 2009). The ESBLs render treatment of β-lactam antibiotics less effective leading to therapeutic failure thus requiring the application of more broad spectrum and costly therapeutic agents (Sageerabanoo *et al.,* 2015).

### 2.11.3 Antibiotic resistant genes related to cephalothin

Bacteria produce the enzyme β-lactamase to prevent cephalothin antibiotics to reach the target site known as penicillin binding proteins (Anacona and Serrano, 2003). The *ampC* gene is responsible for conferring resistance to cephalosporins and cephamycins (Shanthi *et al.,* 2012). The *ampC* gene belongs to β-lactamase class C known as Ambler or group I cephalosporinases (Shanthi *et al.,* 2012; Sageerabanoo *et al.,* 2015). The *amp C* gene, like any other ESBLs mediate resistance to extended spectrum cephalosporins, aztreonam and oxymino β-lactams and they are not affected by inhibitors such as clavulanic acid, sulbactam and tazobactam (Shanthi *et al.,* 2012; Sageerabanoo *et al.,* 2015).

### 2.11.4 Antibiotic resistant genes related to tetracycline

Tetracycline antibiotics are a family of antibiotics that were derived from *S. aureofacine* in the 1940s (Chopra and Roberts, 2001; Procópio *et al.,* 2012). Tetracycline antibiotics are specifically targeted to inhibit bacterial protein synthesis by ensuring aminoacyl-tRNA to the ribosomal receptor (A) site do not come into contact (Chopra and Roberts, 2001). Tetracycline antibiotics are used in both human and veterinary medicine (Xiong *et al.,* 2018). Its application is effective against respiratory tract, skin, urinary tract and intra-sites infections (Draper *et al.,* 2014). They are also used as growth stimulators for livestock (Granados-Chinchilla and Rodríguez, 2017).

Efflux and ribosome protection are the resistant mechanisms developed by bacteria to render tetracycline ineffective (Draper *et al.,* 2014). Bacterial species produce *tet* genes in order to confer resistance to tetracycline antibiotics (Xiong *et al.,* 2018). There are two mechanisms by which bacteria confer resistance to tetracycline antibiotics; (i) efflux pumps which involve *tet* genes such as *tet* (*K)* and *tet* (*L),* (ii) ribosomal protection which involves *tet* genes such as *tet (M), tet (O*), *tet (Q*), *tet (S), tet (T)* and *tet (W)* (Zahid *et al.,* 2018).

### 2.11.5 Class 1 integrons *IntI1* gene

Class 1 integrons genes are associated with the capture and dissemination of antibiotic resistant genes in the environment (Chen *et al.,* 2015b; Koczura *et al.,* 2016). They are characterized into three features: an integron-integrase gene (*inti1),* a recombinant site (*att1)* and a promoter ($P_C$) (Gillings *et al.,* 2015; Wei *et al.,* 2018). Class 1 integrons are involved in the acquisition of gene cassettes that are associated with antibiotic resistance (Koczura *et al.,* 2016). According to Lin *et al*. (2015b), these gene cassettes confer resistance to a wide range of antibiotics such as aminoglycosides, β-lactams, chloramphenicol, etc.

Class 1 integrons pose a threat in water environments since they are not removed by conventional water treatment processes and this increases their chances of being present in drinking water (Gillings *et al.,* 2015; Chen *et al,* 2015b). Lin *et al*. (2015b) state that integrons are present in an estimated 9% of sequenced bacterial genomes. The integrons are associated with mobile elements such as transposons and plasmids that facilitate resistance to antibiotics, heavy metals and disinfectants (Gillings *et al.,* 2015; Koczura *et al.,* 2016).

## 2.12 Antibiotic resistant genes spread to other microorganisms

Water environments serve as a perfect habitat for antibiotic resistant bacteria and a vector for microorganisms to spread antibiotic resistant genes to daughter cells or other microorganisms (Vaz-Moreira *et al.,* 2014; Timi and Adeniyi, 2013). Antibiotic resistance genes are passed from the mother to daughter cells via spontaneous mutation vertical gene transfer (VGT) (Lin *et al.,* 2016a), while other microorganisms belonging to a different family acquire antibiotic resistance characteristics through horizontal gene transfer (HTG) (Li *et al.,* 2017a; Lin *et al.,* 2016a; Rizzo *et al.,* 2013). Horizontal gene transfer between bacterial cells is facilitated by mobile genetic elements such as the integrons, transposons or plasmids (Freitas *et al.,* 2017). Horizontal gene transfer is the defence mechanism developed by bacteria for dissemination of antibiotic resistant genes that enable bacterial cells to survive unfavorable environmental conditions by using other microorganisms' machinery for survival and replication (Stange *et al.,* 2016; Kümmerer, 2009). Heavy metals, biocides such as zinc and triclosan present in aquatic systems enhance horizontal gene transfer (Makowska *et al.,* 2016). There are three mechanisms of horizontal gene transfer which include conjugation, transformation and transduction (Lin *et al.,* 2016a).

## 2.13 Methods used to study water quality parameters and characteristics of microbes

### 2.13.1 Physico-chemical parameters of water

Physical parameters are measured using a mobile multi-meter (PCS Testr 35, Eutech Intruments Pte Ltd, Singapore). Operating this multi-meter is easy and can measure temperature, total dissolved solids, pH, electrical conductivity and salinity on site. Measuring physical parameters is advantageous because tests are conducted while the water samples are still fresh and one does not need to travel to get water quality results.

The Hach Lange DR 2800 spectrophotometer was used to measure chemical parameters of drinking water (Hach Company, 2012). This machine produces results within a short period

of time to ensure drinking water does not contain chemicals that might have health risks to humans (Bisi-Johnson *et al.,* 2017).

### 2.13.2 The membrane filter technique

Membrane filter technique has been in use since the 1950s for microbiological analysis in water samples (Rompré *et al*., 2002). This technique is important in the enumeration of faecal coliform and *E. coli* present in raw and drinking water (Ellis *et al.,* 2017). The advantage of using this method over pour plate technique is that large water sample volume is used and it yield higher count of isolates than the multiple-tube fermentation procedure (APHA *et al.,* 1998). Faecal coliform and *E. coli* recovery is much higher due to the size of the membrane filter with porosity of 0.45 µm and diameter of 47 mm (Palamuleni and Akoth, 2015; Rompré *et al*., 2002).

### 2.13.3 Spread plate

Spread plate is an important isolation technique of bacteria as it yields higher counts of isolates (Allen *et al.,* 2004). This is achieved by preparing a serial dilution of up to $10^{-5}$ and spread each water sample onto a differential media (Sanders, 2012). This technique was used to determine the total colony forming units (CFU) present in drinking water of viable colonies (Sanders, 2012). The viable colony count has a wide range of application in microbiology such as gene cloning, monitoring genetically modified organisms, surveillance of bioremediation effects and examination of the effectiveness of antimicrobials in an antibiotic susceptibility test (Thomas *et al,* 2015). Spread plate technique allows prevention of aerobic organisms getting trapped inside the agar media, formation of isolates on the surface of agar media that are separated and easily distinguishable because colonies formed are unique in colour, shape and size (Sanders, 2012; Thomas *et al,* 2015).

### 2.13.4 Haemolysis test

Hemolysis is the breakdown of the erythrocytes circulation before their normal life span of 120 days (Dhaliwal *et al.,* 2004). Hemolysis test is carried out on the medium that is composed mainly of trypticase soy and sheep blood agar (Egwuatu *et al.,* 2014; Yeh *et al.,* 2009). Trypticase soy enriches the nutrition of medium to create a favourable condition for bacterial growth. Supplementing the medium with 5% sheep blood is essential diagnostics as bacteria produce haemolytic patterns (Egwuatu *et al.,* 2014; Yeh *et al.,* 2009). HPC bacteria that are β or α haemolytic on 5% sheep blood have to be subjected to extracellular enzymes tests (Yeh *et al.,* 2009; Pavlov *et al.,* 2004). If HPC bacterium produce two or more

extracellular enzymes is considered as potential pathogen (Prinsloo, 2014). Extracellular enzymes are cytotoxic to the host cell (Horn *et al.,* 2016).

### 2.13.5 Deoxyribose nuclease (DNase)

DNase is a group of enzymes that are responsible for the breakdown of the nucleic acid into oligonucleotides (Palmer *et al.,* 2012). This lead to weakening of the host immune response by allowing the liberation of nucleotides that are responsible for growth (Palmer *et al.,* 2012; Sumby *et al.,* 2005). Furthermore, DNase minimizes the production of purulent exudate that form part of the host cell immune response, responsible for the inhibition of the spread of bacteria to vital tissues (Palmer *et al.,* 2012; Sumby *et al.,* 2005).

### 2.13.6 Lecithinase

Lecithinase enzymes catalyse the hydrolysis of the connection that joins glycerol and phosphate in lecithin into diglyceride and phosphorylcholine (Ali *et al.,* 2016; El-baz *et al.,* 2016). The invasion of lecithinase producing bacteria inside a host result is associated with toxicity such as destruction of reproductive tissues, erythrocytes and cell membranes (Sharaf *et al.,* 2014).

### 2.13.7 Lipases

Lipase is a group of enzymes that catalyse the hydrolysis of long-chain triglycerides to glycerol and fatty acids (Bharathi *et al.,* 2018; Knapp *et al.,* 2016; Ayinla *et al.,* 2017). Lipases are pathogenic to the host cell, since they reduce the immune response of the host cell by attacking non-pathogenic bacteria, *Propionibacterium acne* and *Staphylococcus epidermidis* present on the human skin responsible for protection against pathogenic bacteria (Park *et al.,* 2013). *Staphylococcus epidermidis* is known as human cutaneous commensal microorganism that is responsible for human skin protection against foreign bodies from the environment (Stehr *et al.,* 2003).

### 2.13.8 Gelatinase

Gelatinase catalyse the hydrolysis of gelatin and other compounds such as pheromone, collagen, casein and fibrinogen (Balan *et al.,* 2012). Gelatinase belonging to proteases hydrolyse gelatin only, whereas extracellular mettallo-endopeptidases hydrolyse gelatin and other coumpounds (Ekpenyong *et al.,* 2017; Balan *et al.,* 2012). Production of gelatinase in water environments is associated with biofilm formation and while in the host it facilitates translocation of HPC bacteria throughout the intestinal cell layer (Park *et al.,* 2007).

### 2.13.9 Proteinase

Proteinase is a group of enzymes that catalyses the hydrolysis of protein into short peptides and amino acids (Sharma *et al.,* 2017). Bacterial species produce proteinase to infect the host and cause disease (Culp and Wright, 2017; Yang *et al.,* 2007). Proteinase producing bacteria weakens the immune system of humans by inactivating the immunoglobulin A (IgA) and subsequently making the host cell susceptible to more pathogens in the environment (Collin and Olsén, 2003).

## 2.14 Kirby-Bauer disk diffusion test

The Kirby Bauer disk diffusion test was developed in the 1950s for susceptibility test (Hudzicki, 2009). At the time the method was introduced, there were no standards which led to lot of discrepancies (Hudzicki, 2009; Vineetha *et al.*, 2015). As a result, the method was standardized by the World Health Organization in 1961 (WHO) (Gautam *et al.,* 2013; Vineetha *et al.*, 2015; Hudzicki, 2009). The Clinical and Laboratory Standards Institute (CLSI) highly recommends this method to determine effectiveness of antimicrobials against a pathogenic aerobic or facultative anaerobic bacterium; it determines whether a bacterium is sensitive or intermediate or resistant to a particular antimicrobial (Vineetha *et al.*, 2015). Testing how microorganisms react to antibiotics is essential for prescription of medicine. This test is cost effective and can be conducted with ease as compared to other dilution methods (Joseph *et al.,* 2011; Gautam *et al.,* 2013). In the current study a total number of 12 antibiotics discs (Mast Diagnostics) impregnated with different concentrations were used.

## 2.15 Detection of antibiotic residues in the environment

The SPE-DEX system (Horizon technology Salem, NH, USA) was used for the extraction of antibiotics (Horizon Technology, 2015). The ultra-high liquid chromatography (UHPLC) was used to detect antibiotics in water (Ferguson *et al.,* 2013). The detection of antibiotics in water environments is not often associated with harmful effects to human (Ebele *et al.,* 2017). However, antibiotics in water environment are used to assess their influence on the ecosystem (Ferguson *et al.,* 2013).

## 2.16 Molecular techniques

Anciently, bacterial species were previously identified and classified based on their phenotypic characteristics, this method was replaced by early molecular technique in which classification was based on GC content, plasmid profiling and compatibility to genetic transformation (Barghouthi, 2011). The discovery of the of the polymerase chain reaction (PCR) in the 1980s by a Nobel prize winner Dr. Kary Mullis drastically transformed biological

sciences (Garibyan and Avashia, 2013; Valones *et al.,* 2009; Yu *et al.,* 2017). PCR is a method of *in vitro* enzymatic synthesis that allows amplification of small amount of specific DNA segment into many DNA copies in a semi-conservative way and exponential manner (Rahman *et al.,* 2013; Valones *et al.,* 2009). PCR development has contributed greatly in genome sequencing, gene expression in recombinant systems, molecular genetic analyses including detection of antibiotic resistant genes (Valones *et al.,* 2009). PCR is used for analysis of microbial detection from human, veterinary medicine and various environments including water. To run a successful PCR protocol, DNA template, primers, nucleotides, Taq DNA polymerase and buffer are required (Rahman *et al*., 2013; Garibyan and Avashia, 2013).

### 2.16.1 Identification of HPC bacteria

Polymerase chain reaction (PCR) that specifically targets the amplification of the 16S rRNA gene is an important technique for confirmatory identification of unknown bacterial species and phylogenetic tool for bacterial diversity, evolution and taxonomy (Jenkins *et al.,* 2012; Patel, 2001; Hongoh *et al.,* 2003). The 16S rRNA gene consists of two regions; (i) conserved region amplified by universal primers; (ii) variable region amplified by genus or species-specific primers (Hassan *et al.,* 2014). In this study, universal PCR primers are used to target the specific conserved region of the bacterial genome. The PCR products were run on the gel to confirm whether the PCR was successful (Devereux and Wilkinson, 2004). Successful amplicons will be subjected to sequencing for identification to determine the genus and species name of unknown bacterial isolates with the aid of Basic Local Alignment Search Tool (BLAST) software (Hassan *et al.,* 2014; Barghouthi, 2011).

The 16S rRNA gene is used for identification and phylogeny because it is advantageous over other genes; (i) 16S rRNA gene is a universal gene present in almost all bacterial species which occurs as either a multigene family or operons; (ii) The function of the 16S rRNA gene still remains the same even after a long period of time, suggesting that this gene has survived mutations that are associated with changes of the gene sequences that could result in the change in the molecule function; (iii) finally, 16S rRNA gene has a large length of approximately 1500 base pair (bp) that is essential in informatics and statistical purposes (Janda and Abbott, 2007; Patel, 2001).

### 2.16.2 Detection of antibiotic resistant genes

ARGs are environmental contaminants that are present everywhere. It is therefore important to detect these environmental contaminants in raw and drinking water. ARGs may be detected in water environments are by sensitive molecular techniques (Fernando *et al.,*

2016; Xi *et al.,* 2009; Zhang *et al.,* 2009). These techniques provide learning tools that enable scientists to study and understand potential impact of ARGs on the environment, as well as people (Choi *et al.,* 2018; Shanthi *et al.,* 2012; Fernando *et al.,* 2016). Subsequently, to come up with new water treatment technologies those are effective in the activation of ARGs (Guo *et al,* 2017). In this study, *ampC, tetM, ermB, ermF,* $bla_{TEM}$ and *intl 1*were subjected to PCR, to target and amplify specific regions of these genes (Fernando *et al.,* 2016; Xi *et al.,* 2009; Zhang *et al.,* 2009). Specific primers (Inqaba biotec) and PCR cycling conditions are used for the detection of each gene (Lachmayr *et al.,* 2009).

## 2.17 Environmental DNA

Traditional laboratory culture-based methods have a limitation since they do not meet suitable conditions to allow growth of viable but nonculturable cells (VBNC) (Fakruddin *et al*. 2013). Traditional laboratory culture-based methods only allow analysis of up to 1% of the total population of bacteria in natural habitats (Fakruddin *et al*., 2013). Therefore, the study of environmental DNA (eDNA) is becoming recognised worldwide as it provides a better understanding of species biodiversity that is important in the fields of ecology, biological conservation and biogeography (Ficetola *et al.,* 2008; Yamanaka *et al.,* 2016). The study of biodiversity is achieved through the extraction of genomic DNA directly from the environmental samples and subjecting it to molecular techniques to determine the diversity of microorganisms present in that specific location (Djurhuus *et al.,* 2017).

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Study area

The WC-A treatment system has two sources and thus two treatment plants. There is the DPR plant that uses treated wastewater as source and a combination of conventional and advanced water purification processes such as, nanofiltration, advanced oxidation and reverse osmosis to produce the drinking water (Figure 1). The reclaimed water (30%) is blended with treated dam and borehole water (70%) and transported to various reservoirs and then into the distribution networks (Matthews, 2015). The DPR has a capacity of producing 2.5 Ml/d of drinking water but is currently operated to produce only 1.1 Ml/d (DWS, 2012b).

Plant WC-F depends on dam and borehole for production of drinking water (Figure 2). The water is mixed prior to treatment and assembles in a dam off the drinking water production premises. Raw water is treated using conventional water treatment processes as indicated in Figure 2. The system Design Capacity of plant WC-F is 8 Ml/d and the operational capacity is at 52.50 % (DWS, 2012b).

## 3.2 Sampling

At WC-A samples were collected from:

- Wastewater (Raw 4)
- Borehole water (Raw 1)
- Dam water (Raw 3)
- Reclaimed water (RW)
- Treated water (AT)
- Distribution networks(D)

At WC-F samples were collected from:

- Borehole water (Raw 1)
- Mixed raw water (Raw 2)
- Dam water (Raw 3)
- Treated water (AT)
- Distribution networks (D)

Water samples were collected in sterile 1L bottles (Duran Schott, Germany). Water samples ar WC-F were collected in June 2017 and September 2018, whereas at WC-A samples were collected in June and November (2017) and September 2018. Water samples were transported in ice to North-West University laboratories. Sampling was done as instructed by DWAF sampling guide (DWAF, 2000). Water sample analysis was done within 24 hours after sampling (O'Reilly, 2012). The September 2018 sampling was to collect water for eDNA and antibiotic residue analysis only.

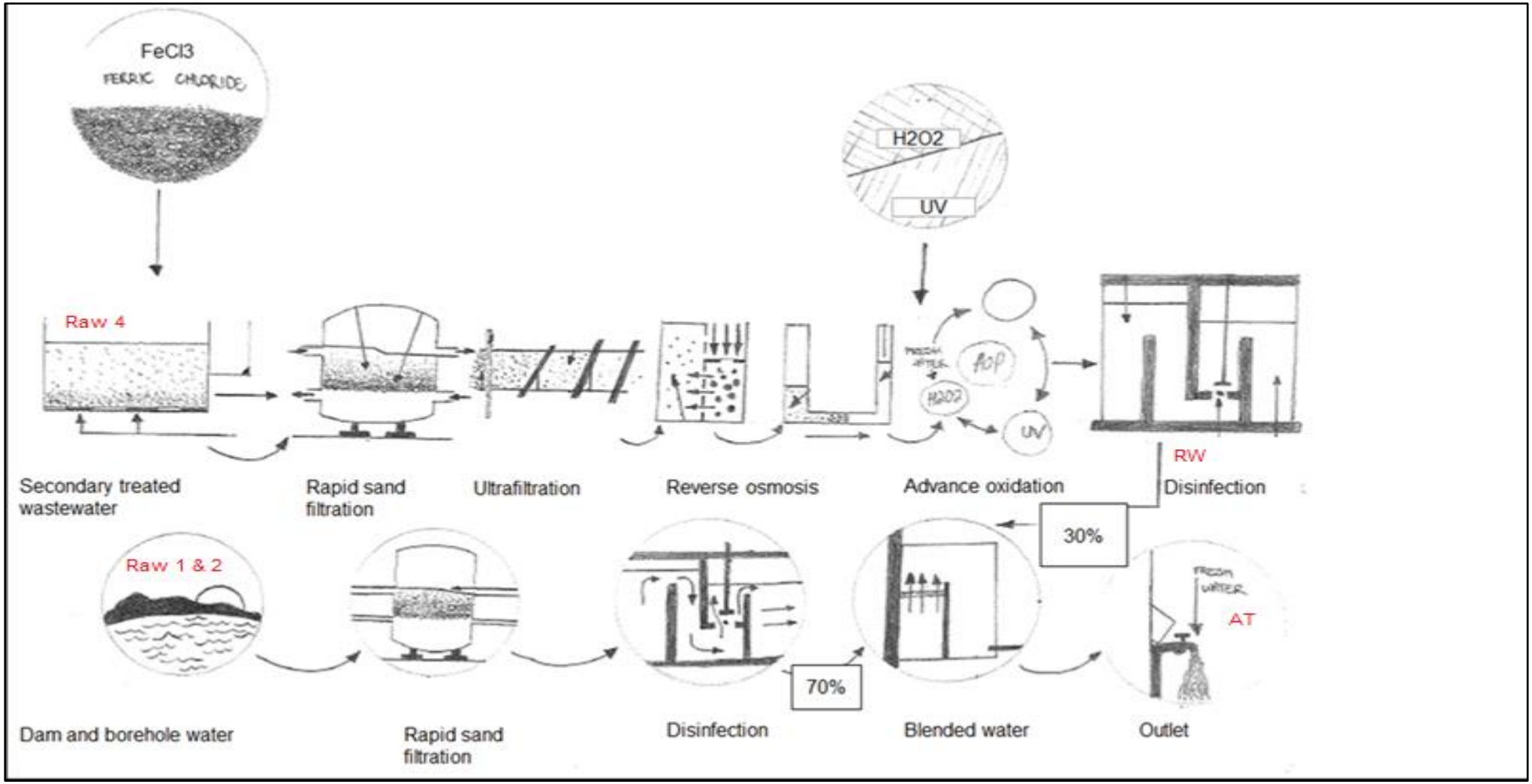


**Figure 1: Schematic representation of WC-A indicating the two water sources and associated treatment processes as well as the blending regime.**

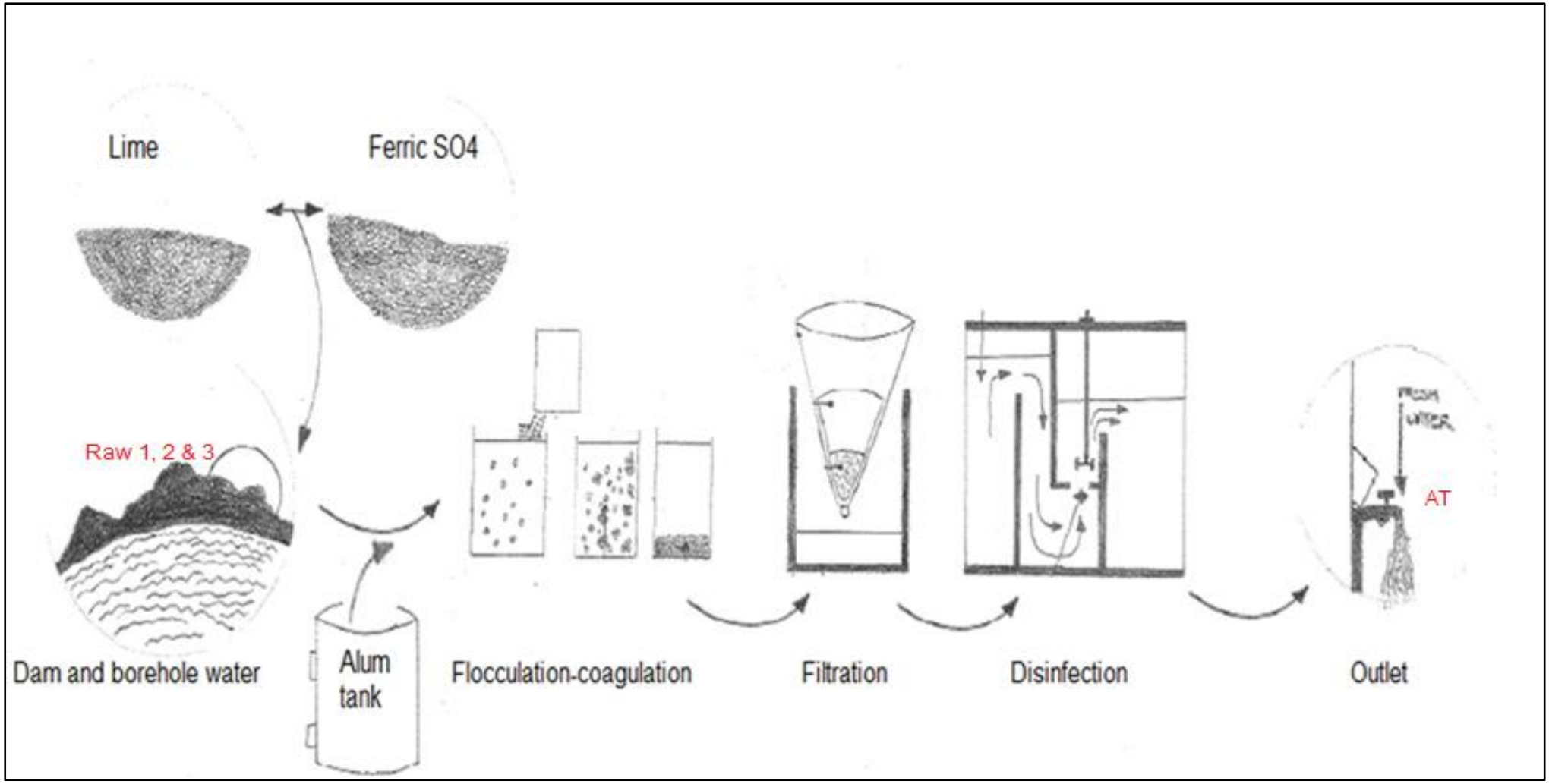


**Figure 2: Schematic representation of WC-F**

## 3.3 Physico-chemical analysis

A multimeter (PCSTestr 35, Eutech Instruments Pte Ltd. Singapore) was used to measure the pH, salinity, electrical conductivity, total dissolved solids (TDS) and temperature on site. The multimeter and beaker were rinsed with distilled water before and after taking the measurements at each sampling site. Upon arrival at North-West university laboratories, chemical parameters were measured (O'Reilly, 2012). The concentrations of nitrate (method 8039), nitrite (method 8153), phosphorous (method 8178) and free chlorine (method 8021) were measured using the Hach Lange DR 2800 spectrophotometer (Hach Company, 2007). Turbidity was measured using the Hach 2100P Portable Turbidimeter (Hach, GmB). Chemical parameters were measured according to the manufacturer's protocols (Hach Germany). Both chemical and physical parameters measured were compared to the South African National Standards (SANS 241:2015) for drinking water. This was only done for the drinking water.

## 3.4 Detection of faecal coliforms and *E. coli*

Membrane filtration technique was used for the detection of faecal coliforms and *E. coli* from water samples. The EZ-Stream water filtration system (Millipore SAS, France) was sterilized with 70% ethanol and washed with sterile distilled water. Sterile filter membranes (PALL Life Sciences, Mexico) with 0.45 µm porous size were placed on the filtration membrane using sterile forceps. Water samples (100 ml) were filtered through sterile membranes. Each sample was analysed in triplicate (Mulamattathil *et al.,* 2015). These membranes were aseptically placed on the surface of Membrane Lactose Glucuronide Agar (MLGA; Oxoid, England). The MLGA was incubated at 37°C for 24 hours. After incubation, *E. coli* appeared green on MLGA (Eccles *et al.*, 2004) and were counted. The other colonies were an indication of total coliforms.

## 3.5 Isolation and enumeration of heterotrophic plate count (HPC) bacteria

The spread plate technique was used for isolation and enumeration HPC bacteria. A dilution series of $10^{-1}$ up to $10^{-5}$was prepared for each water sample. Using the spread plate technique, 100 µl of water samples were spread on the Reasoner 2A (R2A) agar (Lab M Ltd., UK) from $10^{-2}$ up to $10^{-5}$ dilution series. The medium was prepared according to the manufacturer's instructions. This was done in triplicate. Spread plates were incubated for 4-7 days at room temperature. After incubation, results obtained were compared to the SANS 241:2015 for drinking water. Colonies were then grouped according to their morphological characteristics observed on the medium. Each group was streaked out on a clean R2A

media and incubated at room temperature for 4-7 days at room temperature. This step was repeated for at least three times to ensure colonies were pure (Jordaan and Bezuidenhout, 2016).

### 3.6 Burke Gram staining method

After incubation, a smear was prepared and heat-fixed. Gram stain was conducted as described by Willey *et al*. (2013). This method was used to determine whether colonies were pure and classify them as Gram-positive or Gram-negative. Only pure colonies were subjected to Kirby-Bauer disk diffusion method.

### 3.7 Kirby-Bauer disk diffusion method

Pure colonies were inoculated into R2A broth (Lab M Ltd., UK) and incubated at 30°C for 24 hours. After incubation, 100 µl of the culture was spread on Mueller-Hinton agar (MHA) (Merck, RSA). The medium was prepared according to the manufacturer's instructions. A total number of twelve commercially prepared antibiotic disks (Mast Diagnostics, UK) were placed on MHA that has a bacterial culture. Antibiotic disks were impregnated with different concentration; ampillicin (AMP) 10 µg, cephalothin (KF) 30 µg, chloramphenicol (Chl) 30 µg, ciprofloxacin (CIP) 5 µg, erythromycin (Ery) 15 µg, kanamycin (Kan) 30 µg, neomycin (Neo) 30 µg, oxytetracycline (O-T) 30 µg, penicillin G (Pen-G) 10 µg, streptomycin (S) 25 µg, trimethoprim (TM) 5 µg and vancomycin (Van) 30 µg. MHA plates with antibiotic disks were incubated at 37°C for 24 hours. After incubation, inhibition zones were measured (in millimetres). The measured values were compared to Performance Standards for Antimicrobial Susceptibility Testing (2014) provided by the Clinical and Laboratory Standards Institute (CLSI). This was done to determine whether HPC bacteria were susceptible, intermediate or resistant to antimicrobials (Terfassa and Jida, 2018; Sangeetha *et al*., 2014).

Data generated from Kirby-Bauer disk diffusion method was used to calculate Multiple Antibiotic Resistant (MAR) indices The MAR is used to determine whether water sampling sites were contaminated with antibiotic resistance and it is represented by the following equation (Chitanand *et al.,* 2010).

MAR indices = a/(b x c) per sample

a = total amount of resistance to antibiotics

b = amount of antibiotics used

c = number of isolates in sample

## 3.8 Detection of antibiotic residues

Antibiotic residues from water samples were extracted using SPE-DEX system (Horizon Technology, Salem, NH, USA). This system is automated for solids phase extraction (SPE) and conditioning of the disks. One litre water sample were loaded and automatically passed through the disks. Antibiotic residues were concentrated 2 000 times. Oasis HLB disks were used due to their effectiveness of extracting analytes with wide range of pH and various polarities (Pedrouzo *et al*., 2011). HLB-L was well-suited for this experiment due to low organic nature of the sample. This disk is used in the US EPA method 1694 for analysis of antibiotic residues. The application note was followed for the HLB extraction disks (47 mm, Horizon Technology). Gentle stream of nitrogen gas was used to concentrate the eluent. Methanol was used to reconstitute samples which were subjected to UPLC-QTOF for analysis.

The UPLC was used to carry out for screening of analytes. The UPLC system used consist of an Agilent 1290 Infinity Binary pump (G4220A); 1290 Infinity Autosampler (G4226A); and 1290 Infinity Thermostatted Column Compartment (G1316C) coupled to an Agilent 6540 Accurate mass Q-TOF/MS (G6540A) (Agilent Technologies, Santa Clara, CA, USA). The desolvation and ionisation of samples were achieved by positive and negative electrospray ionisation (ESI) enhanced with Agilent Jet Stream (AJS) technology. The QTOF was set to scan from 50 to 950 m/z and the instrument was set to extended dynamic range (2 GHz). Software used was MassHunter Data Acquisition (version B.05.00), MassHunter Qualitative Analysis (version B.05.00), and Quantitative Analysis for QTOF (version B.05.01). Mass axis calibration of QTOF was performed daily for positive and negative ionisation with tuning mixes (G1969-85000, Agilent). A reference solution with masses of 121.050873 [M+H] and 922.009798 [M+H] were constantly infused as accurate mass references.

## 3.9 Haemolysin test

Blood agar (BA) medium (Selecta-media, RSA) supplemented with 5% sheep blood was used to test the ability of HPC bacteria to break down red blood cells. HPC isolates were streaked on BA and incubated at 37°C for 24 hours. The following results were recorded after incubation; beta-haemolytic isolate was represented by a complete clear zone around the colony, alpha-haemolytic isolate was represented by a partial clear zone and gamma-haemolytic isolate had no clear zone but growth (Russell *et al.,* 2006).

## 3.10 Extracellular enzyme assays

### 3.10.1 DNase

DNase medium was composed of DNase agar (Merck, Germany), prepared according to the manufacturer's instructions. The medium was supplemented with 0.01% toluidine blue (Sigma, Germany). Toluidine blue acts as a dye and substrate by binding to the hydrolysed DNA. Plates were inoculated and incubated at 37 ºC for 24-48 hours. Bacterial species that hydrolyse DNA are represented by a clear around or colour change around the colony (Sunita *et al.,* 2015; Sánchez and Colom, 2010)

### 3.10.2 Lipase

Lipase extracellular enzyme test was carried out on trypticase soy agar (Merk, Germany), prepared according to the manufacturer's instructions. The medium was supplemented with 1% Tween 80 (Sigma, Germany). Tween 80 acts as a substrate. Isolates were inoculated and incubated at 37 ºC for 72 hours. Bacterial species that hydrolyse lipids were indicated by a turbid halo around the colonies (Prinsloo, 2014; Pavlov *et al.,* 2004).

### 3.10.3 Gelatinase

Gelatinase medium was composed of 120 g/L gelatine powder (Merck, Germany), mixed with 3 g meat extract (Lab M Ltd., UK). Five-gram peptone (Merck, Germany) and 15 g agar (Merck, RSA) in 1000 ml distilled water. The pH of the medium was adjusted to 6.8 and autoclaved. Isolates were inoculated on slants and incubated for 37°C for 48 hours. After incubation, the medium was put in a 4°C fridge for 10 to 15 min. Positive isolates were represented by a liquefied medium. (Biswas and Paul, 2013; Balan *et al.,* 2012).

### 3.10.4 Proteinases

The ability of bacterial species to hydrolyse proteins was tested on 3% (v/v) skimmed milk agar (Oxoid, UK) and Brain heart infusion broth (BHIB) (Merck, Germany) prepared according to the manufacturer's instructions. The Media were prepared separately. Fifteen gram per litre of bacteriological agar was added to the BHIB. Skimmed milk agar and BHIB were autoclaved separately according the manufacturer's instructions. Both media were allowed to cool down then mixed together. Isolates were inoculated and incubated at 37°C for 48 hours. Bacterial species that hydrolyse protein were indicated by a clear zone around the colonies (Prinsloo*,* 2014).

### 3.10.5 Lecithinase

Lecithinase media was composed of McClung Toabe agar base (Remel) prepared according to the manufacturer's instructions and autoclaved. After autoclaving, the media was allowed to cool down and 100 ml of the 50% egg yolk enrichment (Merck, Germany) was added. Isolates were inoculated and incubated at 37°C for 72 hours. A positive test was indicated by a white precipitate around or beneath the inoculum spot (Rossignol *et al.,* 2009; Edberg *et al.,* 1996).

## 3.11 Molecular techniques

Molecular techniques are important in the identification of bacterial species based on the amplification of the 16S rRNA gene sequencing. The presence of antibiotics resistant genes was also detected by end-point PCR and sequencing to confirm their identity.

### 3.11.1 DNA isolation

Pure colonies were inoculated on R2A broth and incubated at 28°C for 24 hours. After incubation, Chemagic Bacterial DNA/RNA kit (PerkinElmer®, RSA) was used for isolation of the nucleic acid. The nucleic acid of bacterial species was isolated as instructed on the manufacturer's protocol. The quality and integrity of isolated DNA was determined using agarose gel electrophoresis.

### 3.11.2 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out on 1.5% agarose gel (Lonza, USA) (w/w) in 1 x TAE buffer (20 mM acetic acid, 40 mM Tris and 1 mM EDTA at pH 8.0). Ten microliters of ethidium bromide (Bio-Rad, UK) was added. The gel was allowed to solidify. Two microliters l of 6x orange loading dye (Thermo Scientific, US) was premixed with 3 µl of the isolated DNA and loaded into the wells of the gel. The 1 kb O'Generuler DNA ladder (Thermo Scientific, US) was loaded in the first well of the gel to measure the size of the DNA product in base pair (bp). Electrophoresis was for 45 minutes at 80 V. The ChemiDoc MP imaging System (Bio-Rad, US) was used to generate and capture the image of the gel for analysis of isolated DNA and PCR products.

### 3.11.3 Nanodrop

The concentration (ng/µl) of successful extracted DNA was measured using the NanoDrop™ ND-1000 Spectrophotometer (NanoDrop Technologies, USA). DNA was diluted to 20 ng/µl for downstream PCR analysis. The nanodrop was also used to determine the purity ($A_{260nm}$:$A_{280nm}$ ratio) of the DNA (Coertze and Bezuidenhout, 2018).

### 3.11.4 Endpoint PCR

DNA samples were subjected to endpoint PCR according to Manaka *et al*. (2017) and Jordaan and Bezuidenhout (2013). Endpoint PCR was used for amplification of the 16S rRNA gene using universal primers 27 F (AGA GTT TGA TCM TGG CTC AG) and 1492 R (GG TTA CCT TGT TAC GAC TT). Primers amplified the V3 and V4 region of the 16S rRNA gene. The total volume of the PCR reaction mixture was 25 µl, aliquots were as follows; 12,5 µl Dream Taq PCR master mix (5 U/µl Taq DNA polymerase in reaction buffer, 2 mM $MgCl_2$, 0.2 nM of each dNTP) (Thermo Scientific), 1 µl of each primer (reverse and forward; Inqaba biotec, RSA), 1 µl of 20 ng/µl DNA template and 9.5 µl nuclease free water. Reaction mixtures for no template DNA control was also prepared. The Techne$^{TM}$ PCRmax Alpha Cycler 1 PCR (Fisherscientific, UK) was used for the amplification of the nucleic acid products, using the following thermal cycling condition; initial denaturation at 95ºC for 5 minutes, 35 cycles of denaturation at 95ºC for 30 seconds, annealing at 53ºC for 30 seconds, extension at 72ºC for 1 minute and final extension at 72°C for 10 minutes. After completion of 35 cycles, the PCR products were analysed on a gel as described in the gel electrophoresis section (Section 3.11.2).

### 3.11.5 Sequencing

Successful amplicons were sent to Inqaba Biotechnology Industries (Pty) Ltd – Pretoria, South Africa for sequencing. Bacterial sequences from Inqaba Biotechnology Industries were analysed using Finch TV (Version 1.4.0). Nucleic acid sequences were analysed in BLAST software (www.blast.ncbi.nlm.nih.gov/Blast.cgi) and EzTaxon software (www.ezibiocloud.net) to identify bacterial species.

### 3.11.6 Endpoint PCR: Detection of ARGs

This study focused on the detection of *ampC, bla$_{TEM}$, intI1, tetM, ermF* and *ermB* ARGs*.* The total volume of the reaction mixture for each gene was 25 µl. The reaction mixture contained 12,5 µl Dream Taq PCR master mix (5 U/µl Taq DNA polymerase in reaction buffer, 2 mM $MgCl_2$, 0.2 nM of each dNTP) (Thermo Scientific), 1 µl of each primer (reverse and forward) (Inqababiotec, RSA), 1 µl of 20 ng/µl DNA template and 9.5 µl nuclease free water. The sequences for the various primers used are provided in Table 1.

#### 3.11.6.a *Bla$_{TEM}$*

The PCR conditions for detection of *bla$_{TEM}$* genes were as follow: initial denaturation at 95ºC for 5 minutes, 30 cycles of denaturation at 95ºC for 60 seconds, annealing at 60ºC for 60

seconds, elongation at 72ºC for 60 seconds and final extension at 72°C for 5 minutes (Costa *et al*., 2007).

### 3.11.6.b *ermF and ermB*

The thermal cycling conditions for the detection of *ermF* and *ermB* gene were performed as described by Fourie (2017) and Chung *et al*. (1999). The PCR cycling conditions were as follows: initial denaturation at 95ºC for 5 minutes, 35 cycles of denaturation at 95ºC for 30 seconds, annealing (*ermF* at 50ºC for 30 seconds and *ermB* at 48ºC for 1 minute), elongation at 72ºC for 2 minutes and final extension at 72°C for 10 minutes.

### 3.11.6.c *TetM*

For detection of *tetM* genes the PCR protocol was used as described by Aminov *et al*. (2001). PCR conditions were as follow: initial denaturation at 95ºC for 5 minutes, 35 cycles of denaturation at 95ºC for 45 seconds, annealing at 55ºC for 45 seconds, elongation at 72ºC for 45 seconds and final extension at 72 for 7°C minutes.

### 3.11.6.d *Intl1*

The PCR conditions for detection of *Intl1* genes were as follow: initial denaturation at 95ºC for 5 minutes, 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 64ºC for 30 seconds, elongation at 72ºC for 60 seconds and final extension at 72°C for 5 minutes. Successful amplicons have a length product of 473 bp (Labbate *et al.* 2008; Coertze and Bezuidenhout, 2018).

### 3.11.6.e *ampC*

The following thermal cycling condition were used: initial denaturation at 94ºC for 5 minutes, 33 cycles of denaturation at 94ºC for 30 seconds, annealing at 49ºC for 30 seconds, elongation at 72ºC for 60 seconds and final extension at 72 for 7 minutes. Successful amplicons have a length product of 550 bp (Coertze and Bezuidenhout, 2018; Schwartz *et al.,* 2003).

## 3.12 eDNA

In the current study, the Ma *et al.* (2017a) methodology for water collection was adjusted for eDNA extraction. LifeStraw filter consisting of a hollow fibre filter (Vestergaard Frandsen, Switzerland) were modified so that it was able to fit onto a tap and withstand the water pressure. Water sample of approximately 1000 L was filtered through the hollow fibre filter. The hollow fibre filters were cut and placed inside a Schott bottle containing 300 ml

autoclaved distilled water. Water sample was sonicated for 10 minutes. Water samples were filtered through sterile filter membranes (PALL Life Sciences, Mexico) with the porous size of 0.45 µm. The protocol of the Powerwater kit (Qiagen, Netherlands) was followed for the extraction of eDNA. Methodologies described in Sections 3.11.2 to 3.11.6, except for 3.11.5 were followed for further analysis of eDNA.

**Table 1: Oligonucleotide primers for PCR amplification of 16S rDNA, *bla*$_{TEM}$, *ermF, intI1, ermB, tetM, and ampC* genes. F- Forward primer and R- Reverse primer**

| Target gene | Name | Sequence (5'…..3') | Size (bp) | Reference |
|---|---|---|---|---|
| **16S rDNA** | 27F | AGA GTT TGA TCM TGG CTC AG | 1 465 | Jiang *et al*., 2006 |
| | 1492R | GG TTA CCT TGT TAC GAC TT | | |
| ***bla*$_{TEM}$** | TEM-F | ATT CTT GAA GAC GAA AGG GC | 1 150 | Costa *et al*., 2007 |
| | TEM-R | ACG CTC AGT GGA ACG AAA AC | | |
| ***erm*F** | ermF1 | CGG GTC AGC ACT TTA CTA TTG | 466 | Fourie, 2017 |
| | ermF2 | GGA CCT ACC TCA TAG ACA AG | | |
| ***erm*B** | ermB-F | GAA AAG GTA CTC AAC CAA ATA | 638 | Fourie, 2017 |
| | ermB-R | AGT AAC GGT ACT TAA ATT GTT TAC' | | |
| ***IntI1*** | HS463A | CTG GAT TTC GAT CAC GGC ACG | 473 | Labbate *et al.,* 2008 |
| | HS464 | ACA TGC GTG TAA ATC ATC GTC G | | |
| ***ampC*** | AmpC-F | TTC TAT CAA MAC TGG CAR CC | 550 | Coertze and Bezuidenhout, 2018 |
| | AmpC-R | CCY TTT TAT GTA CCC AYG A | | |
| ***tetM*** | tetM-FW | ACA GAA AGC TTA TTA TAT AAC | 171 | Aminov *et al,.* 2001 |
| | tetM-RV | TGG CGT GTG TCT ATT GAT GTT CAC | | |

### 3.13 Statistical analysis

Microsoft excel 2016 was used to calculate the average and standard errors of physical and chemical parameters (O'Reilly, 2012). Redundancy analysis (RDA) was done to determine the correlation between microbiological agents and physico-chemical parameters in water environments from the DWPFs. The RDA triplots were constructed using Canoco 4.5 for windows (Jordaan and Bezuidenhout, 2013). These diagrams were used to compare the correlation between microbiological agents and physico-chemical parameters data from the DWPFs to distribution networks (O'Reilly and Bezuidenhout, 2012; Jovanović *et al.,* 2017; Fourie*,* 2017; Bringué *et al.,* 2015).

The 16S rRNA sequences were trimmed and copied into a Microsoft word document. Reference sequences obtained from the EzTaxon software (www.ezibiocloud.net) were also copied into a documented that has 16S rRNA sequences (Du *et al.,* 2015). The document was converted into a FASTA file so that it could be recognised by Mega 7 software. The DNA sequences were aligned by Mega 7 software. Once the sequences were aligned, a phylogenetic tree was constructed to determine how closely related are the identified species (Coertze and Bezuidenhot, 2018).

# CHAPTER 4

# RESULTS AND INTERPRETATION

## 4.1 Physical parameter

The physical parameters such as temperature, pH, TDS and salinity were taken on site using a portable multimeter and turbidity was taken upon arrival at North-West University laboratory. The measured physical parameters from WC-A and WC-F are shown in Tables 2 and 3, respectively. These tables represent the average and the standard errors of physical parameters from each sampling site. However, temperature recorded in WC-A from November 2017 samples was not recorded in triplicates. As a result, the standard errors were calculated. Dam water samples were not collected in November 2017 at WC-A. The measured parameters were compared to the South African National Standards of (SANS 241:2015) of drinking water. These standards are not application for raw water samples. Salinity and turbidity were not measured in June 2016 sampling run at WC-F.

### 4.1.1 Temperature

Water samples from June 2017 sampling at WC-A were collected in winter season. Hence the temperatures were lower than water samples collected in November 2017 (summer). The lowest temperature recorded at WC-A was 12.3ºC from the wastewater sample (surface water in a lagoon) for June 2017 sampling. The highest temperature of 21.0°C was recorded in treated water sample from November 2017 sampling.

Water samples from WC-F for June 2016 and June 2017 were collected in the winter season. The temperature of water samples from both sampling runs were lower than 20°C, except for borehole watersamples that were 20.5°C for June 2016 sampling and 21.3ºC for June 2017 sampling. The lowest temperature recorded at WC-F was 13.0°C from treated water sample for June 2017 sampling.

In the case of both plants and systems the temperatures measured will allow bacteria to survive and even propagate. It seemed that ground water from WC-F maintained a relative constant temperature.

### 4.1.2 pH

There was no general trend observed in the measured pH at WC-A. The pH was fluctuating from raw to drinking water samples for June and November 2017 sampling. However, the lowest pH from each sampling run was recorded in final water from the direct potable reclaim system. The pH was 6.37 during June 2017 sampling and 7.47 in the November 2017 sampling. The highest pH from both sampling runs of 8.26 was recorded in dam water sample collected in June 2017.

A general trend in the pH was observed at WC-F for June 2016 and June 2017. There was an increase in the pH from raw to treated water. The elevated pH was recorded in distribution networks from each sampling run. The measured pH in distribution networks from June 2016 sampling was 7.2 and in June 2017 it was 7.55. The lowest pH was recorded in borehole water samples from each sampling run. The pH from all sampling sites were within the SANS 241: (2015) for drinking water (pH between 5 and 9.7).

### 4.1.3 TDS

The recorded TDS values were elevated in raw water that was used for reclamation and borehole water samples for both the June and November 2017 sampling runs at WC-A. The highest TDS value of 987 ppm was measured in November 2017 sampling. The lowest TDS values were recorded in reclaimed water samples. The TDS values for the reclaimed water were 144 ppm and 218 ppm for June and November 2017, respectively. TDS values in drinking water distribution network for the November 2017 sampling was higher than June 2017.

There was an increase in the TDS values in mixed raw water, treated water and distribution networks from June 2016 to June 2017 at WC-F. The elevated levels of TDS were recorded in borehole water samples from each sampling run. The dam water sample from June 2016 had the lowest TDS values of 221 ppm recorded at WC-F. The TDS from all sampling sites were within the SANS 241:2015 of drinking water. According to standards drinking water should have TDS ≤1200 ppm.

### 4.1.4 Salinity

Elevated salinity levels were measured in raw water samples of WC-A. The levels of salinity measured in June 2017 was higher than the November 2017 sampling period for all water samples. The salinity of drinking water ranged from 85 ppm to 439 ppm for June sampling and 131 ppm to 560 ppm for the November 2017 sampling. The lowest salinity value of 85 ppm in drinking water was recorded in reclaimed water from June 2017 sampling period. The

salinity levels of treated water were higher than of distribution networks for June 2017 sampling.

The average salinity of raw water samples from the borehole and mixed raw water was 232 ppm for June 2017 sampling for WC-A. Raw water samples had lower salinity levels compared to drinking water samples. The measured salinity in distribution network was 328 ppm. This value was the highest recorded from WC-F. There is no guideline in the SANS 241: (2015) for drinking water. The main inference for salinity levels is TDS or electrical conductivity. For these two inter-related parameters there are SANS 241 (2015) guidelines.

### 4.1.5 Turbidity

The dam water sample of WC-A was the most turbid. The measured turbidity value in dam water from June sampling was 9.58 NTU. At the time when sampling was done the dam levels were already critically low due to the drought in the region. Water samples could not be taken in November 2017 since the dam dried up completely just prior to the sampling. The lowest turbidity in raw water was measured in borehole water samples for each sampling run. The readings were as follows; 0.55 NTU for June 2017 sampling and 0.21 NTU for November 2017 sampling run. Drinking water samples had turbidity levels below 0.6 NTU.

Raw water samples from WC-F had elevated turbidity levels. The highest turbidity was measured in borehole water samples, with the value of 9.99 NTU. However, treated water and distribution network samples had relatively low levels (0.21 NTU and 0.31 NTU, respectively). Turbidity was not measured in water samples from June 2016 at WC-F The turbidity from all sampling sites were within the SANS 241:2015 of drinking water. According to SANS, drinking water should have turbidity ≤1 NTU.

**Table 2: Results of physical parameters for June and November 2017 at WC-A**

| Date | | Temperature (°C) | pH | TDS (ppm) | Salinity (ppm) | Turbidity (NTU) |
|---|---|---|---|---|---|---|
| | **SANS 241:2015** | - | ≥5 to ≤9.7 | ≤1200 | - | ≤1 |
| **June 2017** | **Raw 4** | 12.3±0.24 | 7.55±0.02 | 956±3.93 | 692±1.67 | 4.39±0.10 |
| | **Raw 1** | 18.5±0.29 | 7.74±0.02 | 924±1.52 | 710±0.89 | 0.55±0.01 |
| | **Raw 3** | 15.2±0.15 | 8.26±0.01 | 356±0.33 | 214±0.33 | 9.58±0.06 |
| | **RW** | 12.6±0.17 | 6.37±0.01 | 144±0.58 | 85±0.19 | 0.08±0.01 |
| | **AT** | 16.6±0.09 | 7.75±0.01 | 729±1.45 | 447±2.52 | 0.47±0.01 |
| | **D** | 15.8±0.12 | 7.80±0.01 | 714±0.58 | 439±0.58 | 0.57±0.01 |
| **November 2017** | **Raw 4** | 17.0 | 8.14±0.02 | 987±0.89 | 618±2.96 | 4.62±0.03 |
| | **Raw 1** | 19.1 | 7.70±0.05 | 950±5.33 | 764±13.29 | 0.21±0.01 |
| | **RW** | 17.2 | 7.47±0.15 | 218±0.58 | 131±0.5 | 0.26±0.07 |
| | **AT** | 21.0 | 7.98±0.05 | 850±8.64 | 531±5.31 | 0.37±0.03 |
| | **D** | 17.5 | 7.93±0.05 | 906±3 | 560±0.577 | 0.47±0.029 |

Raw 4-wastewater; Raw 1-borehole water; Raw 3-dam water; RW-reclaimed water; AT; treated water D-distribution networks; TDS-Total Dissolved Solids; EC-Electrical conductivity; Ave-average; SE-standard error; N/D-not determined; SANS-South African National Standards

**Table 3: Results of physical parameters for June 2016 and 2017 at WC-F**

| DATE | | Temperature (°C) | pH (pH values) | TDS (ppm) | Salinity (ppm) | Turbidity (NTU) |
|---|---|---|---|---|---|---|
| | **SANS 241:2015** | - | ≥5 to ≤9.7 | ≤1200 | - | ≤1 |
| **June 2016** | **Raw 1** | 20.5 | 5.2 | 455 | N/D | N/D |
| | **Raw 2** | 18.5 | 5.5 | 307 | N/D | N/D |
| | **Raw 3** | 17.1 | 5.4 | 221 | N/D | N/D |
| | **AT** | 16.8 | 6.5 | 385 | N/D | N/D |
| | **D** | 16.0 | 7.2 | 387 | N/D | N/D |
| **June 2017** | **Raw 1** | 21.3±0.15 | 5.64±0.01 | 422±0.33 | 259±0.33 | 3.13±0.08 |
| | **Raw 2** | 13.1±0.06 | 7.16±0.01 | 371±30 | 204±0.33 | 9.99±0.00 |
| | **AT** | 13.0±0.06 | 7.23±0.01 | 391±0.00 | 234±0.00 | 0.21±0.06 |
| | **D** | 15.8±0.04 | 7.55±0.03 | 394±1.05 | 328±0.49 | 0.31±0.02 |

Raw 1-borehole water; Raw 2-mixed raw water; Raw 3-dam water; AT; treated water; D-distribution networks; TDS-Total Dissolved Solids; EC-Electrical conductivity; Ave-average; SE-standard error; N/D-not determined; SANS-South African National Standards

## 4.2 Chemical parameters

The measured chemical parameters from WC-A and WC-F are shown in Tables 4 and 5, respectively. The measured parameters were compared to the SANS 241:2015 of drinking water. These standards were not applied to raw water samples. All above mentioned chemical parameters were not measured in June 2016 at WC-F.

### 4.2.1 Free chlorine

Free chlorine concentrations detected in drinking water samples of WC-A were generally low. Most water samples contained free chlorine <0.1 mg/L. The reclaimed water had higher (0.3 mg/L) free chlorine levels. This was also the case for the free chlorine levels in the distribution network. Free chlorine levels at WC-F were also relatively low (<0.01 mg/L). The levels of free chlorine were higher in the distribution system. Even though low, the free chlorine from all sampling sites was within the SANS 241:2015 for drinking water (≤ 5 mg/L).

### 4.2.2 Phosphate

The phosphate levels for the WC-A plant and distribution system was low (2.49 mg/L in reclaimed water for the November sampling and 5.28 mg/L was measured in the dam water) A similar scenario was observed for the WC-F system. In this case the highest phosphate concentration of 3.81 mg/L was recorded in treated water sample. The levels were lower (2.28 mg/L) in the distribution network. Once again no SANS 241 92015) guidelines are available. However, the measurable levels of phosphates in drinking water indicate that nutrients for the maintenance and propagation of heterotrophic organisms are present.

### 4.2.3 Nitrates and nitrites

As anticipated, the nitrates levels in the wastewater sample were elevated at WC-A. Nitrates were not detected in dam water sample (June 2017). High nitrate concentration in drinking water was detected in reclaimed water for each sampling run. The distribution network samples and water samples immediately after mixing had the lowest nitrate concentration for the June and November 2017 sampling. Nitrite levels were relatively low in all water samples site for June and November 2017at WC-A. The highest nitrate level measured was 0.57 mg/L from wastewater for June 2017 sampling. The nitrite concentrations from other sampling sites were higher than 0.2 mg/L. Nitrites were not detected in treated water samples during both sampling runs. In DWF WC-F, the highest nitrate concentration was measured in borehole water sample. The lowest was measured in dam water. The nitrate levels in drinking and distribution network was also low. Nitrites were not detected in any of

the water samples of WC-F. The nitrates and nitrites from all sampling sites were within the SANS 241: (2015) for drinking water (≦ 11 mg/L and ≤0.9 mg/L respectively).

**Table 4: Results of chemical parameters for June and November 2017 at WC-A**

| Date | | Free Chlorine (mg/L) | Phosphates (mg/L) | Nitrates (mg/L | Nitrites (mg/L) |
|---|---|---|---|---|---|
| | **SANS 241: 2015** | ≤5 | - | ≤11 | ≤0.9 |
| **June 2017** | **Raw 4** | 0.05±0.02 | 4.00±0.12 | 9.45±0.89 | 0.57±0.00 |
| | **Raw 1** | 0.05±0.02 | 4.74±0.67 | 0.47±0.03 | 0.03±0.00 |
| | **Raw 3** | 0.13±0.02 | 5.28±0.10 | 0.00±0.00 | 0.01±0.01 |
| | **RW** | 0.04±0.02 | 4.11±0.61 | 2.37±0.22 | 0.18±0.01 |
| | **AT** | 0.03±0.02 | 3.34±0.04 | 1.13±0.24 | 0.00±0.00 |
| | **D** | 0.07±0.02 | 3.36±0.26 | 0.77±0.12 | 0.11±0.03 |
| **November 2017** | **Raw 4** | 0.03±0.00 | 3.50±0.00 | 4.60±0.10 | 0.01±0.00 |
| | **Raw 1** | 0.07±0.04 | 4.45±0.65 | 1.13±0.03 | 0.01±0.00 |
| | **RW** | 0.33±0.03 | 2.49±0.36 | 2.30±0.06 | 0.07±0.01 |
| | **AT** | 0.03±0.01 | 3.6±0.23 | 0.97±0.07 | 0.00±0.00 |
| | **D** | 0.04±0.01 | 3.74±0.47 | 1.27±0.06 | 0.10±0.09 |

Raw 4-wastewater; Raw 1-borehole water; Raw 3-dam water; RW-reclaimed water; AT; treated water D-distribution networks; Ave-average; SE-standard error; N/D-not determined; SANS-South African National Standards

**Table 5: Results of physical parameters for June 2017 at WC-F**

| Date | | Free Chlorine (mg/L) | Phosphates (mg/L) | Nitrates (mg/L) | Nitrites (mg/L) |
|---|---|---|---|---|---|
| | **SANS 241: 2015** | ≤5 | - | ≤11 | ≤0.9 |
| **June 2017** | **Raw 1** | 0.07±0.02 | 3.00±0.29 | 0.37±0.07 | 0.00±0.00 |
| | **Raw 2** | 0.00±0.00 | 3.07±1.06 | 0.10±0.06 | 0.00±0.00 |
| | **Final** | 0.01±0.01 | 3.81±0.72 | 0.33±0.01 | 0.00±0.00 |
| | **D** | 0.04±0.02 | 2.28±0.36 | 0.17±0.05 | 0.00±0.00 |

Raw 1-borehole water; Raw 2-mixed raw water; Raw 3-dam water; AT; treated water D-distribution networks; Ave-average; SE-standard error; N/D-not determined; SANS-South African National Standards

## 4.3 Detection of faecal coliforms and *E. coli*

Water samples were filtered through sterile filter membranes with 0.45 µm porous size for the detection of faecal coliforms and *E. coli.* The filter membranes were placed on the surface of MLGA and incubated. After incubation, there was no *E. coli* detected in borehole water, dam, reclaimed water, treated water and distribution networks from WC-A and WC-F. Faecal coliforms were also not present at those sites either, except in dam water for June 2017 sampling at WC-A. There was only one faecal coliform detected in dam water. Wastewater samples contained high levels of *E. coli* and faecal coliforms at WC-A for June and November 2017 sampling. Another site that contained *E. coli* and faecal coliforms was mixed raw water at WC-F. However, the presence of these microorganisms was expected. Water samples from WC-F collected in June 2016 were not subjected to this test.

## 4.4 Isolation of HPC bacteria

The spread plate technique was conducted for the enumeration and isolation of HPC isolates from WC-A (June and November 2017) and WC-F (June 2017). HPC isolates were not detected in water samples from the boreholes and distribution networks during the June 2017 sampling. This was the case for both WC-A and WC-F. However, HPC bacteria were successfully isolated in all water samples from November 2017 sampling at WC-A.

## 4.5 Correlation of physico-chemical parameters and microbiological agents

The RDA triplots were constructed to determine the correlation of microbiological factors and physico-chemical parameters for each sampling run, except for samples collected in June 2016 at WC-F. Figures 3 and 4 represent the RDA triplots from WC-A for June and November 2017 sampling, respectively. While Figure 5 represents the RDA triplots of water sample collected in June 2017 at WC-F.

The trend observed at WC-A and WC-F in which turbidity, and to a certain extend pH, were closely associated with the occurrence (levels) of HPC isolates (Figure 3 to 5). This was particularly obvious in the case of WC-A water sampled in November and WC-F water sampled in June 2017. In these cases, *E. coli* and faecal coliforms also associated with these physical parameters. In the case of WC-A nitrates also impacted the microbiological parameters in water samples. The sampling point that is associated with many of the listed parameters (nitrates, microbiology, particularly *E. coli* and faecal coliforms levels) was wastewater effluent.

At WC-A, the reclaimed water (RW), treated water and water in the distribution formed a cluster with borehole water (June 2017). This indicates that quality of the water from the various sources were relatively similar. This was the case during both sampling periods (except distribution water in November, Figure 4). Temperature and free chlorine were the physical and chemical parameters that had the most profound effect on these mentioned water samples. During the November 2017 sampling the water quality in the distribution was different but no information is available to describe why this was so.

In the case of WC-F, the quality of the dam water and the water in the distribution system was similar and also impacted by temperature and free chlorine (Figure 5). Water quality of the mixed raw water was different and impacted by faecal indicator bacteria, nitrates and turbidity. The quality of water immediately after treatment was different from the water from other compartments and was impacted by phosphates.

All the physico-chemical parameters measured were at such levels that either would maintain or promote heterotrophic bacterial population growth. Some of these parameters were strongly correlated with bacterial levels. Water quality at the various sites also correlated with specific physico-chemical parameters.

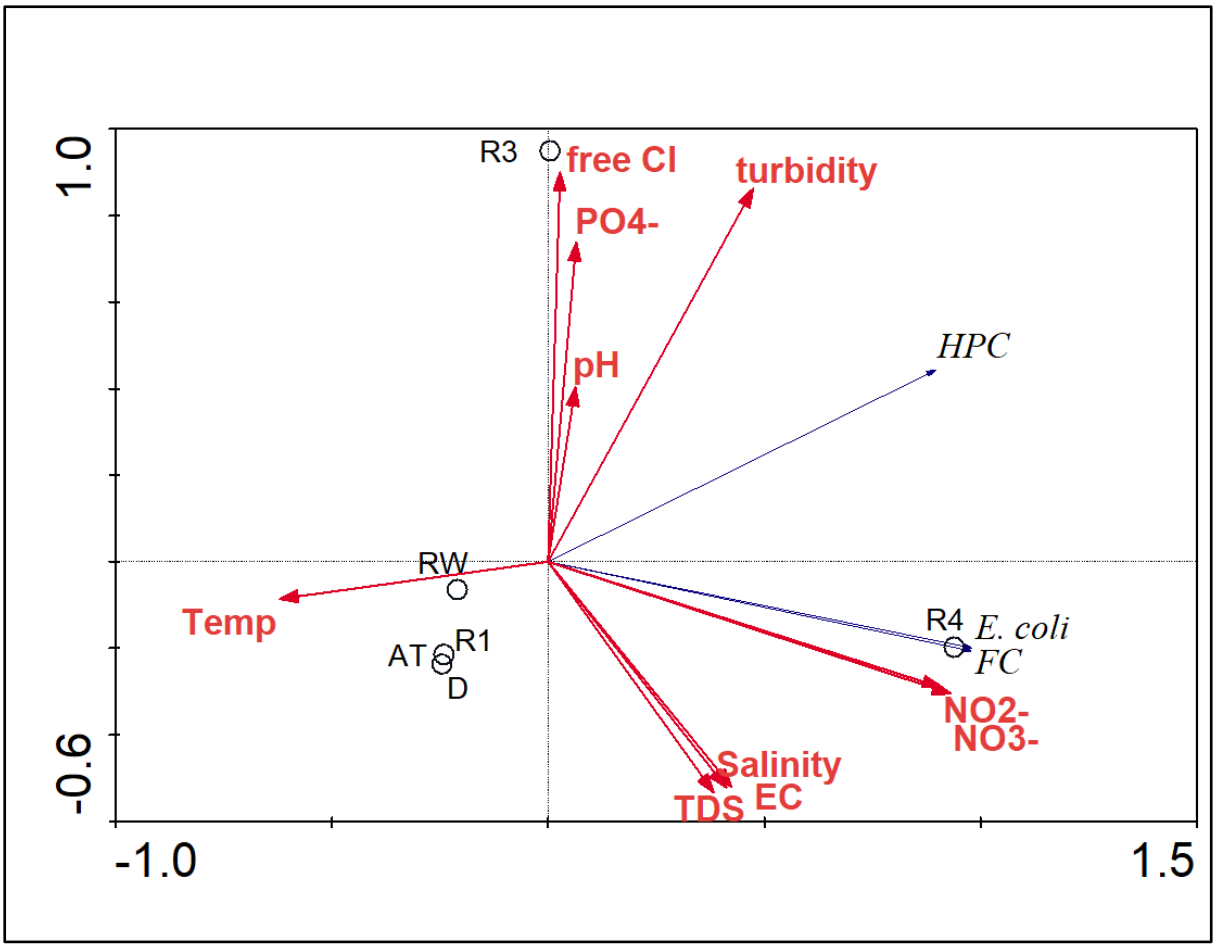


**Figure 3: RDA triplots representing the correlation between the microbiological agents and physico-chemical parameters from June 2017 sampling at WC-A**

R4-wastewater; R1-borehole water; R3-dam water; RW-reclaimed water; AT; treated water D-distribution networks; Temp-temperature, $PO_4^{-}$phosphates; $NO_2$-nitrates; $NO_3^{-}$-nitrites; EC-electrical conductivity, TDS-Total dissolved solids; FC- faecal coliforms, free Cl-free chlorine; HPC-heterotrophic plate count bacteria

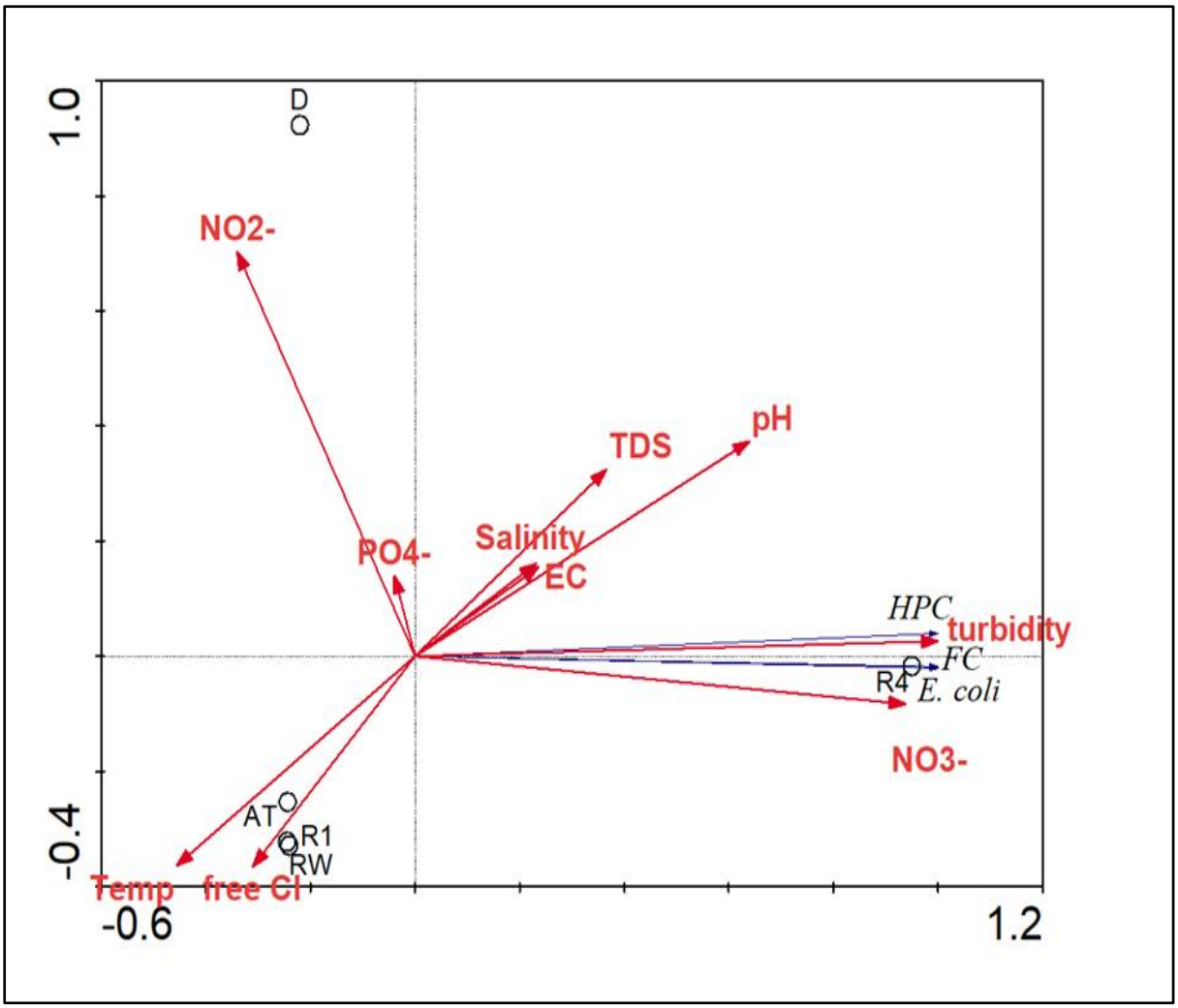


**Figure 4: RDA triplots representing the correlation between physico-chemical parameters and microbiological agents from November 2017 sampling at WC-A.**
R4-wastewater; R1-borehole water; R3-dam water; RW-reclaimed water; AT; treated water D-distribution networks; Temp-temperature, $PO_4^{-}$phosphates; $NO_2$-nitrates; $NO_3^{-}$-nitrites; EC-electrical conductivity, TDS-Total dissolved solids; FC- faecal coliforms, free Cl-free chlorine; HPC-heterotrophic plate count bacteria

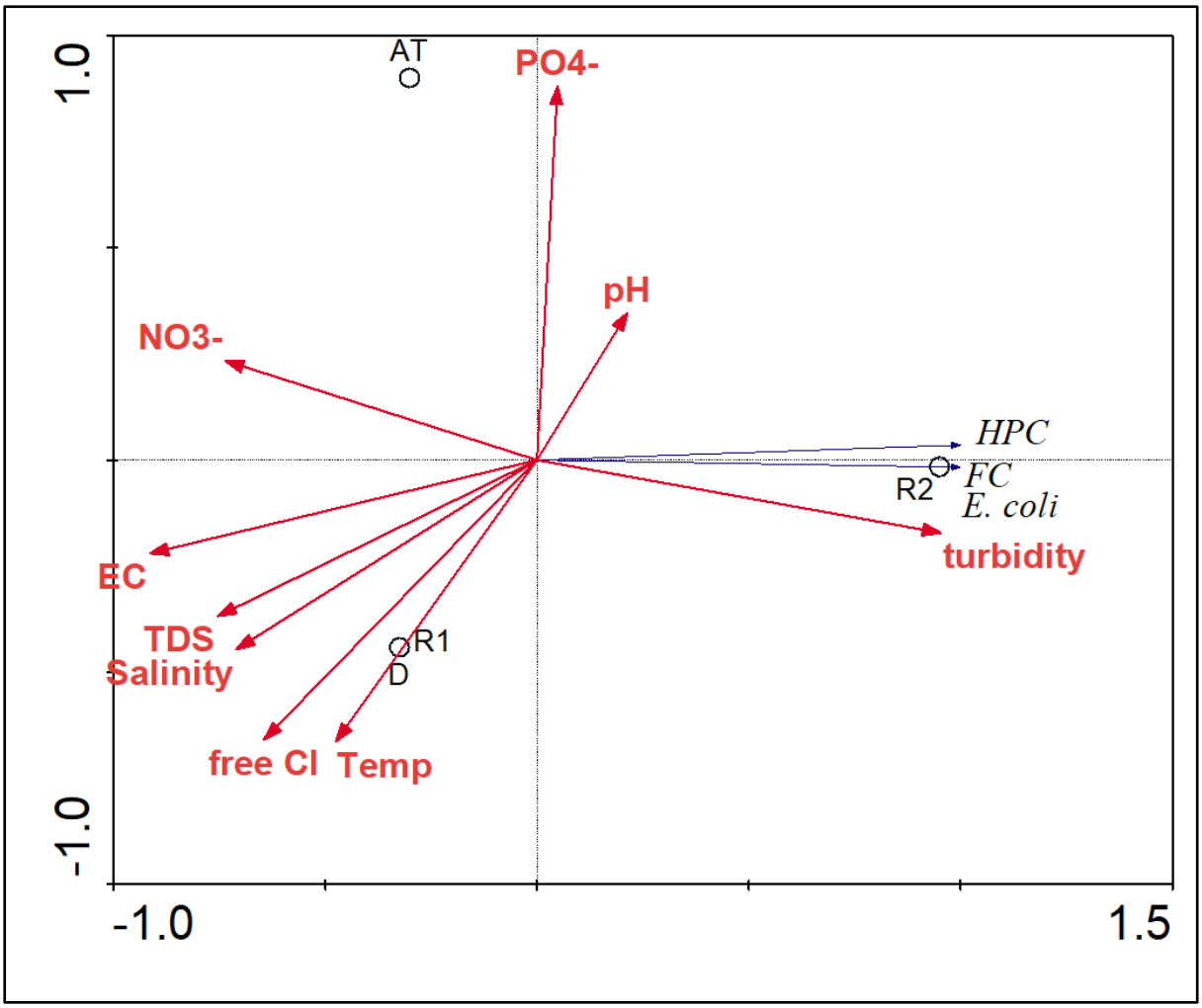


**Figure 5: RDA triplots representing the correlation between the microbiologica agents and physico-chemical parameters from June 2017 sampling at WC-F.**

R1-borehole water; R2-mixed raw water; AT; treated water D-distribution networks; Temp-temperature, $PO_4^{-}$phosphates; $NO_2$-nitrates; $NO_3^{-}$-nitrites; EC-electrical conductivity, TDS-Total dissolved solids; FC- faecal coliforms, free Cl-free chlorine; HPC-heterotrophic plate count bacteria

## 4.6 Kirby-Bauer susceptibility test

Pure colonies confirmed by Gram stain test were subjected to the Kirby-Bauer antibiotic susceptibility test. Figure 6 and 7represent the percentages of ARB for each sampling site from WC-A (June and November 2017 sampling) and WC-F (June 2016 and June 2017 sampling) that were resistant to the various antibiotics. Antibiotic resistance pattern of HPC bacteria from WC-A and WC-F are shown in Appendix A.

### 4.6.1 Resistance patterns among isolates from water of WC-A

Data for the WC-A isolates from June (e.g Raw 4_06; Raw 3_06; RW_06; AT_06) and November (Raw 4_11; Raw 3_11; RW_11; AT_11; D_11) are provided in Figure 6. A large percentage of raw water isolates from the wastewater effluent (Raw 4) and dam water (Raw 3) were resistant to beta-lactam antibiotics (ampicillin and cephalothin). This ranged between 55 and 100%. The pattern was similar for isolates from both sampling periods. In addition, 55% and 90% of isolates (Raw 3_06 and Raw 4_06, respectively) were resistant to trimethoprim. Between 70 and 80% of isolates from the June (Raw 4_06) isolates were resistant to chloramphenicol, erythromycin and oxy-tetracycline. In the case of the November isolates (Raw 4_11) lower percentage isolates were resistant to the same antibiotics. Among these isolates 82% were resistant to streptomycin. Some of the isolates from the dam water were also resistant to a number of the other antibiotics including kanamycin and neomycin.

The percentage of isolates from the drinking water (RW, AT and D) that were resistant to antibiotics were lower. Once again, resistance to ampicillin (>10 to >65%) was observed amongst the isolates from all the compartments (RW, AT and D) and sampling periods (June and November). None of the isolates after reclamation were resistant to cephalothin, however, resistance to this antibiotic was observed in the after treatment (>10 to 50%) and in the distribution system (>65%). The percentage of isolates resistant to trimethoprim among the isolates from the distribution system was also considerably high (75%).

### 4.6.2 Resistance patterns among isolates from water of WC-F

Antibiotic resistance data for the WC-F isolates from June 2016 (e.g Raw 1_16; Raw 3_16; Raw 2_16; AT_16; D_16) and June 17 (Raw 1_17; AT_17) are provided in Figure 7. From this data it is evident that a large percentage (55 to 100%) of raw water isolates were resistant to beta-lactam antibiotics (ampicillin and cephalothin) and trimethoprim (55 to 100%). This was also the case for chloramphenicol (72 to 82%). Varying numbers (percentages) were resistant to several of the other antibiotics.

Amongst the drinking water isolates (AT_16; AT_17; D_16) a similar pattern was observed. A large percentage was again resistant to the beta-lactam antibiotics (66 to 100%), chloramphenicol (33 to 85%) as well as trimethoprim (55 to 100%). In this case 33 to 70% were resistant to oxy-tetracycline.

In both systems overlapping antibiotic resistance patterns were observed between raw water and drinking water. It is evident that in both cases beta-lactam resistance, resistance to chloramphenicol and trimethoprim were dominant phenotype.

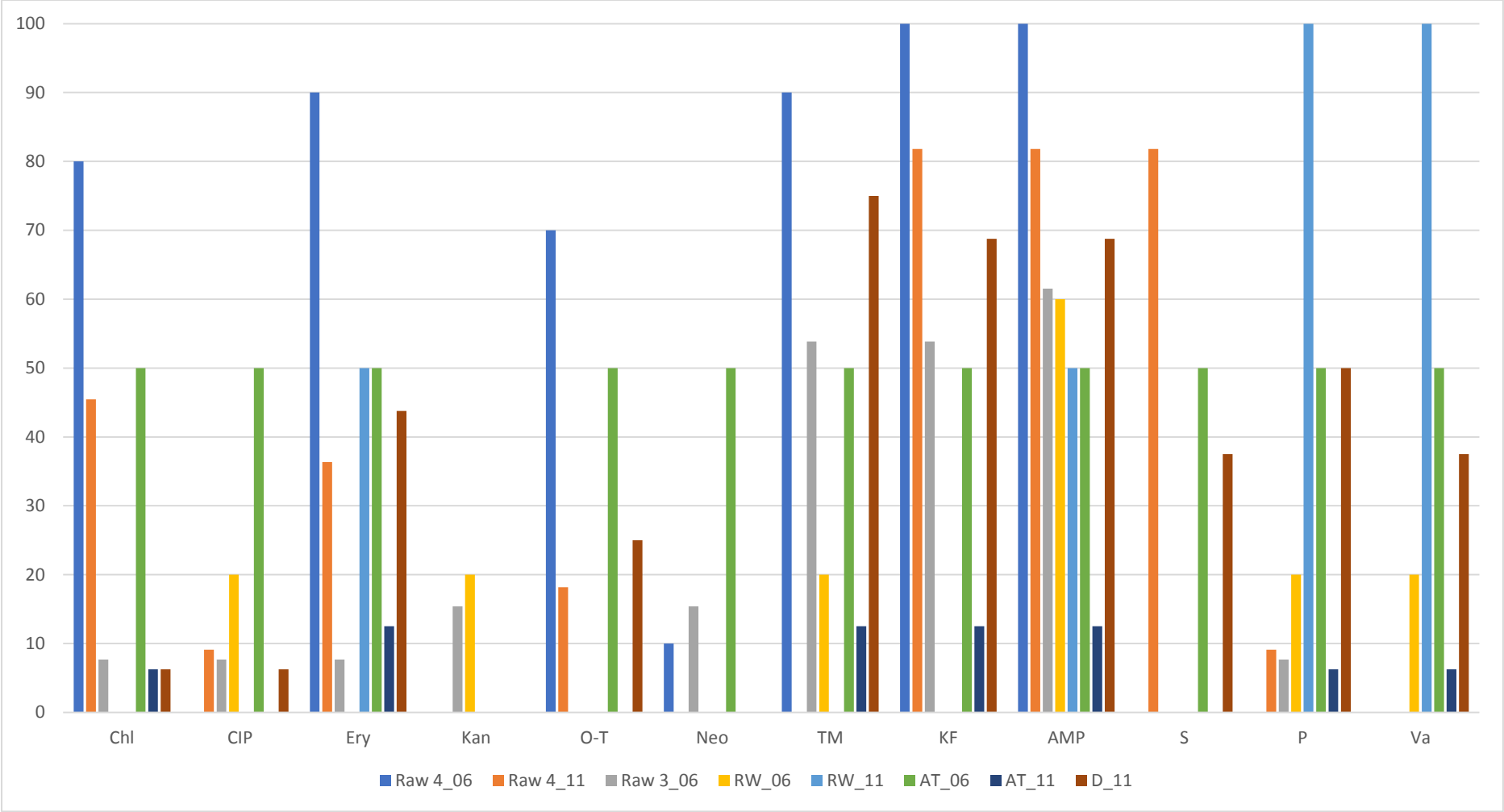


**Figure 6: Percentage of HPC bacteria that were resistant to antibiotics at WC-A**

Raw 4-wastewater; Raw 1-borehole water; Raw 3-dam water; RW-recycled wastewater; AT; treated water D-distribution networks; AMP-Ampicillin; KF–Cephalothin; Chl–Chloramphenicol; CIP-Ciprofloxacin; Ery-Erythromycin; Kan–Kanamycin; N-Neomycin; O-T–Oxy-tetracycline; P-Penicillin G; S-Streptomycin; TM–Trimethoprim; Va-Vancomycin

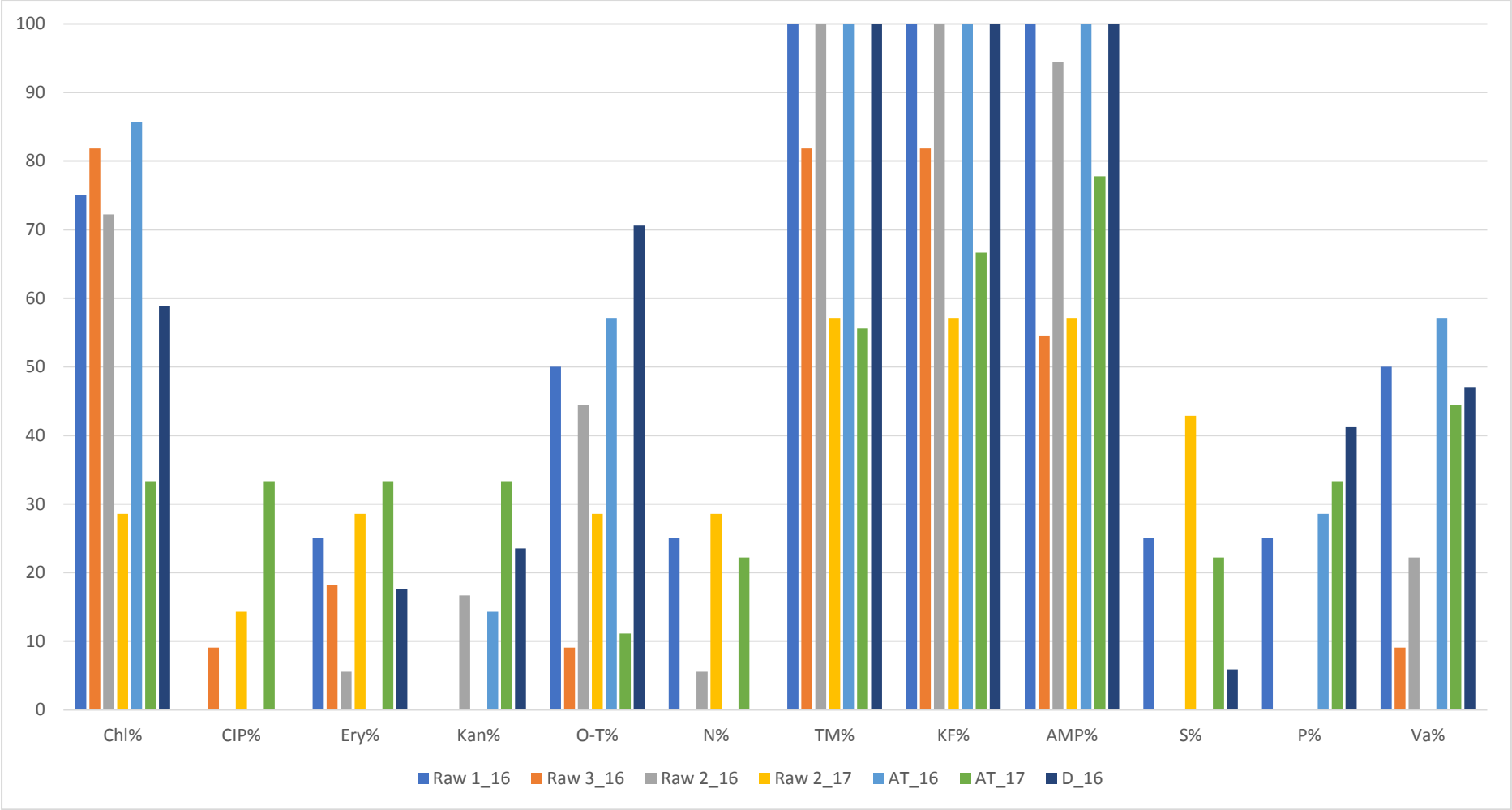


**Figure 7: Percentage of HPC bacteria that were resistant to antibiotics at WC-F**
Raw 1-borehole water; Raw 2-mixed raw water; Raw 3-dam water; AT; treated water D-distribution networks; AMP-Ampicillin; KF–Cephalothin; Chl–Chloramphenicol; CIP-Ciprofloxacin; Ery-Erythromycin; Kan–Kanamycin; N-Neomycin; O-T–Oxy-tetracycline; P-Penicillin G; S-Streptomycin; TM–Trimethoprim; Va-Vancomycin

## 4.7 MAR indices

The data generated in Kirby-Bauer disk diffusion method was also used to determine MAR indices of each sampling site as shown in Tables 6 and 7 for WC-A and WC-F, respectively. In the case of WC-A, the effluent from the WWTP that is used for water reclamation, had a high MAR index (0.45 and 0.30) for both June and November 2017. This is a result that could be anticipated. The MAR index for the treated water (mixed water) and in the distribution system also had a high MAR index (0.46 and 0.34). Lower MAR values (0.13 and 0.19) were observed for reclaimed water and for water from the dam, respectively.

**Table 6: MAR indices for June and November 2017 sampling at WC-A**

| Sampling date | Raw 4 | Raw 1 | Raw 3 | RW | AT | D |
|---|---|---|---|---|---|---|
| **Jun-17** | 0.45 | N/A | 0.19 | 0.13 | 0.46 | N/A |
| **Nov-17** | 0.30 | 0 | N/A | 0.25 | 0.46 | 0.34 |

Raw 4-wastewater; Raw 1-borehole water; Raw 3-dam water; RW-reclaimed water; AT; treated water D-distribution networks

In the case of WC-F (Table 7) for June 2016 and June 2017 MAR indices were all higher than 0.2. The highest MAR index (0.48) at WC-F was recorded in borehole water from June 2016 samples. MAR indices for after treatment and in distribution networks were also high (0.45 and 0.47, respectively).

**Table 7: MAR indices for June 2016 and June 2017 sampling at WC-F**

| sampling date | Raw 1 | Raw 2 | Raw 3 | AT | D |
|---|---|---|---|---|---|
| **Jun-16** | 0.48 | 0.38 | 0.29 | 0.45 | 0.47 |
| **Jun-17** | N/A | 0.26 | N/A | 0.39 | N/A |

Raw 1-borehole water; Raw 2-mixed raw water; Raw 3-dam water; AT; treated water D-distribution networks

MAR values greater than 0.2 indicate that the isolates were recently or constantly exposed to antibiotics. It is thus a reflection on the antibiotic exposure history of the isolates from a particular site. The results presented here thus generally indicate that the various HPC isolates that were isolated from the raw and drinking water had overlapping antibiotic exposure histories.

## 4.8 Identification

The identity of the HPC bacteria was determined by 16S rRNA gene sequencing. Figure 8 represents a 1.5% (w/v) agarose gel of successfully amplified 16S rRNA gene fragments. The size of amplicons is approximately 1465 bp. Amplicons had no non-specific bands and primer dimers present. A no template DNA (NT) had no band. It could be concluded that this test was free from contamination and suitable for sequencing. Sequencing was performed by Inqaba Biotechnology Industries (Pty) Ltd – Pretoria, South Africa.

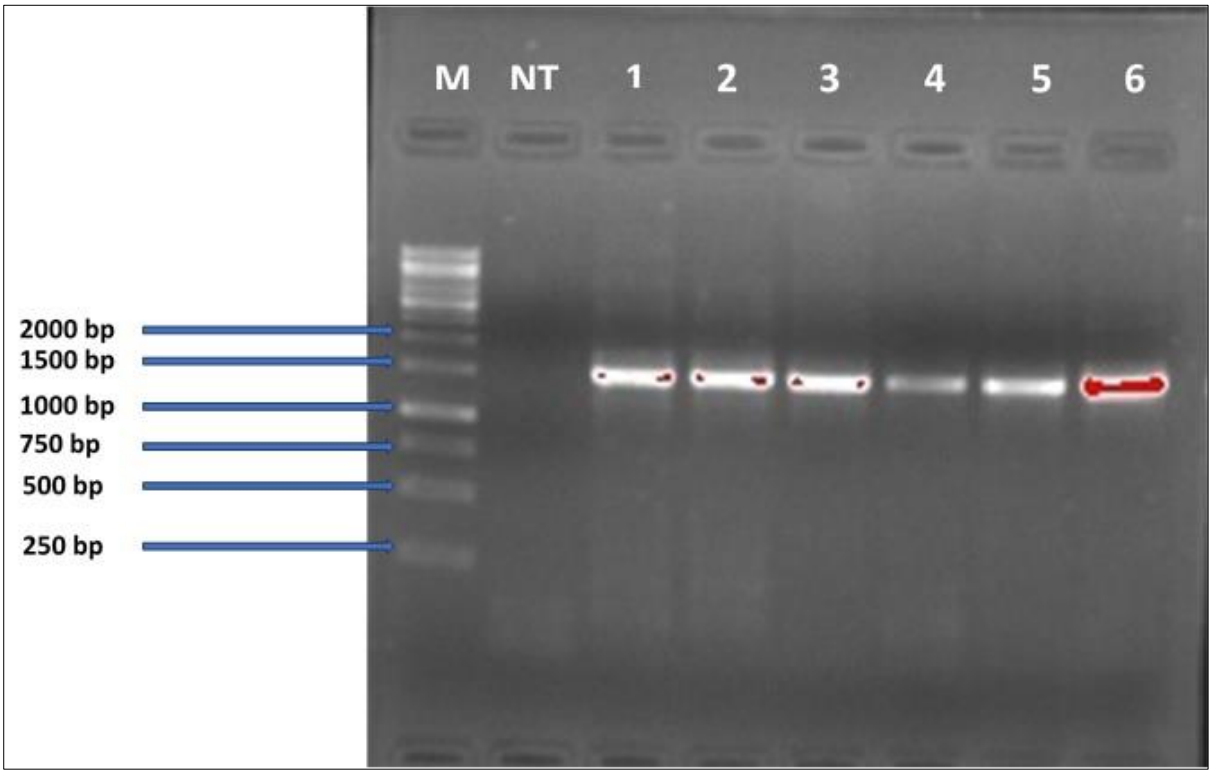


**Figure 8: Gel electrophoresis of a 1.5% (w/v) agarose gel with 6 selected successful 16S rDNA amplifications with the expected size of 1 465 bp. The lanes marked M and NT shows a 1 kb molecular weight marker (GeneRuler™ 1 kb DNA ladder, Fermentas, US) and the no template DNA control, respectively.**

Finch TV (version 1.4.0) was used to extract the nucleic acid sequences of HPC bacteria. The generated nucleic acid sequences were exported to Eztaxon software (www.ezibiocloud.net) for identification of HPC isolates in raw and drinking water. A total of 109 bacterial species were identified in this study. Appendix B represents bacterial species identified in WC-A and WC-F, respectively.

There was a total of 52 different bacterial species identified in raw and drinking water from WC-A and WC-F. Figures 9 to 10 presents the identity of bacterial species in raw and drinking from WC-A and WC-F, respectively. As expected there were more bacterial species

in raw water than drinking water. *Pseudomonas* sp. and *Bacilli* sp. were prevalent in raw water samples. Most of the bacterial species were successfully eliminated by the DPR plant and DWPFs. However, after treatment, *Bacillus wiedmannii, Bacillus mobilis* and *Pseudomonas protegens* were present in both and raw drinking water at WC-A, while *Chyseobacterium hispanicum, Bacillus cereus* as well as *Bacillus wiedmannii* were present in both and raw drinking water at WC-F. *Bacilli* sp. were prevalent in drinking water samples from WC-A and WC-F.

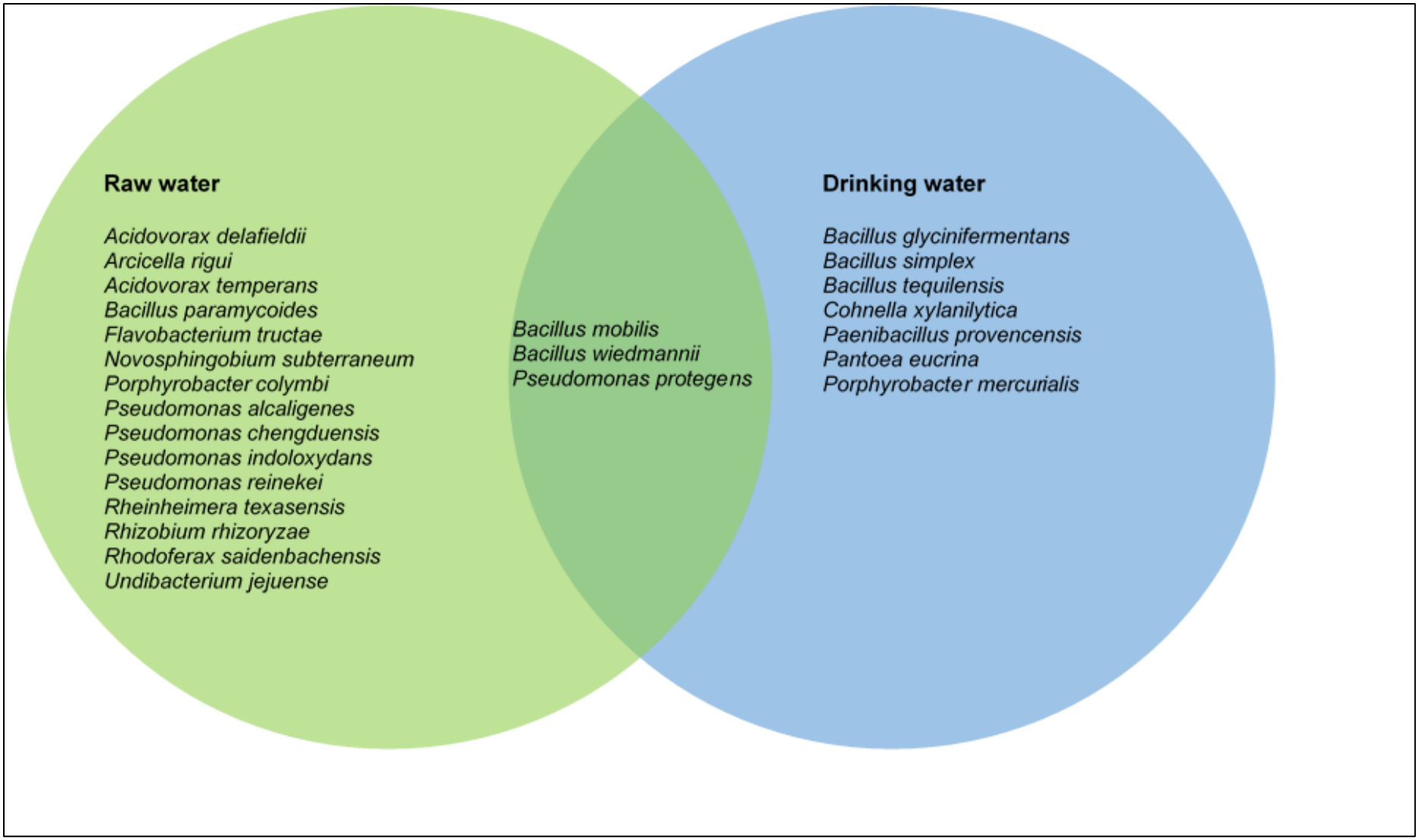


**Figure 9: Identity of bacterial species in raw and drinking water from WC-A**

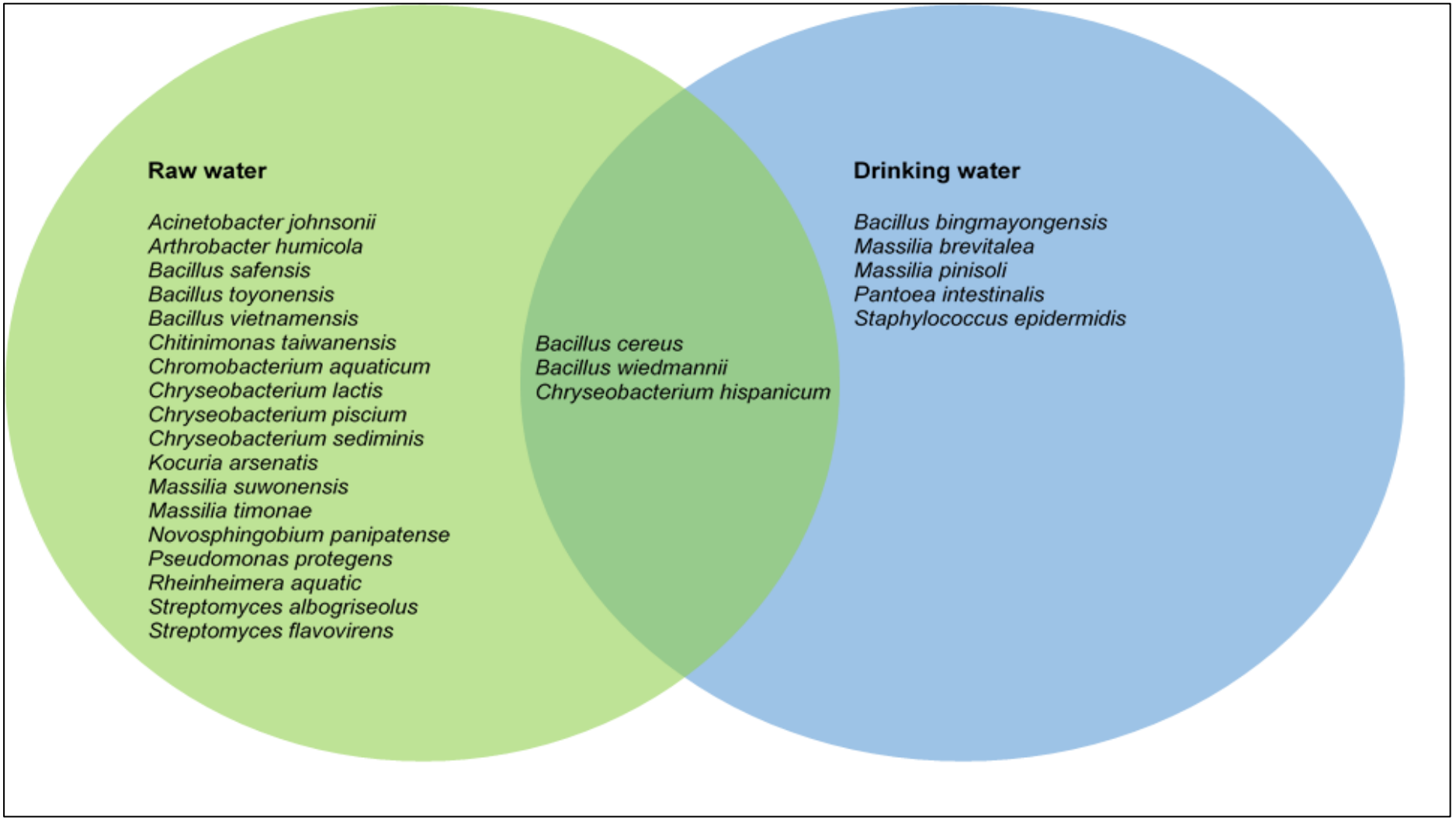


**Figure 10: Identity of bacterial species in raw and drinking water from WC-F**

## 4.9 Phylogenetic tree

The 16S rRNA sequences of identified HPC isolates and their reference sequences obtained from the EzTaxon software (www.ezibiocloud.net) were exported to Mega 7 for the construction of the phylogenetic tree. *E. coli* (M96394) sequence obtained from the GenBank (www.ncbi.nlm.nih.gov) was used as an outlier. Identified bacterial species were grouped into three phyla, namely *Proteobacteria, Firmicutes* and *Bacteroidete*s. Figure 11 represents a phylogenetic tree of 16S rRNA gene sequences samples of raw and drinking water from WC-A (June and November 2017) and WC-F (June 2017). However, this phylogenetic tree does not include the sequences of HPC bacteria collected in June 2016 at WC-F.

The phylogenetic tree shows that most of the bacterial species identified belong to the phylum *Proteobacteria.* This phylum consisted mostly with *Pseudomonas,* followed by *Massilia* species. Other species belonging to this phylum include *Pantoea, Rhodoferax, Rheinheimera, Rhizobium, Porphybacter, Novosphingobium, Undibacterium* and *Acidovorax* species. *Firmicutes* was the second largest phylum in the current study. It consisted mostly with *Bacilli* spp. and only one *Cohnella* spp... *Bacteroidete* was the smallest phylum which consisted of *Hymobacter seosoulenisis, arcicella rigui* and *Flavobacterium tructae.*

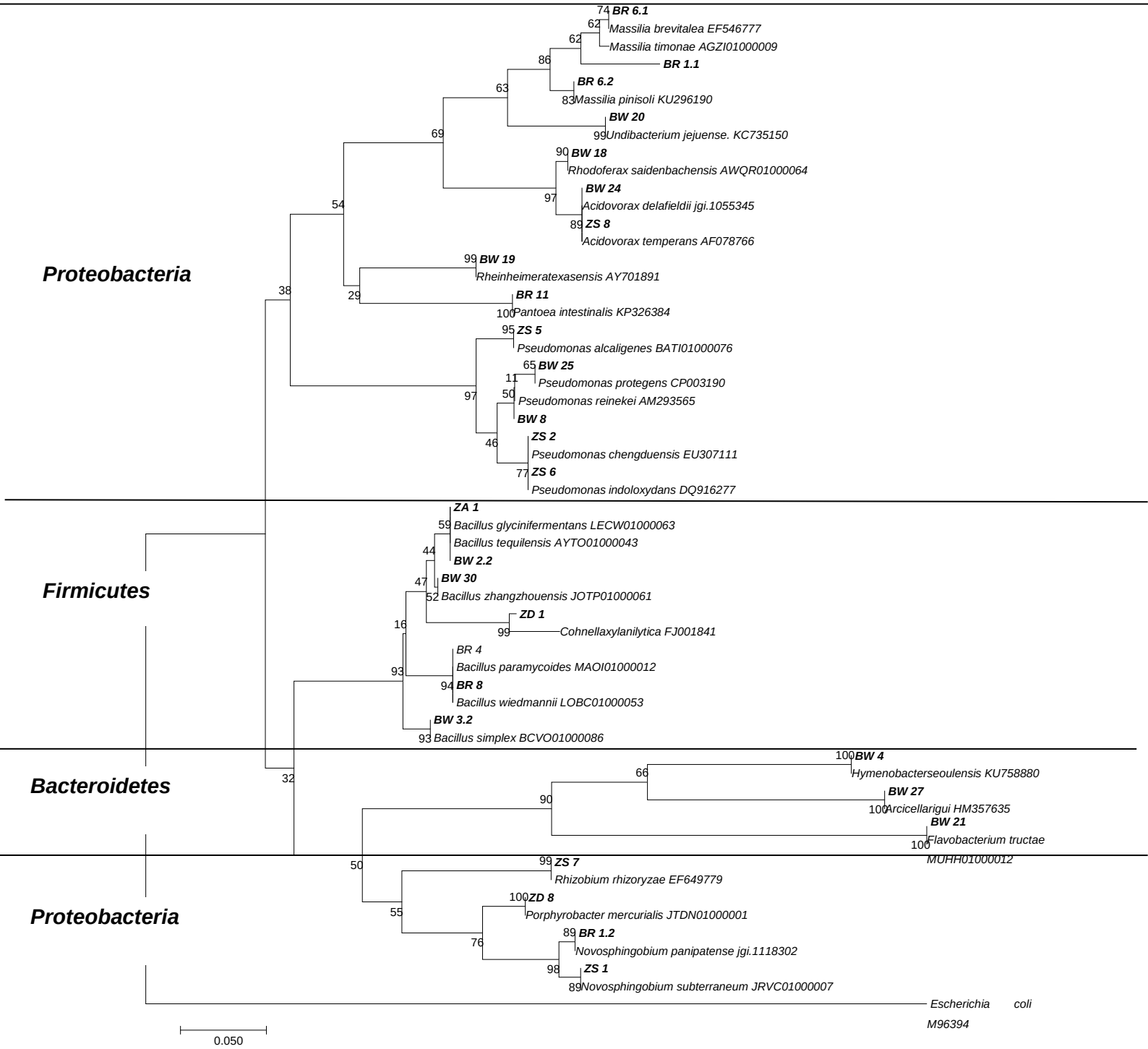


**Figure 11: Phylogenetic tree of 16S rRNA gene sequences from samples of raw and drinking water from WC-A (June and November 2017) and WC-F (June 2017)**

## 4.10 Detection of ARGs

There were 83 HPC isolates subjected to endpoint-PCR for the detection of $bla_{tem}$, *ermB, Intl1, ampC* and *tetM* ARGs from WC-A (June and November 2017) and WC-F (June 2017). Only 39 identified Gram-negative bacteria were subjected to endpoint-PCR for detection of *ermF* ARG. The $bla_{TEM}$ and *tetM* ARGs were not detected among the isolates from WC-A and WC-F for all the sampling periods. Figure 12 represents a 1.5% (w/v) agarose gel to demonstrate the sizes of successfully amplified ARGs**.** Tables 8 and 9 represent summary of the ARGs detected among the isolates from WC-A and WC-F, respectively. The HPC bacteria from WC-F (June 2016) were not included in these tests.

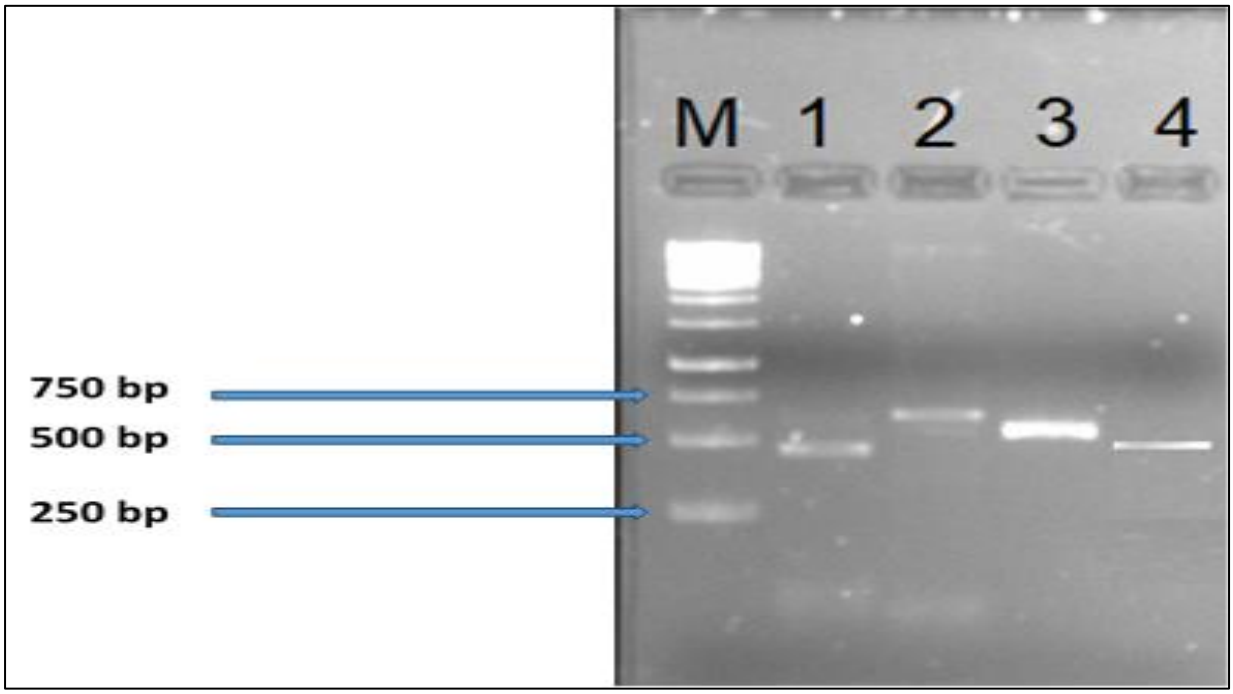


**Figure 12: Gel electrophoresis of 1.5% (w/v) agarose gel with the representative of the 4 ARGs that were successfully amplified. Lane 1 represents *ermF,* lane 2 represents *ermB,* lane 3 represents *ampC* and lane 4 represents *intl 1.* The lane marked M represents a 1 kb molecular weight marker (GeneRuler™ 1 kb DNA ladder, Fermentas, US).**

ARGs were mostly detected among the HPC bacteria from the WC-A water samples. A total of 33 HPC bacteria each from June and November 2017 sampling period were tested. The prevalent ARG in water sample was *ermB*. This gene was detected in 17 of the 33 isolates. Species that harboured most *ermB* were *Bacillus spp.* (41.18%) and *Pseudomonas spp*. (29.42%).

The *ermB* was the only gene detected in HPC bacteria from WC-F (June 2017). The *ermB* gene was detected in 7 out of 17 HPC bacteria. This gene was present in *Bacillus* sp. (4) and *Massilia* sp. (3). The *ermB* (71.42%) was most prevalent in treated water.

The *IntI1* gene was only detected in the DNA of six of the HPC bacteria from November 2017 at WC-A. Five HPC bacteria were from the distribution networks and one from wastewater effluent. This gene was detected in *Bacillus* sp. (4), *Porphyrobacter* spp. (1) and an unidentified isolate (1). The *ampC* gene was detected in drinking water sources from June and November 2017 sampling at WC-A. The *ampC* gene was detected in treated water (June 2017) and distribution network (November 2017) and was detected in *Bacillus wiedmannii* only.

A total of 31 Gram-negative HPC isolates were tested for the presence of *ermF* gene from WC-A. The *ermF* was detected in 3 HPC isolates from June 2017. This gene was present in one isolate each from the dam, treated water and reclaimed water. This gene was not present in wastewater effluent isolates. The *Rhodoferax saidenbachensis, Paenibacillus provencensis* and *Pseudomonas protegens* are responsible for carrying the *ermF* gene.

**Table 8: ARGs detected in WC-A for June and November 2017 sampling**

| Date | Site | Isolate | Identity | ARG |
|---|---|---|---|---|
| **June 2017** | Raw 4 | **2** | *Pseudomonas reinekei* | *erm B* |
| | | **8** | *Pseudomonas reinekei* | *erm B* |
| | | **10** | *Pseudomonas reinekei* | *erm B* |
| | Raw 3 | **3** | *Rhodoferax saidenbachensis* | *erm F* |
| | | **5** | *Undibacterium jejuense* | *erm B* |
| | | **6** | *Flavobacteriumtructae* | *erm B* |
| | | **10** | *Pseudomonas protegens* | *erm B* |
| | RW | **4** | *Paenibacillus provencensis* | *erm F* |
| | AT | **2** | *Pseudomonas protegens* | *erm F, amp C* |
| **November 2017** | Raw 4 | **2** | *Pseudomonas chengduensis* | *erm B* |
| | | **11** | *Bacillus wiedmannii* | *erm B, int1* |
| | Raw 1 | **2** | *Bacillus wiedmannii* | *erm B* |
| | RW | **1** | *Bacillus mobilis* | *erm B* |
| | D | **2** | N/D | *Intl 1* |
| | | **4** | *Bacillus wiedmannii* | *erm B, intl1* |
| | | **6** | *Bacillus wiedmannii* | *erm B* |
| | | **7** | *Bacillus wiedmannii* | *erm B, intl1* |
| | | **8** | *Porphyrobactermercurialis* | *intl 1* |
| | | **9** | *Bacillus wiedmannii* | *erm B* |
| | | **10** | *Bacillus wiedmannii* | *erm B* |
| | | **12** | N/D | *erm B* |
| | | **14** | *Bacillus wiedmannii* | *intl 1* |
| | | **18** | *Bacillus wiedmannii* | *amp C* |
| | AT | **2** | *Bacillus wiedmannii* | *erm B* |

Raw 4-wastewater; Raw 1-borehole water; Raw 3-dam water; RW-reclaimed water; AT; treated water D-distribution networks; N/D-not determined

**Table 9: ARGs detected in WC-F for June 2017 sampling**

| site | Isolate | Identity | ARG |
|---|---|---|---|
| Raw 2 | 4 | *Bacillus wiedmannii* | *erm B* |
| | 5 | *Massilia timonae* | *erm B* |
| AT | 1 | *Massilia brevitalea* | *erm B* |
| | 2 | *Massilia pinisoli* | *erm B* |
| | 3 | *Bacillus wiedmannii* | *erm B* |
| | 9 | *Bacillus wiedmannii* | *erm B* |
| | 10 | *Bacillus wiedmannii* | *erm B* |

Raw 1-borehole water; Raw 2-mixed raw water; Raw 3-dam water; AT; treated water D-distribution networks

## 4.11 Haemolysis and Extracellular enzyme assays

Purified HPC bacteria were streaked out on media containing 5% sheep blood agar for the haemolysis test. This test is essential for the classification of HPC isolates as α, β or Ɣ haemolytic and thus assessing potential pathogenicity. Appendix C represents haemolysis test results for WC-A and WC-F. The HPC isolates from June 2016 sampling at WC-F were not included in this test. A total of 83 isolates were from raw and drinking water at WC-A and WC-F. Of these 48 (58%) were beta-haemolytic (ability to completely lyse red blood cells). Amongst the isolates that were beta haemolytic 26 (31%) were from raw water and 22 (26%) from drinking water. Most of the HPC bacteria from raw water came from the wastewater effluent (Raw 4; 11/26), dam water (Raw 3; 10/26) and mixed raw water for WC-F (5/26). Several of the HPC isolates (23%) were alpha-haemolytic. A total of 67 (81%) of the HPC bacteria were haemolytic and thus potential pathogens.

Beta and alpha haemolytic bacteria were further subjected to extracellular enzyme assays and the results were as follow; 82.48% lecithinase, 77.99% DNase, 66.80% proteinase, 56.44% gelatinase and 41.67% lipase, as shown in Appendix C. Tables 10 and 11 represent summarised potential pathogenic HPC bacteria that harbour at least one ARG, with the ability to confer resistance to antibiotics and breakdown (α and β haemolysis) red blood cells were selected and their extracellular enzymes are discussed below. *Pseudomonas protegens* (WC-A) and *Bacillus wiedmannii* (WC-F) isolated from drinking water displayed the most pathogenic characteristics than HPC bacteria from raw water. *Massilia timonae, Massilia pinisoli, Rhodoferax saidenbachensis, Undibacterium jejuense* and *Porphyrobacter mercurialis* produced more than two extracellular enzymes.

**Table 10: Identification and virulence characteristics of each isolate from WC-A**

| . | Site | Isolate | Accession no. | Antibiotic resistance | ARG | Haemolysis | Extracellular assays | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Dn | G | Li | Le | P |
| WC-A June 2017 | Raw 4 | *Pseudomonas reinekei* | AM293565 | 2 | *ermB* | β | X | | | X | X |
| | | *Pseudomonas reinekei* | AM293565 | 6 | *ermB* | α | X | | | X | X |
| | Raw 3 | *Rhodoferax saidenbachensis* | AWQR01000064 | 4 | *ermF* | β | X | X | | X | |
| | | *Undibacterium jejuense* | KC735150 | 3 | *ermB* | β | X | | X | X | |
| | | *Pseudomonas protegens* | CP003190 | 5 | *ermB* | β | | X | X | | |
| | AT | *Pseudomonas protegens* | CP003190 | 11 | *ermF, ampC* | β | X | X | X | X | X |
| WC-A Nov 2017 | Raw 4 | *Pseudomonas chengduensis* | EU307111 | 5 | *ermF* | α | | | | X | |
| | | *Bacillus wiedmannii* | LOBC01000053 | 6 | *ermB, intI1* | α | X | | | | |
| | RAW 1 | *Bacillus wiedmannii* | LOBC01000053 | 3 | *ermB* | α | | X | | X | |
| | RW | *Bacillus mobilis* | KJ812449 | 2 | *ermB* | α | X | | | X | X |
| | AT | *Bacillus wiedmannii* | LOBC01000053 | 6 | *ermB* | α | X | | X | X | X |
| | D | *Bacillus wiedmannii* | LOBC01000053 | 6 | *ermB, intI1* | α | X | | X | | |
| | | *Bacillus wiedmannii* | LOBC01000053 | 7 | *ermB* | β | X | | X | X | |
| | | *Bacillus wiedmannii* | LOBC01000053 | 4 | *ermB, intI1* | β | X | | | X | X |
| | | *Porphyrobacter mercurialis* | JTDN01000001 | 7 | *intI1* | β | X | X | | X | X |
| | | *Bacillus wiedmannii* | LOBC01000053 | 6 | *ermB* | β | X | X | | X | X |
| | | *Bacillus wiedmannii* | LOBC01000053 | 3 | *ermB* | β | X | X | | X | X |

Raw 4-wastewater; Raw 1-borehole water; Raw 3-dam water; RW-recycled wastewater; AT; treated water D-distribution networks Dn - Dnase; G – Gelatinae, Li – Lipase, Le – Lecithinase, P - Proteinas

**Table 11: Identification and virulence characteristics of each isolate from WC-F**

| | Site | Isolate | Accession no. | Antibiotic resistance | ARG | Haemolysis | Extracellular assays | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Dn | G | Li | Le | P |
| WC-F | Raw 2 | *Massilia timonae* | AGZI01000009 | 5 | *ermB* | β | X | X | X | X | X |
| June | AT | *Massilia pinisoli* | KU296190 | 1 | *ermB* | β | X | | X | X | X |
| 2017 | | *Bacillus wiedmannii* | LOBC01000053 | 3 | *ermB* | β | X | | | X | X |
| | | *Bacillus wiedmannii* | LOBC01000053 | 10 | *ermB* | β | X | X | X | X | X |

Raw 2-mixed raw water; AT; treated water Dn - Dnase; G – Gelatinae, Li – Lipase, Le – Lecithinase, P - Proteinase

## 4.12 eDNA

The successfully extracted eDNA was subjected to endpoint PCR for the amplification of ARGs. Water samples that were subjected to endpoint PCR included wastewater samples collected immediately after rapid sand filtration in the DPR plant and treated water from DWPF at WC-A. Also, mixed raw water and treated water samples from WC-F. The 16S rRNA amplification of eDNA was used as quality control to demonstrate that the DNA was free from inhibitors. The eDNA was subjected to another endpoint PCR for the detection of $bla_{tem}$, *ermB, Intl1, ampC* and *tetM* ARGs from environmental samples. The $bla_{tem}$, *ampC* and *tetM* ARGs were not detected in environmental samples. Figure 12 represents 1.5% (w/v) agarose gel of successfully amplified ARGs with different length sizes. The *ermB* and *Intl1* ARGs were detected in only wastewater eDNA from WC-A and mixed raw water from WC-F. However, these ARGs were not detected in drinking water samples from WC-A and WC-F.

## 4.13 Detection of Antibiotic residues in water samples

Water samples from WC-A were collected from the DPR plant only. Water samples include wastewater effluent that underwent rapid sand filtration and reclaimed water. While mixed raw water and treated water samples were collected in the DWPF at WC-F. Table 12 represents the results obtained from screening antibiotics from WC-A and WC-F.

Ciprofloxacin was detected in raw and drinking water samples from WC-A and WC-F. Penicillin G was detected in raw water samples (WC-F) and treated water samples (WC-A and WC-F). Trimethoprim detected in wastewater effluent and reclaimed water from the DPR plant at WC-A. Streptomycin, oxy-tetracycline and chloramphenicol were detected only in wastewater effluent from WC-A. All the antibiotics mentioned were detected in small quantities. Antibiotics that were not detected in water samples include ampicillin, cephalothin, erythromycin, kanamycin, neomycin, benzylpenicillin (penicillin G) and sulfamethoxazole.

**Table 12: Detection of antibiotic residues in water from WC-A and WC-F**

| List of antibiotics | WC-A | | | WC-F | |
|---|---|---|---|---|---|
| | Raw 4 | RW | AT | Raw 2 | AT |
| Ampicillin | | | | | |
| Cephalothin | | | | | |
| Chloramphenicol | X | | | | |
| Ciprofloxacin | X | X | X | X | X |
| Erythromycin | | | | | |
| Kanamycin | | | | | |
| Neomycin | | | | | |
| Oxytetracycline | X | | | | |
| Benzylpenicillin (Penicillin G) | | | | | |
| Penicillin | | | X | X | X |
| Streptomycin | X | | | | |
| Trimethoprim | X | X | | | |
| Sulfamethoxazole | | | | | |

Raw 2-mixed raw water; Raw 4-wastewater; RW-reclaimed water; AT; treated water

# CHAPTER 5

# DISCUSSION

## 5.1 Physico-chemical parameters

A general trend was observed where the physico-chemical parameters, except for pH, were lower after water purification. At the WC-A plant, dam water was not collected during November 2017. This was due to an extensive drought and the dam was completely dry. According to the DWS (2018b), the current dam water level at WC-A is 0 $M^3$. As a result, the WC-A municipality has a water restriction of level 5 and WC-F municipality has level 3 (Western Cape Government, 2018). The measured physico-chemical parameters of drinking water were all within the SANS 241 (2015) at WC-A and WC-F. Drinking water that is meant for human consumption should have physico-chemical parameters that meet the standards proposed by the country and WHO (Bensoltane *et al.,* 2018).

### 5.1.1 Temperature

Water samples collected in June 2017 (winter) at WC-A and WC-F had lower temperature than samples collected in November (summer). The recorded temperatures during the winter (range between 12.3°C and 21.3°C) and summer season (range between 17.0°C and 21.0°C) have no adverse effect on microbial populations in the water distribution systems. Temperature is an essential ecological and physical factor which has big impact on biotic and abiotic factors of the environment (Palamuleni *et al.,* 2015). The temperatures observed in the present study would thus be able to maintain HPC bacterial populations.

### 5.1.2 pH

Measuring pH is essential for determining whether water is acidic or alkaline (Mulamattathil *et al*., 2015). pH values higher or lower than limit standards may give water a peculiar taste. Water with low pH may taste sour while water with high pH may taste bitter or soapy (Mulamattathil *et al*., 2015; Patil *et al.,* 2012). Drinking water samples from WC-F had a lower pH compared to samples from WC-A. However, all samples were within the SANS 241 (2015) levels for drinking water. Lower pH values were recorded at WC-F. At this DWPF lime is added to increase the pH of the source water. It plays an important role in the adjustment of pH in drinking water production due to its solubility (Evuti *et al.,* 2012; Al-Mutaz and Al-Ghunaimi, 2001). Raw and drinking water sources from WC-A and WC-F had the pH range between 6.37 and 8.14. This was an indication that water from WC-A and WC-F were mostly neutral. pH is one of the factors that facilitates the biological activities and the maintenance

of HPC bacterial populations (Palamuleni *et al.,* 2015). There are no health risks that are directly associated with pH, unless extremely acidic or alkaline (WHO, 2007).

### 5.1.3 Total Dissolved Solids (TDS)

The TDS levels in June 2017 (wet season) at WC-A was lower than the November 2017 (dry season) levels. The TDS concentrations of water were just below 1000 ppm at WC-A (range between 144 ppm and 956 ppm) and WC-F (221 ppm and 455) (ranges). Mulamattathil *et al*. (2015) measured TDS ranging from 223 to 364 ppm in North West DWPFs. The measured TDS of drinking water samples from the current study were higher than these as well as those measured by Rahmanian *et al*. (2015). In their study, the TDS concentration ranged from 18 ppm to 40 ppm indicating very soft drinking water. Excess TDS concentrations in drinking water may pose health problems to humans (Gupta *et al.,* 2017). The SANS 241 (2015) limit for TDS is 1200 ppm and none of the measured values exceeded this limit.

### 5.1.4 Salinity

There a clear decrease in the salinity concentration from raw to treated water samples at WC-A and WC-F. The DPR plant (WC-A) was most efficient in the removal of salinity from water samples. This is understandable since advanced treatment and filtration processes including nanofiltration and reverse osmosis are applied (Rahmanian *et al.,* 2015). The other drinking water samples had the salinity levels that range from 439 and 560 ppm. A drinking water study was done to investigate the effects of drinking water on blood pressure in the Costal of Bangladesh (Talukder *et al.,* 2016). The study showed that high salt concentrations in drinking water pose health hazards such as systolic and diastolic blood pressure in young adults (Talukder *et al.,* 2016). Another drinking water study conducted in the same area further concluded that excess concentration of salinity may cause cardiovascular health risks and high intake of sodium accounts for 20% of all stroke deaths in Bangladesh (Scheelbeek *et al.,* 2017). The two drinking water production facilities in the present study produced water that would not be a health risk to any of the consumers.

### 5.1.5 Turbidity

High levels of turbidity were recorded in source (WWTP effluent) and dam water samples from WC-A, as well as mixed raw water samples from WC-F. This was expected. High turbidity concentrations in raw water samples were recorded in other studies as well (Singh and Lin, 2015). It has been reported that high levels of turbidity in raw water sample enhance the ability of microorganisms to resist water treatment processes (Gupta *et al.,* 2017; Adekola *et al.,* 2015). According to Gupta *et al*. (2017) water filter membranes do not

operate optimally on water that has elevated levels of turbidity. This must be reduced prior to membrane filtration as is done in the case of WC-A. Drinking water from WC-A and WC-F had turbidity concentrations less than 0.6 NTU. This is lower than the SANS 241 (2015) standard of 1.0 NTU. This shows that drinking water treatment processes used in WC-A and WC-F were effective in treating turbid water. Even though turbid water has no direct adverse human health effects, it may affect drinking water production efficiency and is an important parameter (Roos *et al.,* 2007).

### 5.1.6 Free chlorine

Free chlorine residues inhibit bacterial regrowth in the distribution networks and prevent contamination of pathogenic bacteria in treated water (Bensoltane *et al.,* 2018; Dippong *et al.,* 2014; Zheng *et al,* 2015). In the present study the free chlorine levels in drinking water at was generally low, with the exception of reclaimed water collected in November 2017 at WC-A. It is important to control free chlorine in drinking because high free chlorine concentrations are not good in drinking water. High concentration of free chlorine forms toxic disinfection by products such as trihalomethane and other carcinogenic compounds (Hoque *et al.,* 2017). The SANS 241 (2015) standard for free chlorine is 5 mg/L. There is thus a trade-off in maintaining sufficient free chlorine in drinking water but to ensure that the levels are not extreme. No free chlorine as well as very high free chlorine could be associated with human health risks.

### 5.1.7 Phosphate

Phosphate concentration was lower in the treated water samples compared to the raw water. However, in the reclaimed water at WC-A (June 2017 sampling) higher phosphates were measured compared to the WWTP effluent. The highest value of 4.74 mg/L was recorded in dam water at WC-A. The presence of phosphate in water is most often associated with agricultural runoffs (Goody *et al,* 2017). However, excess concentration of phosphates in raw water samples promotes plant growth resulting in dense growth of algae and other plants in the water body (Singh and Lin, 2015; Choudhary *et al.,* 2014). Drinking water sources with high phosphate concentrations pose health threats such as muscle damage, breathing problems and kidney failure in humans (Gupta *et al.,* 2017; Nduka *et al* 2008). Extreme, dangerously high levels of phosphates were not measured in the drinking water or even the raw water in the present study, even though one of the source waters was WWTP effluent. However, the presence and levels of phosphates is sufficient to allow for the maintenance an HPC bacterial population.

### 5.1.8 Nitrates and Nitrites

There were lower nitrate levels in the drinking water samples at WC-A and WC-F when compared to the raw water sources. High levels of nitrates in treated water samples are an indication of sewage contamination through seepage or ineffective treatment processes (Orogu *et al.,* 2017; Qureshimatva *et al.,* 2015).

Drinking water samples had nitrites lower than 0.1 mg/L, except for reclaimed water. Nitrites concentrations in raw water recorded by Toure *et al.,* (2018) were all higher than 0.1 mg/L. Nitrites may contaminate treated water through leakage points of water channels (Orogu *et al.,* 2017).

Extremely high nitrate (50 mg/L) and nitrite concentrations (3 mg/L) in drinking water pose health effects to children under the age of 3 (WHO, 2017; Kumar and Puri, 2012). High nitrite levels are also associated with health risks in humans particularly linked to certain cancers (WHO, 2017)

## 5.2 Faecal coliforms and *E. coli*

Faecal coliforms and *E. coli* were more prevalent in wastewater effluent from WC-A and dam water from WC-F. These are raw water sources and this could be expected. In previous studies, faecal coliforms and *E. coli* were also prevalent in raw water samples (Mulamattathil *et al.,* 2015; Samie *et al.,* 2011). In the present study, faecal coliforms and *E. coli* were not detected in drinking water samples. This indicates the effective treatment, particularly disinfection.

Birks *et al*. (2004) conducted a study to investigate the efficacy of a direct reclamation plant at the Millennium Dome, London investigating the capacity to remove indicator microorganisms using ultrafiltration and reverse osmosis for water treatment process (same processes used in the current study). The results have shown that advanced treatment processes are effective in the removal of coliforms (99%) and *E. coli* (100%). In a study by Edokpayi *et al*. (2018), *E. coli* was not detected in municipal tap water from Limpopo Province, South Africa. However, faecal coliforms were detected in drinking water from that study. Fadaei (2014) also detected faecal coliforms in drinking water. The presence of these faecal indicator microorganisms in drinking may not pose a direct health risk, but they are indication of the efficacy of DWPFs. It could imply that the removal of other pathogens such as protozoa parasites and viruses that were not directly subjected to this test are also not removed (Edokpayi *et al.,* 2018). Indicator microorganisms help to minimize risks associated with waterborne diseases that result from consumption of contaminated water (Mann, 2016).

The SANS 241 (2015) standard for drinking water is 0 cfu/100 mL and all the drinking water samples in the present study adhered to this standard.

## 5.3 HPC bacteria

The levels of HPC bacteria were reduced from raw to drinking water samples at WC-A and WC-F. High levels of HPC bacteria were recorded in wastewater effluent, and dam water samples from WC-A and mixed raw water samples from WC-F. However, HPC bacteria were not detected in borehole water samples in WC-A and WC-F from June 2017 sampling. Elevated levels of HPC bacteria were recorded in the wet season, except for distribution networks in the case of WC-A. Similar results were recorded by Shakoor *et al*. (2018). It was demonstrated by various studies that HPC bacteria may pose health risks to water consumers, particularly high risk (Chowdhury, 2011; Xi *et al.,* 2009). Waterborne diseases caused by poor sanitation and contaminated water sources contribute to 80% of the world's health problems (Bedada *et al.,* 2018; Abera *et al.,* 2011). The drinking water from WC-A and WC-F had HPC levels that were within the SANS 241 (2015). This is once again an indication that the drinking water from the two plants provided water of a very high quality.

## 5.4 Correlation of physico-chemical parameters and microbiological agents

There were various studies aimed to determine the influence of environmental parameters on the presence of microbiological agents in raw and drinking water (Bisi-Johnson *et al*., 2017; Amanidaz *et al*., 2015; Fajri *et al*., 2015). These studies provided important information that give scientists an insight of what causes elevated levels of microbiological agents in water and data generated is essential to remedy waterborne diseases. In the present study RDA plots were constructed to determine the correlation of physico-chemical parameters and microbiological agents in raw and drinking water.

The correlation between free chlorine and microbiological agents was not strong, except for samples collected in June 2017 at WC-A. The free chlorine recorded in June 2017 sample at WC-A was closely related with HPC bacteria. There was a higher free chlorine concentration in reclaimed water November 2017 samples compared to the June 2017. This could have been due to operational condition. However, the higher free chlorine levels had an associated lower HPC levels. This was in accordance with the findings of Chowdhury (2011). However, these findings did not comply with other results obtained from other sampling sites from the current study. Free chlorine concentration in other drinking water was insignificant and could not be correlated with lower HPC levels.

There was also a strong correlation of pH and microbiological agents (HPC bacteria). These results were in similar with the results obtained by Amanidaz *et al*. (2015). pH also plays an important role in survival and growth of microbiological agents in water (Palamuleni *et al.,* 2015). Microbiological biological activities are sensitive to pH changes (Palamuleni *et al.,* 2015).

The clustered formed between various sites from WC-A such as reclaimed water, treated water and distribution works, as well as borehole from WC-F were closely related with temperature. Other water samples did not have correlation with temperature. A study by Habuda-Staníc *et al*. (2013) showed that temperature did not have strong effects on the microbiological parameters. However, in a study where temperature strongly correlated with microbiological agents it was suggested that temperature is important in the control of microbiological agents in water (Amanidaz *et al.,* 2015). It was suggested that low temperatures are essential for the control of *E. coli* and faecal coliforms in the absence of free chlorine (Amanidaz *et al.,* 2015; Fajri *et al.,* 2015).

There was a strong correlation between turbidity and microbiological agents in raw water samples from WC-A and WC-F. Similar correlation results were recorded by Obi *et al*. (2008). The authors suggest that turbidity between 3.8 and 84 NTU interferes with the disinfection, leading to increased number of HPC bacteria. The measured turbidity in the current study of raw samples was greater than 3.8, except for borehole water samples. Turbid water creates favourable condition for the growth of microorganisms because nutrients needed are absorbed by suspended particles (Bisi-Johnson *et al*., 2017).

Phosphates recorded in the current study were closely related to the HPC bacteria. Similar results were recorded by Miettinen *et al*. (1997). The authors suggest that even a slight increase in the phosphate concentration influences the growth of microorganisms in water. This was in accordance with data obtained in reclaimed water and treated water samples from WC-A from the present study. A study by Chu *et al*. (2005) supports this finding. In their study they evaluated the growth of microbiological agents before and after phosphate and nitrate were added into the water. The results obtained showed that HPC bacteria growth was rapid in water samples where there was high concentration of phosphates and nitrates. A strong correlation was observed in the present study between nitrates and microbiological parameter at WC-A from November 2017 samples. The HPC bacterial levels was higher in borehole water where there were high nitrate levels in November 2017 compared to June 2017 at WC-A. Phosphates and nitrates reach the water environment through human waste and agricultural runoff (Naylor *et al*., 2018; Gupta *et al*., 2017; Goody *et al*., 2017) but these

nutrients, as shown also in this study are reduced, but not completely removed by the water purification processes. Their levels are still sufficient to maintain heterotrophic microbial populations in drinking water distributions.

## 5.5 Kirby-Bauer susceptibility test

HPC bacteria isolated in borehole water sample from November 2017 were all resistant to trimethoprim, ampicillin and cephalothin. Ogu *et al*. (2017) also found that most bacteria were resistant to ampicillin. Borehole water serves as one of the major sources of drinking water in Western Cape, as the province has low water levels. Boreholes are becoming more recognised in Africa as the main water sources used in the production of drinking water (Chidinma *et al.,* 2016). It is therefore important for borehole water to undergo water treatment processes in the DWPF, to ensure it is safe for human consumption (Samie *et al.,* 2011).

The DWPFs depend on dam and borehole water for production of drinking water. Raw mixed water in the DWPFs contain high microbial density (Li *et al.,* 2017a). The alarming issue is the presence and maintenance of ARB within the DWPFs (Zhang *et al.,* 2016). In the present study, mixed (borehole and dam) raw water was collected only at WC-F for June 2016 and June 2017 sampling. HPC bacteria from WC-F were all resistant to trimethoprim and cephalothin from June 2016 samples. Most of the HPC bacteria were resistant to ampicillin (94.44%) and chloramphenicol (72.22%). Ferro *et al*. (2017) suggested that DWPFs serve as hotspots for antibiotic resistance and that purification processes select for these bacteria. ARB are able to pass their antibiotic determinants to humans through consumption of water that is not properly treated and this pose a great public risks to the public (Xiong and Hu, 2013). Furthermore, even if treated properly, water containing ARBs may pass these to consumers.

Traditional water sources are not sufficient to supply adequate water to the communities in WC-A due to low rainfall and a long term drought in the Province (Grimmer and Tuner, 2013). This has led to the establishment of the plant that reclaims sewage for production of drinking water to compensate for the normal to high demand of drinking water (Matthews, 2015). This wastewater effluent is generally contaminated with waste from homes, hospitals and other small industries. Hence, this wastewater source is expected to be contaminated with high levels of ARB (Garner *et al.,* 2018). HPC bacteria isolated from June 2017 samples showed that all isolates were resistant to cephalothin and ampicillin. Many of the isolates (70%) were also resistant to ciprofloxacin, erythromycin, oxy-tetracycline and trimethoprim.

Similar observations were made by Obayiuwana *et al*. (2018) who studied wastewater and concluded that human medicine was a major source of the observed ARB.

The conventional drinking water treatment processes are not designed to remove ARB from water. Hence, the wastewater effluent in WC-A is subjected to advanced treatment processes (Garner *et al.,* 2018; Matthews, 2015). The advanced water treatment processes include processes such as reverse osmosis and advanced oxidation by the combination of $UV/H_2O_2$. The least ARB in drinking water samples were recorded from recycled wastewater at WC-A for June and November 2017. The detection of high levels of vancomycin, ampicillin and penicillin G (Gram positive bacteria) in reclaimed water serves as an indication of these bacteria may survive and eventually land in the final water (Nwachukwu and Emeruem, 2007).

Treated water at WC-A is produced by blending 30% of reclaimed water from the DPR direct reclamation plant and 70% treated dam and borehole water from DWPF. While WC-F, treated water is produced by mixing dam and borehole water only. The detection of high levels of ARB is an indication of that the conventional treatment processes applied at the DWPFs are ineffective in the removal of ARB (Bergeron *et al.,* 2015). A general trend was observed from WC-A and WC-F in which HPC bacteria were resistant chloramphenicol, trimethoprim, cephalothin and ampicillin in treated water. Similar results found by Mulamattathil *et al*. (2014b), who stated that this could be due to continuous exposure of HPC bacteria to these antibiotics in human and veterinary medicine.

There were higher levels of antibiotic resistant HPC bacteria in the distribution networks compared to water immediately after treatment. Similar results were recorded by Xu *et al*. (2016). In the present study, ARB in the distribution system was resistant to ciprofloxacin, erythromycin, oxy-tetracycline, trimethoprim, cephalothin, ampicillin, and vancomycin. Resistance to larger numbers of antibiotics in distribution networks may be caused by bacterial regrowth (Xu *et al.,* 2016). Bacterial regrowth occurs as a result of the formation of biofilm in the pipes that deliver drinking water to various communities (Srinivasan *et al.,* 2008). Another alarming concern is that distribution networks are not adequately protected against bacterial recontamination, particularly when corroded pipes are replaced. This could result in an increased number of waterborne diseases (Chowdhury, 2011).

It is evident that raw and drinking water are contaminated with ARB. Therefore, it was important use MAR indices to give an overview of the antibiotic exposure history. The MAR index is an important tool to assess whether water environments have low or high antibiotic

use (Davis and Brown, 2016). HPC bacteria from water environments are considered to have a history of constant antibiotic exposure if the MAR is greater than 0.2 (Chitanand *et al.,* 2010). A low MAR index in drinking water for reclaimed water from June and November 2017 sampling was observed. The MAR index for June 2017 sampling was 0.13 and this number was higher (0.25) in November 2017 at WC-A. The MAR indices from other drinking water sources such as treated water and distribution networks range between 0.34 and 0.46. The MAR indices from raw water samples were lower compared to drinking water. Environmental water environments with MAR index greater than 0.4 are considered to be polluted with human faeces and samples with less than 0.4 are from nonhuman faecal pollution (Poonia *et al.,* 2014). This could not be applied to the drinking water in the present study. Another deduction is required for drinking water. It was however, evident that the ARB data from the present study indicated that these had a history for exposure to antibiotics and that the drinking water production processes may be selecting for ARB survival in the treated water.

## 5.6 Identification of HPC bacteria and phylogenetic associations

There was a reduction in the HPC bacteria from raw to drinking water at WC-A and WC-F, as was expected. *Pseudomonas* spp. and *Bacillus* spp. were prevalent in raw and drinking water samples. Similar results were recorded by Burtscher *et al*. (2009). The presence of HPC bacteria in drinking water may due to low free chlorine residues. It is therefore important to increase the free chlorine concentrations to control and prevent recontamination of HPC in drinking water (Bensoltane *et al*., 2018). However, according to the United States Environmental Protection Agency USEPA (2011), free chlorine concentration should not exceed the limits 0.8 mg/L. Excess free chlorine in drinking water may pose health risks such as production of disinfection by-products that could be carcinogenic (Chowdhury, 2011).

Most of the isolated bacterial species in water samples were from the Phylum *Proteobacteria*. Similar observations were made in various studies (Ma *et al.,* 2017b; Liao *et al.,* 2014; Chen *et al.,* 2013; Williams *et al.,* 2004). Since *Proteobacteria* were present in raw and drinking water sources, a special attention is needed to control the occurrence of the species from this Phylum. Various studies demonstrated that they are associated with antibiotic resistance and pathogenicity which may pose public health threats (Liao *et al.,* 2014; Vaz-Moreira *et al.,* 2013). However, a decrease of *Proteobacteria* from raw to drinking water is an indication that bacteria special are susceptible to the activity of chlorination (Ma *et al.,* 2017b).

Special attention should also be diverted to the *Firmicutes,* since some bacterial species from such as *Bacillus wiedmannii* are resistant disinfection. Similar results were recorded by Chen *et al*. (2013) which show that the presence of *Bacillus* spp. in drinking water may lead to potential health risks to humans. Furthermore, *Firmicutes,* such as *Cohnella xylanilytica, Bacillus zhangzhouensis, Bacillus tequilensis,* and *Bacillus glycinifermentans*are also of great concern since they were only isolated in drinking water. Similar results were obtained by Eichler *et al*. (2006) who observed that more than half phyla were isolated in drinking water were not present in raw water sources. Bacteria species from phylum *Bacterioides* were only detected in raw water samples The persistence and growth of antibiotic resistant microorganisms in drinking water is a worldwide problem since they are the major sources of waterborne infections (Bedada *et al.,* 2018; Abera *et al.,* 2011).

## 5.7 Pathogenicity: haemolysis and extracellular enzyme assays

The HPC bacteria isolated from water samples at WC-A and WC-F are potential pathogens based on their haemolysis and extracellular assay tests. HPC bacteria in drinking and raw water samples are mostly β-heamolytic. β-heamolytic bacteria are associated with the complete breakdown of red blood cells (Horn *et al.,* 2016). While some are α-haemolytic, this denotes incomplete breakdown of the red blood cells (Pakshir *et al*., 2013) but ability to grow in the presence of blood cells. As shown in the results section (Tables 16 and 17), HPC bacteria could be classified as potential pathogens based by displaying genotypic, and phenotypic resistance and ability to breakdown (α and β haemolysis) red blood cells. HPC bacteria that harbour at least one ARG, with the ability to confer resistance to antibiotics and breakdown (α and β haemolysis) red blood cells were selected and their extracellular enzymes are discussed below:

### 5.7.1 *Pseudomonas* spp.

The virulence of *Pseudomonas* spp was investigated in several studies (Mulamattathil *et al.,* 2014b; da Silva *et al.,* 2008). In the present study, potential pathogenic *Pseudomonas* spp. were detected in raw and drinking samples from WC-A. All *Pseudomonas reinekei* isolated in wastewater effluent produced the same extracellular enzymes, which included DNase, gelatinase and lecithinase. While, *Pseudomonas chengduesis* tested positive to lecithinase only. A study by Georgescu *et al.* (2016) shown that *Pseudomonas* spp. from clinical specimens produce lipase, gelatinase, lecithinase and DNase. *Pseudomonas* spp. are able to survive extreme environmental conditions and their presence in drinking water may lead cystic infection, septicaemia, pneumonia, endocarditis, otitis and keratitis (Mulamattathil *et al.,* 2015). Finding such species in drinking water is thus of concern.

### *5.7.2 Rhodoferax saidenbachensis*

*Rhodoferax saidenbachensis* belonging to the family *Comamonadaceae* is a Gram-negative, rod-shaped and polar flagellated bacterium (Kaden *et al.,* 2014). *Rhodoferax saidenbachensis* was detected in dam water from WC-A. *Rhodoferax saidenbachensis* was isolated in water environment from various studies (Vandermaesen *et al.,* 2018; Kaden *et al.,* 2014). The pathogenicity tests revealed that this bacterium is beta-haemolytic which was in accordance with findings of Kaden *et al*. (2014). This bacterium tested positive to DNase, gelatinase and lecithinase. However, the extracellular enzyme assays of *Rhodoferax saidenbachensis* was not found on the searched database. This species was not isolated from drinking water. However, since the selection of isolates was a random process, it may be possible that is was missed and it can thus not be concluded that it was not present in the drinking water.

### 5.7.3 *Undibacterium jejuense*

*Undibacterium jejuense* belonging to the family *Oxalobacteraceae*is are Gram-negative, rod-shaped, oxidase-positive, non-motile and non-spore forming species (Du *et al.,* 2015; Kämpfer *et al.,* 2007). *Undibacterium jejuense* was detected in dam water from WC-A. This bacterium was also detected in surface water as well as drinking water from the DWPF that uses rapid sand filtration (Vandermaesen *et al.,* 2018). It produced DNase, lipase and lecithinase. A study conducted by Kim *et al*. (2014) shows that *Undibacterium jejuense* hydrolyse lipase as shown in the current study, but however, it was DNase negative and lecithinase tests were not done. It thus appears that this species has the ability to survive the treatment processes.

### 5.7.4 *Bacillus* spp.

Potential pathogenic *Bacillus* spp. were more frequent in drinking water, from WC-A and WC-F. Similar results were obtained from various studies (Chen *et al.,* 2013; Pavlov *et al.,* 2004). DNase (90.91%) was produced by most of the *Bacillus* spp., followed by lecithinase (81.82%). Proteinase was produced by 63.64% HPC bacteria. A low number (36.36%) of isolates produced gelatinase and lipase. *Bacillus* spp. that produce gelatinase and lipase were not detected in wastewater effluent. The study by Pavlov *et al*. (2004) indicates that *Bacillus* spp. produce DNase, gelatinase, proteinase, as well as fibrinolysin. The latter enzyme was not tested in the present study. However, lecithinase producing bacteria were not detected in their study.

DNase and lecithinase were the more prevalent in water samples. An equal number of 18 out of 21 DNase and lecithinase positive virulent bacteria were recorded in water samples. The DNase enzyme evade the innate immune response in the host by degrading neutrophil extracellular traps (NETs) and also has the capability to kill bacteria by interfering with DNA synthesis (Sumby *et al.,* 2005). Furthermore, DNase facilitates the spread of infectious bacteria by liquefying pus (Sumby *et al.,* 2005). The DNase enables the pathogenic bacteria to overcome the host's innate immune response (Anbalagan and Chaussee, 2013). The high level of lecithinase has a large influence on the host's immune response (Thomas *et al.,* 2014). Lecithinase leads to the break down of erythrocytes, myoctes, fibroblasts platelets and white blood cells (Sharaf *et al.,* 2014).

Gelatinase and lipase were each produced by 42.86% of the HPC bacteria. Gelatinase enzymes play a major part in biofilm formation and also weaken the host immune respond through degradation of collagen, fibrinogen, fibrin, endothelin-1, bradykinin, LL-37, and complement component C3 and C3a (Thurlow *et al.,* 2010). Lipase enzymes enable the bacteria to survive in environments where carbohydrates are limited (Thomas *et al.,* 2014; Park *et al.,* 2013). In these environments, lipids are the sole carbon source and facilitate bacterial adhesion to the host tissue (Thomas *et al.,* 2014; Park *et al.,* 2013). Lipase weakens the host immune response (Park *et al.,* 2013). High proteinase positive isolates were detected in drinking water samples. The enzyme proteinase is associated with the invasion of the host cell with the purpose of causing damage to their tissues and subsequently weakening the host-defence (Thomas *et al.,* 2014). It is thus with concern that it is reported that bacteria that produced the various extracellular enzymes were present in the drinking water of the two drinking water distribution systems.

## 5.8 Detection of ARGs

Wastewater treatment plants (WWTPs) and DWPFs are not designed to remove or inhibit the survival of ARB and ARGs, as indicated by various studies (Yang *et al.,* 2017; Ju *et al.,* 2016). WWTPs and DWPFs serve as hotspots for ARB to share and incorporate their ARGs which are taken up (via various processes such transformation, transduction etc.; Lu *et al.,* 2018; Tehrani and Gilbride, 2017; Shi *et al.,* 2013). Shakoor *et al*. (2018) stated that although the overall trends in quantity of ARB may correlate with ARGs, the potential role of drinking water in transmission and acquisition of antibiotic resistance in human remains unknown. ARGs were investigated in raw and drinking samples from WC-A and WC-F (June 2017 sampling only). However, traditional laboratory culture based methods have limitations; they do not allow cultivation of viable but non-culturable cells (VBNC; Fakruddin *et al.,* 2013). The study by Horn *et al*. (2016) suggested that the more studies should be conducted for the

detection and analysis of the eDNA. VBNC might pose health risks and since they cannot be detected through traditional laboratory methods their effects are often neglected.

### 5.8.1 *ampC*

The *ampC* gene was not detected in eDNA. The *ampC* was only detected in drinking water isolates from WC-A. A total of 2 *ampC* positive colonies were isolated from treated water and distribution networks. Several studies also detected this gene in drinking samples (Fernando *et al.,* 2016; Shi *et al.,* 2013). Fernando *et al*. (2016) went further to investigate the occurrence of this gene in raw water samples. This gene was not detected in raw water samples from that study. These results correspond with the results obtained from the present study. However, the *ampC* gene was also detected in wastewater samples by Schwartz *et al*. (2003). The *ampC* gene confers resistance to cephalosporins and cephamycins (Shanthi *et al.,* 2012). This gene is associated with clinical environments. Therefore, the detection of this gene plays a vital role in the surveillance and managing activity of antibiotic resistance to curb the occurrence of infections (Khari *et al.,* 2016)

### 5.8.2 *bla$_{TEM}$*

The *bla$_{TEM}$* gene was not detected in eDNA and HPC bacteria from WC-A and WC-F. However, several studies have detected the presence of *bla$_{TEM}$* gene in water samples (Adesoji and Ogunjobi, 2016; Xi *et al.,* 2009). This gene was detected in raw and drinking water samples by Xi *et al*. (2009). Adesoji and Ogunjobi (2016) detected this gene in distribution networks only. The *bla$_{TEM}$* encodes resistance for ampicillin (Muhammad *et al.,* 2014). The *bla$_{TEM}$* gene may enter environment through sewage effluent (Lachmayr *et al.,* 2009).

### *5.8.3 tetM*

The *tetM* gene was not detected in eDNA and HPC bacteria. The *tetM* was also not detected in other studies as well (Stange *et al.,* 2016; Yuan *et al.,* 2015). The resistance mechanism of this gene is ribosomal protection protein (Tehrani and Gilbride, 2017; Chen *et al.,* 2017). The spread of *tetM* is associated with the facilitated by the conjugative transposons of the mobile elements (Haubert *et al.,* 2015).

### 5.8.4 *Intl1*

The *Intl1* gene was detected in eDNA isolated in wastewater from WC-A and mixed raw water from WC-F. The *Intl1* gene was mostly prevalent in drinking water at WC-A in HPC bacteria. A total of 3 out 4 *Intl1* genes were detected in distribution networks. However,

Obayiuwana *et al*. (2018) detected *Intl1* gene mostly in wastewater samples. This gene is associated with anthropogenic activities and its presence in drinking water makes it good indicator that measures human pollution (Koczura *et al.,* 2016). The presence of this gene in water environment increases the change of ARG to spread and incorporate its resistance determinants to other bacterial species, including non-pathogenic ones (Obayiuwana *et al.* 2018).

### 5.8.5 *ermB and ermF*

The *ermB* gene was detected in eDNA were from wastewater at WC-A and mixed raw water at WC-F. While, the *ermB* was the most abundant gene in HPC bacteria in raw and drinking water samples from WC-A and WC-F. This gene was also detected in high levels by Shi *et al*. (2013). The *ermF* was detected in both raw and drinking water. However, it was more prevalent in drinking samples. The *ermB* and *ermF* encode resistance to many classes of antibiotics that include macrolides, lincosamides and streptogramin B (Bergeron *et al.,* 2015). These genes confer resistance to antibiotic by methyltransferase which is involved in the modification 23S rRNA of the bacterial ribosome (Dzyubak and Yap, 2016; Bergeron *et al.,* 2015).

In summary, the most commonly used disinfection process, chlorination, selects for antibiotic resistance in water environment (Yuan *et al.,* 2015). In the current study ABR were more prevalent in treated samples than raw water samples. The general trend of antibiotic resistance was as follows; raw water < treated water < distribution networks. The presence of ARB and their associated genes in drinking water is a major public health concern and global challenge (Naylor *et al.,* 2018; Adefisoye and Okoh, 2016; Lin *et al.,* 2016b). ARB and their associated genes colonize the gastrointestinal tract of humans, through the food chain (Khan *et al.,* 2016). Once ARB have colonized the gastrointestinal tract they may be involved conjugal transfer of antibiotic resistance to the normal flora in the leading to more multiple antibiotic resistance (Tagoe *et al.,* 2011). Consumption of drinking water that is contaminated with ARB and ARGs has a negative impact on the antibiotic therapeutic treatment such as prolong treatment of waterborne diseases and possibly treatment failure (Barancheshme and Munir, 2018; Zhang *et al.,* 2017). Individuals who are at risk to antibiotic resistance include children, immunocompromised patients and elderly people (Khan *et al.,* 2016; Xiong and Hu, 2013). This is major section of the community of the Western Cape population and processes should be put in place to protect them from ARBs and ARGs.

## 5.9 Detection of antibiotic residues in water samples

A total of 7 out 13 antibiotic residues were not detected in water samples. The ones detected included trimethoprim, penicillin, streptomycin, oxy-tetracycline, and chloramphenicol Antibiotics such as erythromycin and sulfamethoxazole were absent in the current but present in the study by Asghar *et al*. (2018). Most of the antibiotic residues were detected in wastewater samples from the DRP plant as compared mixed raw sample at WC-F that comprises of dam and borehole water. Similar results were produced in a study where wastewater samples harboured more antibiotic resistance residues than surface and drinking water (Ferrer *et al.,* 2010; Batt *et al.,* 2008). Results produced by Asghar *et al*. (2018) show that antibiotic residues are present low concentrations in water environments. Similar results were recorded in the present study. Antibiotic residues have physiological effect in organisms inhabiting water environments (Ebele *et al.,* 2017; Ferguson *et al.,* 2013). However, the impacts they pose on human is unknown (Ebele *et al.,* 2017).

# CHAPTER 6

# CONCLUSION AND RECOMMENDATIONS

## 6.1 Conclusion

The aim of the study was to determine the incidence and characteristics of antibiotic resistant bacteria in raw and drinking water from two Western Cape water production facilities. To achieve this goal, the following specific objectives were achieved:

### 6.1.1 Physico-chemical and the general microbiological quality of water

There were no irregularities with the measured physico-chemical parameters at WC-A and WC-F. The physico-chemical parameters of drinking water at these two sites were within the limits set by the SANS 2015:241. This is an indication of the treatment processes are working effectively. However, the relatively low free chlorine in treated water and distribution networks may increase the survival chances of microbiological agents from raw to drinking water (Zheng *et al,* 2015).

There were no alarming concerns with the microbiological parameters in drinking water. Faecal coliforms and *E. coli* were not detected in drinking water. The absence of indicator bacteria in drinking water shows that the DWPFs and DPR plants do not harbour other pathogens (Edokpayi *et al.,* 2018). There was a general trend observed, where the number of HPC bacteria decreased after water treatment at WC-A and WC-F. HPC bacteria were not detected in distribution networks at WC-A and WC-F for June 2017 sampling. The reduction of HPC bacteria in drinking water sample is an indication that the water treatment processes employed in the DWPFs and DRP plant are effective.

### 6.1.3 Antibiotic resistant patterns and their associated genes

There was an increase in the levels ARB as follows; raw water < treated water < distribution networks, except for reclaimed water at WC-A. This trend may be caused by biofilm formation and bacteria regrowth (Xu *et al,* 2016; Srinivasan *et al.,* 2008). High numbers of ARBs in drinking water shows that the commonly used disinfection process, chlorination selects for antibiotic resistance in water (Yuan *et al.,* 2015). Antibiotics that were associated with resistance include chloramphenicol, trimethoprim, cephalothin and ampicillin. Resistance associated with these antibiotics may render them ineffective when treating waterborne diseases (Nwachukwu and Emeruem, 2007). The MAR indices revealed that most of HPC bacteria from sampling sites were exposed to antibiotics. Furthermore, ARGs were detected in both eDNA and HPC bacteria. A pattern was observed in the ARGs

detected in the current study. The *ermB, ermF, intl 1* and *ampC* detected in HPC bacteria were prevalent in drinking water samples. The *ermB* and *intl1* were only raw water samples only from the eDNA.

### 6.1.4 Antibiotic resistant HPC bacteria

Water samples harboured variety of microorganisms, of which *Bacillus* and *Pseudomonas* were the most prevalent genera in water samples. HPC bacteria that were identified in drinking water include *Bacillus wiedmanni, Massilia pinisoli, Massilia brevitalea, Pseudomonas protegens, Novosphingobium panipatense, Staphylococcus epidermisis, Chryseobacterium hispanicum, Bacillus cereus, Porphyrobacter mercurialis, Pantoea eucrina, Cohnella xylanilytica, Bacillus, glycinifermentans* and *Bacillus mobilis.* Many of these are known as pathogens/opportunistic pathogens.

HPC bacteria were classified as potential pathogens based on their ability to display phenotypic and genotypic antibiotic resistance, lyse red blood cells and synthesize at least one extracellular enzyme. A total of 21 HPC bacteria from this study were classified as potential pathogens. *Bacilli were* the most potential pathogens identified in this study, followed by *Pseudomonas sp..* Extracellular enzymes are defence mechanisms produced by bacterial species which have harmful effects to the host cell by damaging host defence macromolecules such as mucus, lipoprotein membranes, DNA, proteins and immunoglobulins (Pavlov *et al.,* 2004). It is thus with concern that it is noted that the isolated HPC bacteria in the present study was resistant to several antibiotics and that these produced various extracellular enzymes that is associated with virulence.

### 6.1.5 Antibiotic residues in raw and drinking water

Antibiotic residues that were detected in water samples include trimethoprim, penicillin, streptomycin and chloramphenicol. These antibiotic residues in the water environment are not associated with health risk effects to human but could influence the organisms in the water environment (Ebele *et al.,* 2017; Ferguson *et al.,* 2013).

In summary, the aim and objectives of the study were achieved. It was determined that the DWPFs produce good quality drinking water that has physico-chemical parameters that are within the SANS 2015:241 for drinking. However, drinking water contains emerging contaminants of concern such as ARGs, ARB and antibiotic residues (albeit in low concentrations). The incidence of emerging contaminants of concern in drinking water is due to the improper and extensive usage of antibiotics in veterinary medicine, human medicine, and agriculture. As shown in the results and discussion section, HPC bacteria in drinking

water are potential pathogens that are associated with weakening the host's immune response. Individuals who are more susceptible are children, elderly people and immunocompromised patients. Therefore, proper drinking water practices are implemented because South Africa has the population of 11.2% of people who are living with AIDS; therefore, it is important to ensure that the DWPFs produce water that is safe for these consumers (Horn *et al.,* 2016).

## 6.2 Recommendations

Due to the data gathered and as parameters have shown a further investigative study is necessary to look in depth into the health related impacts of the bacterial species which were identified and also found that they contain virulence factors thus causing a public health concern. Policy makers of the SANS of drinking water and governmental sectors should aid in funding such a study so as to protect the public but also save costs that may arise if such scientific evidence is not given enough/urgent attention.

A similar or continuation of the current study is necessary. One of the objectives must be focused on the quantification of ARGs by real-time PCR. Real-time PCR is used for its supremacy to determine the initial concentration of ARGs detected in raw water and their final concentrations in drinking water from HPC bacteria and eDNA. This technique is essential in determining whether the DWPFs are effective in the removal of ARGs.

Rapid and economical enzyme-linked immunosorbent assays (ELISAs) must be conducted for quantification antibiotic residues in water samples. ELISAs are sensitive and can detect very low antibiotic residues, which are essential in the determining whether DWPFs and DPR plants are effective in the removal of antibiotic residues in drinking water.

Furthermore, with such substantial data that was gathered from the current study there is a need for whole-genome sequencing, which will not only give insight into the world of these identified bacterial species but also be able to trace their lineage and possibly find innovative remediation solutions.

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# APPENDIX A

**Table 13: Antibiotic susceptibility patterns of the HPC bacteria from WC-A (June 2017)**

| site | isolate | Chl | CIP | Ery | Kan | O-T | Neo | TM | KF | AMP | St | P | Va |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Raw 4** | 1 | R | I | R | S | R | S | R | R | R | S | | |
| | 2 | S | nd | nd | S | S | S | S | R | R | nd | | |
| | 3 | R | S | R | S | R | R | R | R | R | I | | |
| | 4 | S | S | R | S | R | S | R | R | R | I | | |
| | 5 | R | S | R | S | I | S | R | R | R | S | | |
| | 6 | R | S | R | S | I | S | R | R | R | S | | |
| | 7 | R | S | R | S | R | S | R | R | R | I | | |
| | 8 | R | S | R | S | R | S | R | R | R | S | | |
| | 9 | R | S | R | S | R | I | R | R | R | I | | |
| | 10 | R | S | R | S | R | S | R | R | R | S | | |
| **Raw 3** | 1 | S | I | S | I | S | S | R | R | R | S | | |
| | 3 | S | S | S | S | S | S | R | R | R | S | R | S |
| | 4 | S | S | S | S | S | S | S | S | S | S | | |
| | 5 | S | S | S | S | S | S | R | R | R | I | | |
| | 6 | S | S | S | R | S | I | R | R | R | S | | |
| | 7 | S | S | S | S | S | S | R | R | R | S | | |
| | 8 | S | S | S | S | S | S | S | R | R | S | | |
| | 9 | S | S | S | S | S | S | S | S | S | S | | |
| | 10 | S | R | R | R | S | R | R | S | S | I | | |
| | 11 | S | I | S | S | S | R | R | R | R | S | | |
| | 12 | S | S | S | S | S | S | S | S | R | S | | |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 13 | R | S | S | S | S | S | nd | nd | nd | S | | |
| | 14 | S | S | S | S | S | S | nd | nd | nd | S | | |
| **RW** | 1 | S | S | S | S | S | S | S | I | I | S | | |
| | 4 | S | S | S | S | S | S | S | I | R | S | | |
| | 5 | S | S | S | S | S | S | S | I | R | S | R | R |
| | 6 | S | S | S | R | S | I | I | S | S | S | | |
| | 7 | S | R | S | S | S | S | R | S | R | S | S | S |
| **AT** | 1 | I | S | I | S | S | S | S | S | S | S | | |
| | 2 | R | R | R | ed | R | R | R | R | R | R | R | R |

Raw 4-wastewater; Raw 3-dam water; RW-reclaimed water; AT; treated water D-distribution networks; AMP- Ampicillin; KF – Cephalothin; Chl – Chloramphenicol; CIP- Ciprofloxacin; Ery- Erythromycin; Kan – Kanamycin; Neo- Neomycin; O-T – Oxy-tetracycline; P - Penicillin G; St- Streptomycin; TM – Trimethoprim; Va- Vancomycin; S – Susceptible; R – resistance; I – Intermediate resistant; nd – no growth; RO final treated reclaimed water; AT - After treatment, mixed water

**Table 14: Antibiotic susceptibility patterns of the HPC isolates from WC-A (November 2017)**

| site | isolate | Chl | CIP | Ery | Kan | O-T | Neo | TM | KF | AMP | St | P | Va |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Raw 1** | 1 | S | S | S | S | S | S | S | R | R | R | | |
| | 2 | R | S | R | S | I | S | S | R | R | R | | |
| | 3 | R | S | R | S | S | S | S | R | R | R | | |
| | 4 | R | S | R | S | I | S | S | R | R | R | | |
| | 5 | R | I | I | S | I | S | S | R | R | R | | |
| | 6 | R | S | R | S | R | S | S | R | R | R | | |
| | 7 | S | S | S | S | S | S | S | S | S | S | | |
| | 8 | S | S | S | S | S | S | S | R | R | S | | |
| | 9 | I | S | I | S | I | S | S | R | R | R | | |
| | 10 | S | S | S | S | S | S | S | I | S | R | | |
| | 11 | S | R | S | S | R | S | I | R | R | R | R | S |
| **RAW 2** | 2 | S | S | S | S | S | S | R | R | R | S | | |
| | 3 | S | I | I | S | I | S | R | R | R | S | | |
| **RO** | 1 | S | S | S | S | S | S | S | S | S | S | R | R |
| | 2 | S | S | R | S | S | S | S | S | R | S | R | R |
| **AT** | 1 | R | S | R | S | S | S | R | R | R | S | | |
| | 2 | S | I | R | S | S | S | R | R | R | S | R | R |
| **D** | 1 | S | S | S | S | S | S | S | S | S | S | | |
| | 2 | R | S | R | S | R | S | R | R | I | R | | |
| | 3 | S | S | R | I | S | I | S | S | S | R | | |
| | 4 | S | S | R | S | I | S | R | R | R | S | R | R |
| | 5 | S | I | R | S | S | S | R | R | R | R | R | S |
| | 6 | S | I | R | S | R | S | R | R | R | S | R | R |
| | 7 | S | S | I | S | R | S | R | R | R | S | | |
| | 8 | S | S | R | S | I | S | R | R | R | R | R | R |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 9 | S | I | S | S | R | S | R | R | R | S | R | S |
| 10 | S | I | S | S | I | S | R | R | R | S | | |
| 11 | S | S | S | S | S | S | S | S | S | S | R | R |
| 12 | S | I | S | S | I | S | R | R | R | R | R | R |
| 13 | I | R | R | I | S | I | I | S | S | R | S | S |
| 14 | S | I | S | S | S | S | R | R | R | S | | |
| 16 | S | I | S | S | S | S | R | R | R | S | | |
| 18 | S | S | S | S | S | S | R | I | R | S | R | R |

Raw 4-wastewater; Raw 1: Borehole; Raw 3-dam water; RW-reclaimed water; AT; treated water D-distribution networks; AMP- Ampicillin; KF – Cephalothin; Chl – Chloramphenicol; CIP- Ciprofloxacin; Ery- Erythromycin; Kan – Kanamycin; Neo- Neomycin; O-T – Oxy-tetracycline; P - Penicillin G; St- Streptomycin; TM – Trimethoprim; Va- Vancomycin; S – Susceptible; R – resistance; I – Intermediate resistant; nd – no growth

**Table 15: Antibiotic susceptibility patterns of the HPC isolates from WC-F (June 2016)**

| Site | Isolate | Chl | CIP | Ery | Kan | O-T | Neo | TM | KF | AMP | St | P | VA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Raw 1** | 3 | S | S | S | S | S | S | R | R | R | S | | |
| | 4 | I | S | S | I | I | S | R | R | R | S | R | R |
| | 6 | S | S | S | S | S | S | R | R | R | S | R | R |
| | 9 | S | S | R | S | R | R | R | R | R | I | | |
| **Raw 3** | 1 | S | S | S | S | S | S | R | R | R | S | | |
| | 4 | nd | nd | nd | S | nd | nd | nd | R | nd | nd | | |
| | 5 | S | S | R | S | S | S | R | R | S | S | | |
| | 6 | S | S | S | S | S | S | R | R | R | S | | |
| | 7 | S | S | S | S | I | S | R | R | R | S | | |
| | 9 | S | S | S | S | I | S | R | R | R | S | | |
| | 11 | S | S | S | I | S | S | R | R | R | S | | |
| | 13 | S | R | R | I | S | I | R | R | R | I | | |
| | 18 | S | S | S | S | S | S | R | R | R | nd | | |
| | 21 | S | S | S | S | S | S | R | S | S | S | R | S |
| **Raw 2** | 1 | S | S | S | S | S | S | R | R | S | S | | |
| | 2 | S | S | R | I | R | I | R | R | R | S | | |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3 | S | S | S | R | R | S | R | R | R | S | | |
| | 4 | S | S | S | I | S | S | R | R | R | S | R | S |
| | 5 | S | S | S | I | I | S | R | R | R | S | | |
| | 6 | S | S | S | S | I | S | R | R | R | S | | |
| | 8 | S | S | S | S | S | S | R | R | R | S | R | S |
| | 9 | I | S | S | R | I | S | R | R | R | S | | |
| | 11 | S | S | S | S | I | S | R | R | R | S | R | R |
| | 12 | S | S | S | S | S | S | R | R | R | S | | |
| | 13 | S | S | I | I | S | S | R | R | R | S | | |
| | 14 | S | S | I | S | S | S | R | R | R | S | | |
| | 15 | S | S | S | S | S | S | R | R | R | S | | |
| | 17 | I | S | S | S | I | S | R | R | R | S | | |
| | 18 | S | S | S | S | I | S | R | R | R | S | R | R |
| | 19 | S | S | I | S | I | S | R | R | R | S | | |
| | 20 | S | S | S | S | I | S | R | R | R | S | | |
| | 21 | S | S | I | R | I | S | R | R | R | S | | |
| **AT** | 1 | I | S | I | I | R | S | R | R | R | S | R | R |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | S | S | S | S | I | S | R | R | R | S | R | R |
| | 3 | S | S | I | I | S | S | R | R | R | S | R | R |
| | 4 | S | S | S | I | S | S | R | R | R | S | | |
| | 5 | S | S | S | I | R | S | R | R | R | S | | |
| | 6 | S | S | S | S | I | S | R | R | R | S | | |
| | 7 | S | S | S | R | R | S | R | R | R | S | R | R |
| **D1** | 1 | S | S | S | S | I | S | R | R | R | S | | |
| | 2 | S | S | S | I | I | S | R | R | R | S | R | R |
| | 3 | S | S | S | S | I | S | R | R | R | S | | |
| | 4 | S | S | I | I | R | S | R | R | R | S | | |
| | 7 | I | S | S | I | I | S | R | R | R | S | | |
| | 8 | S | S | R | I | R | S | R | R | R | S | | |
| | 9 | S | S | S | S | I | S | R | R | R | S | | |
| | 1a | S | S | S | S | I | S | R | R | R | S | | |
| | 2a | S | S | S | S | I | S | R | R | R | S | R | S |
| | 3a | S | S | I | I | R | S | R | R | R | S | R | R |
| | 5a | S | S | R | R | R | I | R | R | R | S | | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6a | S | S | I | I | S | S | R | R | R | S | R | R |
| 7a | S | S | I | S | I | S | R | R | R | S | R | R |
| 9a | S | S | S | I | I | S | R | R | R | S | | |
| 12a | S | S | S | I | I | S | R | R | R | S | R | R |
| 13a | S | S | R | R | R | S | R | R | R | R | | |
| 14a | S | S | S | I | I | S | R | R | R | S | | |

Raw 1-borehole water; Raw 2-mixed raw water; Raw 3-dam water; AT; treated water; D-distribution networks; AMP- Ampicillin; KF – Cephalothin; Chl – Chloramphenicol; CIP- Ciprofloxacin; Ery- Erythromycin; Kan – Kanamycin; Neo- Neomycin; O-T – Oxy-tetracycline; P - Penicillin G; St- Streptomycin; TM – Trimethoprim; Va- Vancomycin; S – Susceptible; R – resistance; I – Intermediate resistant; nd – no growth

**Table 16: Antibiotic susceptibility patterns of the HPC isolates from WC-F (June 2017)**

| Site | Isolate | Chl | CIP | Ery | Kan | O-T | N | TM | KF | AMP | St | P | Va |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Raw 2** | 1 | S | S | S | S | S | S | R | R | R | S | | |
| | 2 | S | R | S | S | R | I | R | R | R | R | | |
| | 3 | I | S | R | S | S | S | S | S | S | S | | |
| | 4 | I | S | S | S | S | S | S | S | S | S | S | S |
| | 5 | S | S | S | I | S | R | R | R | R | R | | |
| | 6 | R | S | S | S | S | S | S | S | S | S | | |
| | 7 | R | S | R | S | R | R | R | R | R | R | | |
| **AT** | 1 | S | S | S | S | S | S | S | S | S | S | | |
| | 2 | S | S | S | S | S | S | S | I | R | S | | |
| | 3 | S | R | R | S | nd | S | R | R | R | S | S | S |
| | 4 | S | S | R | S | S | S | S | S | S | S | S | R |
| | 5 | R | S | I | S | S | S | R | R | R | S | S | S |
| | 6 | R | R | R | R | R | R | R | R | R | R | R | R |
| | 7 | S | I | I | R | S | S | R | R | R | S | R | R |
| | 8 | S | S | S | S | S | S | S | R | R | S | S | S |
| | 9 | R | R | nd | R | nd | R | R | R | R | R | R | R |

Raw 1-borehole water; Raw 2-mixed raw water; Raw 3-dam water; AT; treated water; D-distribution networks; AMP- Ampicillin; KF – Cephalothin; Chl – Chloramphenicol; CIP- Ciprofloxacin; Ery- Erythromycin; Kan – Kanamycin; N- Neomycin; O-T – Oxy-tetracycline; P - Penicillin G; St- Streptomycin; TM – Trimethoprim; Va- Vancomycin; S – Susceptible; R – resistance; I – Intermediate resistant; nd – no growth

# APPENDIX B

**Table 17: The identity of the isolates that were determined by 16s rDNA sequencing from WC-A June and November 2017**

| Date | Site | GenBank ID | Accesion number | Similarities % | No. of isolates |
|---|---|---|---|---|---|
| Jun-17 | Raw 4 | *Pseudomonas reinekei* | AM293565 | 98.69 | 9 |
| | Raw 3 | *Bacillus wiedmannii* | JH792383 | 99.07 | 1 |
| | | *Rhodoferax saidenbachensis* | AWQR01000064 | 98.11 | 1 |
| | | *Rheinheimera texasensis* | AY701891 | 98.42 | 1 |
| | | *Undibacterium jejuense* | KC735150 | 97.99 | 2 |
| | | *Flavobacterium tructae* | MUHH01000012 | 99.39 | 1 |
| | | *Acidovorax delafieldii* | jgi.1055345 | 99.88 | 2 |
| | | *Pseudomonas protegens* | CP003190 | 99.53 | 1 |
| | | *Bacillus paramycoides* | KJ812444 | 99.65 | 1 |
| | | *Arcicella rigui* | HM357635 | 99.24 | 1 |
| | | *Bacillus mobilis* | KJ812449 | 99.65 | 1 |
| | RW | *Pseudomonas protegens* | CP003190 | 99.36 | 1 |
| | | *Bacillus tequilensis* | AYTO01000043 | 99.88 | 1 |
| | | *Paenibacillus provencensis* | EF212893 | 98.84 | 1 |
| | | *Bacillus simplex* | BCVO01000086 | 96.94 | 1 |
| | | *Hymenobacter seoulensis* | KU758880 | 99.40 | 1 |
| | AT | *Bacillus zhangzhouensis* | JOTP01000061 | 99.30 | 1 |
| | | *Pseudomonas protegens* | CP003190 | 99.41 | 1 |
| Nov-17 | Raw 4 | *Novosphingobium subterraneum* | JRVC01000007 | 99.50 | 1 |
| | | *Pseudomonas chengduensis* | EU307111 | 98.64 | 2 |
| | | *Pseudomonas alcaligenes* | BATI01000076 | 98.45 | 1 |
| | | *Pseudomonas indoloxydans* | DQ916277 | 99.61 | 1 |
| | | *Rhizobium rhizoryzae* | EF649779 | 98.85 | 1 |
| | | *Acidovorax temperans* | AF078766 | 99.17 | 1 |
| | | *Porphyrobacter colymbi* | AB702992 | 98.74 | 1 |
| | | *Bacillus wiedmannii* | LOBC01000053 | 99.06 | 1 |
| | Raw 1 | *Bacillus wiedmannii* | LOBC01000053 | 99.77 | 1 |
| | RW | *Bacillus mobilis* | KJ812449 | 93.41 | 1 |
| | AT | *Bacillus glycinifermentans* | LECW01000063 | 98.48 | 1 |
| | | *Bacillus wiedmannii* | LOBC01000053 | 98.81 | 1 |
| | D | *Cohnella xylanilytica* | FJ001841 | 97.04 | 1 |
| | | *Pantoea eucrina* | CP009880 | 99.77 | 1 |
| | | *Bacillus wiedmannii* | LOBC01000053 | 99.00 | 9 |
| | | *Porphyrobacter mercurialis* | JTDN01000001 | 98.61 | 1 |
| | | *Pseudomonas protegens* | CP003190 | 99.29 | 1 |

Raw 1-borehole water; Raw 2-mixed raw water; Raw 3-dam water; AT; treated water D-distribution

**Table 18: The identity of the isolates that were determined by 16s rDNA sequencing from WC-F June 2016 and June 2017**

| Date | Site | GenBank ID | Accesion number | Similarities % | No. of isolates |
|---|---|---|---|---|---|
| | | *Acinetobacter johnsonii* | APON01000005 | 97.94 | 1 |
| | | *Bacillus toyonensis* | CP006869 | 100 | 1 |
| | | *Chryseobacterium piscium* | AM040439 | 98.09 | 1 |
| | **Raw 1** | *Streptomyces albogriseolus* | AJ494865 | 100 | 1 |
| | | *Acinetobacter johnsonii* | APON01000005 | 99.03 | 1 |
| | | *Rheinheimera aquatic* | GQ168584 | 100 | 1 |
| | | *Chryseobacterium scophthaimum* | AJ271009 | 98.37 | 1 |
| | | *Chromobacterium aquaticum* | EU109734 | 96.26 | 2 |
| | | *Streptomyces flavovirens* | AB184834 | 99.76 | 1 |
| | **Raw 3** | *Kocuria arsenatis* | KM874399 | 100 | 1 |
| | | *Bacillus cereus* | AE016877 | 99.70 | 5 |
| | | *Chitinimonas taiwanensis* | AY323827 | 96.29 | 1 |
| | | *Bacillus vietnamensis* | CLG48530 | 97.47 | 1 |
| | | *Arthrobacte rhumicola* | AB279890 | 99.88 | 1 |
| | | *Bacillus safensis* | ASJD01000027 | 100 | 1 |
| | | *Massilia suwonensis* | FJ969487 | 99.39 | 1 |
| | | *Chryseobacterium sediminis* | KR349467 | 98.84 | 1 |
| | | *Chryseobacterium lactis* | JX100821 | 98.46 | 1 |
| | **Raw 2** | *Chryseobacterium hispanicum* | AM159183 | 99.52 | 1 |
| | | *Bacillus cereus* | AE016877 | 99.67 | 3 |
| | **AT** | *Chryseobacterium hispanicum* | AM159183 | 99.40 | 1 |
| | | *Bacillus cereus* | AE016877 | 99.82 | 11 |
| | | *Staphylococcus epidermidis* | L37605 | 93.13 | 1 |
| **Jun-16** | **D** | *Bacillus bingmayongensis* | AM159183 | 99.40 | 1 |
| | | *Massilia timonae* | AGZI010 | 95.12 | 2 |
| | | *Novosphingobium panipatense* | jgi.1118302 | 98.75 | 1 |
| | | *Pseudomonas protegens* | CP003190 | 98.55 | 2 |
| | **Raw 2** | *Bacillus wiedmannii* | LOBC01000053 | 99.65 | 1 |
| | | *Massilia brevitalea* | EF546777 | 98.58 | 1 |
| | | *Massilia pinisoli* | KU296190 | 98.93 | 1 |
| | | *Bacillus wiedmannii* | LOBC01000053 | 99.44 | 5 |
| **Jun-17** | **AT** | *Pantoea intestinalis* | KP326384 | 99.53 | 1 |

Raw 1-borehole water; Raw 2-mixed raw water; Raw 3-dam water; AT; treated water D-distribution networks

# APPENDIX C

**Table 19: Haemolysis test results for WC-A (June and November 2017) and WC-F (June 2016)**

| Location and Date | Site | No of isolates tested | Beta-haemolysin | Alpha-haemolysin |
|---|---|---|---|---|
| **WC-A June 2017** | Raw 4 | 10 | 5 | 2 |
| | Raw 3 | 14 | 10 | 0 |
| | RW | 7 | 4 | 1 |
| | AT | 2 | 1 | 0 |
| **WC-A November 2017** | Raw 4 | 11 | 6 | 4 |
| | Raw 1 | 2 | 0 | 1 |
| | RW | 2 | 0 | 1 |
| | AT | 2 | 0 | 2 |
| | D | 16 | 12 | 4 |
| **WC-F June 2017** | Raw 2 | 7 | 5 | 2 |
| | AT | 10 | 5 | 2 |

Raw 1-borehole water; Raw 2-mixed raw water; Raw 3-dam water; AT; treated water D-distribution

**Table 20: Extracellular enzymes assay results from WC-A and WC-F**

| Location and Date | Site | No of isolates tested | Dnase | Gelatinase | Lipase | Lecithinase | Proteinase |
|---|---|---|---|---|---|---|---|
| **WC-A June 2017** | Raw 4 | 7 | 6 | 4 | 2 | 5 | 6 |
| | Raw 3 | 10 | 8 | 4 | 0 | 8 | 4 |
| | RW | 5 | 4 | 4 | 4 | 3 | 3 |
| | AT | 1 | 1 | 1 | 1 | 1 | 1 |
| **WC-A November 2017** | Raw 4 | 10 | 8 | 5 | 4 | 7 | 5 |
| | Raw 3 | 1 | 0 | 1 | 0 | 1 | 0 |
| | RW | 1 | 1 | 0 | 0 | 1 | 1 |
| | AT | 2 | 2 | 1 | 1 | 2 | 2 |
| | D | 16 | 12 | 7 | 5 | 11 | 9 |
| **WC-F June 2017** | Raw 2 | 7 | 5 | 4 | 6 | 5 | 5 |
| | AT | 7 | 6 | 3 | 3 | 6 | 5 |

Raw 1-borehole water; Raw 2-mixed raw water; Raw 3-dam water; AT; treated water D-distribution networks