Characterization of *Clostridium*, *Aeromonas* and heterotrophic bacteria in selected groundwater systems

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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 have been duly acknowledged.

Onalenna Mabeo Date

…… …….

DEDICATION

This dissertation is dedicated to the following:

1.God of creation. Lord you are faithful. Let this be for your glory.

“Exodus 15:2 The Lord is my strength and my song; he has given me victory. This is my God, and I will praise him— my father’s God, and I will exalt him!”

“Deuteronomy 31:8 It is the Lord who goes before you. He will be with you; he will not fail you or forsake you. Do not fear or be dismayed.”

2. My father, Modisaotsile Mabeo, tshwene ya rotwe. Thank you for the motivation, thank you for always reminding me that: “Maleka ga se makgona, makgona ke maboeletsa” and “Nko ya kgomo mogala tshwara ka thata, e sere o utlwa sebodu oa kgaoga”.

3. My Mother (Mangi Mabeo) and grandmother (Esther Mongwegi) for your continuous love and prayers, I am because of you.

4. Late Atiyah Osman-Latib, a very good friend and colleague, whom would’ve also submitted her MSc this year, but God called her home. Ati, this is as much your achievement as it is mine. You were of great help at the beginning of this degree and it saddens me that you won’t be around to see the end. However, I am constantly comforted by the fact that I have gained a genius angel that is cheering me all the way. Continue to rest in perfect peace love, we did it.

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ABSTRACT

Water sources in the North West Province (NWP) are made up of groundwater, surface water and re-useable effluent. However, major water challenges are experienced in the NWP when quality and quantity are considered. The NWP is regarded a water stressed Province, due to its limited water sources. Communities in the NWP rely greatly on ground and surface water for their water provision. Urban areas such as Mahikeng, Lichtenburg, and Coligny rely solely on groundwater. In these regions, groundwater undergoes partial purification treatments. However, communities in rural areas and informal settlements use groundwater untreated. This Province is also known for its great participation in agriculture because of its rich production in crops such as maize, sorghum and sunflower. This also requires a great quantity of water, hence stressing available water sources even more. The aim of this study was to characterize *Clostridium* sp., *Aeromonas* sp. and heterotrophic count bacteria in selected groundwater systems in the NWP. During the course of this study, groundwater was obtained across three seasons: winter, summer and autumn. Groundwater temperature fluctuated between the seasons, with the lowest and highest water temperatures observed during summer autumn, respectively. The pH of the groundwater systems was relatively neutral throughout the seasons. However, parameters such as TDS, salinity as well as CODs were exceptionally high in all seasons. Chemical parameters such as nitrates and phosphates were observed in low concentrations in all seasons. The identification and characterization of *Clostridium* sp., *Aeromonas* sp., and HPCs was achieved utilizing culture-based methods. A total of 91 isolates were enumerated and identified using 16S rRNA PCR and sequencing. HPCs were observed in high levels with *Bacillus* being the most predominant HPC species seen across all three seasons. Other species of concern that were identified include *Citrobacter* sp. and *Escherichia* sp. Furthermore, *Clostridium* sp., and *Aeromonas* sp. were found present in the groundwater systems during summer, however at very low levels. Three *Clostridium* species (*C. perfringens*, *C. sordellii* and *C, tepidum*) and a single *Aeromonas* species (*A. hydrophila*) were identified in this study. Out of all isolates obtained, 80% were beta-hemolytic while 70% of the HPCs were positive for DNase production. Ninety percent of the *Aeromonas* species were positive for gelatinase production whilst none of the isolated microorganisms tested positive for lecithinase production. The production of extracellular enzymes in microorganisms is a clear indication that they could be regarded as opportunistic pathogens. The antibiotic resistance patterns of the isolated microorganisms was achieved using the Kirby-Bauer Disk diffusion method. *Aeromonas* sp., and HPC species were exposed to a total of 11 antibiotics and displayed resistance towards Ampicillin (10µg), Penicillin-G (10µg) and Trimethoprim (5µg). The isolated organisms were also screened for antibiotic resistant genes (ARGs) using endpoint PCR. Additionally, the *Aeromonas* isolates (4) were screened to determine the presence of 10 virulence genes. Of the five ARGs screened, 50 % isolates harboured *ampC* and bla_{TEm}. Whilst none of the screened *Aeromonas* species harboured any of the screened

virulence genes. The physico-chemical quality of the groundwater systems of interest clearly illustrated that the parameters were favourable for bacterial growth. Whilst the presence of pathogenic multi-drug resistant organisms such as *Clostridium*, *Aeromonas*, *Bacillus* and *Escherichia* sp. harbouring ARBs is a cause for concern. Thus, the findings of this study indicate that groundwater systems in the NWP are a potential safety hazard and require special attention If the water is to be used for consumption.

Keywords: Groundwater, *Clostridium* sp., *Aeromonas* sp., Heterotrophic bacteria, Pathogenicity, Antibiotic Resistant Genes (ARGs), Antibiotic Resistant Bacteria (ARBs), and Virulence genes.

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LIST OF ABBREVIATIONS

ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistant Genes
CDI	Clostridium difficile infections
CFU	Colony Forming Unit
COD	Chemical Oxygen Demand
DWAF	Department of Water Affairs
DWS	Department of Water and Sanitation
EC	Electrical Conductivity
HGT	Horizontal Gene Transfer
HPC	Heterotrophic Plate Count Bacteria
NWDC	North-West Development Corporation
NWP	North-West Province
PBP	Penicillin-binding protein
PCR	Polymerase Chain Reaction
SA	South Africa
SRC	Sulphite-reducing Clostridium
TDS	Total Dissolved Solids
WWTP	WasteWater Treatment Plant
WRC	Water Research Commission

CHAPTER 1: INTRODUCTION

1.1. Overview and problem statement

Water is one of the crucial and abundant resources that exist on the surface of the Earth and is known to cover more than 70% of our planet (Hossain, 2017). This resource is highly dependent on by all forms of life. However, water in South Africa (SA) is not as abundant as in other countries as this country is classified as one of the driest and dirtiest countries in the world (Colvin *et al*., 2013; GreenCape, 2017). The scarcity of water in SA is highly influenced by irregular rainfalls that lead to extremely harsh dry (drought) and wet (floods) periods (Colvin *et al.,* 2013; Fourie, 2017). Large quantities of water are required in SA as it is necessary and needed in domestic, recreational, agricultural and personal hygiene purposes. According to the Department of Water and Sanitation (DWS, 2015), South Africa's water is drawn from a variety of sources that are distributed as follows: (i) 77% surface water, (ii) 9% groundwater and (iii) 14% reusing return flows. With increasing population yearly, and the dependence of water in sectors such as agriculture and mining, the quantity of available water is decreasing, causing strenuous pressure on water sources. In 2015 one of South Africa's biggest cities, Cape Town actually experienced "day zero" whereby their drinking water sources had depleted. The whole Western Cape Province experienced drought and had to use alternative methods for water provision such as reusing and recycling wastewater had to be put in place (GreenCape, 2017). This incident caused the country to start evaluating alternative sources of water that can be used, should drought occur and sources such as surface water and drinking water run dry.

Groundwater, a relatively hidden yet crucial source of water can be utilized as an alternative of drinking water, should demand exceed supply. The importance of groundwater in SA is usually miscalculated, whereas the majority of our population depends on groundwater for domestic purposes (Pietersen *et al*., 2011). Majority of water users in South Africa depend on surface water, whereas smaller underdeveloped regions such as rural areas depend on groundwater (WRC, 2011). According to the DWS (2015) groundwater is defined as a strategic resource in many parts of South Africa, especially in rural areas and is considered as a natural water purification system. Groundwater is resistant to the effects of droughts, as it is protected underground and has a low evaporation rate (Mussá *et al*., 2015). This is an indication that boreholes can become a reliable source of water, when rivers and streams have dried up (Mussá *et al*., 2015).

The North-West Province (NWP) is considered a dry Province, as water is also scarce. Majority (80% of rural communities) of communities that live in the NWP depend solely on the use of groundwater for their everyday use (Pietersen *et al.*, 2011). This was observed in the majority of the rural areas including urban areas such as Mahikeng, the capital of NWP. Groundwater is of

importance in this Province, hence the quality thereof is of value. There are a number of anthropogenic activities, to name a few, such as agriculture, urbanization and runoff from sewage that have been implicated as the main pollutants of groundwater. The presence of such pollutants compromises the quality of groundwater as they have the ability to alter the physico-chemical as well as microbiological properties thereof (Oparaocha *et al*., 2011; Rahmanian *et al*., 2015).

Water in general has been known to harbour microorganisms of both pathogenic and non-pathogenic nature (Bedada *et al.,* 2018; Tsholo, 2019). The presence of microbiological parameters such as heterotrophic count bacteria can be used to determine the quality of water and they are usually considered to be harmless, not a threat to human health (Ikonen *et al.,* 2013; Prinsloo, 2014).Whereas the presence of indicator organisms in groundwater such as Clostridium and *Aeromonas* are alarming as they are considered as faecal indicators (WHO, 2011). The detection of these species is due to water runoff from animal and human faecal contamination (Mulamattathil *et al*. 2014). These species have been proven to be toxic to human health, by causing intestinal infections such as diarrhoea, gastroenteritis and soft tissue infections (Popoff and Bouvet, 2013).

According to Hecht (2004) and Fourie (2017) antibiotic resistance in indicator bacteria such as *Aeromonas* and *Clostridium* has been recognized clinically. A study conducted by Peng *et al.* (2017) stated that spore forming organisms (e.g. *Clostridium*) may survive antimicrobial therapy and are known to be resistant to multiple antibiotics, such as aminoglycosides, lincomycin, tetracyclines, erythromycin, clindamycin, penicillins and cephalosporins which are commonly used in the treatment of bacterial infections in clinical settings. *Clostridium* species have acquired several mechanisms for antibiotic resistance and the factors contributing include resistance-associated genes harboured in the bacterial chromosome, mobile genetic elements, and alterations in the antibiotic targets of antibiotics and in metabolic pathways of *Clostridium* and lastly biofilm formation.

Wastewater environment is regarded as a reservoir of antibiotic resistant bacteria and is considered a source of surface and groundwater contamination, which may result in the spread of antibiotic and antimicrobial resistance (Igbinosa and Okoh, 2012).*Clostridium* and *Aeromonas* are opportunistic pathogens that have been documented in both wastewater and drinking water reports however no studies have been conducted to determine their prevalence as well as virulence in groundwater. Groundwater is of outmost importance in many Provinces in SA, especially in the North-West, however it is highly taken for granted and not protected. The quality of groundwater used to be exceptionally good centuries ago, but with increasing factors of toxic pollutants that contaminate this source of water, renders its quality questionable.

Since South Africa is currently undergoing water pressures, the use of groundwater can be seen as an alternative source of water, when our surface water resources are stressed. The vitality of groundwater in South Africa is neglected and measures are needed to preserve its quality. According to WRC (2011) surface water is the most utilized water resource across South Africa. However, in Provinces such as the North-West, which have many smaller communities, groundwater is the main source for water provision. This water resource is relatively abundant. Nonetheless, it is difficult to quantify the amount of available groundwater as it is widely dispersed across the landscape (Department of Rural, Environmental and Agricultural Development, 2013).

1.2. Research aim and objectives

1.2.1. Aim

The aim of the study was to characterize *Clostridium* spp., *Aeromonas* spp and heterotrophic count bacteria in selected ground water in systems.

1.2.2. Objectives

The specific objectives of the study were:

- To determine physico-chemical parameters;
- To isolate and identify *Clostridium* spp., *Aeromonas* spp. and heterotrophic count bacteria found in groundwater;
- To determine haemolysin and extracellular enzymes production of *Clostridium* spp., *Aeromonas* spp. and HPCs
- To determine antibiotic profiles of *Clostridium* spp., *Aeromonas* spp. and heterotrophic count bacteria
- To determine the presence of antibiotic resistance genes in HPCs and virulence genes in *Aeromonas* sp.

CHAPTER 2: LITERATURE REVIEW

2.1. Water in South Africa

South Africa (SA) is a semi-arid country and has a relatively low rainfall (Germs *et al*., 2004). This makes water a scarce resource. According to Colvin *et al*. (2013) and Fourie (2017) the irregular rainfall experienced leads to extensive wet and dry periods, thereby causing either drought or floods, impacting on the scarcity of water. Water as a basic need, is utilized in large quantities that are for domestic, recreational, agricultural as well as personal hygiene uses. According to Africa Check (2018) about 88% of South African households have access to water whereas piped water is available to less than 50% in South African homes. Furthermore, South Africa has less water per person than Botswana and Namibia, as it has just 66% of average annual rainfall and it is also known as the 39th driest countries in the world. The total surface water that is available in South Africa is about 49 200 million m^3 per year of which 4 800 million m^3 per year originates from Lesotho (DWS, 2016). It is estimated that based on recent poor usage trends of available water, SA's water demand is most probably to exceed availability of economically usable freshwater resources by 2025 (DWAF, 2002). This will be impacted by the continuing inclination in industrialization and urbanization of South Africa's population that will increase pressure that already exists on the country's water supply sources (DWAF, 2004; Tewari, 2009).

The quantity of water available for direct use, or to support aquatic ecosystems in SA, solely depends on both the sustainability and availability of the resource (DWS, 2013). According to the bill of rights (1996), every human being has the right to safe and clean drinking water, but due to a rapid increase in the growing human population in South Africa, various pressures are placed on the quality, quantity and accessibility of drinking water.

According to DWAF (1996) water quality describes the physical, chemical, biological as well as the aesthetic properties of water which determine its fitness for a variety of uses. Apart from scarcity of water, South Africa's water also suffers from water quality problems that are caused by a variety of human activities such as agriculture, urban and industrial development, mining and recreation (Edokpayi *et al*., 2017). These human activities can potentially alter the quality of natural waters as well as change the water use potential (DWS, 2013). The main water quality problems faced in South Africa are as a result of eutrophication, faecal pollution, salinization as well as acid mine drainage (DWS, 2013, DWAF 1996). Additionally, the malfunctioning and overloading of wastewater treatment plants (WWTPs) results in effluent that is poor in quality, which inevitably introduces faecal pollution in river systems (Mitchell *et al*., 2014).

2.1.1. Water in North-West Province

The North West Province (NWP) lies on the northern section of South Africa, and is rich in metal (NWDACE, 2008; StatsSA, 2016). This province is an important food basket for the country as agriculture is regarded an important economic activity (Ferreira, 2011; NWDC, 2014). The main crops that are produced within the NWP include sunflower seeds, sorghum, groundnuts, maize and wheat. The agriculture sector of the NWP produces about 2.8% of the provincial GDP (NWDC, 2014). North West is also classified as the fourth-smallest province in the country, accounting for 8.7% of South Africa's land area and with a mid-2018 population estimation of 3 979 million people (NWDACE, 2007; StatsSA, 2016). The North-West Province is predominantly rural and the majority of the people within the province come from villages that have experienced limited economic activities (Department of Rural, Environment and Agriculture development, 2017). This province also consists of an urban population from towns and big cities such as Lichtenburg, Brits, Klerksdorp, Potchefstroom, Rustenburg, Vryburg and the province's capital Mahikeng (Bezuidenhout *et al.,* 2011). According to NWP-SOER (2002) and Bezuidenhout *et al*. (2011) the average rainfall for the western region is less than 300mm per annum, the central region is approximately 550mm per annum, whilst both the eastern and south-eastern receives over 600mm per annum.

According to NWDC (2014) water is not naturally found in abundance in the NWP thus making it a water stressed province. Furthermore, this creates opportunity for water recycling and purification investments, in order to minimise pollution of groundwater caused by both natural and human-induced sources such as mining and industrial activities, agriculture and domestic use. The state of the environment report of 2008 indicated that the rural communities of the NWP require at least 70 million m^3 water per annum, where 25 million m^3 per annum is utilized for domestic consumption and the remaining amount is utilized for livestock water provision and other various agricultural purposes (Bezuidenhout *et al.,* 2011). Surface and groundwater in the NWP give support to various activities such as gold, platinum and chrome mining, related support towards manufacturing industries and has to also account for its both urban and rural population. Cities and towns of this province have the luxury of providing treated drinking water to its residents (Bezuidenhout *et al.,* 2011), but this is not the case for rural communities as they depend on water from directly dams and boreholes. StatsSA (2016) also indicated that about 80% of the rural population of this province depend only on groundwater for their water provision.

2.1.3. Groundwater in North-West Province

Groundwater is an essential natural resource that offers important economic benefits (Ferreira, 2011). Unlike surface water resources, groundwater systems within the Province have not been extensively investigated (Department of Rural, Environmental and Agricultural Development, 2013).

Groundwater is resistant to the effects of droughts, as is protected underground and has a low evaporation rate (Vrba, 2002). This indicates that boreholes can become a reliable source of water, when rivers and streams have dried up. Groundwater quality is usually of good condition and in most cases requires little or no treatment, however not all of groundwater is safe to drink without treatment (DWA, 2010; Department of Rural, Environmental and Agricultural Development, 2013). Although groundwater is safely protected underground, it does not imply that it cannot be vulnerable to contamination. It can be polluted in many ways such as groundwater that is associated with coal deposits usually contains dissolved minerals that can be toxic to animals and humans (EPA, 2004). Pollutants that are not handled with care and dumped onto the ground may leak into the soil, making their way into groundwater thereby contaminating it. The North West Environmental Outlook (2013) indicated that there were notable fluctuation trends in 2012 physico-chemical parameters of various groundwater catchments in the NWP. This report indicated that Electrical Conductivity (EC), Ammonia, Nitrates, Phosphorous and Sulphates were noted to have increased in the 2012 and had exceeded the concentrations stated in the TWQR standards.

There was a study conducted almost ten years ago that covered almost all boreholes in the NWP by Ferreira (2011). Findings of this study indicated that there were several physico-chemical parameters that exceed the standards of TWQR (Target Water Quality Range) with nitrates being the most alarming. According to WHO (2006) and Venter (2003) there are different microorganisms that pose a great threat to the microbiological safety of groundwater and can also lead to disease outbreaks in humans. Ferreira (2011) indicated several microbiological contaminants that play a negative role in the groundwater quality. Ferreira (2011) also highlighted that there were faecal indicator bacteria with pathogenic nature that were present in various groundwater systems in the NWP. These microorganisms included faecal coliforms (*E.coli* predominant), faecal streptococci (*Enterococcus* predominant), presumptive *P. aeruginosa* as well as *S.aureus*. The presence of such opportunistic pathogens in groundwater systems may cause possible health risks to communities that are exposed.

Another study that was conducted in 2006 by Kwenamore related to groundwater in the NWP indicated that there were relatively high bacteria counts of both total and faecal coliforms in

groundwater that was collected from Ditsobotla and Molopo districts. The results yielded from this study indicated the possibility of constant faecal contamination of groundwater in those particular districts. In this study, two opportunistic coliform pathogens that were found were *Klebsiella* spp. and *Citrobacter* spp. and they also indicated multiple resistance toward commonly used antibiotic, of which is a problem (Bezuidenhout *et al*., 2011; Kwenamore, 2006).

2.2. Microorganisms of interest in the study

2.2.1. *Clostrdium* genus

Clostridium is the largest genus within the class *Clostridia* belonging to the family *Clostridiaceae* and consists of relatively many species, five subspecies, with only a few species being considered as opportunistic pathogens to humans (Public health England, 2016). This genus consists of obligate anaerobic, fermentative, Gram-positive bacteria that possess peritrichous flagella for motility (Public Health England, 2016; Willey *et al*., 2017). Most *Clostridia* species cannot grow in aerobic conditions and a slight exposure to oxygen can lead them to fatality (Hatheway, 1990). Due to such conditions, they are able to form endospores of which help them survive harsh conditions posed by the environment (Siegrist, 2011; Fourie*,* 2017). However, the formation of spores in *Clostridium* species, is only possible in anaerobic conditions (Hippe *et al.,*1992). Although most Clostridium species are said to be anaerobic, Public Health England (2016) and Hippe *et al*. (1992) highlighted that there are a few species in the *Clostridium* genus that are aerotolerant, these include *C. carnis*, *C. histolyticum*, *C. tertium* as well as *C. aerotolerans*. According to Law *et al*. (1990) the difference between aerotolerant strains of *Clostridium* from anaerobic strains is that they can tolerate oxygen and can even indicate growth to a certain level in the presence of oxygen.

Several *Clostridium* species are naturally found in the microbiota of humans and wild animals (Haagsma, 1991). However, when humans and animals are exposed to the opportunistic species via the faecal oral route, infections can occur (Siegrist, 2011). According to Sathish and Swaminathan (2009) of all *Clostridium* species that have been identified in the world, thirty species are classified as minor pathogens while thirteen of those are considered opportunistic pathogens. Clostridia possess the ability to produce a variety of toxins that can lead to clinical diseases (Mahon and Mahlen, 2015; Public Health England, 2016). Clinically, *Clostridium* species are a source of illnesses that are highly associated with the toxins they produce (Hatheway, 1990). *Clostridium* species such as *C. tetani* and *C. botulism* are neurotoxic and can cause tetanus (paralysis together with hypertonia of skeletal muscles) (Córdoba *et al.,* 2011; Schiavo *et al.,*

2000) as well as botulism in humans (Montecucco *et al.,* 2006; Fourie, 2017). Other *Clostridium* species such as *C. difficile* and *C. perfringens* are classified as enterotoxic clostridials due to the toxins they produce that are harsh in the intestinal systems and as a result causes enteric disease in both humans and animals (Songer and Uzal, 2005).

Clostridium species are distributed in all types of environments such as soil, sediments, sewage, dust, surface of plants and food. According to Hippe *et al*. (1992) and Public Health England (2016) members of this genus can grow at a broad variety of temperatures because it consists of psychrophilic, mesophilic and thermophilic species. For decades the use of spore-forming and sulphite-reducing clostridium (SRC) species such as *Clostridium perfringens* as a possible faecal pollution indicator in the environment has been studied (Cabelli, 1978; Cabral, 2010 cited by Fourie*,* 2017). *Clostridium* species are the only obligate anaerobic bacterial species that can be used as an indicator of the sanitary quality of water (Cabelli, 1978; Ashbolt *et al.,* 2001). According to Figueras and Borrego (2010) most of these species are present in wastewater, especially of humans and animals because of their ability to survive and multiply in the gastrointestinal tract of humans and warm-blooded animals. However, the use of *Clostridium* as a faecal indicator in water has its limitations, as it is oxygen sensitive (Rhodes and Kator, 1999; Ashbolt *et al.,* 2001). The presence of *Clostridium* in water, especially drinking water can indicate poor treatment of wastewater effluent from wastewater treatment plants (Marcheggiani *et al.,* 2008).

2.2.2. *Aeromonas* genus

Aeromonas species are Gram-negative, non-spore forming, facultative anaerobic, rod shaped and mesophilic bacteria (Li *et al.,* 2014; Igbinosa and Okoh, 2013; Miyagi *et al*., 2016). Members of this genus are able to reside in surface waters (rivers and lakes), sewage, drinking water (tap and bottled mineral), thermal waters as well as sea water (Pablos *et al.,* 2009; Figueira *et al.,* 2011). *Aeromonas* genus comprises of a relatively large group of species that includes 31 species with 12 subspecies, however only a few species have been identified as pathogens of both cold-blooded animals and humans (Martin-Carnahan and Joseph, 2005; Piotrowska and Popowska, 2015). A portion of the species in this genus are recognized in the clinical sector as agents that cause illnesses such as food poisoning, sepsis and wound infections (Janda and Abbott, 1998; Miyagi *et al*., 2016). Various studies have indicated that the other portion of species reside in different kinds of aquatic environments such as lakes, rivers, estuaries, both drinking water and groundwater and lastly in various stages of purification in wastewater (Gordon *et al*., 2008; Moura *et al*., 2012; Piotrowska and Popowska, 2015).

Environmental and clinical relevance has been observed in certain *Aeromonas* species. With regards to environmental relevance, members of this species such as mesophilic *A. hydrophila*, *A. veronii* and psychrophilic *A. salmonicida* are known as fish pathogens and they cause infections like furunculosis as well as epizootic ulcerative syndrome (Burr *et al*., 2005; Dallaire-Dufresne *et al*., 2014; Rahman *et al*., 2002). The same *Aeromonas* species that are found to cause infections, have also been identified as pathogens that are observed in clinical settings with regards to human infection. According to Piotrowska and Popowska (2015) these species have the ability to cause infectious complications in both immunocompromised and immunocompetent individuals. Janda and Abbott (1998) and Ko and Chuang (1998) stated that serious *Aeromonas* infections can occur in exposed patients with diseases such as hepatitis, diabetes and malignant tumors. General *Aeromonas* infections that can be observed in humans are of the digestive system (gastroenteritis) (Holmberg and Farmer 1984; Figueras, 2005), respiratory and genitourinary infections (Bossi-Kupfer *et al*., 2007; Al-Benwan *et al*., 2007), wound infections (infections of both skin and soft tissues) (Jorge *et al*., 1988; Chim and Song, 2007), sepsis (Ko *et al*., 2000; Tsai *et al*., 2006), eye infections (Khan *et al*., 2007) and lastly meningitis (Seetha *et al*., 2004).

Aeromonas can be used as an indicator for the hygienic state of water (Legnani *et al*., 1998). They can be used as an indicator of faecal contamination in water, as it has the ability to cause enteric diseases in children under five years old, elders as well as immunosuppressed people (Cabral, 2010). The presence of Aeromonas in drinking water is due to its ability to resist standard chlorine treatments thereby withstanding inside biofilms (Handfield, 1996; Cabral, 2010).

2.2.3. Heterotrophic bacteria (HPC)

Heterotrophic bacteria comprise both Gram-positive and Gram-negative bacteria that can use organic nutrients for growth and nourishment. These bacteria are relatively present in all types of habitats such as water, soil, food, vegetation as well as air (Allen *et al*. 2004). The genera include a variety of genera such as *Escherichia, Klebsiella, Enterobacter, Citrobacter, Serratia* and*Bacillus.* The population of heterotrophic bacteria that is present in a habitat varies with other habitats (Table 2.1). Most heterotrophic bacteria are harmless and do not necessarily pose a threat to human health, but literature has indicated that a certain portion of the genera are opportunistic pathogens in drinking water. The latter include *Aeromonas* spp., *Pseudomonas* spp. and *Klebsiella* spp. (WHO, 2003).

Opportunistic pathogens observed in the HPC group are usually influenced by hospital-acquired infections and not consumption of drinking water (Allen *et al.,* 2004). Since the medical sector is the main source of pathogenicity in heterotrophic bacteria, it also plays a vital role in the antibiotic resistance that is acquired by these genera. HPC are of importance in aquatic habitats, more especially drinking water as they provide insight on (i) the quality of the source water, (ii) types and efficacy of treatment, (iii) type and concentration of disinfection residuals, (iv) age and the condition of the storage and distribution system as well as (v) the concentration of dissolved organics in the treated drinking water (Allen *et al.,* 2004).

Table 2.1. Examples of HPC genera found in aquatic systems (Allen *et al*. 2004).

Genera	**Genera**	**Genera**
Acinetobacter	*Escherichia coli*	*Proteus*
Actinomycetes	*Flavobacterium*	*Pseudomonas*
Alcaligenes	*Flavobacterium meningosepticum*	*P. cepacian*
Aeromonas	*Gallionella*	*P. fluorescens*
Arthrobacter	*Hafnia alvei*	*P. maltophilia*
Bacillus	*Klebsiella pneumoniae*	*Serratia liquefaciens*
Beggiatoa	*Methylomonas*	*Sphaerotilus*
Citrobacter freundi	*Micrococcus*	*Sphingomonas*
Corynebacterium	*Mycobacterium*	*Staphylococcus*
Crenothrix	*Morexella*	*Streptococcus*
Desulfovibrio	*Nitrobacter*	*Streptomyces*
Enterobacter agglomerans	*Nitrosomonas*	*Yersinia enterocolitica*
Enterobacter cloacae	*Nocardia*	

As indicated, the HPC group includes various genera of microorganisms, some of them can be used as indicator organisms in aquatic ecosystems. According to Amanidaz *et al*. (2015) heterotrophic bacteria can be used in determining coliforms that are present in water. HPCs can be used to determine three types of coliforms, of which are (i) Total coliforms, (ii) faecal coliforms and (iii) *E. coli*. The presence or absence of coliforms in water determines the quality of water, whether it is contaminated or in good condition. Total coliforms can be used to determine the presence of a biofilm or can be used as an indicator of treatment efficiency due to their quick response to chlorine (Verhille, 2013). Faecal coliforms can be used as a reliable indicator of faecal contamination present in water (Amanidaz *et al*., 2015). The detection of faecal coliforms in water, can give insight into the types of contamination sources that are responsible of which in most cases are (i) animal excreta, (ii) wastewater, (iii) sludge, (iv) septage or (v) biosolids (Culbertson *et al*., 2014). The presence of faecal coliforms can also indicate the possible presence of opportunistic pathogens as most of the wastes are excreted in terms of urine and faeces from warm-blooded animals. *E. coli* can also be used as an indicator organism, as it is a reliable indicator of enteric disease and the presence of current faecal contamination in drinking water systems (Verhille, 2013; Culbertson et al., 2014).

2.3. Antibiotic resistance

Antibiotics and antimicrobials have been used as active substances in the eradication of pathogenic organisms and have been effective in the mortality of microorganisms for many years (Coetzee, 2015). However, the widespread use of antibiotics and antimicrobials over the years has become a problem due to microbial resistance that is emerging rapidly (Van den Honert *et al*., 2018). Over the years bacteria have acquired several mechanisms, in order to combat antibiotics and antimicrobials thereby allowing them the ability to become resistant. Emergence of resistance among bacteria that are classified as pathogens has been recognised as a major public health threat, that affects humans worldwide (Munita and Arias, 2016). According to Van den Hornert *et al*. (2018) the increasing rate of antibiotic resistance and the degeneration of new antimicrobial and antibiotic development pose major threats to global health, as it increases high rates of treatment failure and high infection. The main factors contributing towards antibiotic resistant bacteria (ARB) are the misuse and overuse of antibiotics in the agriculture sector and health care sector, of which forms selection pressures that favour the generation of ARB strains.

According Munita and Arias (2016) bacteria have a remarkable genetic plasticity that enables them to react to a wide array of environmental threats, and this includes the presence of antibiotic molecules that may wipe out their entire existence. Microorganisms that share the same habitat

(ecological niche) with antimicrobial-producing organisms have evolved ancient mechanisms allowing to resist the effects of harmful antibiotics and their intrinsic resistance allows them to thrive even when exposed to antibiotics. There are two main genetic strategies that microorganisms utilize (see Figure 2.1.for possible mechanisms utilized) in order to adjust to antibiotic attacks and they are (i) mutations in genes that are associated with the mechanism of action of the compound and (ii) the acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (HGT). During mutational resistance a subdivision of bacterial cells acquired from a susceptible population develop mutations in genes, affecting the drug activity, thereby preserving cells enabling them to survive in the presence of antimicrobials and antibiotics. When the resistant mutant (antibiotic resistant bacteria) emerges, the susceptible population of the bacteria are removed by the antibiotics and the resistant population thrives and reproduces.

Horizontal gene transfer is when microorganisms acquire foreign DNA material and is classified as one of the most important drivers of evolution of microorganisms and is usually responsible for the development of antibiotic/ antimicrobial resistance (Willey *et al*., 2017). Microorganisms gain external genetic material through three main mechanisms (i) transformation (integration of naked DNA), (ii) transduction (usually phage mediated) and conjugation (bacterial sex) (Munita and Arias, 2016). Other resistance mechanisms that microorganisms have included (i)efflux pumps (pump out the antibiotic from the bacterial cell thereby decreasing intracellular antibiotic concentration), (ii)enzyme modifications of antibiotics (renders the antibiotic ineffective), (iii)the degradation of the antimicrobial compounds, metabolic pathways (bacteria uses other metabolic pathways to those that are inhibited by the antibiotic), (iv)overproduction of target enzyme, and (v) modification of antibiotic targets and alteration of cell permeability (inhibits the antimicrobial agent from entering the cell and reaching target sites) (Van den Hornert *et al*., 2018; Bhullar *et al*., 2012).

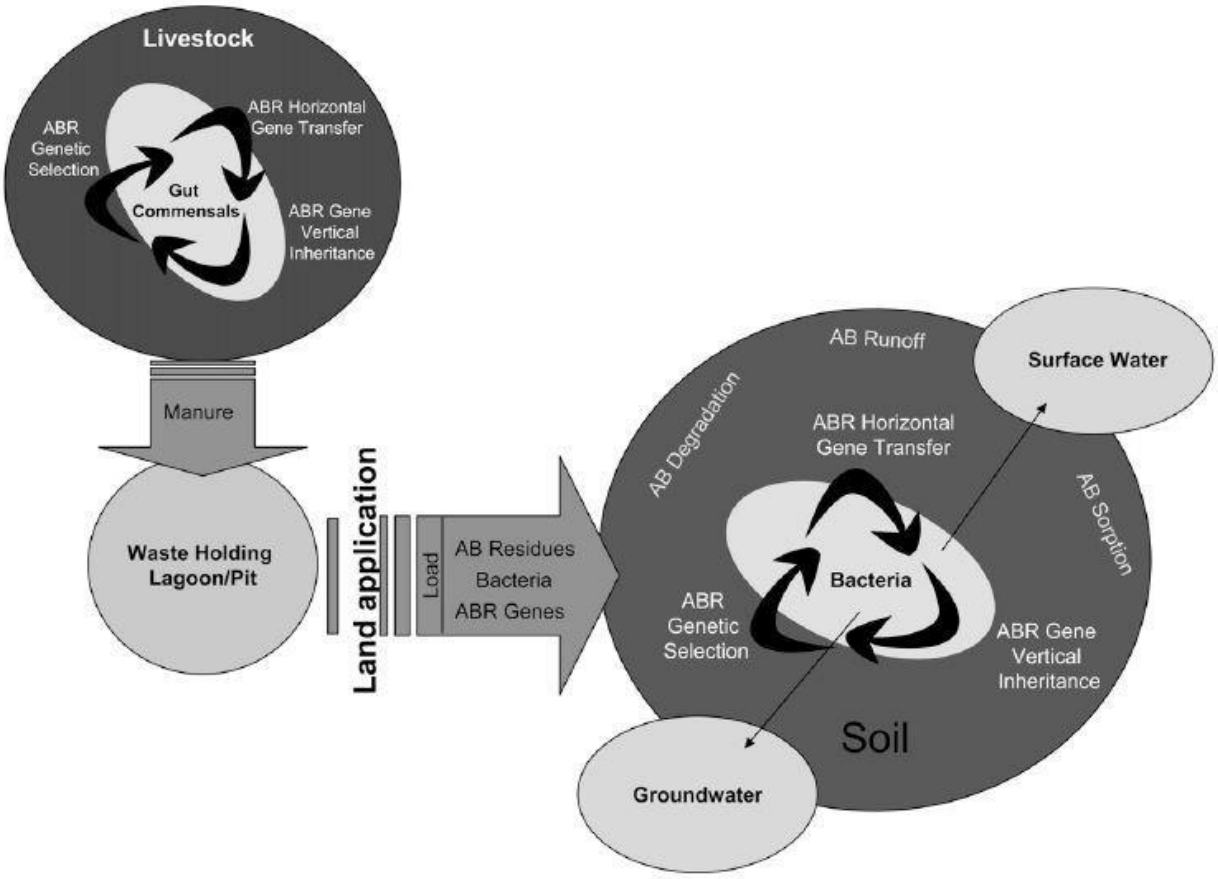

Figure 2.1. Possible fates of antibiotic residues and mechanisms of antibiotic resistance genes acquisition (Chee-Sanford *et al*., 2009).

2.3.1. Different classes of antibiotics

Antibiotics can be classified in various ways, but the most common classification schemes are based on their molecular structures, mode of action as well as spectrum of activity (Calderon and Sabundayo, 2007; Etebu and Arikekpar, 2016). Antibiotics that are considered to be in the same class will indicate similar patterns of effectiveness, toxicity and allergic potential side effects (Etebu and Arikekpar, 2016). According to Van Hoek *et al*. (2011) and Adzitey (2015) some of the different classes of antibiotics based on chemical or molecular structures include Beta-lactams, Macolides, Tetracyclines, Quinolones, Aminoglycosides, Sulphonamides, Glycopeptides and Oxazolidinones, see Table 2.2 and Table 2.3 for classification and summary of these antibiotics.

a. Beta-Lactams

The molecular structure of beta-lactam antibiotics includes a 3-carbon and 1-nitrogen ring that is highly reactive. Members of this antibiotic class interfere with proteins that are important for the synthesis of the bacterial cell wall, by either killing or inhibiting bacterial cell growth within the process (Etebu and Arikekpar, 2016). Microorganisms containing a bacterial enzyme known as the penicillin-binding protein (PBP) are responsible for cross-linking peptide units during peptidoglycan synthesis. Heesemann (1993) stated that members of the beta-lactam class can bind themselves to the PBP enzymes, thereby interfering with peptidoglycan synthesis, in most cases leading to cell lysis and ultimately cell death. Representatives of this class include Penicillins, Cephalosporins, Monobactams and Carbapenems (Etebu and Arikekpar, 2016).

Penicillin is the first antibiotic that was discovered and reported by Alexander Fleming in 1929 and was found later to be among several other antibiotics known as Penicillins (Etebu and Arikekpar, 2016). It was initially discovered and isolated from a fungus known as *P. notatum*. Penicillin molecules consists of the beta-lactam ring, which is important for bioactivity. According to Boundless (2016) members of the Penicillin class include antibiotics such as Penicillin G, Penicillin V, Oxacillin, Methicillin, Nafcillin, Ampicillin, Amoxicillin, Carbenicilin, Piperacillin and Ticarcillin. Although Penicillin is one of the most important antibiotics, especially Penicillin G, it has a narrow spectrum, whereby only Gram-positive bacteria (streptococci) and some Gram-negative bacteria such as Treponema pallidum are sensitive to it (Talaro and Chess, 2008). Recently, despite rapid antimicrobial resistance in the environment, penicillins continue to take on a vital role in the health sector, in modern antibiotic therapy (Eyler and Shvets, 2019).

b. Cephalosporins

Members that exist in the cephalosporin class of antibiotics are similar to penicillins in terms of their structure as well as their mode of action (Etebu and Arikepar, 2016). According to Talaro and Chess (2008) cephalosporins are amongst the most prescribed as well as administered antibiotics in the world. In 1948, the first member of this class was isolated from a fungus *Cephalosporium acremonium* (Willey *et al.,* 2017). This class consists of 7-aminocephalosporanic acid nucleus and a side chain containing 3,6-dihydro-2H-1,3-thiazane rings. According to Pegier and Healy (2007) cephalosporins are usually used to treat bacterial infections and diseases arising from penicillin-producing as well as methicillin-susceptible staphylococci and streptococci. The class of cephalosporins is subdivided into five generation according to their target organisms and later version have been proven to be more effective towards inhibiting Gram-negative

pathogens (Etebu and Arikepar, 2016). They consist of a wide range of side chains that enable them to bind to various penicillin-binding proteins (PBP), to circumvent the blood brain barrier, resist breakdown by penicillinase producing bacterial strains and ionize to facilitate entry into Gram-negative bacterial cells (Abraham, 1987).

c. Monobactams

Monobactams class of antibiotics was discovered by Skyes and co-workers and were obtained from the bacterium *Chromobacterium violaceum* (Etebu and Arikepar, 2016). This class forms part of beta-lactam compounds but is different from other beta-lactams in terms of its beta-lactam ring that stands alone and is not fused into a ring (Sykes and Bonner, 1985; Etebu and Arikepar, 2016). Monobactams only have one antibiotic that is available commercially known as Aztreonam and has a narrow activity spectrum. Members of this class are able to inhibit aerobic Gram-negative bacteria and have been proven to be ineffective against Gram-positive bacteria (especially anaerobes) (Sykes and Bonner, 1985).

d. Carbapenems

Carbapenems were discovered in 1976 when the effectiveness of penicillin was compromised owing to the emergence of beta-lactamase in bacteria (Etebu and Arikepar, 2016). This was problematic as bacterial beta-lactamases enabled resistance in bacteria towards penicillin (Papp-Wallace *et al.,* 2011). According to Eyler and Shvets (2019) structural modifications on the beta-lactam backbone gave rise to the carbapenem class of antibiotics, with a wider spectrum of activity, including activity against beta-lactamase producing Gram-negative bacteria. Members of this class play a vital role in the fight against diseases and infections, as they are able to resist the hydrolytic action of the beta-lactamase enzyme. According to Torres *et al*. (2007) carbapenems consist of a broad spectrum of activity as well as potency against both Gram-positive and Gram-negative bacteria and as a result, this class is known as the “antibiotics of last resort” as they are only administered when patients with infections become very ill or are suspected of harbouring resistant bacteria. The emergence of antibiotic resistance in bacteria has become problematic to an extent that bacterial pathogens indicate resistance towards this life saving class of antibiotics (Etebu and Arikepar, 2016). However, what is more alarming is the fact that bacterial resistance towards carbapenems is increasing globally and becoming an international concern (Livermore *et al*., 2011; Patel and Bonomo, 2011; Papp-Wallace *et al*., 2011).

e. Macrolides

Members of this class are characterized by the 14-, 15-, or 16- membered macrocyclic lactose rings with unusual deoxy sugars L-cladinose and D-desoamine attached. This class of antibiotics has a broader spectrum of antibiotic activity than Penicillins and they can be utilized as an alternative to patients that are allergic to penicillin (Moore, 2015). This class of antibiotics are able to kill or inhibit microbes by successfully inhibiting bacterial protein synthesis. Upon achieving this, they bind to the bacterial ribosome and in the process, preventing the addition of amino acid to the polypeptide chain during protein synthesis (Etebu and Arikepar, 2016). Even though macrolides are of importance, they tend to build up in the body, as the liver is able to recycle it into bile and they also have the ability to cause inflammation, hence it is recommended that it is administered in small doses. Examples of this class include Erythromycin, Azithromycin and Clarithromycin (Hamilton-Miller, 1973).

f. Tetracyclines

According to Sanchez *et al*. (2004) tetracyclines were first discovered in 1945 from a soil bacteria of the genus *Streptomyces* by Benjamin Duggar. Structurally, this class consists of four hydrocarbon rings and they are known as by the suffix ‘cycline’. Members of this class are known to be classified into different generations according to their method of synthesis. The first generation of this class consists of antibiotics that are obtained by biosynthesis, such as Tetracycline, Chlortetecycline, Oxytetracycline and Demeclocycline (Fuoco, 2012; Table 2.2). Antibiotics such as Doxycycline, Lymecycline, Meclocycline, Methacycline and Minocycline belong to the second generation as they are the derivatives of semi-synthesis (Etebu and Arikepar, 2016). This particular class targets the ribosome of bacteria as their mode of action (antimicrobial activity). They disrupt the addition of amino acids to polypeptide chains during protein synthesis within bacterial cells (Sanchez *et al*., 2004; Etebu and Arikepar, 2016).

Table 2.2: summary of all classes of antibiotics as well as their examples based on structure (Khan, 2018).

Classes of antibiotics	**Examples**
Penicillins	1.Natural: Penicillin G, Penicillin-VK, 2. Penicillinase Resistant: Methicillin, Nafcillin, Oxacillin and other, 3. Aminopenicillins: Ampicillin
Fluoroquinolones	First generation: Norfloxacin, Ofloxacin, Ciprofloxacin, Pefloxacin, Second generation; Levofloxacin, Moxifloxacin, Lomefloxacin, Gemifloxacin, Sparfloxacin, Prulifloxacin
Aminogycosides	Streptomycin, Gentamycin, Kanamycin, Tobramycin, Amikacin, Sleomicin, Netlimicin
Monobactams	Aztreonam
Carbapenems	Imipenem, Meropenem, Faropenem, Doripenem
Macrolides	Azithromycin, Clarithromycin, Dirithromycin, Erythromycin, Clindamycin, Roxythromycin
Others	Clindamycin, Vnacomycin, Linezolid, Rifamycin, Tetracycline, Trimethroprim, Chloramphenicol and others

Table 2.3. Classification of antibiotics according to mechanism of action (Khan, 2018)

	Antibiotics classified based on the mechanism of action
Cell wall synthesis	Penicillins, Cephalosporins, Vancomycin, Beta-lactamase

	inhibitors, Carbapenems, Aztreonam, Polymycin, Bacitracin
Protein synthesis inhibitors	Inhibit 30s Subunit: Aminoglycosides (gentamicin) Inhibit 50s Subunit: Macrolides, Chloramphenicol, Clindamycin, Linezolid, Streptogramins
DNA synthesis inhibitors	Fluoroquinolones, Metronidazole
RNA synthesis inhibitors	Rifampin
Mycolic Acid synthesis inhibitors	Isoniazid
Folic Acid synthesis inhibitors	Sulfonamides. Trimethoprim

2.3.2. Antibiotic resistance in *Clostridium*

Clostridium species, especially *Clostridium difficile* are considered one of the major healthcare-associated pathogens that are responsible for a variety of diseases (Lachowicz *et al*., 2015; Tenover *et al*., 2012; Freeman *et al*., 2005). *Clostridium difficile* infections (CDI) are usually influenced by multiple factors such as patient demographics (age and immune status) and most importantly the kind of antimicrobial therapy administered (Tenover *et al*., 2012). For many years antibiotics and antimicrobials have been used in order to inhibit the growth of CDI but as the genera are able to form endospores (enabling them to resist harsh conditions), of which plays a major role in the organism's antibiotic resistance. According to Harnvoravongchai *et al*. (2018) drug resistance in *Clostridium* species has become worse, due to the misuse and inappropriate use of antibiotic adaptations that play a role in driving the evolution for resistance. According to Banawas (2018) antibiotic resistance contributes to the spread of CDI among hospitalized patients, especially in the elderly and immunocompromised individuals.

Clostridium species are resistant towards antimicrobial drugs such as erythromycin, penicillin, clindamycin and fluoroquinolones (Freeman *et al*., 2005; Banawas, 2018); however metronidazole and vancomycin have been suggested as the standard clinical method in order to treat CDI for many years but due to high resistance and reduction in antibiotic susceptibility in Clostridium species has been reported, making it difficult to find treatment options that can be utilized (Harnvoravongchai *et al*., 2018).

2.3.3. Antibiotic resistance in *Aeromonas*

Aeromonas species are considered as opportunistic pathogens as they also raise concerns in the health care sector. In agriculture and abattoir ecosystems *Aeromonas* can cause bacterial infections, of which may result in relatively high antimicrobial resistance, making it difficult to treat infections thereof. In order to treat infections caused by Aeromonas species, administration of antibiotics is required (Igbinosa and Okoh, 2013). These species are relatively multi-resistant towards antibiotics such as penicillin and ampicillin, but reports have proven that they are susceptible to aminoglycosides, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, cephalosporin and quinolones (Stratev and Odeyemi, 2016).

2.3.4. Antibiotic resistance in HPCs

Since the HPC genera is relatively large and comprises of a variety of genera, not all of the families that are included within it are pathogenic, thereby making them susceptible to most of the antibiotics that are used. According to Shakoor *et al*. (2018) indicator organisms in these genera are usually the only ones that are problematic regarding antibiotic and antimicrobial resistance. It is said that there are elevated rates of resistance in thermotolerant *E. coli* than in other pathogens, which may be impacted by several sources, particularly wastewater. In a study conducted by Boon and Cattanach (1999) resistance to ampicillin, chloramphenicol, kanamycin, neomycin and streptomycin was higher in other members of the HPC genera than in *E. coli*. In a more recent study conducted by Lépesováa *et al*. (2019) a high number of antibiotic resistant bacteria were detected in biofilm effluent that indicated abundant resistance from coliform bacteria and *E. coli*, of which portrayed resistant strains towards ampicillin, gentamicin and ciprofloxacin only.

2.3.5. Antibiotic resistance in the environment

The environment is currently being recognized for the role that it plays in the global spread of clinically relevant antibiotic resistance (Singer *et al*., 2016). The role that the environment plays in the transmission of various bacterial pathogens that are often associated with insufficient sewage infrastructure, faecal contamination of water or organic fertilizers has been long recognized for many years (Allen *et al*., 2010; Bengtsson-Palme *et al*., 2018; Larssona *et al*.,2018). According to Larssona *et al*., 2018; D'Costa *et al*., 2011 and Wellingston *et al*., 2013 of late, the understanding has developed and improved that many resistance genes that are obtained in pathogens within the environment originate from bacteria that thrive in environments with antibiotics and antimicrobials present. This is the reason why the environment is considered as a dispersal route as well as a reservoir of resistant pathogens and as an arena for the evolution of resistance (Bengtsson-Palme *et al*., 2018). A literature review conducted by Singer *et al*. (2016) highlighted three characterized classes of resistance driving chemicals and they include (i) antimicrobials, of which is composed of four subclasses known as antibiotics, antifungals, antivirals and antiparasitics (ii) heavy metals and (iii) biocides. This literature also described the three major pathways that are responsible for driving chemicals into the environment (Figure 2.2) and they include (i) Municipal and industrial wastewater, (ii) Land spreading of animal manure and sewage sludge and (iii) Aquaculture.

Municipal and industrial wastewater play an important role as a relevant pathway for antibiotics distribution as a large fraction of antibiotics consumed by humans are excreted in both urine and feces in their biologically active form (Singer *et al*., 2016; Zhang *et al*., 2015). According to Chen *et al*. (2015), Li and Zhang (2010) and Rivera-Utrilla *et al*. (2013) antibiotics that are released from humans in the form excretion will enter WWTPs with one of these fates, (i) biodegradation, (ii) absorption to sewage sludge or (iii) exit in the effluent unchanged and released back into the environment. In terms of the veterinary sector, antibiotics are essential for maintaining the health and welfare of animals and they are often dispensed to treat or prevent infections in herds or flocks (Gelband *et al*., 2015; Singer *et al*., 2016). Animals consume about 30 to 90% of antibiotics and it is released through manure and urine, as in humans (Sarmah *et al*., 2006; Berendsen *et al*., 2015). Animal excretion residues has been shown to contaminate the environment with antibiotic resistant bacteria as well as antibiotics (Wichmann *et al*., 2014; Singer *et al*., 2016). Astudy conducted by Singer *et al*. (2016) and Liu *et al*. (2016) highlighted various factors that make AMR control in veterinary settings hard and these factors include (i) persistence and shedding of the drug into the environment in feces and urine, (ii) selection of antibiotic resistance in the environment, and (iii) transmission of drug-resistant microbes acquired from the environment.

A study conducted by Call *et al*. (2013) indicated the need and importance of keeping animals being treated separated from the herds as this enables the transmission of resistance genes through contaminated cow as well as calf bedding and soil. Apart from transmission between animals, the transmission of antibiotic resistant bacteria and genes from animals to humans, especially in farms has been observed (Smith *et al*., 2013; Wulf *et al*., 2008). The rapid release of antibiotics into WWTPs goes hand in hand with the release of resistance genes. Resistance genes that occur in wastewater are derived from the gastrointestinal tract of humans and animals (Hu *et al*., 2013; Singer *et al*., 2016). According to Xu *et al*. (2015) the coexistence of both antibiotics and ARGs in WWTPs can select for various combinations of AMR that can be shared between microorganisms by HGT on mobile genetic elements, such as plasmids, hence increasing the prevalence as well as the combination of multi-drug resistance within the microbial community. The environment that exist in WWTPs give rise to favourable conditions that enable the amplification of genes as well as the creation of a series of resistance genes or genomic assemblages (Zhang and Zhang, 2011). Groundwater quality is affected as antibiotics found in manure or sludge-amended agricultural soils enter groundwater systems through rainfall, irrigation and various human activities (Hirsch *et al*., 1999; Sui *et al*., 2015). However very little has been reported regarding the influence that antibiotic residues in groundwater have on the recreation and generation of AMR in pathogens (Haznedaroglu *et al*., 2012; Singer *et al*., 2016).

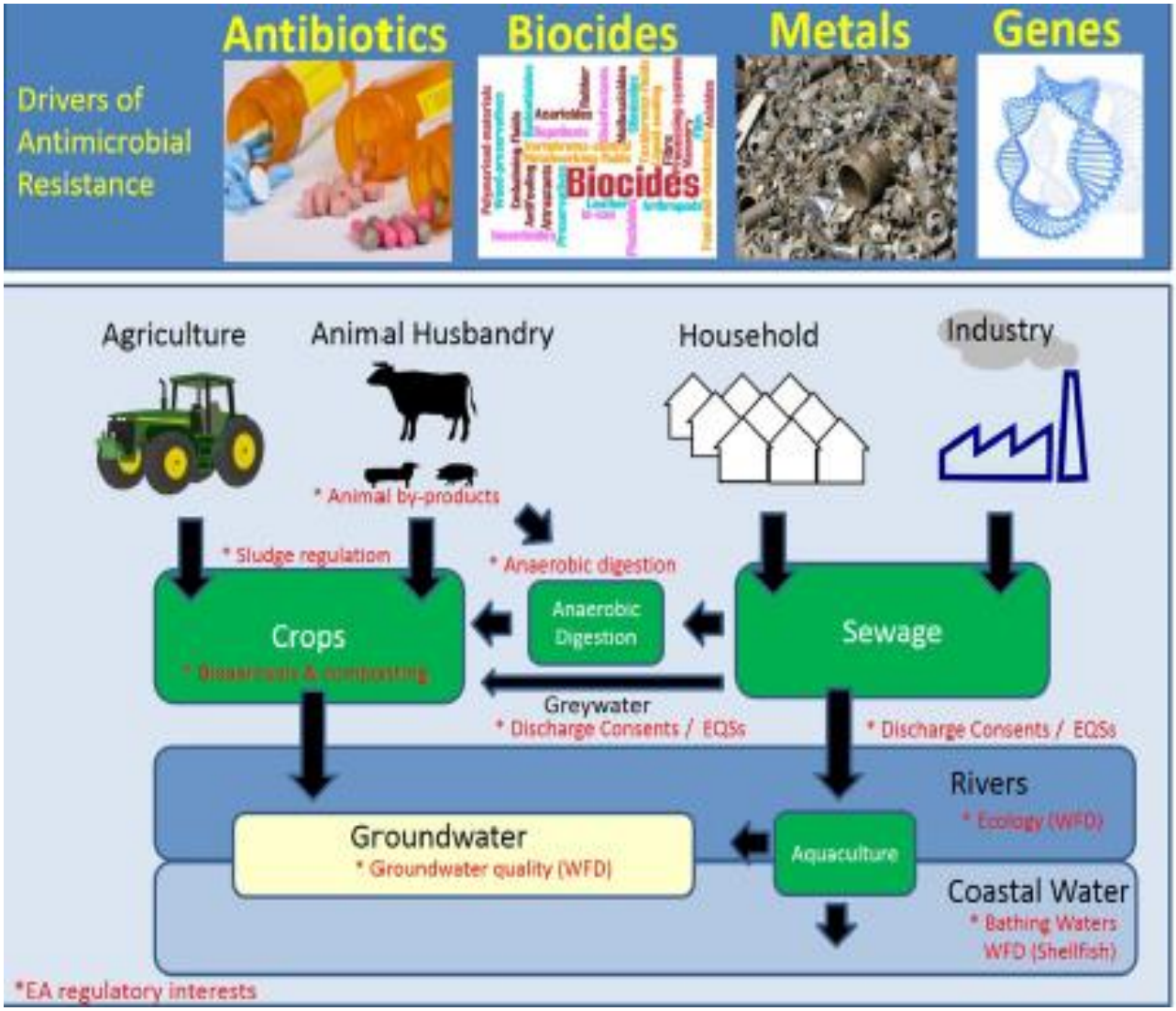

Figure 2.2. Diagram of the hospots and drivers of antimicrobial resistance (AMR) (Singer *et al*., 2016).

2.4. Bacterial virulence

According to Batt *et al*. (2016) virulence can be described as the ability of bacteria to cause diseases and infections in humans and animals. In most cases virulence factors or genes can be observed in opportunistic pathogenic strains. Although virulence genes are not excessively identified in non-pathogenic bacteria, Gennari *et al*. (2012) explained that many of the bacterial virulence genes are situated in mobile genetic elements, therefore the acquisition of mobile DNA could introduce virulence genes or virulent strains in species that are considered as non-pathogenic. For many years, bacterial strains from the environment were considered to lack virulence genes, as their presence has widely been documented and observed in clinical strains. However, in recent studies conducted by Sechi *et al*. (2000) and Xie *et al*. (2005) indicated that these genes could be present in environmental strains and their inheritance could have been

caused by the use of many mechanisms bacteria have such as mobile gene elements exchange between environmental and clinical strains in the aquatic environment. The virulence genes that are present in microorganisms of both clinical and environmental strains are positively correlated with the microorganism's pathogenicity (Casadevall and Pirofski, 2009). Various studies have highlighted that many of the virulence genes have been widely detected in *Vibrio* species that were isolated from fish and were associated with causing infections in humans (Schets *et al*., 2006; Austin, 2010; Gennari *et al*., 2012). In fish farms, antibiotics are excessively used to maintain the health of fish, and this plays an important role in microbial communities, as such fish exposure towards antibiotics, and enables them to become potential reservoirs of antibiotic resistant microorganisms (Alcaide *et al*., 2005; Pedersen *et al*., 2008).

In the present study, the virulence of *Aeromonas* species was of interest. *Aeromonas* species are known as the common inhabitants of both the aquatic environment and the digestive system of humans (Janda and Abbott, 1998). Species belonging to this genus are pathogens and their pathogenicity that is observed in both humans and fish is highly of scientific and economic interest (Borrell *et al*., 1997; Chacon *et al*., 2003). According to Heuzenroeder *et al*. (1999) it is of importance to check both clinical and environmental samples for thedetection of virulence genes present in Aeromonas species, as they will give more information regards to the depth of its potential pathogenicity. Alves da Silva, *et al*. (2017) highlighted that many of the virulence factors involved in *Aeromonas* species are associated and released in different steps of infection mechanisms. The virulence factors that can be seen in *Aeromonas* species include; (1) Flagella, fimbriae as well as capsules that will enable attachment when in contact with the host surface, (ii) enzymes and toxins (e.g. protease, elastases, lipases and haemolysins) that have the ability to damage cells and tissues, (iii) secretion enzymes that can allow attack against immune response of the host, (iv) siderophore that act as iron predators in the host cell, (v) a capsule containing S-layer, lipopolysaccharide as well as porins that comprise of the host’s defenses, (vi)biofilm formation (allow attachment to cell surface) and (vii) quorum sensing system (modulate bacterial virulence gene expression) (Batra *et al.,* 2016; Janda and Abbott, 2010; Tomas, 2012). Above all the mentioned virulence factors, the most frequently studied in various studies are aerolysin and haemolysin (Chopra *et al*., 1996; Wang *et al*., 2003;) and the most frequently studied genes in *Aeromonas* species include heat liable (alt), cytotonic heat- stable enterotoxins (ast), cytotoxic enterotoxin (hlyA), cytolysin aerolysin (aerA), DNase (exu), lipase (lip) and flagellin (fla) as thy can determine the pathogenicity potential of *Aeromonas* species (Alves da Silva *et al*., 2017).

2.5. Principles of methodologies

2.5.1. Physico-chemical parameters

Water is one of the most important and rich resources that is essential in all life forms. Natural waters are vulnerable to contamination with various toxic contaminants caused by man-made activities. According to Patil *et al*. (2012) the availability of good quality water is an indispensable feature for preventing diseases as well as improving quality of life. Water used for drinking purposes should be assessed regularly to determine its safety and quality, because due to the use of contaminated drinking water, human population can be vulnerable to different water-borne diseases (Adefemi and Awokunmi, 2010). In order to assess the safety and quality of water physical parameters (used to assess the physical state) such as Temperature, pH, turbidity using a multimeter and the chemical parameters using a Hach machine (used to assess the chemical state) such as COD, nitrates and phosphates are used.

a. pH

pH of natural waters is the determination of the acid-base equilibrium of several compounds that have dissolved within the water environment, and as such are a result of the equilibrium that exists between carbon dioxide-bicarbonate-carbonate of which involves several constituent equilibria (DWAF, 1996). pH is one of the important factors that can determine the corrosive state of natural waters, the lower the pH value, the higher the corrosive state of water (Patil *et al.,* 2012). However, the stabilization of pH in natural waters can be affected by various sources such as temperature, toxic heavy metals as well as the protonation or deprotonation of other ions.

b. Temperature

According to Delpla *et al*. (2009) and Prinsloo *et al*. (2014) temperature is one of the important parameters that play a crucial role in the physico-chemical equilibriums and biological reactions in natural waters. It has the ability to affect physical parameters such as pH, dissolved oxygen as well as microbial activity directly or indirectly (Park *et al*., 2010; Prinsloo, 2014). Elevated temperatures in natural waters may cause excessive microbial growth leading to eutrophication, of which may impact water quality negatively. However, a decrease in the temperature of natural waters may lead to low microbial growth (Zamxaka *et al*., 2004).

c. Total Dissolved Salts (TDS)

Total dissolved solids (TDS) are the determination of the quantity of several inorganic salts that have penetrated natural waters. Concentration TDS is directly proportional to the electrical conductivity (EC) of water (Agriculture water use, 1996). TDS includes compounds such as ions of sodium, calcium, bicarbonates, chlorides, magnesium, potassium as well as a small percentage of organic matter (WHO, 2011; Heydari and Bidgoli, 2012; Prinsloo, 2014). Natural waters usually contain different concentrations of TDS, because of factors such as dissolution of minerals in rocks, soils and decomposing plant material. This is the main reason that TDS of natural waters is dependent on the characteristics of the geological formations of which the water is in close contact with (Agriculture water use, 1996). Continuous exposure to TDS through water consumption can be toxic towards human health (Hohls *et al*., 2002).

d. Electrical Conductivity

The ability of water to be able to conduct electricity is solely reflected by its electrical conductivity (DWAF, 1996). Electrical conductivity is an indication of a significant relationship between ten parameters such as temperature, pH value, alkalinity, total hardness, calcium, total solids, total dissolved solids, chemical oxygen demand, chloride as well as iron concentration of water (Patil *et al*., 2010). It is indicated that water consisting of high salinity levels can conduct electricity more efficiently (Prinsloo, 2014). Increased levels of electrical conductivity in water can cause several health implications such as altering the balance between salts and water in patients suffering from heart failure, infants as well as individuals with high blood pressure (Memon *et al*., 2008).

e. Chemical Oxygen Demand (COD)

COD is described as the amount of dissolved oxygen that is essential to cause chemical oxidation of organic matter in water. It can be determined by measuring organic material contamination in water and is measured in mg/L (Patil *et al*. 2010). Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) are both vital indicators that can be used to determine the environmental health of a surface water supply.

f. Nutrients (Nitrates and phosphates)

Nitrates and phosphates are nutrients that are mostly released into the aquatic environment and are utilized by plants, algae and cyanobacteria (Dallas and Day, 2004; Correll, 1999). There are many sources that contribute to the provision of nutrients in natural waters and these could include climatic factors (weathering, erosion, rainfall and variability of runoff) and catchment characteristics (surface geology and land form) (Dallas and Day, 2004). Another source contributing is the application of different fertilizers in the environment (soil) by farmers, with the aim to enhance crop growth of which reach water systems (rivers and dams) in the form of agricultural run-off (Yang *et al*., 2007).

Increased levels of these nutrients can contribute to eutrophication, impacting water quality poorly. Nitrogen in natural waters can be presented in three ions forms of which are ammonium (NH_4^+), nitrite (NO_2^-) and Nitrate (NO_3^-). As bacteria convert nitrogen into these ions, it undergoes a process known as nitrification that can pose threats to human and animal health (Yang *et al*., 2007; Sutton *et al*., 2011). Phosphates are a reactive form of a plant nutrient known as phosphorus and is important in the management of rivers and lakes as it can be used to avoid eutrophication (Webster *et al*., 2001; Dallas and Day, 2004).

2.5.2. Microbiological parameters

a. Heterotrophic plate count method

The concept of using HPC as a water quality parameter was initially proposed by Robert Koch in 1883 (Bartram *et al*., cited by Gensberger *et al*., 2015). The HPC method can be described as the enumeration of growth of heterotrophic culturable microorganisms on a non-selective solid medium under specific conditions (Gensberger *et al*., 2015). Over the years the heterotrophic plate count method has been exposed to widespread series of changes in order to attain the best possible recovery of heterotrophic organisms from various environments (Allen *et al*., 2004). R2A agar that was developed by Reasoner and Geldreich (2004) can be used for the enumeration as well as cultivation of HPCs in water as it contains casein acid hydrolysate, yeast extract, proteose peptone, dextrose, starch, di-potassium phosphate, magnesium sulphate, sodium pyruvate and agar (Coetzee *et al*., 2015). This media/agar is a low nutrient medium and is incubated at lower temperatures and longer incubation time (Tsholo, 2019). It can be utilized in the form of pour plates, spread plates and membrane filter media (Gensberger *et al*., 2015). In treated water, R2A media can promote the growth of fast-growing bacteria and inhibit the growth of slow-growing or stressed bacteria.

b. Membrane filtration

According to WHO (2003) membrane filtration method provide a direct count of bacteria of interest (depending on the media to be used) present in a water sample. A total volume of 100ml of water sample is filtered in a sterile container, under a vacuum, through a cellulose acetate membrane of uniform pore diameter (usually 0.45 µm). The bacteria are retained on the surface of the membrane, which will be placed on a suitable selective media of interest, in a sterile container and incubated at a specific temperature according to the selective media used. This technique however is appropriate for the use of natural waters that contain high levels of suspended materials (such as sludge and sediments) as they could block pores in the filter membrane before a desired volume of water has filtered through.

2.5.3. Primary identification

a. Gram staining technique

Gram staining was first developed in 1884 by the Danish physician Christian Gram and has turned out to be one of the most used staining methods in bacteriology. Gram staining is a differential staining procedure that is able to differentiate between organisms based on their staining properties (Willey *et al*., 2017). This staining technique divides bacteria into two groups, known as Gram-positive and Gram-negative based on their cell wall properties. This procedure begins with a heat-fixed smear stained with a primary dye (crystal violet) then followed by treatment with gram's iodine, of which functions as a mordant. The smear will then undergo decolourization by washing with alcohol-acetone. Finally, the smear undergoes a counterstain, safranin (Willey *et al*., 2017).

b. Endospore staining technique

Endospore staining is a type of a staining technique that is used to recognize the presence of spores in bacterial vegetative cells (Oktari *et al*., 2017). This staining technique requires a stain that can penetrate wall thickness of bacterial spore, hence it requires heat to penetrate dye into an endospore (Willey *et al*., 2017; Oktari *et al*., 2017). Bacterial endospores are a mode of a mechanism that bacteria use to overcome adverse effects posed by the external environment. There are two endospore staining techniques, namely the Schaeffer Fulton technique and the Klein technique. The only difference between these two techniques, is the types of dyes that they utilize. In the Schaeffer Fulton technique, the primary stain used is Malachite Green and the

counterstain used thereof is Safranin. The Klein technique utilizes Carbol Fuchsin as the primary stain and Methylene Blue as the counterstain (Oktari *et al*., 2017). In this study, the Schaeffer Fulton endospore staining technique was used.

2.5.4. Pathogenicity characterization

According to Willey *et al*. (2017) pathogenicity refers to the ability of a microorganism to cause diseases and an opportunistic pathogen refers to an organism that can infect a host, especially hosts with compromised immune systems. According to Janda and Bottone (1981), Yuk and Marshall (2004) and Prinsloo, (2014) microorganisms that are consumed that have the ability to cause gastrointestinal disease share several virulence characteristics such as (i) secretion of extracellular enzymes, (ii) cytotoxicity of cells and adherence to cells and (iii) survive passing through the gastric fluids of the stomach. Bacteria have the ability to secrete extracellular enzymes that act as toxins and these toxins are for pathogenicity and can cause diseases in a host (Prinsloo, 2014). Microorganisms have the ability to produce two kinds of toxins, and they are known as endotoxins and exotoxins. Endotoxins are lipopolysaccharides that are associated with the cell and exotoxins are soluble heat-labile proteins (Kashid and Ghosh, 2010; Prinsloo, 2014).

a. Haemolysin

According to Willey *et al*. (2017) haemolysin is responsible for lysis of erythrocytes and make iron available for microbial growth. Haemolysins are virulence factors implicated in several types of bacterial infections (Ali-Vehmas *et al*., 2001). Haemolysin can be used as a screening test, to determine and differentiate among opportunistic pathogenic microbes and non-pathogenic microbes. According to Prinsloo, (2014) haemolysin is one of the first toxins that are tested when an organism is screened for pathogens or pathogenic potential. In this procedure, microorganisms are streaked and grown onto blood agar containing sheep blood cells, in order to determine whether they are able to lyse red blood cells. According to Payment *et al*. (1994) there are three types of haemolysin reactions and they are (i) alpha, (ii) beta and (iii) gamma. The difference between the three haemolysin reactions is that gamma haemolysin does not lyse blood cells, alpha haemolysin breaks down the blood cells partially and beta haemolysin are responsible for full lysis of blood cells.

b. DNase

Deoxyribonucleases (DNases) are the enzymes which can break phosphodiester linkages of deoxyribonucleic acid. DNases are an essential part of each and every cell, but there are a number of microorganisms that can produce DNases extracellular (Shelkh and Hosseini, 2013). DNase is DNA specific and has the ability to induce nucleic acids degradation (Pavlov *et al*., 2004). For many decades, bacterial extracellular DNase activity has been reported in haemolytic streptococci (Tillet *et al*., 1948; Sunita *et al*., 2015). Pathogenic bacteria have been known to produce extracellular DNase, but the advantage of utilizing its enzyme activity has not been completely understood (Sunita *et al*., 2015). In a study conducted by Fox and Holtman (1968) suggested that DNase facilitate the dissemination and spread of infecting bacteria in hosts as well as providing nucleotides for rapid growth of bacteria by means of hydrolysing DNA. In another study conducted by MacFaddin (1985) hypothesized that pathogens then utilize the degraded DNA as a source of energy.

c. Lecithinase

The phospholipid lecithin is one of the important components of cell membranes and it can easily be damaged by lecithinase to produce diglyceride and phosphorylcholine, thereby causing toxicity (Ghannoum, 2000; Sharaf *et al*., 2014). Lecithinase has the ability to degrade lecithin in plasma membranes allowing pathogens to spread in the form of pore formation in the membrane, making it easier for bacteria to gain entry within the cell (Hoult and Tuxford, 1991; Willey *et al*., 2017; Prinsloo *et al*., 2015). In microorganisms, lecithinase can break down the lecithin to an insoluble diglyceride that results in an opaque halo, surrounding the colony when it is grown on the yolk agar medium (Sharaf *et al*., 2014). According to Bates and Liu (1963) lecithinase activity can be used to identify as well as differentiate between bacterial species.

2.5.5. Kirby-Bauer disk diffusion

The Kirby-Bauer method is the most used disk diffusion test for the determination of antibiotic susceptibility (Willey *et al*., 2017). According to Coetzee (2015) and Willey *et al*. (2017) after the discovery of antibiotics by Alexander Flemming in the 1928 of which was regarded as a milestone, more and more antimicrobials and antibiotics were created in order to treat various infectious diseases. The Kirby-Bauer disk diffusion method was firstly developed in the early 1960s by William Kirby, A.W. Bauer and colleagues (Willey *et al*., 2017; Hudzicki, 2012). The principle of

this method begins with fresh grown bacteria plated out on an entire surface of Mueller-Hinton agar plate. Antibiotic disks (paper-like disks impregnated with varying concentrations of antibiotics) are placed onto the bacteria spreaded on the Mueller-Hinton agar and incubated at 37°C for 24 hours. Following incubation, inhibition zones indicated by clear zones around the disk is measured and compared to the Antibiotic disk susceptibility test standards (Hudzicki, 2012; Coetzee, 2015).

2.5.6. Molecular methods for bacteria identification

It is of importance to be able to determine the identity of a bacteria. Although phenotypic methods give information about the phenotype (colour, shape, arrangement, size), Gram positive or negative, the presence of endospore as well pathogenicity of the microorganisms. All of these are important, but it is inadequate to be able to identify the species of the bacteria that is being used. According to Prinsloo (2014) the conventional culturing methods may not be helpful in the identification of fastidious bacteria that require specific culturing conditions. The use of molecular techniques is advantageous as it provides a new and rapid facilities for the detection of pathogens that are involved in nosocomial infections (Pindi *et al*., 2013). Purifying genomic DNA from bacterial cultures provides foundation for molecular analysis and it is achieved by using commercially available kits (Wright *et al*., 2017).

Polymerase chain reaction (PCR) is an in-situ DNA replication method that enables the exponential amplification of target DNA in the presence of oligonucleotide primers and a thermostable DNA polymerase (Wang, 2000; Adzitey *et al*., 2013). This method involves a wide range of varying concentrations that include DNA templates (5-25 ng), Taq DNA polymerase (0.6-1.25 U), primers (0.11-10 µM) and temperature cycles (45-95.8 °C and running for about 30-40 cycles) (Boonmar *et al*., 2007; Su *et al*., 2011; Adzitey *et al*., 2013). According to Adzitey *et al*. (2013) other components that are included in the PCR reaction such as deoxyribonucleotide triphosphates (dNTPs), magnesium and buffer solutions are used in different concentrations an increase in detection limits. In order to determine the identity of bacteria, 16S ribosomal RNA gene is targeted for amplification as it is present in most prokaryotes (Janda and Abbott, 2007; Prinsloo, 2014).

CHAPTER 3: MATERIALS AND METHODS

3.1. Study site and sample collection

Figure 3.1 below is a map indicating the location of the study sample sites. The study site consisted of eight groundwater systems in the North-West Province. Five of the systems are located within the premises of the North-West University (Potchefstroom) while three are located in neighbouring areas. Groundwater samples were collected during one cold-dry and two warm-rainy seasons. The coordinates of each sampling site were obtained using Global Positioning System (GPS) (Garmin, USA), specific coordinates of the sites are attached in Annexure A. The map (Figure 3) was created using ArcGIS (10.5.1) developed by ESRI (Environmental Systems Research Institute) and is commercially available at (www.esri.com).

Figure 3.1. Map indicating the geographical location of groundwater systems of interest (Image created by Bredenhann, 2018).

Groundwater samples were collected in June 2018, October 2018 and March 2019. This was done in order to determine the physico-chemical and microbiological seasonal trends of the selected groundwater systems. Prior to sample collection, one litre bottles were autoclaved, marked according to site names and placed in a cooler box with ice. The groundwater systems selected had an outlet pipe pumping water into a pool (water used for irrigation purposes) and sampling was conducted following instructions from the DWAF groundwater sampling guide (DWAF, 2000). During sample collection, gloves were worn, and 70% Ethanol was sprayed on the outlet pipe for sterilization purposes. After collection of water samples, one litre bottles were kept on ice and transported to the laboratory. All water samples were analysed within six hours of collection (Molale, 2012).

3.2. Physico-chemical analysis

Groundwater was collected in a glass beaker and the physical parameters [temperature (°C), pH, electrical conductivity (ppm), salinity (ppm) and total dissolved solids (ppm)] were determined on site using a Oakton PCS testr™ 35 calibrated waterproof field multi-meter probe (Thermo Fisher Scientific, US). To reduce cross contamination and for accurate results both the beaker and multi-meter probe were rinsed with distilled water before and after each sample (O'Reilly, 2012). The chemical parameters [chemical oxygen demand (Cat. 2125815), phosphates (Cat. 2106069) and nitrates (Cat. 2106169)] were measured in mg/L in the laboratory using a HACH DR 2800™ (HACH, US) machine following the manufacturer's instructions. The physical and chemical parameters results obtained were compared to the TWQR standards (DWAF, 1996).

3.3. Microbiological parameters

3.3.1. *Clostridium* species isolation

The membrane filtration technique was used to isolate *Clostridium* species using 0.45 µm sterilized filter membranes (Separations, UK) (APHA, 1998). The filtering system used was rinsed with 70% ethanol and distilled water before and after filtering each sample. Sterile forceps were used to place filter membranes on the filtration system (Tsholo, 2019). Triplicates of 100ml water samples were filtered and membranes aseptically placed on TSC perfringens agar plates (Oxoid, UK) supplemented with: 25ml egg yolk emulsion (Merck, Germany) and 1 vial of TSC supplement (Oxoid, UK) per 500ml. To maintain anaerobic growth conditions the plates were placed in an Anaerojar (AG0025; Oxoid) containing an AnaeroGen sachet (AN0025; Thermo Scientific) and

incubated for 24 hours at 44°C. Post incubation, *Clostridium* isolates indicated by black round colonies were counted and noted as colony forming units per 100ml (cfu/ml). Pure cultures of *Clostridium* were obtained by aseptically sub-culturing selected black colonies at least three times using the streak plate technique on TSC agar plates while maintaining the afore described incubation conditions.

3.3.2. *Aeromonas* species isolation

Aeromonas species were also isolated using the membrane filtration method described in 3.3.1. The obtained membranes were aseptically placed onto Membrane Lactose Glucuronide Agar (MLGA; Oxoid, England) and incubated at 37°C for 24 hours. Post incubation, potential *Aeromonas* species were indicated by blue colonies and were counted and noted as cfu/ml (Environment Agency, 2008). Pure cultures were obtained by means of the streak plate technique performed on MLG agar and incubated at 37°C for 24 hours. All sub-culturing was performed thrice.

3.3.3. Heterotrophic plate count bacteria isolation and enumeration

Isolation and enumeration of HPCs was achieved using the spread plate technique. A dilution series of 10^{-1} to 10^{-4} was performed on each sample. Thereafter 100 µl of water was collected from one of the dilution bottles and spread onto Reasoner 2A (R2A) agar (Lab M Ltd, UK). Spread plates were performed from 10^{-2} to 10^{-4} dilutions, in triplicates. Plates were incubated at 30°C (alternatively at room temperature) for four to seven days. Post incubation, colonies were counted and noted as cfu/ml and clustered according to their morphology. Pure HPCs cultures were obtained by streaking individual colonies onto R2A agar and incubated at 30°C for four to seven days. The latter was done three times (Jordaan and Bezuidenhout, 2016).

3.4. Gram staining and Endospore staining

The Burke Gram staining technique was used to determine the purity and cell morphology of isolates as well as to classify them as Gram-positive or Gram-negative. Bacterial cultures were grown for 24 hours prior to use at 37°C. Bacterial smears were made and heat fixed. Briefly, slides were dyed with 1% crystal violet for one minute, rinsed with water, dyed with Gram's iodine for one minute again, decolourised with acetone alcohol and finally dyed with safranin for one minute

then rinsed with water (Willey *et al*. 2017; Pandolfi and Pons, 2004). The slides were dried and viewed under 100x (oil immersion) magnification objective lens using a light microscope (Nikon E200, China). Schaeffer and Fulton's method for endospore detection was used and conducted according to Willey *et al*. (2017). This method was done in order to determine if the *Clostridium* species had the ability to produce endospores when subjected to harsh conditions. Bacterial smears were made, and heat fixed. Slides were placed on top of a beaker with boiling water and smears covered with filter paper. Malachite green dye was added on top of the filter paper for five minutes (making sure the filter paper does not dry out). After five minutes, slides were rinsed with water and again stained with safranin for 60 seconds, rinsed with water again then observed under a microscope.

3.5. Extracellular enzymes

3.5.1. Haemolysin

Pure *Clostridium*, *Aeromonas* and HPC isolates were streaked onto blood agar supplemented with 5% sheep blood (Selecta-media, RSA). This was done in order to determine whether the isolates could lyse blood cells. The plates were incubated for 24 hours at 37°C (Xiao *et al*, 2009; Prinsloo, 2014). The results were noted as alpha (α), beta (β) and gamma (Y) haemolytic. Isolates that were classified as β-haemolytic displayed a clear zone around the inoculated or streaked area as well as a metallic green growth, α-haemolytic isolates displayed a partial clear zone and gamma-haemolytic isolates had no clear zone, but growth was present (Russell *et al.,* 2016).

3.5.2. DNase

Clostridium, *Aeromonas* and HPC isolates were streaked onto DNase agar (Merck, Germany) supplemented with 0.01% toluidine blue (Sigma, Germany) and incubated for 24-48 hours at 37°C. The toluidine blue acts as a dye and processes properties of binding to hydrolysed DNA (Tsholo, 2019). Positive results were indicated by a clear zone around the inoculated area (Sunita *et al.,* 2015).

3.5.3. Lecithinase

A 9:1 ratio solution of McClung Toabe agar base (Remel, Country) supplemented with 50% egg yolk enrichment (Merck, Germany) was used to determine the production of lechitinase. *Clostridium*, *Aeromonas* and HPC isolates were streaked and incubated at 37°C for 72 hours. A positive result was indicated by formation of a white precipitate around or beneath the inoculated area (Rossignol *et al.,* 2009).

3.6. Kirby-Bauer disk diffusion test

The Kirby-Bauer disk diffusion method (developed by Kirby and co-workers, 1966) was used to determine the antibiotic susceptibility of *Aeromonas* and HPC isolates. Overnight cultures were grown in Muller-Hinton broth (Merck, RSA) for 24 hours at 37°C. Using the spread plate technique, 100 µl of the overnight culture was spread plated evenly on the surface of Mueller-Hinton (Merck; RSA) agar plates. The plates were left to dry for five minutes, then antibiotic disks of interest were placed on the plates, using sterilized forceps. Plates were incubated (uninverted) at 37°C for 24 hours. After incubation, inhibition zones were measured (in mm) and compared to Performance Standards for Antimicrobial Susceptibility Testing (2018) provided by the Clinical and Laboratory Standards Institute (CLSI). Antibiotics used were commercially prepared antibiotic disks (Mast Diagnostics, UK) of varying concentrations (Tsholo, 2019).The antibiotics of interest were: ampicillin (AMP, 10 µg) , chloramphenicol (C, 30 µg) , ciprofloxacin (CIP, 5 µg), erythromycin (E, 15 µg) , kanamycin (K, 30 µg), neomycin (NE,30 µg), oxytetracycline (OT, 30 µg), penicillin (Pen-G, 10 µg), streptomycin (S, 25 µg), trimethoprim (W, 5 µg) and vancomycin (VA, 30 µg).

Multiple Antibiotic Resistant (MAR) indices were calculated using the results generated from the Kirby-Bauer disk diffusion tests. According to Chitanand *et al*. (2010) the MAR index is used to establish whether the sampled sites are contaminated with antibiotic resistance and can be calculated using the following equation:

MAR indices = x/(y x z) per sample

x = total amount of resistance to antibiotics

y = amount of antibiotics used

z = number of isolates in sample

3.7. Molecular analysis

3.7.1. DNA extraction

Purified colonies of *Aeromonas* and HPCs were streaked onto nutrient agar and grown at 37°C for 24 hours. Post incubation, colonies were inoculated into nutrient broth and incubated at 37°C for 24 hours for DNA extraction. A Chemagic Bacterial DNA/RNA extraction kit (PerkinElmer®, RSA) was used according to the manufacturer's instructions to extract DNA.

Clostridium DNA was isolated using the microwave method described by Jordaan and Bezuidenhout (2016). *Clostridium* isolates were streaked and grown overnight at 44°C. Colonies were collected using a sterile toothpick and suspended in a 1.5ml Eppendorf tube containing 15µl of Nuclease free PCR water. Eppendorf tubes were sealed with plastic sealers, placed in an open rack then microwaved for two minutes at maximum power (1 000 W). The tubes were then centrifuged for ninety seconds at a maximum speed (13 400 rmp) and placed on ice for further analysis.

3.7.2. Nanodrop

It is of importance to determine the concentration of the DNA obtained, as gel electrophoresis does not provide sufficient insight for polymerase chain reaction purposes. Concentration of successfully extracted DNA was determined using the NanoDrop™ ND-1000 Spectrophotometer (NanoDrop Technologies, USA) (Tsholo, 2019). Isolates that had DNA concentration above 20 ng/µl were diluted with double distilled water down to 20 ng/µl for PCR analysis. According to Coetzee and Bezuidenhout (2018) the NanoDrop also determines the quality or purity of the DNA obtained by the (A260nm/A280nm ratio).

3.7.3. Endpoint PCR amplification

A total amount of 2µl of DNA was used to perform PCR. The PCR reaction consisted of (i)12.5 µl of Dream Taq master mix (5 U/µl Taq DNA polymerase in reaction buffer, 2 mM MgCl2, 0.2 nM of each dNTP) (Thermo Scientific), (ii) 8.5 µl Nuclease free water (Fermentas Life Sciences, US), (iii) 1 µl (10µm/pmol) 27F primer (5'- AGA GTT TGA TCM TGG CTC AG- 3') and 1 µl (10µm/pmol) 1492R (5'- GG TTA CCT TGT TAC GAC TT- 3') (InqabaBiotec; SA) (Jiang *et al,* 2006), 2 µl (20 ng/ul) DNA product, making up a total volume of 25 µl. An amplicon size of 1465 bp is expected when using 16S rDNA primers (Weisburg *et al*., 1991). The ICycler Thermocycler (Bio-Rad, US)

was used for amplification with the following conditions: Initial denaturation step of 95°C for 5 minutes, followed by 35 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 10 minutes.

3.7.4. Gel electrophoresis

Post PCR amplification of DNA templates**,** amplicon sizes of PCR products were determined using gel electrophoresis. The gel was prepared as follows: 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) and was allowed to solidify. Two microlitres of 6x Orange Loading Dye (Thermo Scientific, US) premixed with GelRed (Biotium, US) was mixed with three microlitres of the DNA/PCR product. The mixture was then loaded onto a 1.5% (w/v) agarose gel. To determine the amplicon size, a 1 kb molecular marker (Fermentas; US) was loaded into the first well. Electrophoresis was then carried out at 80 volts for 45 minutes. A Bio-Rad Gel Doc imaging system (Bio-Rad, UK) was used to view the amplicons that were loaded and ran on the gel and the presence of a band, on a specific mark (according to the gene being investigated) yielded a positive result. Gel Visualization was achieved by the ChemDoc MP imaging (Bio-Rad, US) that was used to analyse and capture the image of the gel.

3.7.5. Sequencing

Amplicons that were successful were delivered for sequencing to Inqaba Biotechnology Industries (Pty) Ltd- Pretoria, South Africa. The collected data sequences of isolates from Inqaba were analysed using Finch TV (Version 1.4.0). Sequences obtained from the Finch TV were further analysed in BLAST software (www.blast.ncbi.nlm.nih.gov/Blast.cgi) and EzTaxon software (www.ezibiocloud.net) to identify bacterial species (Tsholo, 2019).

3.7.6. Endpoint PCR: Detection of ARGs

In this study, five antibiotic resistance genes (ARGs) (*ampC, bla$_{TEM}$, ermB, ermF and tetM*) were investigated. Table 2 indicates the primer sequence information of the ARGs. Each PCR mixture contained 12.5 µl Dream Taq master mix (5 U/µl Taq DNA polymerase in reaction buffer, 2 mM MgCl2, 0.2 nM of each dNTP) (Thermo Scientific), 8.5 µl nuclease free water, 1 µl (10 µm/pmol) of each forward and reverse primers (Inqababiotec, RSA) and 2 µl of isolated DNA template (20 ng/µl), making up a total reaction mix of 25 µl. Below in Table 1 is the description of the thermal

cycler conditions of each gene. All of the conditions began with an initial denaturation of 95°C for five minutes.

Table 3.1. Thermal cycler conditions of ARGs investigated.

Gene	Denatura-tion	Annealing	Elongation	Final extension	Cycle-s	Reference
ampC	94°C (30 sec)	49°C (30 sec)	72°C (60 sec)	72°C (7 min)	30	Coetzee and Bezuidenhout, 2018; Schwartz *et al*., 2003
bla$_{TEM}$	95°C (60 sec)	60°C (60 sec)	72°C (60 sec)	72°C (5 min)	30	Costa *et al*., 2007 Tsholo, 2019
ermB	95°C (30 sec)	48°C (60 sec)	72°C (2 min)	72°C (10 min)	35	Chung *et al*., 1999 Fourie, 2017
ermF	95°C (30 sec)	50°C (30 sec)	72°C (2 min)	72°C (10 min)	35	Chung *et al*., 1999 Fourie, 2017
tetM	95°C (45 sec)	55°C (45 sec)	72°C (45 sec)	72°C (7 min)	35	Aminov *et al*., 2001

Table 3.2. Oligonucleotide primers for PCR amplification of 16s rDNA, *ampC*, *bla$_{Tem}$*, *ermB*, *ermF* and *tetM* (Fourie, 2017; Tsholo, 2019).

Gene name	Forward primer Sequence (5'-3')	Reverse primer Sequence (5'-3')	size (bp)	Reference
16S rDNA	AGA GTT TGA TCM TGG CTC AG	GG TTA CCT TGT TAC GAC TT	1 465	Jiang *et al*., 2006
ampC	TTC TAT CAA MAC TGG CAR CC	CCY TTT TAT GTA CCC AYG A	550	Coertze and Bezuidenhout, 2018

bla$_{TEM}$	ATT CTT GAA GAC GAA AGG GC	ACG CTC AGT GGA ACG AAA AC	1 150	Costa *et al*., 2007
ermB	GAA AAG GTA CTC AAC CAA ATA	AGT AAC GGT ACT TAA ATT GTT TAC'	638	Fourie, 2017
ermF	CGG GTC AGC ACT TTA CTA TTG	GGA CCT ACC TCA TAG ACA AG	466	Fourie, 2017
tetM	ACA GAA AGC TTA TTA TAT AAC	TGG CGT GTG TCT ATT GAT GTT CAC	171	Aminov *et al*,. 2001

3.7.7. Endpoint PCR: Virulence genes

A total of ten virulence genes were investigated in confirmed *Aeromonas* isolates. Virulence genes of interest were *act*, *ast*, *aer*, *alt*, *fla*, *hly, ser*, *ela*, *exu* and *lip.* Single PCR reaction mixtures with a final volume of 25 µl were prepared in a laminar flow cabinet (Bioflow I). Each PCR reaction mix contained 12.5 µl of Dream Taq master mix (5 U/µl Taq DNA polymerase in reaction buffer, 2 mM MgCl2, 0.2 nM of each dNTP) (Thermo Scientific), 8.5 µl nuclease free water, 1 µl forward primer (10µm/pmol) (Inqababiotec, RSA), 1 µl reverse primer (10 µm/pmol) (Inqababiotec, RSA) and 2 µl of DNA template (20 ng/ul). The primer sets used in this study are provided in Table 3. The thermal cycling conditions began with an initial denaturation step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 5 min, annealing (see table 3 for specific annealing temperatures) for 30 seconds and extension at 72°C for 7 minutes (Ruhil Hayiti *et al*., 2015).

Table 3.3. Primer sequences used for the detection of virulence genes, A.T indicating the annealing temperature of each primer.

Gene	**Forward primer sequence (5' – 3')**	**reverse primer sequence (5' – 3')**	**A.T (°C)**	**product size (bp)**	**References**
Act	GAGAAGGTGACCAC CAAGAACA	AACTGACATCGGCCTT GAACTC	65	232	Sreedharan *et al*., 2012
Ast	ATCGT CAGCGACAGCTTCT T	CTCATCCCTTGGCTT GTTGT	63	504	El-Barbary, 2010

Aer	CAAGAACAAGTTCA AGTGGCCA	ACGAAGGTGTGGTTCC AGT	63	309	Wang *et al*., 2003
Alt	TGACCCAGTCCTGG	GGTGATCGATCACC	64	442	Hu *et al*., 2012
fla	TCCAACCGTYTGAC CTC	GMYTGGTTGCGRATGG T	55	608	Sen and Rodgers, 2004
Hly	GCCGAGCGCCCAGA AGGTGAGTT	GAGCGGCTGGATGCG GTTGT	65	130	Wang *et al*., 2003
Ser	ACGGAGTGCGTTCT TCCTACTCCAG	CCGTTCATCACACCGT TGTAGTCG	57	211	Nam and Joh, 2007
Ela	ACACGGTCAAGGAG ATCAAC	CGCTGGTGTTGGCCAG CAGG	59	540	Sen and Rodgers, 2004
Exu	AGACATGCACAACC TCTTCC	GATTGGTATTGCCCTG CAAC	61	323	Chacon *et al*., 2003
Lip	GACCCCCTACCTGA ACCTGAGCTAC	AGTGACCCAGGAAGTG CACCTTGAG	63	155	Nam and Joh, 2007

3.7.7. Statistical analysis

MS Excel was used to generate averages and standard deviations in both physico-chemical and microbiological parameters (O'Reilly, 2012). MS Excel was also used to generate pie charts as well as bar graphs. Canoco 4.5. for windows was used to generate Principal Component Analysis (PCA) and Redundancy Analysis (RDA) plots in order to determine the correlations between the physico-chemical and microbiological parameters (Jordaan and Bezuidenhout, 2016). The statistical association of the physico-chemcial and microbiological parameters was determined using basic statistic correlation matrices and marked correlations were significant at $p < 0.05$ using Staistica 13.3.

CHAPTER 4: RESULTS

Introduction

A brief overview of results obtained from methodologies outlined in chapter 3 will be represented in this chapter. Both the physico-chemical and microbiological parameters were investigated to determine the quality of groundwater systems of interest. The layout of the results was separated and illustrated seasonally (winter, summer and autumn). The separation of seasons was done in order to observe changes and impacts that seasons have on both physico-chemical and microbiological parameters. Using microbiological parameters, the following sub-parameters were observed; (i) antibiotic resistance patterns, (ii) presence of ARGs, (iii) extracellular enzymes activity and (iv) identification and associations in species (phylogenetic tree). This was also done to sketch a picture of the current state and quality of groundwater systems that were of interest in the NWP.

4.1. Physical parameters

4.1.1. Temperature

Table 4.1 below shows both the physico-chemical and microbiological parameters obtained from winter, summer and autumn seasons. Temperature is a sensitive parameter as it determines how cold or hot the environment is. Overall trends for the duration of the study indicated that the highest (25.7°C) and lowest (15.53°C) temperatures were both observed at site FA during autumn and winter respectively. Groundwater temperatures, below 20°C, were generally observed during the winter season with the exception of site BG. However, four sites (MD, GM, LR and DK) also displayed water temperatures below 20°C during summer. Additionally, as depicted in Table 4.1, the groundwater temperatures measured at the above-mentioned sites (MD, GM, LR and DK) were lower during summer as compared to winter. The highest (21.13°C) and lowest (16.9°C) groundwater temperatures measured during summer were observed at sites FA and GM respectively. The warmest, above 21°C, groundwater temperatures were observed during autumn. The highest and lowest groundwater temperatures measured during autumn were observed at sites FA (25.7°C) and B4 (21.63°C) respectively. An increase in groundwater temperatures was observed from summer to autumn, at all sampled sites. Out of all the sites, Botanical Gardens (BG) was constant throughout all the seasons with temperatures above 20°C.

4.1.2. pH

The pH determines the acidity of the water environment. Any value that is less than 5 is considered acidic, and a value higher than 9.5 is considered basic according to the standards set by the TWQR (DWAF, 1996). During the the study period, pH levels displayed in Table 4.1 were within the recommended TWQR standards (5.0- 9.5) for all seasons. The overall lowest pH levels observed was at 6.52 at site MD and the overall highest pH obtained was 8.14 at site GM. Groundwater pH levels that were measured during winter were above pH 7.0 except for site BG that had a relatively low pH (6.77) as compared to other sites. The highest pH measured in this season was 7.8 at site GM. In the summer season, groundwater depicted pH levels that were above 7.0 throughout all sites. During this season groundwater seemed to be more basic (alkaline) as the highest pH measured was 8.14 at site GM and the lowest pH measured was 7.01 observed at site BG. pH levels that were measured in the autumn season were slightly lower than pH levels in winter. As depicted in Table 4.1 the lowest pH level measured in autumn was 6.52 at site MD and the highest was 7.51 which was measured at sites GM and LV. Site GM displayed high levels of pH in all the seasons, whereas site BG displayed low levels of pH in winter and summer seasons.

4.1.3. Total Dissolved Salts (TDS)

Total dissolved solids are a measurement of both organic and inorganic substances that are present in water. Table 4.1 shows that during the study period, measured TDS in groundwater systems was higher than the recommended limits (0-450 ppm) by TWQR standards (DWAF, 1996). During the course of this study, the lowest TDS level was 350.67 ppm at site BG and the highest level was 898.66 ppm at site LR. Compliance with the recommended TWQR standards was observed in the winter season only. The highest TDS levels measured were observed at site LR (898.66 ppm). Elevated TDS levels were generally observed in both summer and autumn seasons respectively. In summer high TDS levels were measured at site LV (898.33 ppm), and the lowest TDS levels were measured at site BG (503.33 ppm). In autumn the highest TDS levels were observed at site LR (878.66 ppm) and the lowest TDS level were displayed at site BG (494 ppm). In all the seasons, site BG depicted the lowest TDS levels (below 550 ppm) amongst other sites. Groundwater sites LR, LV and B4 indicated high levels of TDS, above 800 ppm throughout the duration of this study.

4.1.4. Salinity

Salinity is the measurement of salt that is present in water. High salts in water affect the binding of dissolved oxygen, thereby decreasing the amount of dissolved oxygen within the water. There are no TWQR standards limitations available for Salinity levels in terms of groundwater used for irrigation purposes. Table 4.1 depicts various ranges of salinity measured in groundwater during the study period. Overall trends observed during this study indicated the lowest salt levels (213 ppm) at site BG and the highest salt levels (438.33 ppm) were at site LR. Site BG displayed the lowest salt levels in winter (213 ppm), summer (243.33 ppm) and autumn (238.66 ppm) respectively. Throughout the entire duration of the study, two sites displayed high levels of salt in groundwater. These two sites include, site LR that had high salt levels in both winter (445.66 ppm) and autumn (430.66 ppm) seasons as well as site LV which had high levels of salt in the summer (438.33 ppm) season.

Table 4.1. Physico-chemical and microbiological parameters of the winter (2018), summer (2018) and autumn (2019) seasons.

Sites		Temperature (°C)	pH	TDS (ppm)	SALT (ppm)	COD (mg/L)	PO_4^{3-} (mg/L)	NO_3^- (mg/L)	HPCs cfu/100ml	*Clostridium* cfu/100ml	*Aeromonas* cfu/100ml
TWQR		**N/A**	**5.0-9.5**	**0-450**	**N/A**	**N/A**	**N/A**	**<10**	**N/A**	**N/A**	**N/A**
FA	Winter	15.53 ± 0.25	7.52 ± 0.21	513 ± 9.54	312±4.58	0±0	5.2 ±2.27	0.06 ± 0.06	0 ± 0	0	0
	Summer	21.13 ± 0.350	7.59 ± 0.03	782.33 ± 5.51	382± 4	12 ±2.65	4.42 ± 0.32	0 ± 0	293333 ± 7023	1	1
	Autumn	25.7± 0.6	6.94 ± 0.07	784.66 ±3.21	387.66± 3.05	30.66 ±27.02	2.63 ± 1.35	1 ± 1.73	0 ± 0	0	0
MD	Winter	19.73± 0.32	7.15 ± 0.1	413.33± 2.52	248.66 ±4.16	8.33 ±4.04	3.88 ± 0.82	1.63 ± 0.25	0 ± 0	0	0
	Summer	19.4± 0.6	7.29 ± 0.06	592.33 ±1.53	282 ±5.29	24 ±10.58	4.43± 0.95	0.36 ± 0.25	104000 ± 5291	3	0
	Autumn	23.96± 0.55	6.52 ± 0.02	598.66 ±2.31	288.66 ± 3.21	0± 0	3.51± 1.91	1.33 ±2.31	0 ± 0	0	0
BG	Winter	20.97 ± 0.25	6.77 ± 0.06	350.67 ± 0.58	213 ± 1.73	2.33 ± 3.21	3.64 ± 0.94	1.83 ± 0.12	0 ± 0	0	0
	Summer	21.33 ± 0.51	7.01 ± 0.08	503.33±1.15	243.33 ± 1.15	5.66 ± 4.93	4.32 ± 0.78	0.1 ± 0.1	0 ± 0	0	0
	Autumn	23.86 ± 0.23	7.05 ± 0.02	494 ±1	238.66 ± 2.31	0 ± 0	0.82 ± 0.46	0.66 ± 1.15	365000 ± 15000	0	0
GM	Winter	19.6± 0.2	7.8 ± 0.08	378.66 ± 1.53	231.33 ± 0.06	0.33 ± 0.57	4.61 ± 0.81	1.16 ± 0.21	0 ± 0	0	0
	Summer	16.9 ± 0.1	8.14 ± 0.01	516.66 ± 1.53	245.66 ± 2.52	60 ± 14.18	4.35 ± 1.26	0.6± 0.3	165000 ± 13228	0	1
	Autumn	25.16± 0.21	7.51 ± 0.07	527.33 ± 7.02	250.33 ± 7.63	16.33 ± 14.57	3.88± 0.6	1± 1.73	57500 ±11605	0	0
LR	Winter	18.8 ± 0.26	7.36 ± 0.05	898.66 ± 8.5	445.66 ± 3.79	16.33 ± 14.22	0.19 ± 0.01	0.83 ± 0.06	80000 ± 10000	0	0
	Summer	18.23 ± 0.26	7.7 ± 0.04	824.33 ± 3.78	399.66 ± 4.73	5.66 ± 4.35	4.46 ± 1.04	1.93 ± 0.3	5000 ± 3464	0	0
	Autumn	22.26 ± 0.35	6.81 ± 0.04	878.66 ± 1.53	430.66 ± 4.16	10.66 ±9.29	1.75 ± 1.36	0.66 ± 1.15	0 ± 0	0	0
LV	Winter	19.53 ± 0.4	7.42 ± 0.02	813.33 ± 1.53	397.66 ± 0.58	21.33 ± 5.13	0.35 ± 0.01	2.36 ± 0.15	352500 ± 2500	0	0
	Summer	20.4 ± 0.25	7.31± 0.05	898.33 ± 2.08	438.33 ± 3.51	5± 0.57	3.65 ± 0.31	0.6 ± 0.66	12500 ± 2645	0	2
	Autumn	22.2 ± 0.26	7.51 ± 0.03	874 ± 1	429.66 ± 1.53	0 ± 0	0.93 ± 0.54	0± 0	13500 ± 5634	0	0
B4	Winter	17.9 ± 0.46	7.41 ± 0.03	849.66 ± 0.58	415.66 ± 2.08	11.33 ± 2.08	0.19 ± 0.01	1.73 ± 0.06	230000 ± 20000	0	0
	Summer	20.16 ± 0.06	7.33 ± 0.03	841.66 ± 19.35	417.33 ± 5.51	6.33 ± 0.58	4.39 ± 1.67	0.86 ± 0.35	280000 ± 10000	2	0
	Autumn	21.63 ± 0.4	7.29 ± 0.03	846 ± 3.61	408.33 ± 11.59	0 ± 0	0.36 ± 0.04	0.01 ± 0.01	0 ± 0	0	0
DK	Winter	18.06 ± 0.15	7.32 ± 0.07	628.66 ± 0.58	415.66 ± 2.88	15.33 ± 6.35	0.11 ± 0.01	0.53 ± 0.06	206666 ± 40414	0	0
	Summer	17.23 ± 0.25	7.56 ± 0.05	633.66 ± 2.52	300.33 ± 5.03	8.66 ± 1.15	5.15 ± 0.45	0.46 ± 0.12	35000 ± 13228	0	0
	Autumn	22.73 ± 0.65	6.66 ± 0.05	687± 7.93	322.33 ± 20.42	0.33 ± 0.58	0.72 ± 0.55	0.66 ± 1.15	193333 ± 7289	0	0

BG- Botanical gardens, **MD**- Mediclinic, **LV**- LaVaria, **LR**-Lareus, **GM**- Gerhard minnebrone, **DK**-Draak, **FA**- Fanie; **COD**- Chemical Oxygen Demand, **HPCs**- Heterotrophic plate count bacteria, **NO_3^-** - Nitrates, **PO_4^{3-}**-phosphates, **TDS**- Total dissolved solids, **TWQR**- Target Water Quality Range

4.1.5. Chemical Oxygen Demand (COD)

Chemical Oxygen Demand is the total amount of oxygen that is taken up in order to oxidize organic contaminants to inorganic by products. In Table 4.1 COD levels during winter, summer and autumn can be observed. The overall lowest and highest COD levels were observed at site GM with levels of 0.33 mg/L and 60 mg/L respectively. During this study, interesting COD levels were observed. Site LV depicted high COD levels (21.33 mg/L) during the winter season and displayed relatively low COD levels (5 mg/L) during the summer season. During the autumn season, sites MD, BG, LV and B4 did not display any COD levels, whereas sites FA, GM, LR and DK had varying levels of COD. However, the lowest COD levels were displayed at site B4 (0.36 mg/L) and the highest COD levels were displayed by FA (30 mg/L) during autumn. Although site FA showed high levels of COD during the autumn season, no COD levels were recorded at this site in winter season.

4.1.6. Nitrates and Phosphates

Nitrates are commonly known as salts that can naturally be found in groundwater. Groundwater nitrate levels, as seen in Table 4.1, were all within the TWQR standards limitations <10 mg/L. The nitrate levels observed in groundwater was relatively low. Overall, the lowest nitrate levels were seen at site FA (0 mg/L) and the highest at site LV (2.36 mg/L). Site FA displayed low nitrate levels measurements in both winter (0.06 mg/L) and summer (0 mg/L). The highest nitrate levels measured in winter were observed at site LV (2.36 mg/L). During the summer season, elevated levels of nitrates were measured at site LR (1.93 mg/L). As seen in Table 4.1, the lowest levels of nitrates were seen in site LV (0 mg/L) and the highest levels of nitrates were observed at site MD (1.33 mg/L) respectively.

Phosphates are important to plant life but excess levels of it in water, may cause eutrophication, thereby decreasing the levels of oxygen in the water medium. Phosphate levels that were detected during the course of this study were below 10 mg/L in all seasons; however, there are no TWQR standards recommendations available for comparison purposes. Although phosphate levels obtained were generally low, the overall lowest was observed at site DK (0.11 mg/L) and the highest was observed at FA (5.2 mg/L). Table 4.1 depicts that site DK had both the lowest and highest phosphate levels in winter (0.11 mg/L) and summer (5.15 mg/L) respectively. The highest phosphate levels that were measured in the winter season were seen at site FA (5.2 mg/L). Phosphate levels measured during summer were higher than those observed in winter and autumn as the lowest phosphate levels were 3.65 mg/L in site LV, whereas the lowest levels in

the other seasons were below 0.5 mg/L. The phosphate levels that were obtained in the autumn season were by far the lowest observed. During this season, lowest phosphate levels measured were seen at site B4 (0.36 mg/L) and the highest phosphate levels were seen at site GM (3.88 mg/L).

The physico-chemical parameters that were observed during this study were evident to promote and maintain the growth of microorganisms. However, parameters such as TDS, Salinity and CODs portrayed very high concentrations and could be of concern.

4.2. Microbiological parameters

In this section, microbiological parameters will be illustrated as per sampling season. Microbiological parameters of interest include *Clostridium* sp., *Aeromonas* sp, as well as HPCs. *Clostridium* and *Aeromonas* species can be used as additional indicators of faecal pollution as they are opportunistic pathogens. The heterotrophic bacteria group is a rather large group comprising of a variety of microorganisms, some of which are considered as faecal indicators such as *E. coli*.

4.2.1. Heterotrophic count bacteria

As displayed in Table 4.1, HPCs were identified and enumerated. During the winter season, HPCs that were identified at four sites are (LR, LV, B4 and DK). In general, site LR depicted the lowest HPCs levels (5000 cfu/ml) and site BG depicted the highest HPCs levels (365000 cfu/ml) overall. The lowest and highest levels of HPCs observed during winter were 80000 cfu/ml and 352500 cfu/ml at sites LR and LV respectively. During summer, HPCs were identified at all sites with varying HPC levels between sites. The lowest levels of HPCs obtained during summer were 5000 cfu/ml and the highest 293333 cfu/ml at sites LR and FA respectively. Autumn season results indicated a similar trend that was observed in the winter season only four sites (BG, GM, LV and DK) having the presence of HPCs, whilst other sites showed no trace of HPCs. The lowest levels that were obtained were 13500 cfu/ml at site LV and the highest levels were 365000 cfu/ml. Interestingly, HPCs were not identified at site BG during winter and summer but were identified during autumn with the highest HPC cfu/ml noted throughout the study (365000 cfu/ml). Additionally, sites, LV and DK were the two sites where the presence of HPCs was observed throughout all three seasons

4.2.2. *Clostridium* and *Aeromonas*

Clostridium and *Aeromonas* were investigated to determine the quality of groundwater systems and to determine if they had the presence of opportunistic pathogens. *Clostridium* and *Aeromonas* can be used as indicators as their presence in water indicates faecal pollution. These two organisms were only detected in the summer season.

Clostridium was detected at three sites: FA, MD and B4. The lowest *Clostridium* level obtained was 1 cfu/ml at site FA and the highest levels were 3 cfu/ml at site MD. Similarly, *Aeromonas* were also detected at three sites FA, GM and LV. The lowest *Aeromonas* level obtained was 1 cfu/ml at two sites (FA and GM) and the highest levels obtained were 2 cfu/ml at site LV. The enumeration of these two species was relatively low at all sites.

4.3. Correlations observed between physico-chemical and microbiological parameters

The statistical association of the physico-chemical and microbial parameters was determined using basic statistics correlation matrices and marked correlations were significant at $P < 0.05$. Principal Component Analysis (PCA) biplots were generated for winter and autumn seasons analysis while a Redundancy Analysis (RDA) triplot was generated for only the summer season. The RDA that was generated in the summer season was due to the presence of both *Clostridium* and *Aeromonas* that was evident. However, the presence of *Clostridium* and *Aeromonas* was observed in winter and autumn seasons, hence the use of PCAs.

4.3.1. Winter (Cold and Dry) season

Figure 4.1 below is a PCA biplot showing correlations observed between the physico-chemical and microbiological parameters observed in the winter season.

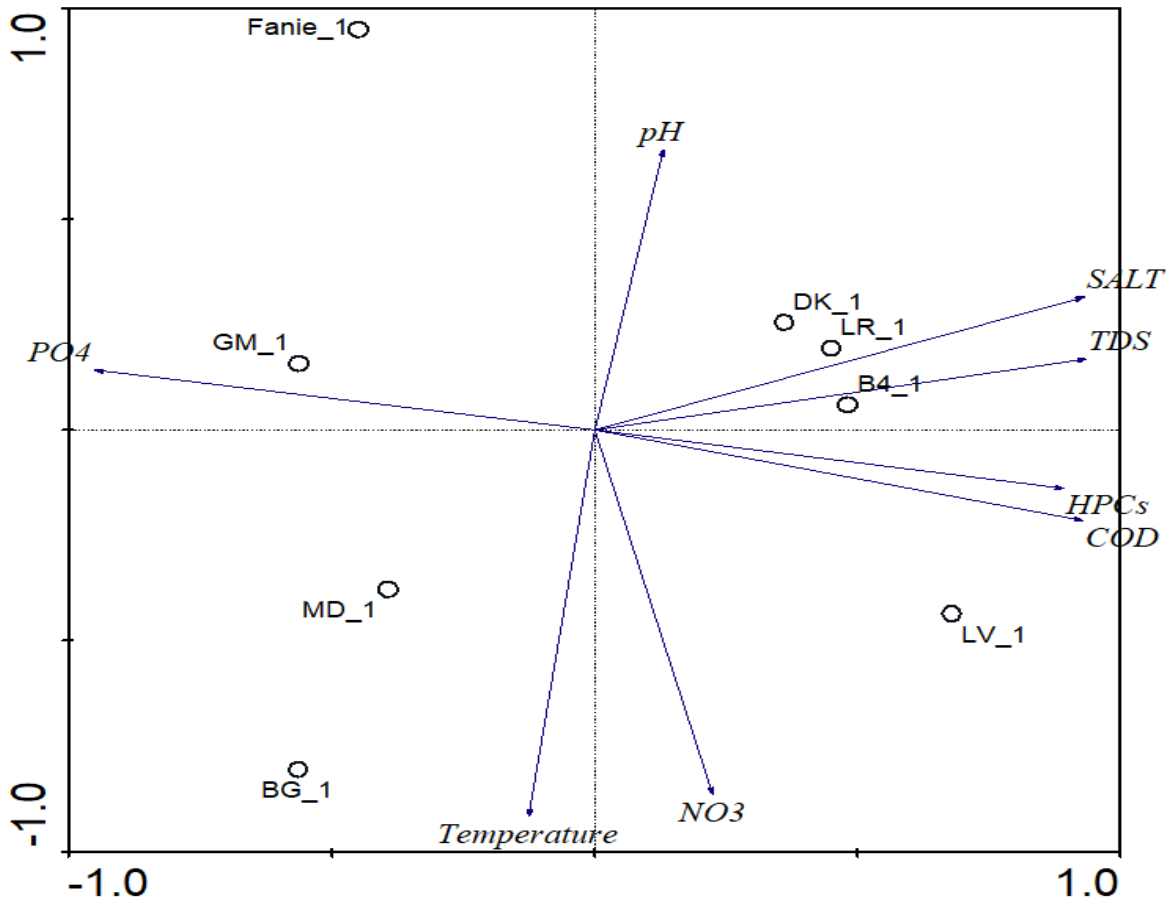

Figure 4.1. Principal Component Analysis (PCA) biplots indicating the correlations between physico-chemical parameters (Temperature, pH, TDS, SALT, COD, NO_{3}^{-}, PO_4^{3-}) and microbiological parameters (HPCS) in varying groundwater systems during the winter season. The blue arrows represent both physico-chemical and microbiological parameters.

BG- Botanical gardens, **MD**- Mediclinic, **LV**- LaVaria, **LR**-Lareus, **GM**- Gerhard minnebrone, **DK**-Draak, **FA-** Fanie; **COD**- Chemical Oxygen Demand, **HPCs**- Heterotrophic plate count bacteria, **NO_3^-** - Nitrites, **PO_4^{3-}**- phosphates, **TDS**- Total dissolved solids.

As seen in Figure 4.1 there was a strong correlation between NO_3^- and temperature (p-value of 0.041). Nitrate (NO_3^-) was the only parameter that showed a positive relationship with temperature and vice versa, whereas the relationship with other parameters was significantly negative for both of them, as the p-value was higher than 1. Six statistically significant correlations were observed between four parameters (TDS, SALT, COD and PO_4^{3-}). The associations were as follows; (i) TDS and SALT (p-value= 0.000), (ii) TDS and COD (p-value= 0.017), (iii) TDS and PO_4^{3-} (p-value = 0.007), (iv) SALT and COD (p-value = 0.017), (v) SALT and PO_4^{3-} (p-value = 0.004) and finally (vi) COD and PO_4^{3-} (p-value = 0.002) respectively. As displayed in Figure 4.1 HPCs were influenced by four physico-chemical parameters. A statistically positive correlation was observed between HPCs and physico-chemical parameters (SALT, TDS, COD and PO_4^{3-}). Significant positive correlations were as follows; (i) HPC and TDS (p-value =0.039), (ii) HPC and SALT (p-value = 0.041), (iii) HPC and COD (p-value = 0.010) and (iv) HPC and PO_4^{3-} (p-value = 0.013) respectively. HPCs were clearly not influenced by temperature, pH and NO_3^- and therefore indicated no correlation.

4.3.2. Summer (Warm and Wet) season

Figure 4.2 below depicts an RDA triplot representing the association observed between the physico-chemical parameters and microbiological parameters during the summer season. As seen in Figure 4.2 temperature showed a statistically significant positive correlation with pH (p-value = 0,031). However, a negative correlation was observed with the rest of the parameters that were investigated in this season. pH displayed another statistically significant positive association with COD (p-value= 0.004). A statistically significant strong correlation was observed between TDS and SALT (p-value = 0.000). In terms of microbiological parameters, a positive correlation was observed between *Aeromonas* and PO_4^{3-} (p-value= 0.049) respectively. There were no significant positive correlations that were observed for NO_3^-, HPCs and *Clostridium*.

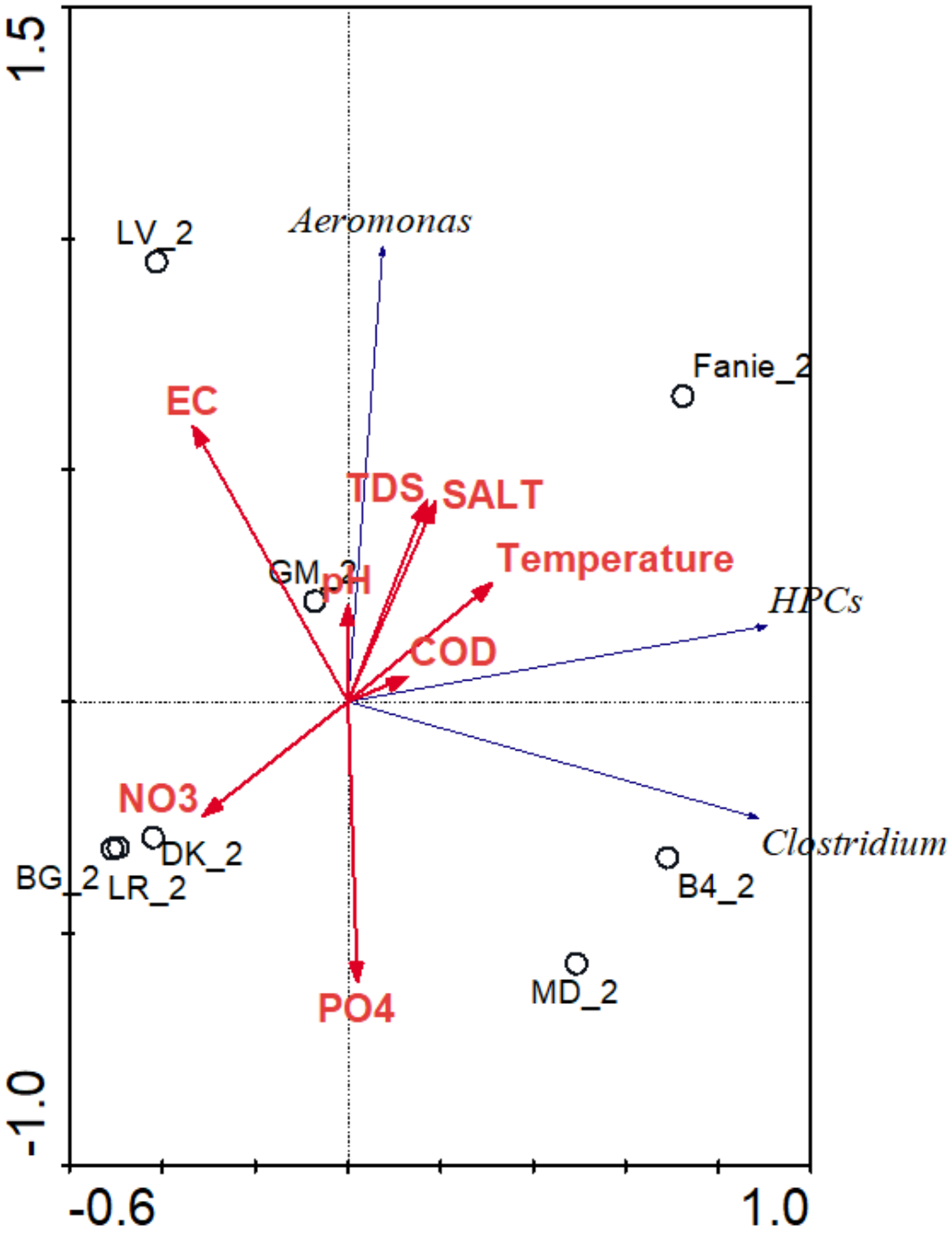

Figure 4.2. Redundancy Analysis (RDA) triplot representing the relationship between varying microbiological species diversity and physico-chemical parameters in various sites in the summer season.

BG- Botanical gardens, **MD**- Mediclinic, **LV**- LaVaria, **LR**-Lareus, **GM**- Gerhard minnebrone, **DK**-Draak; **COD**- Chemical Oxygen Demand, **HPCs**- Heterotrophic plate count bacteria, **NO_3^-** - Nitrites, **PO_4^{3-}**- phosphates, **TDS**- Total dissolved solids.

4.3.3. Autumn (Warm and Wet) season

Figure 4.3 below represents a PCA biplot of correlations observed between the physico-chemical parameters and microbiological parameters during the autumn season. Temperature had a statistically significant correlation with three parameters and the following relationships were observed; (i) Temperature and COD (p-value= 0.042), (ii) Temperature and NO_3^- (p-value= 0.027) and, (iii) Temperature and PO_4^{3-} (p-value= 0.034) respectively. Furthermore, a statistically significant positive correlation was displayed between TDS and SALT (p-value= 0.000). Figure 4.3 shows that for chemical parameters, COD had a positive correlation with only one parameter, of which was temperature. Whereas NO_3^- and PO_4^{3-} had a strong positive correlation (p-value= 0.013) with each other. In addition, NO_3^- (p- value= 0.027) and PO_4^3 (p-value= 0.034) had a statistically significant correlation with temperature. Although correlations were observed for most parameters, pH and HPCs yielded no correlation with other parameters.

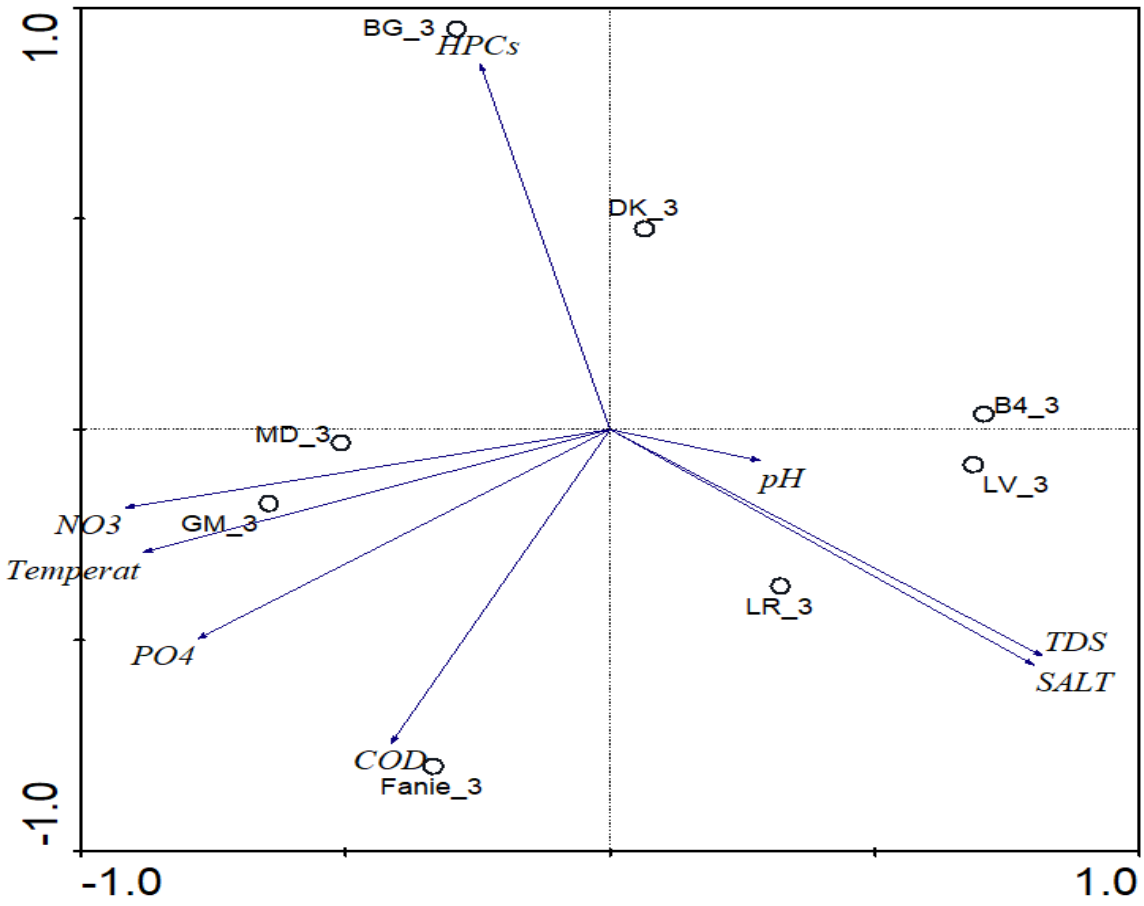

Figure 4.3. Principal Component Analysis (PCA) biplots obtained from the autumn season representing association between physico-chemical parameters and microbiological parameters.

BG- Botanical gardens, **MD**- Mediclinic, **LV**- LaVaria, **LR**-Lareus, **GM**- Gerhard minnebrone, **DK**-Draak; **COD**- Chemical Oxygen Demand, **HPCs**- Heterotrophic plate count bacteria, **NO_3^-** - Nitrites, **PO_4^{3-}**- phosphates, **TDS**- Total dissolved solids and **temperat**- temperature.

4.4. Antibiotic Resistance profiles

4.4.1. Resistance profiles

Table 4.2 below represents the raw percentages of antibiotic resistance profiles obtained in all seasons and Figure 4.5 below is a bar graph representing the summary of antibiotic profiles obtained from all seasons.

Table 4.2. Percentages of antibiotic resistance profiles throughout three seasons.

Season	Percentage (%)	AMP 10 µg	C 30 µg	CIP 5 µg	E 15 µg	K 30 µg	NE 30 µg	OT 30 µg	Pen-G** 10 µg	S 25 µg	W 5 µg	VA** 30 µg
Winter	R	94.4	5.55	0	5.55	27.7	0	61.1	100	0	100	18.1
	IR	0	22.3	11.2	5.55	44.4	0	27.7	0	0	0	54.5
(n=18)	S	5.56	72.2	88.8	88.8	27.7	100	11.1	0	100	0	27.3
Summer	R	96.9	3.13	3.13	3.13	9.38	0	65.6	100	6.25	100	28.6
	IR	0	15.6	0	0	15.6	0	12.5	0	0	0	14.3
(n=32)	S	3.13	81.3	96.9	96.9	75	100	21.9	0	93.8	0	57.1
Autumn	R	100	0	2.5	17.5	15	2.5	25	100	0	100	30
	IR	0	5	30	5	80	7.5	57.5	0	0	0	42.5
(n=41)	S	0	95	67.5	77.5	5	90	17.5	0	100	0	27.5

Amp- ampicilin, **C**-Chloramphenicol, **CIP**- Ciprofloxacin, **E**- Erythromycin, **K**- Kanamycin, **NE**, Neomycin, **OT**- Oxy-tetracycline, **Pen-G**- Penicillin G, **S**-Streptomycin, **W**-Trimethoprim, VA-Vancomycin; **N**- number of isolates, **R**- Resistant, **IR**- Intermediate resistant, **S**- Susceptible.

The results obtained were converted to percentages as per antibiotic. The antibiotics with "**" were only tested in Gram-positive isolates. The winter season yielded a total of 18 isolates and was subjected to varying concentrations of different antibiotics to determine the isolates' resistance patterns. According to the percentages obtained in Table 4.2, high resistance was evident for four antibiotics namely; Ampicillin (94.4%), Oxy-tetracycline (61.1%), Penicillin-G (100) and Trimethoprim (100%). This was also observed in the summer season (32 isolates) results with the exception of the resistance percentage of Ampicillin increasing to 96.9%. Autumn (41 isolates) the isolates obtained indicated complete resistance towards three antibiotics: Ampicillin, Penicillin-G and Trimethoprim.

Isolates were susceptible to five antibiotics namely: Chloramphenicol (72.2%), Ciprofloxacin (88.8%), Erythromycin (88.8%), Neomycin (100%) and Streptomycin (100%) during the winter season. Isolates showed complete susceptibility with percentages of 100% to Neomycin and Streptomycin. During summer isolates were susceptible to seven antibiotics in total but indicated highest susceptibility towards: Neomycin (100%), Ciprofloxacin (96.9%) and Erythromycin (96.9%). In autumn trends similar to winter were observed. Wherein isolates showed high susceptibility percentages towards five antibiotics.

Percentages obtained from Kanamycin and Vancomycin suggested that they had the highest percentages of isolates that were intermediate resistant. This was observed for both winter and autumn season (winter- K= 44.4% & VA= 54.5% and autumn- K=80% & VA= 42.5%). Isolates obtained in the autumn season also indicated a high percentage of intermediate resistance to Oxy-tetracycline (57.5%) that was initially regarded as resistant for both winter and summer seasons. The summer season did not have any isolates that were intermediate resistant to any antibiotics.

Observations that were made for Table 4.2 were supported by Figure 4.4 below, where all seasons were combined, and a brief outline of resistance patterns was made. Overall isolates indicated high resistance as well as multi-drug resistance to Ampicillin (98%), Penicillin-G (100%) and Trimethoprim (100%) had the highest resistance indicating multi-drug resistance in isolates obtained. This was also observed in Table 4.2 as indicated by percentages. Of the three resistant antibiotics, Penicillin-G and Trimethoprim were the predominant antibiotics with the highest resistance overall. According to Table 4.2 and Figure 4.4 isolates were generally susceptible towards five antibiotics namely Chloramphenicol, Ciprofloxacin, Erythromycin, Neomycin and Streptomycin. One antibiotic had the highest percentage of intermediate resistance, Kanamycin (49%) and Vancomycin had the same percentage for both intermediate resistance and susceptibility of which was 36%.

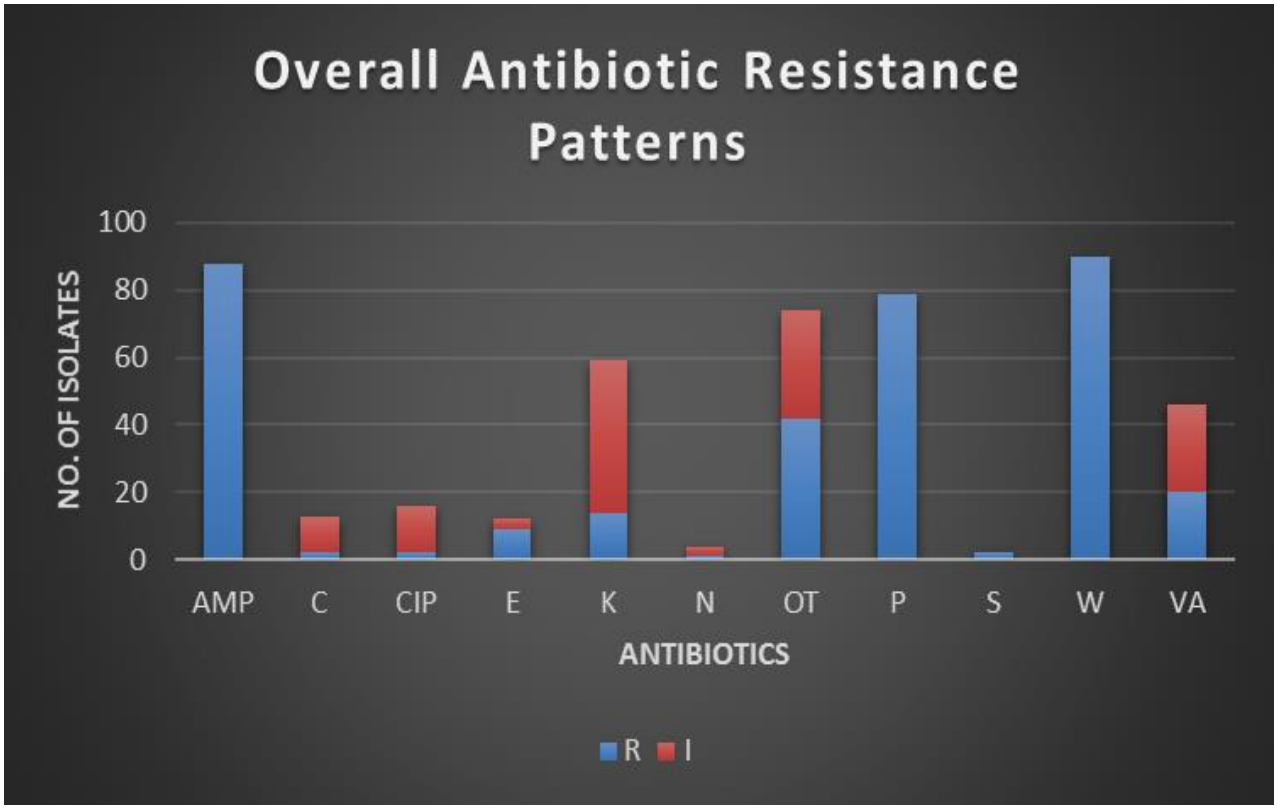

Figure 4.4. Summary of antibiotic profiles obtained from isolates obtained from winter, summer and autumn seasons combined.

Amp- Ampicillin, **C**- Chloramphenicol, **CIP**- Ciprofloxacin, **E**- Erythromycin, **K**- Kanamycin, **N**- Neomycin, **OT**- Oxy-tetracycline, **P**- Penicillin-G, **S**- Streptomycin, **W**- Trimethroprim, **VA**- Vancomycin; **R**- Resistant, **I**- Intermediate.

4.4.2. MAR Indices

The multiple resistance (MAR) indexing is a cost effective and valid method that can be used for bacteria source tracking (Sandhu *et al*., 2016). The MAR index can be calculated as a ratio of the number of antibiotics microorganisms are resistant to the total amount of antibiotics that they were exposed to. According to Sandhu *et al*. (2016) a MAR index value that is greater than 0.2 show that the microorganisms were recently exposed to an environment that was contaminated with antibiotics. Table 4.3 below shows the MAR indices generated from the Kirby-Bauer results obtained during the entire period of the study.

As can be seen in Table 4.3 in the winter season, the MAR index was greater than 0.2 (indicating all sites were recently exposed to antibiotics) in all sites. Sites LR and LV had the highest MAR index of 0.39. Sites BT, FA, GM and MD did not have any isolates hence a MAR index of zero.

Summer season MAR index was also greater than 0.2 for all sites, except BT of which did not have any isolates. The highest MAR index observed was in site DK and MD with 0.36. The MAR index observed for the autumn season showed that all sites had a MAR greater than 0.2, except for the sites that did not have isolates and are represented by NT (Not tested). The highest MAR was observed in site LR with 0.55. In all the seasons, the predominant MAR phenotype observed was Ampicillin, Penicillin-G and Trimethoprim. Findings that were obtained indicate that the sites may have a history with antibiotics, especially site LR both winter and summer season it had the highest MAR. Site MD is situated next to a hospital, hence the high MAR in the summer season.

Table 4.3. MAR indices generated from the disk diffusion test results obtained from winter, summer and autumn seasons.

Sites	B4	BT	DK	FA	GM	LR	LV	MD
MAR (Winter)	0.37	0	0.35	NT	NT	0.39	0.39	NT
	MAR phenotype AMP-PEN-W							
MAR (Summer)	0.27	NT	0.36	0.35	0.32	0.27	0.32	0.36
	MAR Phenotype AMP-PEN-W							
MAR (Autumn)	NT	0.36	0.31	NT	0.35	0.55	0.36	NT
	MAR Phenotype AMP-PEN-W							

BT- Botanical gardens, **DK**- Draak, **FA**- Fanie, **GM**- Gerhard minnebrone, **LR**- Lareus, **LV**- LaVaria, **MD**- Mediclinic; **Amp**- Ampicillin**, Pen-G**- Penicillin G, **W**- Trimethoprim.

4.5. 16S rRNA PCR

Figure 4.5 depicts an agarose gel image of *bla*$_{TEM}$ PCR that was conducted on a total of seven isolates. The DNA template that was used for this particular PCR ranged from a concentration of 20 µg/ml to 40 µg/ml. The purity of the DNA (A_{260}/A_{280}) was relatively good, ranged from 1.7 to 1.9. The lane indicated by M represents the 1kb molecular marker (GeneRuler™ 1 kb DNA ladder, Fermentas, US) and the lane indicated by C represents negative control (no template). The size of the obtained amplicons was 1150bp. Lanes 2 and 6 indicate unsuccessful PCR reactions.

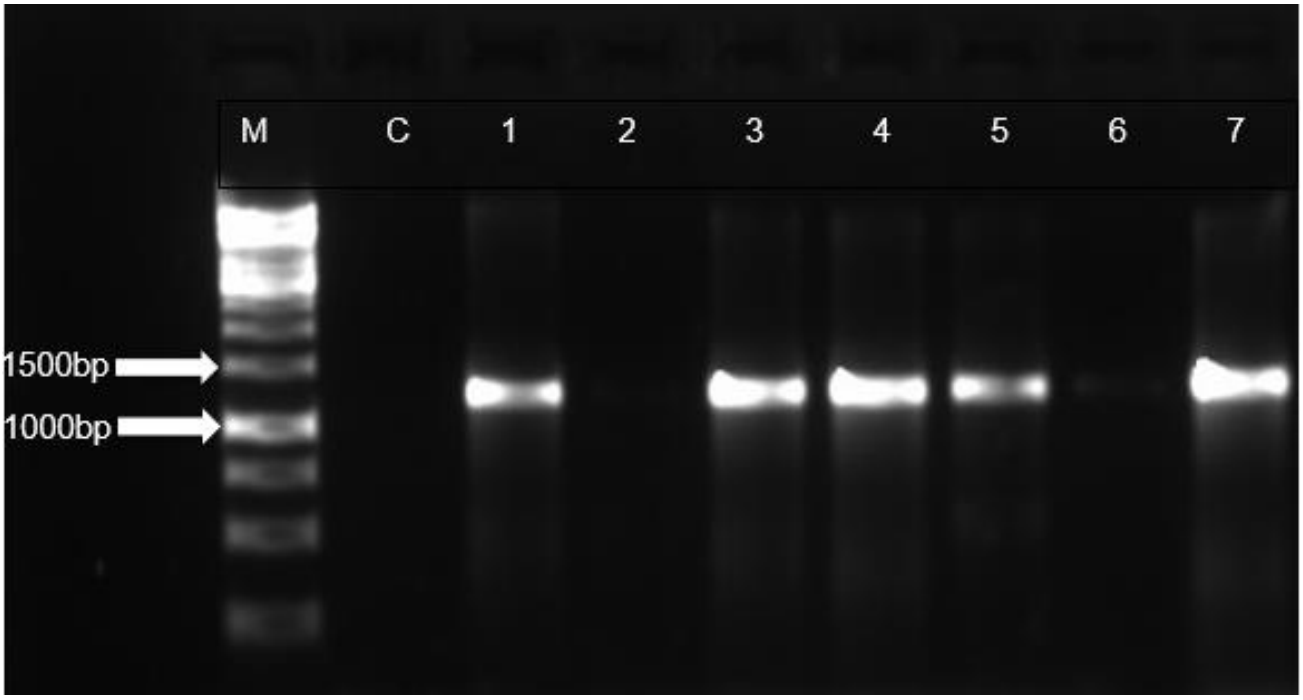

Figure 4.5. 1.5% (w/v) agarose gel, indicating five successful *bla*$_{TEM}$ amplicons, with a size of 1150bp. The lane that is indicated by M is a 1 kb molecular weight marker (GeneRuler™ 1 kb DNA ladder, Fermentas, US) and the lane indicted by C was used as a negative control (No DNA template).

4.6. Species identification

Table 4.4 below shows the identification of various species that were obtained from each site during the course of the study. Species amplified DNA sequence were identified using methodology in explained in Chapter 3. As seen in Table 4.4 in the species column, accession numbers are included in the brackets. This was achieved by submitting the obtained sequences to GenBank in order to acquire original accession numbers for species identified. This was only

possible for species with a 95% similarity when blasted in NCBI GenBank database for identification. Species that do not have any accession numbers in Table 4.4, were below 95% similarity when blasted.

Table 4.4. Identification of various species per site in the winter, summer and autumn seasons.

Season	**Site**	**Species**	**No of isolates**
Winter	**B4**	*Bacillus thuringiensis (*MN514254)	2
		*Virgibacillus halodenitrificans (*MN514260, MN514258)	2
		*Bacillus cereus (*MN514262, MN514261, MN514257)	3
		*Bacillus wiedmannii (*MN514249)	1
	DK	*Vigiobacillus halodenitrificans (*MN514253, MN514256)	2
		Bacillus thuringiensis	1
	LR	*Virgibacillus halodenitrificans (*MN514251, MN514250, MN5142590	3
			3
	LV	*Bacillus cereus (*MN514252)	1
		*Bacillus proteolyticus (*MN514252)	

Summer	**B4**	*Bacillus mobilis (*MN368043)	1
		Clostridium perfringens	2
	DK	*Bacillus cereus (*MN368037)	1
		*Bacillus endophyticus (*MN368041)	1
		*Bacillus mobilis (*MN368033)	1
		*Bacillus subtilis (*MN368078)	1
		*Bacillus thuringiensis (*MN368032)	2
	FA	*Aeromonas hydrophila*	1
		Bacillus mobilis	1
		*Bacillus pumilus (*MN368042)	1
		*Bacillus saferensis (*MN368079)	1
		*Bacillus subtilis (*MN368036)	1
		*Bacillus tropicus (*MN368034)	1
		Clostridium perfringens	1
	GM	*Aeromonas hydrophila*	1
		Bacillus cereus	1
		*Bacillus cucumis (*MN368035)	1
		Bacillus kochii (MN368085)	1
			1

		Bacillus mobilis (MN368040)	1
		*Bacillus proteolyticus (*MN368030)	1
		*Bacillus subtilis (*MN368031)	1
		Bacillus thuringiensis	
	LR	*Bacillus paramycoides (*MN368084)	1
	LV	*Aeromonas hydrophila*	1
		Bacillus cereus	1
		*Bacillus proteolyticus (*MN368086, MN368083)	2
		*Bacillus wiedmannii (*MN368081)	1
		*Flavobacterium (*MN368080)	1
		Plesiomonas shigelloides	1
	MD	*Bacillus cereus*	1
		*Bacillus megaterium (*MN368039)	1
			1

		*Bacillus subtilis (*MN368038)	1
		Bacillus thuringiensis	1
		Clostridium perfringens	1
		Clostridium sordellii	1
		Clostridium tepidum	1
		*Staphylococcus succinus (*MN368044)	1
		Paenibacillus lautus (MN368082)	
Autumn	**BT**	*Bacillus aerius (*MN396342)	1
		*Bacillus cereus (*MN396343, MN396344, MN396345, MN396346, MN396347, MN396348, MN396350, MN396351, MN396352, MN396357)	11
		*Bacillus thuringiensis (*MN396358, MN396359, MN396349)	3
		*Citrobacter freundii (*MN414236, MN414237, MN414238, MN414234)	4
		*Escherichia coli (*MN414235)	1
		Stenotrophomonas maltophilia	1

	DK	*Bacillus cereus (*MN414221, MN414222, MN414224, MN414225)	4
		*Bacillus simplex (*MN414223)	1
	GM	*Bacillus thuringiensis (*MN396354, MN396355)	2
		*Bacillus toyonensis (*MN396353)	1
		*Citrobacter freundii (*MN414229, MN414230, MN414231, MN414233)	4
		*Escherichia coli (*MN414232)	1
		*Plesiomonas shigelloides (*MN396356)	1
		Salmonella bongori	1
	LR	*Bacillus thuringiensis(*MN396360)	1
	LV	*Bacillus cereus (*MN414227, MN414228)	2
		Bacillus megaterium (MN414226)	1

BG- Botanical gardens, **DK**- Draak, **LR**- Lareus, **LV**- LaVaria, **MD**- Mediclinic, **GM-** Gerhard minnebrone, **FA**- Fanie

Table 4.4 shows that in the winter season isolates were only identified in four sites. Identification was made possible with the aid of chromatograms and Genbank database. During the winter season, only four sites had HPC presence and site DK had the most isolates and different species. Bacillus species were the only species found in this season. *Virgibacillus halodenitrificans* was the dominant species with seven isolates, followed by *Bacillus cereus* that had six isolates, *Bacillus thuringiensis* with three isolates and *Bacillus wiedmannii* and *Bacillus proteolyticus* had single isolates.

In the summer season, a total of seven sites had isolates and seven genera were observed namely; *Aeromonas*, *Bacillus*, *Clostridium*, *Flavobacterium*, *Plesiomonas*, *Paenibacillus* and *Staphylococcus*. The site with the most isolates was MD with nine isolates and nine species. This site had all three *Clostridium* species (*C. sordellii*, *C. perfringens* and *C. tepidum*), four *Bacillus* species (*B. cereus*, *B. megaterium*, *B. subtilis* and *B. thuringiensis*), one *Staphylococcus* species (*S. succinus*) and *Paenibacillus* species (*P. lautus*). Site GM was the second with microbial community and had 8 species. Most of the species were *Bacillus* species (*B. cereus*, *B. cucumis*, *B. kochii*, *B. mobilis*, *B. proteolyticus*, *B. subtilis* and *B. thuringiensis*) and a single *Aeromonas* species (*A. hydrophila*). Both FA and LV had the same number of isolates but different species. Site FA had three genera, namely; *Aeromonas* sp. (*A. hydrophila*), *Bacillus* sp. (*B. mobilis*, *B. pumilus*, *B. saferensis*, *B. subtilis* and *B. tropicus*) and *Clostridium* sp. (*C*. *perfringens*). Site LV had four genuses of which were *Aeromonas* genus (*A. hydrophila*), *Bacillus* genus (*B. cereus*, *B. proteolyticus* and *B. wiedmannii*), *Flavobacterium* genus and *Plesiomonas* (*P. shigelloides)*. Site DK had 6 isolates, all of which were *Bacillus* species (*B. cereus, B. endophyticus, B. mobilis, B. subtilis and B. thuringiensis*). The remaining two sites, B4 had 3 isolates, two of which were *Clostridium perfringens* and *Bacillus mobilis* and LR that had one isolate that was identified as *Bacillus paramycoides*.

The autumn season yielded only four sites that had isolates. Site BT had a total of 21 isolates present and four genera was observed. The first genus observed was *Bacillus* (*B. cereus, B. aerius and B. thuringiensis*) and was the predominant species, followed by *Citrobacter* (*C. freundii*), *Escherichia* (*E. coli*) and *Stenotrophomonas* (*S. maltophilia*). The next site were more isolates were observed was GM, it had 10 isolates with 5 genera present. The genera obtained were *Bacillus* (*B. thuringiensis*, *B. toyonensis*), *Citrobacter* (*C. freundii*), *Escherichia* (*E. coli*),

Plesiomonas (*P. shigelloides*) and *Salmonella* (*S. bongori*). Sites LR and LV had the least number of isolates and all of the isolates belonged to the *Bacillus* genus.

4.6.1. Microbial diversity

Figure 4.4 below is a pie chart displaying the overall microbial diversity obtained from different seasons for the the duration of this study. In this study three groups of microorganisms were investigated. Figure 4.4 indicates all species obtained from all seasons. *Bacillus* species were the most predominant species observed in all seasons (73%). The second predominant species observed was *Citrobacter* (8%) and *Clostridium* (6%) species. *Aeromonas* species were the third dominant species (5%). *Escherichia* (2%), *Flavobacterium* (1%), *Plesiomonas* (2%) *Salmonella (1%)*, *Staphylococcus* (1%) and *Stenotrophomonas* (1%) were present in relatively low numbers. *Bacillus*, *Citrobacter*, *Escherichia*, *Flavobacterium*, *Plesiomonas*, *Salmonella*, *Staphylococcus* and *Stenotrophomonas* were grouped as HPCs representatives and were the predominant group in all seasons. The number of *Clostridium* (6%) and *Aeromonas* (5%) species was relatively less when compared to HPCs.

In Table 4.5 the overall microbial community obtained in Figure 4.5 was depicted in table form with the respective sites they were obtained from. This was done to determine the prevalence of species in different sites. As can be seen in Table 4.5 *Bacillus* species were great in numbers and their highest levels obtained at sites, BG and DK with a percentage of 15% each. The lowest percentages (3% each) were seen in sites, LR and B4. The second biggest genus, *Citrobacter* was prevalent in high percentages at site BG and GM with 4% each. The lowest levels thereof were 0% at other sites. *Virgibacillus* genus, not included in Figure 4.5 was another genus, belonging to the family of Bacillaceae. This genus was present at three sites: LR (3%), B4 (2%) and DK (2%.) *Clostridium* and *Aeromonas* genus were present in low numbers. The highest percentage obtained for *Clostridium* genus was 3% at site MD and the highest percentage for *Aeromonas* observed was 2% at site LV. The lowest percentages obtained for these two genera was 1% at sites FA and GM (*Aeromonas*) and FA (*Clostridium*). The remaining genera's percentages were either present in 1% or 2%. Of all sites, GM was the only site that had a variety of genera and they were *Aeromonas*, *Bacillus*, *Citrobacter*, *Escherichia*, *Plesiomonas* and *Salmonella*.

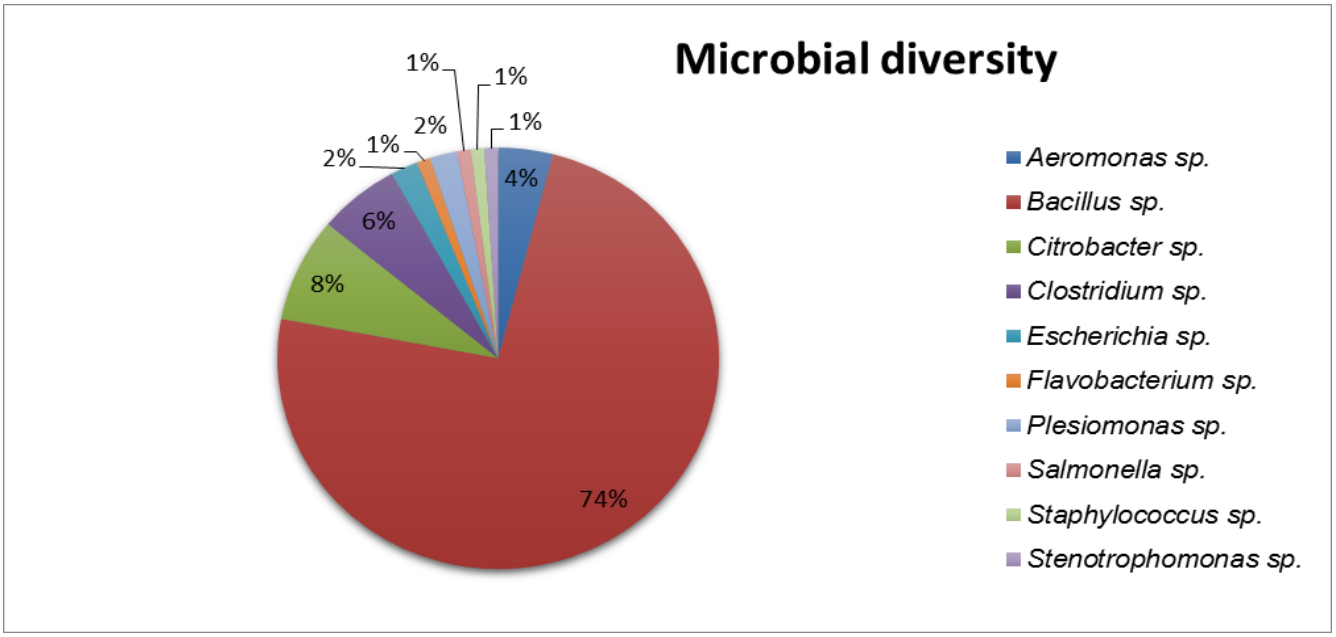

Figure 4.6. An overview of the microbial diversity obtained from winter, summer and autumn seasons.

Table 4.5. Supplementary results showing HPC prevalence in the period of the study.

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Sites	*Aeromonas sp.* %	*Bacillus sp.* %	*Citrobacter sp.* %	*Clostridium sp.* %	*Escherichia sp.* %	*Flavobacterium sp.* %	*Plesiomonas sp.* %	*Salmonella sp.* %	*Staphylococcus sp.* %	*Stenotrophomonas sp.* %	*Vigiobacillus sp.* %
FA	1	5	0	1	0	0	0	0	0	0	0
MD	0	5	0	3	0	0	0	0	1	0	0
BG	0	15	4	0	1	0	0	0	0	1	0
GM	1	10	4	0	1	0	1	1	0	0	0
LR	0	3	0	0	0	0	0	0	0	0	3
LV	2	11	0	0	0	1	1	0	0	0	0
B4	0	3	0	2	0	0	0	0	0	0	2
DK	0	15	0	0	0	0	0	0	0	0	2
Total (100)	4	67	8	6	2	1	2	1	1	1	7

BT- Botanical gardens**, DK-** Draak**, FA-** Fanie**, GM-** Gerhard minnebrone**, LR-** Lareus**, LV-** LaVaria**, MD-** Mediclinic.

4.7. Phylogenetic tree

Phylogenetic trees are used to determine the evolutionary association between different species as well as common ancestry (Hall, 2013; Fourie, 2017). The chromatograms obtained from 16S rRNA were identified and exported to Mega 7 as well as their reference sequences in order to construct a phylogenetic tree. In this study, phylogenetic trees were constructed for each season, based on the species identified.

4.7.1. Winter season phylogenetic tree

Figure 4.8 is a neighbour-joining tree constructed using sequences obtained from the winter season. *Clostridium perfringens* (NC008261) was used as an outgroup and the sequence thereof was exported from the GenBank database (www.ncbi.nlm.nih.gov) . All species obtained in winter season were used to construct this tree and as shown in Figure 4.8, few species were identified, and the numbers were low as well. According to Figure 4.7, two clusters belonging to the phylum Firmicutes were evident. The one main cluster was further divided into smaller clusters, such as Cluster A was divided into A1 and A2 subclusters and Cluster B remained as is. Subcluster A1 consisted of four species (*B. wiedmannii, B. thuringiensis, B. cereus* and *B. proteolyticus*) respectively. It appears that *B. wiedmannii* and *B. thuringiensis* were closely related as their bootstrap value was 88%. Close similarities were observed between the *B. wiedmannii* plus *B. thuringiensis* branch and a single branch of *B. wiedmannii* as it displayed a bootstrap value of 86% relatedness. However, there were less species relatedness observed between the three species formed branch and the *B. cereus* branch as the bootstrap value was 34% similarities. *B. proteolyticus* showed 72% similarity with all of the *Bacillus* species observed in the A1 sub-cluster. Sub-cluster A2 consisted of a single species, of which *B. cereus* indicated a bootstrap value 86% species similarity. A 99% similarity was observed between sub-cluster A1 and A2. Cluster B consist of a single species known as *Virgibacillus halodenitrificans.* The bootstrap values obtained for this single species was 99% similarities.

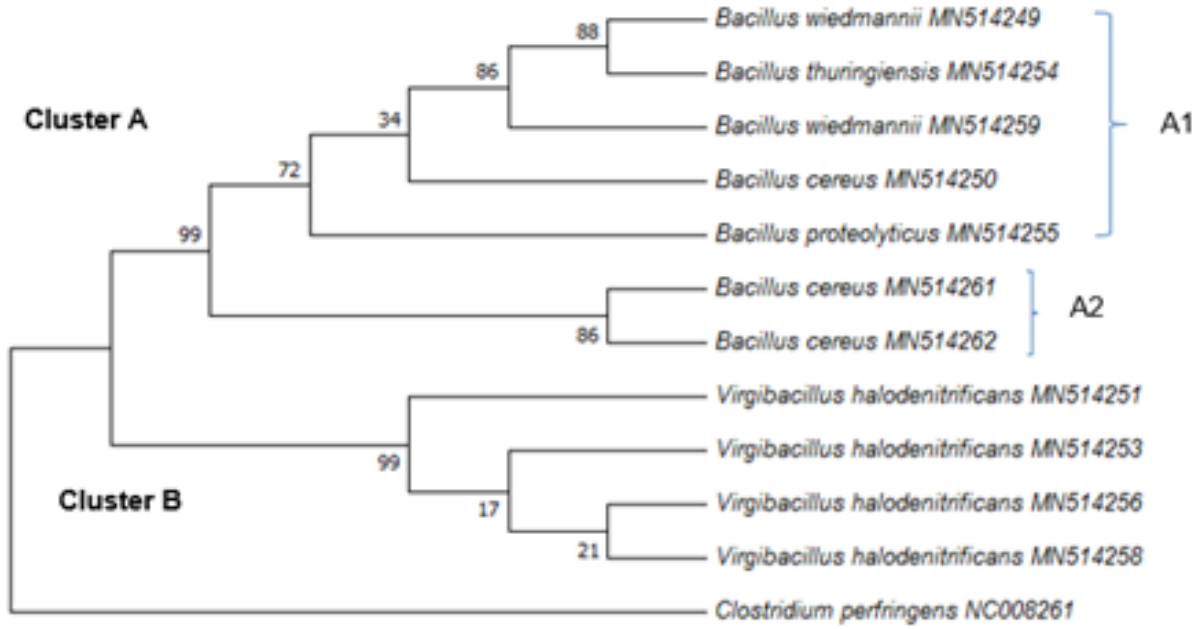

Figure 4.7. Neighbour-Joining tree illustrating the phylogenetic association of a Genbank sequences and sequences obtained from the winter season in 2018. Sequences used to create the tree were from a partial 16S rRNA gene.

4.7.2. Summer season phylogenetic tree

Figure 4.8. indicates a neighbour-joining tree association obtained using summer season species sequences. This tree was constructed using reference sequences exported from the GeneBank database. A total of 20 species were obtained in this season and their sequences had too many gaps and bootstrap values were relatively low hence it was difficult to construct a phylogenetic tree with the original sequences. According to Figure 4.9 two clusters (cluster A and B) are evident. Cluster A consists of three subclusters (A1, A2 and A3). All species obtained in cluster A were all from the phylum Firmicutes, genus *Bacillus*. In subcluster A1 80% similarities can be observed between *B. cucumis*, *B. endophyticus*, *B. kochii* and *B. megaterium*. Furthermore, *B. cucumis* and *B. endophyticus* indicated 62% similarity and 42% similarity with *B. kochii*. Subcluster B depicted 100% species relatedness between *B. pumilus*, *B. subtilis* and *B. saferensis*. Close similarity of 99% was also evident between *B. pumilus* and *B. saferensis*. Subcluster A3 indicated 100% species similarities between six Bacillus species, namely; *B. paramycoides*, *B. cereus*, *B. mobilis*, *B. wiedmannii*, B*. thuringiensis* and *B. proteolyticus*. Amongst the six species, four species (*B. mobilis*, *B. wiedmannii*, B*. thuringiensis* and *B.*

proteolyticus) indicated species relatedness of 65%. Cluster B consisted of three subclusters (B1, B2 and B3) as well as three phyla, namely Firmicutes Proteobacteria and Bacteroides. Subcluster B2 consists of Firmicutes belonging to the genus *Clostridium*. 100% similarities were observed between *C. perfringens* and *C. tepidum*, whereas the overall similarities amongst the three species was 86%. In subcluster B3, 62% similarity was observed between *A. hydrophila* and F. *cucumis. S. succinus* and *P. lautus* were regarded as subcluster B1 with no evident similarity amongst each other, however they indicated 98% species similarities with cluster A. *H. salinagrum* belonging to phylum Archaea was used as an outgroup in this phylogenetic tree.

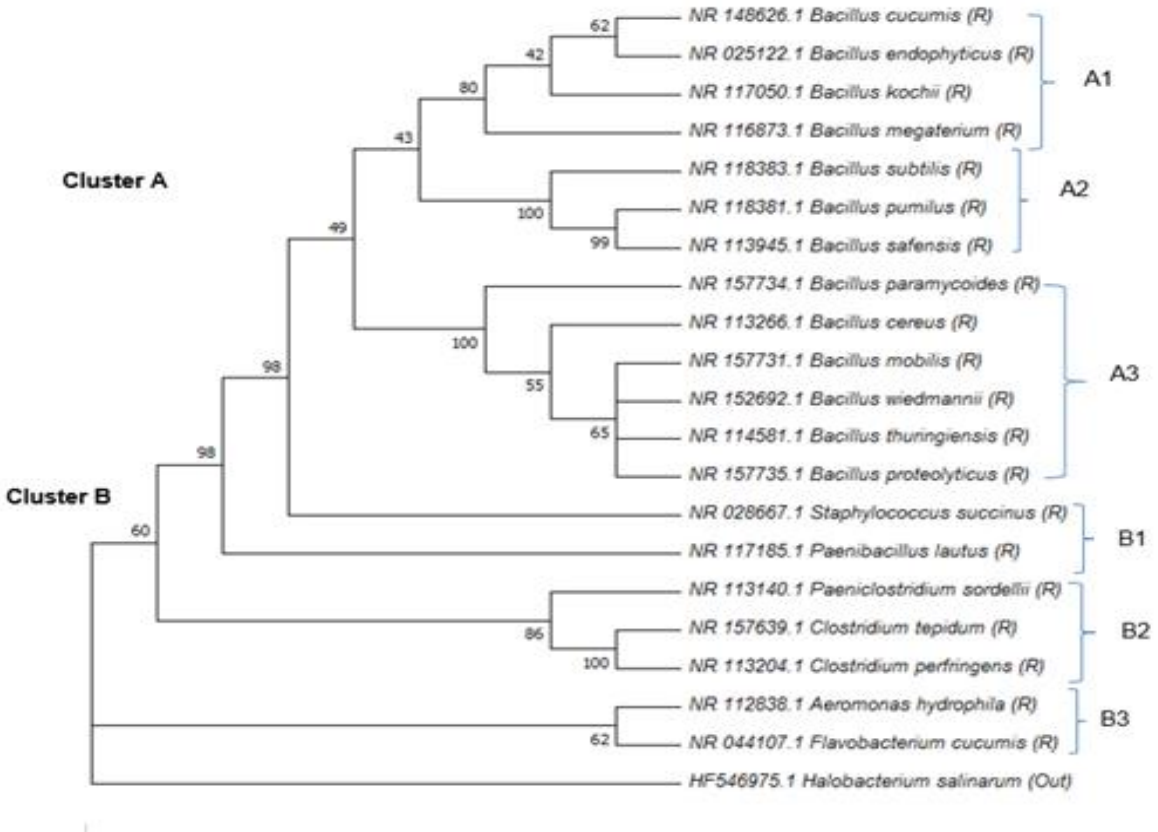

Figure 4.8. Neighbour-Joining tree illustrating the phylogenetic association of a Genbank sequences similar to the sequences obtained from the summer season in 2019. Sequences used to create the tree were from a partial 16S rRNA gene. **R**- Reference sequence, **Out**- outgroup.

4.7.3. Autumn season phylogenetic tree

Figure 4.9 indicatess a neighbour-joining tree constructed using sequences obtained from autumn season. *Candida albicans* (SC514) was used as an outgroup in this tree and was exported from GenBank database. In Figure 4.9 not all species were used, two representatives of each species were used for tree construction. Two clusters were evident, Cluster A representing phylum firmicutes and cluster B representing phylum proteobacteria. Cluster A was divided into sub-cluster A1 and Cluster B was regarded as sub-cluster B2. The sequences for this Figure were relatively short and that could explain the low bootstrap values observed. In sub-cluster A1, the similarity observed between *B. cereus* species and *B. thuringiensis* showed little similarity with a bootstrap value of 42%. The lowest bootstrap value was obtained between the relationships between *B. cereus, B. thuringiensis* and *B. toyonensis*, of which indicated only 18% similarity. Another species 35% similarities were observed between *B. simplex*, *B. cereus* species and *B. thuringiensis.* All the species clustered in subcluster A1 had an overall similarity of 38%. In cluster B, sub-cluster B1 consisted of three species. The similarities between *E. coli* and *P. shigelloides* was indicated by a bootstrap value of 69%. Another similarity was seen between *C. freundii* of which indicated similarity of 88% amongst its two species. All species that were in sub-cluster B1 had an overall similarity of 69%. In this figure, *Candida albicans* were used as an outgroup.

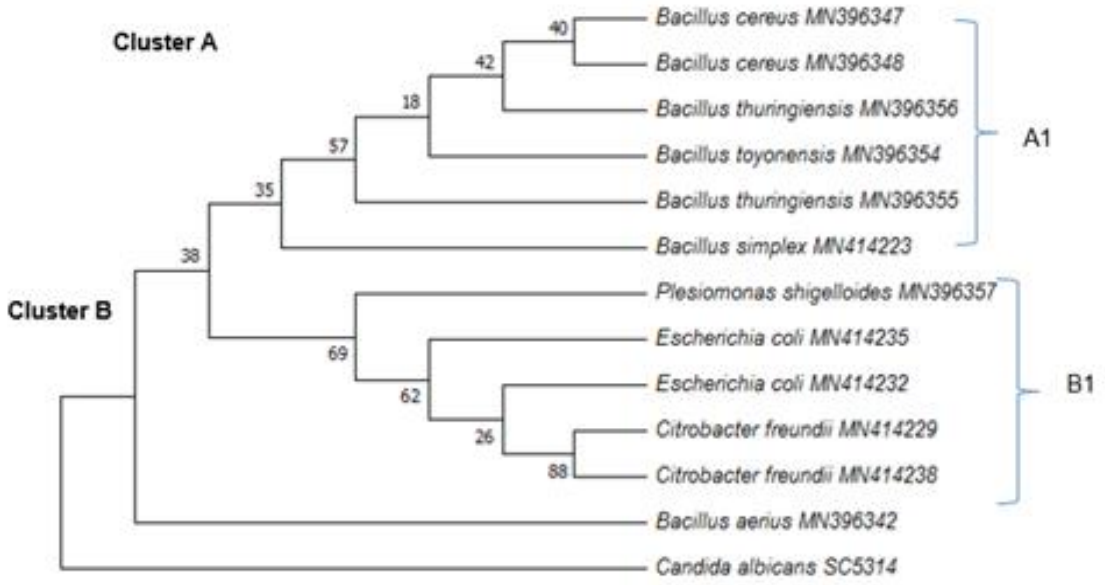

Figure 4.9. Neighbour-Joining tree illustrating the phylogenetic association of a Genbank sequences and sequences obtained from the Autumn season in 2019. Sequences used to create the tree were from a partial 16S rRNA gene.

4.8. Extracellular enzymes and ARGs in *Clostridium* sp., *Aeromonas* sp., and various species of HPCs

Figure 4.10 below depicts the extracellular enzyme activity and ARGs obtained from HPCs per species. HPCs are a relatively large group of varying species of microorganisms. Hence all the HPCs species obtained in this study were not combined with *Aeromonas* and *Clostridium* species in terms of extracellular enzymes and the presence of ARGs results. A total of 26 HPC species were obtained and will be discussed as per species. *Bacillus cereus* was the most predominant species observed in all of the HPCs. This species had the highest number of isolates, beta hemolytic (ability to completely lyse blood cells) and DNase (able to hydrolyse DNA) positive isolates as can be observed in Figure 4.10. Three of the isolates in this species tested positive when screened for the *ampC* gene and seven were positive in the bla_{TEM} gene screening. *Bacillus thuringiensis* was the second predominant species. Members of this species were all beta hemolytic and more than half of the isolates were able to hydrolyse DNA, therefore making them positive for the DNase test. Two of the isolates were positive for bla_{TEM} screen and none were positive for *ampC* gene screening.

Citrobacter freundii was the third species observed to have high numbers of isolates. About 90% of these isolates were beta-hemolytic and all of them tested positive for *ampC* gene screening. None of the Citrobacter species were positive for DNase and bla_{TEM} gene. *Virgibacillus halodenitrificans* had seven isolates, four which were beta-hemolytic and five were positive for DNase. None of the isolates were positive for any ARG. *Bacillus mobilis*, *Bacillus proteolyticus* and *Bacillus subtilis* had the same isolate number. All isolates of both *B. mobilis* and *B. subtilis* were beta hemolytic and only two were DNase positive. Whereas 90% of *B. proteolyticus* isolates were beta-hemolytic and all isolates were DNase positive.

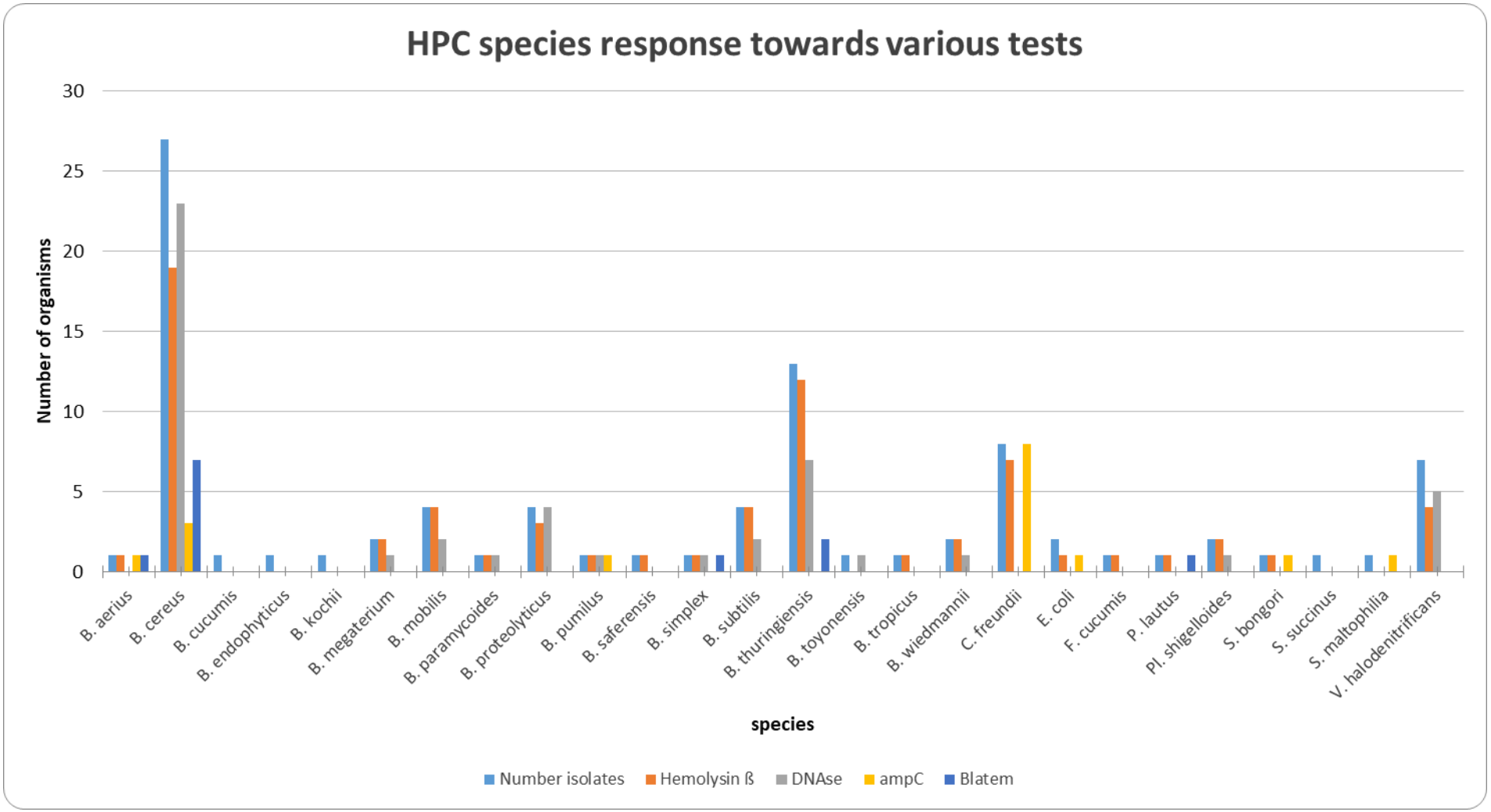

Figure 4.10. Extracellular enzymes and ARGs in HPCs per species.

Table 4.7 below indicates the results obtained from extracellular enzyme activity of both *Clostridium* and *Aeromonas* as well as the virulence genes observed in *Aeromonas*. The identified *Aeromonas* and *Clostridium* species were subjected to extracellular enzyme tests for pathogenicity. A total of four *Aeromonas* positive isolates were obtained. In the four, three of the isolates were beta-hemolytic and only two of the isolates were positive for gelatinase. *Clostridium* positive isolates obtained were six and all of them were beta hemolytic. However, none of the isolates were positive for the other extracellular tests investigated. This result indicate that the two genera may be opportunistic pathogens. *Aeromonas* and *Clostridium* species were also screened for ARGs and VIR, but none of them tested positive for the genes of interest indicated in chapter 3.

Table 4.7. Extracellular enzymes and genes results obtained from *Aeromonas* and *Clostridium* species.

			Extracellular enzyme activity			**Virulence genes**
			Hemolysin (beta)	**Dnase (positive)**	**Gelatinase (positive)**	**10 VIR GENES** *(act, ast, aer, alt, fla, hly, ser, ela, exu* and *lip)*
		No. of isolates (N)	**n (%)**	**n (%)**	**n (%)**	**n (%)**
***Aeromonas* Species**	*Aeromonas sp.*	2	1 (50.0)	0	0	0
	Aeromonas hydrophila	2	2 (100)	0	2 (100)	0
Total		**4**	**3**	**0**	**2**	**0**
***Clostridium* Species**	*Clostridium sordellii*	1	1 (100)	0	0	0
	Clostridium perfringens	4	4 (100)	0	0	0
	Clostridium tepidum	1	1 (100)	0	0	0
Total		**6**	**6**	**0**	**0**	**0**

CHAPTER 5: DISCUSSION

This study was conducted in geographically separated selected groundwater systems in the NWP. It is of common knowledge that groundwater is considered a reliable and renewable source of water, and for years has been known for its exceptional good quality (Cadraku *et al*., 2016; Prasanth *et al*., 2012). In the current study, the quality of groundwater systems was investigated both physico-chemically and microbiologically. A general trend was observed in terms of physico-chemical and microbiological parameters throughout the entire seasons. The trends observed highlighted elevated levels of TDS, Salt, nutrients and HPCs as well as low prevalance of *Clostridium* sp. and *Aeromonas* sp. during the course of the study. The trends went further to indicate signs of opportunistic pathogenicity, resistance towards Ampicillin, Penicillin-G and Trimethoprim and lastly the presence of ARGs in the microbial diversity observed.

5.1. Physico-Chemical parameters

5.1.1. Temperature

In this study, three sets (each set in triplicates) of temperature were observed over three seasons. Furthermore, temperature was measured in triplicate during three seasons. In the winter season seven groundwater systems had water temperatures lower than 20°C, whereas only one system had a temperature higher than 20°C. During this season, the weather in the NWP was cold and dry, hence temperature in the groundwater systems was expected to be lower. Schneider (1961) explained that the cold temperatures that are often observed in winter in groundwater systems are influenced by both air and soil temperature. The study further explained that during winter, relatively cold air temperatures are evident and this plays a significant role in the soil temperature. In relatively cold countries, this causes a frost in soil layers, thereby causing a freezing effect on groundwater, resulting in low temperatures. In the summer season temperature did not increase in all systems, four of the systems had temperatures that were below 20°C and the remaining four systems had temperatures above 20°C. This observation was also made by Yasukawa et al. (2004). In that study the subsurface temperature was higher in winter compared to summer and was probably due to the intrinsic physical properties of water. According to the authors, this kind of phenomenon is normal in temperate climate regions such as Asia, Africa and South America.

Higher temperatures impact groundwater negatively, as they act as a catalyst in the degradation of organic pollutants. This was elaborated in a study conducted by Ni *et al*. (2016) in which an increase of reductive dechlorination was observed after groundwater temperature increased from 10°C to 25°C. Interestingly in the winter season, groundwater system FA had the lowest

temperature, as well as GM in the summer season when compared to other sites. These two sites are situated next to surface water (river/stream). This was in line with observations made by Hayashi and Rosenberry (2002) and Menberg *et al*. (2014). According to the aforementioned authors, groundwater systems that are under surface water, are influenced by events that occur in surface water. Six of the groundwater systems that were investigated in this study were located further from surface water catchments, hence the temperatures observed in these settings could not be linked with surface water effects. However, the temperature variations observed could have been impacted by activities occurring in the environment around them. According to Riedel (2019) aquifers have natural temperatures that range from 5°C to 20°C. However, a slight decrease can have an impact on the quality of the groundwater.

LeChevallier (1996) stated that bacterial coliforms (total coliforms and faecal coliforms) and HPCs were significantly higher in water temperatures that were higher than 15°C in drinking water. This supports the findings of the current study. Various studies also stated that increased temperatures in any water system can cause die-off of pathogens, thereby decreasing the concentration of bacterial growth (An *et al*., 2002; Walters *et al*., 2011; Vermeulen and Hofstra, 2013).

Groundwater temperature is prominently known as one of the important drivers for water quality (Greene *et al*., 2011; Sharma *et al*., 2012; Menberg *et al*., 2014), and for this reason, it is considered to be a crucial parameter for groundwater resource quality management (Figura *et al*., 2011). According to Bates *et al*. (2008) and Greene *et al*. (2011) atmospheric climate change has a significant impact on both the subsurface hydrological and thermal processes, thus can also play a role in altering groundwater temperature. The irresponsible use of various resources that impact climate change, leading to global warming, continues each year and this becomes a problem worldwide, as it affects all four elements of life, particularly water in this study as it has the ability to change the hydrological cycle. According to Anderson and Emanuel (2008) and Taniguchi *et al*. (2007), global-warming affects both the aquifer temperatures as well as the biogeochemical cycles that exist within the soil's surface primarily overlaying the aquifers. Different aquifers (groundwater systems) have different temperatures, for instance, aquifers that are located beneath rivers usually mimic the surface water's temperature as the two are intertwined together and in most cases are of cold temperatures (Hayashi and Rosenberry, 2002; Menberg *et al*., 2014). Taylor and Stefan (2009) also stated that the temperature of shallow groundwater systems positively responds to ground surface temperature that depend highly on land use and climate conditions.

5.1.2. pH

During this study the pH ranged between 6.52 and 8.14, which indicate a slightly acidic to slightly basic, but fairly stable. The results obtained were thus in line with Nilsson and Sanberg (2017) and Andersson *et al*. (2000) and indicated that the pH within the groundwater systems was stable and not affected by environmental factors. Like temperature, pH is one of the most important parameters that influences both the occurrence and distribution of microbes (Jin and Kirk, 2018). Thompson *et al*. (2017) stated that pH is positively associated with the growth of microorganisms influenced by a wide variety of biogeochemical processes. According to Pullanikkatil *et al*. (2015) neutral pH observed in water environments plays a major role in the maintenance as well as growth of microorganisms, especially HPCs. Neutrophiles are known as microorganisms that are able to grow at pH levels from 5 - 9 and include microorganisms such as HPCs as well as opportunistic human pathogens (Horikoshi *et al*., 1999; Baker-Austin and Dopson, 2007; Jin and Kirk, 2018). The current study observed various species of HPCs, some of which were opportunistic human pathogens and is supported with evidence from afore-mentioned authors.

pH is an important parameter, as it can be used to indicate if water is affected by chemicals or not (Nilsson and Sanberg, 2017). Additionally, the pH of water can be used to determine both the solubility and biological availability of nutrients (phosphorous, nitrogen and carbon) and heavy metals (lead, copper, etc) that exist within the environment (Islam *et al*., 2017). In most cases, groundwater pH is changed by pollution, for example, aquifers that are situated beneath mines, will be affected by the effects that take place within the mine and the pH thereof will be low (acidic) (McCarthy, 2011). This happens because of the residues from metals mined. Low pH in groundwater is problematic as it allows the solubility of heavy metals, hence metals may become bioavailable thus affecting bacterial growth.

5.1.3. Total dissolved solids (TDS)

In the winter season only three systems had TDS levels lower than 450 ppm and five systems had TDS levels greater than 450 ppm. In both the summer and autumn seasons, all the systems had TDS that was greater than 450 ppm which was regarded as high TDS levels in water according to the TWQR standards (DWAF, 1996). Very high levels of TDS in groundwater was also observed by Aydin (2007). High TDS in water entails that there are more cations and anions present (Harter, 2003). The groundwater systems used in this study were situated in an environment that is used for agricultural and recreational purposes. The soil in the agricultural environments are constantly fed fertilizers in order to maintain crop production, especially site

GM, hence the elevated TDS levels observed. EPA (2001) stated that water with elevated levels of TDS can deteriorate water clarity by means of harbouring microorganisms that are of health significance. Bisi-Johnson *et al*. (2017) further explains that high levels of TDS in water are a result of pollution, however this could be highly beneficial for microbial growth. TDS that is greater than 500 ppm is considered harmful for consumption and is not recommended for drinking purposes, but it is suitable for irrigation. Water sources analysed in the present study were all used for irrigation purposes.

Total dissolved solids are a composition of inorganic salts as well as organic matter that exists in a water solution (WHO, 2003; Islam *et al*., 2017). TDS in water could come from natural sources, agricultural, sewage and urban run-off, as well as industrial wastewater (WHO, 2003). According to Harter (2003) another contributing source of elevated TDS levels in groundwater also originates from precipitation of surface water that recharges aquifers. The elevated levels of TDS in the present study should be regarded as a cautionary should these water sources be considered for future potable use.

5.1.4. Salinity

Salinity levels increased significantly in both summer and autumn seasons. In winter increased salinity concentrations were observed in four out of eight sites (LR, LV, B4 and DK). All of the groundwater systems investigated in the study, were exposed to various fertilizers, to maintain various agriculture purposes and this could be the reason for elevated salinity levels observed. Anthropogenic processes play a significant role in the salinity of water environments, but in the absence of such processes, natural processes such as (i) catchment weathering, as a result of geological catchments and precipitation and (ii) small amounts of salts that exists in rainwater can influence levels of salinity in both surface water and groundwater (Caňedo-Arguelles *et al*., 2013). Furthermore, according to NWPG (2002) the rainfall in the NWP increases predominantly in the summer seasons resulting in higher concentrations of parameters such as salinity, nitrates and EC.

A study done by Carstens (2014) observed a significant increase in salinity, nitrate as well as EC during the winter period. According to the author, this could be a result of decreased levels of recharge in groundwater in the province. Carstens (2014) also indicated that the groundwater systems investigated were exposed to a constant source of pollution, hence the increased levels of certain parameters in their study. Rozemeijer *et al*. (2009) investigated the association between groundwater quality and meteorological events and came to a conclusion that precipitation

contributed to the chemical constituent's concentrations in groundwater. According to NWPG (2002) the rainfall in the NWP increases predominantly in the summer seasons and as a result higher concentration of parameters such as salinity, nitrates and EC can be observed. This was supported by findings obtained in this study. According to Stanley and Morita (1968) salinity in water plays a vital role in controlling the temperature at which microorganisms will grow. Furthermore Logares *et al*. (2013) indicated that the distribution regarding the abundance as well as the presence of specific microbial taxa is strongly influenced by ranging salinity concentrations.

5.1.5. Chemical Oxygen Demand (COD)

In the present study, COD levels fluctuated between sites and seasons. For instance, during the winter season, COD levels ranged from 0 mg/L to 21.3 mg/L, whereas in the summer season levels ranged from 5 mg/L to 60mg/L. In the autumn season, COD levels ranged from 0 mg/L to 30.66 mg/L. Kritzinger (2019) observed alarming COD concentrations in drinking water ranging from 6 mg/L to 63 mg/L in warmer seasons. Similar levels were also observed in the current study. According to Trivedy and Goel (1984) high the COD concentrations in water depict high pollution levels.

COD measures the quantity of the oxygen that is needed for chemical oxidation of organic substances in water (Ngang and Agbazue, 2016; Sirajedeen *et al*., 2014). COD also highlights the strength that pollutants have on polluted water and can be used as an organic pollution indicator (Ngang and Agbazue, 2016; Koda *et al*., 2017). In wastewater treatment plants, COD concentrations are amongst the important parameters tested to determine the health of both the influent and effluent (Wu *et al*., 2012; Chen *et al*., 2015). In groundwater systems, the main contaminant that influences CODs are landfill leachates (Koda *et al*., 2017). Although high levels of COD in drinking water suggest a potential introduction of toxic pollutants in the water environment and are detrimental to human health, this however is not the case with microorganisms. Microorganisms have a remarkable way of reacting towards these pollutants. They use them as energy sources, by breaking them into simpler forms and secondly, they are able to utilize the energy thereof for cell synthesis (Khan *et al*., 2014). This is a clear indication that microorganisms thrive and reproduce in environments with slightly higher levels of CODs.

5.1.6. Nitrates and Phosphates

In the present study, the nitrate concentrations observed ranged from 0 mg/L to 2.36 mg/L in all the seasons and they were within the standards (<10 mg/L). According to EPA (2010) natural nitrate levels in groundwater are very low and in the range of (<10 mg/L). This was a clear indication that the environmental activities that took place in various groundwater systems, did not affect the natural levels of nitrates. Nitrates form part of nutrients that exist in natural waters. According to Mahler *et al*. (2007) natural occurring nitrates in groundwater are considered a contaminant when at higher concentrations (<10 mg/L). Nitrates in groundwater are affected by agricultural activities, especially those that involve fertilizers and animal waste that are deposited into the soil. Majority of the groundwater systems investigated were in proximity with constantly fertilizer infused soils, however this was not reflected in nitrates concentrations in this study, as they were relatively low. This could be a possible indication of assimilatory nitrate reduction. According to Rivett *et al*. (2008) heterotrophic microorganisms can incorporate nitrates into their biomass for growth especially in the absence of ammonium. Furthermore, since nitrates are more mobile than ammonium, in the vicinity of plant roots, nitrates are more absorbed than ammonium, as it is a vital source for plant growth.

Excess nitrate levels affect all forms of water bodies (groundwater, surface water and drinking water) one way or the other. In groundwater high concentrations of nitrates could have a significant effect on the microbiological biome, as it will affect aerobic microorganisms thereby restricting their growth and enhancing growth of anaerobic microorganisms. In surface water, the most alarming effect that excess nitrates have is eutrophication, which is detrimental to aquatic life. However according to WHO (2017) and Kumar and Puri (2012) high nitrate concentrations in drinking water pose health effects in immunocompromised individuals (children under three years old and the elderly). This was also supported by Omer and Alzahrane (2013) and stated that high concentrations of nitrates in the water is detrimental to pregnant women and infants that are younger than six months as it has the ability to cause methemoglobinemia (commonly known as blue-baby) syndrome that disrupts the blood's ability to carry enough oxygen. Once again, if these groundwater sources are in future to be considered for drinking water purposes then they should also be tested for nitrate levels and if these reach excessive levels, remediation steps should be considered.

In natural water phosphorus occurs in the significant form known as phosphates (Fadiran *et al*., 2008). Phosphates form part of nutrients that exist in water and are essential for both plant and microorganism growth. In this study, phosphate levels were lower than 10 mg/L in all sampling seasons. The highest phosphate levels observed per season was 5.2 mg/L in the water obtained from site FA (winter season), 5.15 mg/L at site DK (summer season) and 3.88 mg/L in site GM

(autumn). According to Domagalski and Johnson (2012) naturally, phosphates bind to soil by a process known as adsorption and is only released into the aquifer only if the adsorption capacity of the soil exceeds. The concentration of phosphates that observed throughout seasons was enough to allow and maintain the growth of HPCs (Tsholo, 2019). According to Fadiran *et al*. (2008) natural concentration levels of phosphates in groundwater as obtained in the present study, are not harmful to human, animal and environmental health. However, excess concentrations of phosphates in groundwater, used for drinking purposes can have negative health implications in humans such as digestive problems, muscle damage as well as kidney failure (Gupta *et al*., 2017; Fadiran *et al*., 2008). There are a number of factors that contribute to elevated levels of phosphates in both surface water and groundwater and they include; fertilizers, animal waste, industrial discharge, phosphate mining and etc (Nolan and stones, 2000; Fadiran *et al*., 2008). This parameter (phosphate levels) is thus important when the water quality is monitored. Although it does not provide health risk information, it may provide some data with respect to pollution trends.

5.2. Microbiological parameters

5.2.1. Heterotrophic count bacteria

Heterotrophic bacteria are natural inhabitants of environmental habitats and they are used as an accessory indicator to determine water quality (Amanidaz *et al*., 2015). Groundwater systems, such as drinking water do not have as high HPC levels present when compared to surface water and wastewater. In the present study, HPCs levels varied between the sites and seasons. In the winter and autumn season, HPCs were isolated from four sites. In summer, seven sites indicated the presence of HPCs andthis was expected as it was a rainy season. According to Kaushik *et al*. (2014) HPC bacteria are largely carried in the water systems from agricultural areas during rainfall events.

However out of eight sites, one site (BG) did not have any presence of HPCs and this was also observed in the winter season. Overall it was evident that more HPCs were recorded in the warm and wet season. The latter observation was also reported by Shakoor (2018) and Tsholo (2019). There are no standards of HPC presence in groundwater used for irrigation purposes, however there are restrictions for drinking water use. According to Amanidaz *et al*. (2015) HPC concentrations in drinking water may vary from less than 1 cfu/ml to 10000 cfu/ml and this highly depends on water temperature, chlorine residues and organic matter. Lillis and Bissonnette (2001) suggested the limit for HPC should be set at 500 cfu/ml (max) due to the fact that several

species of HPC are opportunistic bacteria and also, elevated levels may hinder with procedures used for coliform detection. Since the HPC group consists of a large variety of bacteria (e.g. aerobes and anaerobes) studies have illustrated that this group of bacteria in drinking water possess the ability to be a health threat, especially to individuals with compromised immune systems (Xi *et al*., 2009; Chowdhury, 2011). Levels of HPC in groundwater is thus also an important parameter when the suitability of groundwater is considered for drinking water purposes.

5.2.2. *Clostridium* and *Aeromonas*

In the present study, *Clostridium* representatives were not as prevalent as HPC representatives. *Clostridium* was only detected in the summer season in low levels. The *Clostridium* levels ranged from 1 cfu/ml to 3 cfu/ml in three sites. Clostridia were not detected in the winter and autumn seasons. Similar trends were observed by Francy et al. (2000). According to Francy *et al*. (2000) and Gerord and Stelma (2018) these low levels of *Clostridium* detected could be due to the small volume of water used for enumeration. However, a similar study by Potgieter *et al*. (2006) found Clostridium isolates in boreholes during the dry cold season. According to the latter author, Clostridium isolates identified in the study were isolated form boreholes that had unprotected casings as well as corroded casings.

According to Num and Useh (2014) the *Clostridium* genus comprises of opportunistic pathogenic species that cause diseases in both humans and animals, hence making its slight presence in water alarming. Microbial indicators such as total coliforms and faecal coliforms have been used for years in order to determine the strength of contamination as well as the health risks they might pose (Payment and Franco, 1993). *Escherichia coli* is one of the microorganisms that has been widely used as a faecal indicator in all different spectrum of water bodies. However, the use of *E. coli* has its limitations and is not reliable, for instance in drinking water treatment, *E. coli* and other non-spore forming organisms can be inactivated during the chlorination stage thereby leaving more resistant pathogens unaffected (Araujo *et al*., 2004). Hence Payment and Franco (1993) suggested that *Clostridium*, specifically *C. perfringens* can be used as an indicator of faecal pollution in water as they have the ability to form spores that enable them to resist harsh conditions.

In the present study, *Aeromonas* species were not in abundance as HPCs. These organisms were only detected in the summer (warm and wet) season with levels ranging from 1 cfu/ml to 2

cfu/ml. Several studies have also reported that *Aeromonas* are more prevalent in summer months (Burke *et al*., 1984; Chauret *et al*., 2001; Razzolini *et al*., 2010). Similar to *Clostridium* species, *Aeromonas* species were not detected in the winter and autumn seasons. Kerslers *et al*. (1995) also reported low levels of *Aeromonas* in untreated groundwater. According to Borchardt *et al*. (2003) *Aeromonas* are rare in groundwater. These authors suggest these low levels could also be influenced by the sample size and suggested more water to be used for enumeration purposes. *Aeromonas* have been largely reported in surface water systems subjected to wastewater effluent (Chauret *et al*., 2001)

Several health implications that pose a threat to human health which are associated with the presence of Aeromonas in water used for consumption purposes. Researchers likeJanda and Abbott (1998) as well as Joseph and Carnahan (2000) indicated that certain strains among the genus are opportunistic human enteric pathogens. Although each species poses different health threats, all species are diarrheagenic (Borchardt *et al*., 2003). *Aeromonas* species, commonly known as aeromonands are usually widely found in aquatic environments worldwide and have been recognized as pathogens affecting both fish and reptiles (Egorov *et al*., 2011). Recently, this aquatic genus has been found and isolated in chlorinated drinking water in many well developed and developing countries (Razzolini *et al*., 2008; Pablos *et al*., 2009). According to Gerba *et al*. (2003) this group of bacteria is highly susceptible to chlorine disinfection that is employed in drinking water facilities and a study conducted by Bomo *et al*. (2004) indicated that they have persistence that enables them to grow in distribution systems biofilms. *Aeromonas* representatives can also be detected in groundwater and within a single system, the same species can survive for many years (Kuhn *et al*., 1997; Borchardt *et al*., 2003).

5.3. Correlations between physico-chemical parameters and microbiological parameters seasonally

Physico-chemical parameters that exist within water bodies are important in maintaining and determining the microbial population of water. According to Basu *et al*. (2013) physico-chemical parameters are one of the factors that are used to determine both the microbial composition and its distribution. The correlations that exist between physico-chemical parameters and microbiological parameters during different seasons is significant, as it draws a picture of how different physico-chemical parameters are associated with microbiological parameters seasonally. Principal Component Analysis (PCA) biplots and Redundancy analysis (RDA) triplots were constructed with the aim of determining the correlations observed between physico-chemical and microbiological parameters, in different sites, during three different seasons. This

is important as it determines the relationship between two or more parameters, if they directly affect one another as one increases or decreases. A strong positive correlation between two parameters was observed by a small angle that exists between them and a relationship between any two parameters that had an angle greater than 90° was regarded as a negative correlation (Rahman *et al*., 2008 cited by Molale, 2012).

During the course of this study, it was evident that TDS and SALT indicated strong significant correlations in all of the seasons investigated. According to Williams and Sherwood (1994) salinity that exists within an aquatic environment is greatly influenced by the TDS concentration present. In the winter and autumn seasons, positive associations were observed between TDS, SALT and HPCs. According to Phyllis *et al*. (2007) an increase in TDS leads to an increase in salinity, this however alters the composition of natural waters. Phyllis *et al*. (2007) went to explain that this makes natural waters to be toxic and impacts the microbiological biota negatively or positively. In the winter season, the high levels of TDS and SALT impacted HPCs negatively, as low levels were obtained. Whereas during the autumn season, TDS and SALT impacted HPCs positively and the highest levels of HPCs were evident. HPCs indicated a statistically significant positive correlation with COD and phosphates (in winter and only phosphates in summer). Tsholo (2018) and Miettinen *et al*. (1997) also observed similar correlations between phosphates and HPCs. Miettinen *et al*. (1997) highlighted that a slight increase in phosphate concentrations in water can greatly influence the growth of the microorganisms. This was supported by a study that was conducted by Chu *et al* (2005) which indicated that HPC bacteria grow fast in water that has elevated levels of both phosphates and nitrates.

The correlation observed between COD and HPCs suggested that CODs played a significant role in the growth of HPCs. CODs are known to reflect the available concentrations of pollutants in water and higher COD concentrations indicate higher concentrations of organic pollutants present in water (Alam, 2015; Fourie, 2017). Temperature is regarded as one of the most important physical parameters that is known to play a crucial role in the survival and growth of bacteria in aquatic environments. Pachepsky and co-workers (2014) investigated the relationship that exists between temperature and microbiological parameters and concluded that temperature indeed plays a role in microbiological parameters. This was also supported by several studies conducted by Fourie (2017), Tsholo (2019) as well as Amanidaz *et al*. (2015). This was indicated by statistically strong significant correlations that were observed in these studies. However, this was not observed in the current study. The relationship between temperature and microbiological parameters was insignificant throughout the course of this study. This was also observed by Habuda-Stanic *et al*. (2013). Furthermore, temperature strongly correlated with pH, COD, nitrates as well as phosphates.

5.4. Antibiotic resistance patterns

In the present study, antibiotic resistance patterns were investigated seasonally. All isolates obtained from three seasons were screened against eleven antibiotics that are commonly administered in the health sector. In the winter season, relatively low isolates were obtained, and they indicated high resistance levels towards four antibiotics, namely Ampicillin, Oxy-tetracycline, Penicilli-G and Trimethoprim. The same resistance patterns were observed for the summer season isolates. However, the resistance patterns depicted by the isolates obtained in the autumn season showed resistance to only three antibiotics, Ampicillin, Penicillin-G and Trimethoprim. Results obtained from the three seasons were combined in order to determine the overall antibiotics profile (Figure 4.5). The findings indicated that there was a great resistance overall to three antibiotics; Ampicillin (98%), Penicillin-G (100%) and Trimethoprim (100%). In this study, HPC species were predominant than *Clostridium* and *Aeromonas* and hence reigned supreme in terms of high antibiotic resistance patterns. The Antimicrobial Stewardship Guidelines Governance in SA (2017) reported that antibiotics that are widely administered in high volumes include: amikacin, amoxycillin-clavulanate, ampicillin, azithromycin, ceftriaxone, clindamycin, cloxacillin, ciprofloxacin, gentamicin, metronidazole and penicillin. The report further listed antibiotics that are highly consumed orally, these include: amoxycillin-clavulanate, amoxycillin, azithromycin, ciprofloxacin, clindamycin, flucloxacillin, and metronidazole. This explains the high resistance patterns observed for both ampicillin and penicillin. Nonetheless although the reports clearly state that ciprofloxacin is amongst the commonly orally consumed antibiotic, findings in the current study showed that it was amongst the highest susceptibility rates.

There are a number of sources that contribute to antibiotics pollution in groundwater and it is difficult to quantify how much is present, hence the MAR indices was used in order to obtain an overview of antibiotic exposure history in boreholes investigated (Tsholo, 2019). According to Davis and Brown (2016) the MAR index is significant in determining whether water environments have been exposed to high or low levels of antibiotics. MAR index values that are greater than 0.2 show that the environment in question has been exposed to antibiotics and that they are frequently used (Sandtu *et al*.2016; Osundiya *et al*., 2013). In the present study, MAR index obtained were all greater 0.2 in three seasons and the highest MAR index value obtained was 0.55. According to Poonia *et al* (2014) environmental water that has a MAR index that is lower than 0.4 is contaminated by non-human feces (animal feces) and MAR index that is greater than 0.4 might be contaminated by human feces.

Penicillin was the first antibiotic to be discovered by Alexander Fleming in 1929 and the discovery of ampicillin followed shortly thereafter (Etebu and Arikekpar 2016). This goes to show that penicillin and ampicillin have been used for centuries to counter infections caused by

microorganisms. This is mainly attributed to the fact that these antibiotics have been long released into the environment, resulting in exposure to microorganisms. This however enabled microorganisms to slowly gain resistance towards them. In the current study it was also illustrated that a number of isolates obtained had intermediate resistance towards kanamycin, vancomycin and oxy-tetracycline. This is a cause for concern because frequent exposure to these antibiotics enable them to acquire resistance leading to complete resistance. According to Amarasiri *et al*. (2019) the water environment serves as an ideal environment for acquisition and dissemination of antibiotic resistance as well as human exposure to antibiotic resistant bacteria.

Two other groundwater studies that were conducted in the North-West Province recorded antibiotic resistance profiles depicted by the microbial population. The first study was conducted by Ferreira (2011) from 2009-2010 indicated that the microbial population of particular systems that were investigated had resistance towards Cephalosporin (CEP), Amoxicillin (AMX), Ampicillin (AMP), Oxy-Tetracycline (OXY-TET) and Trimethoprim (W). The second study that was conducted by Carstens (2012) indicated high resistance towards Cephalosporin, Amoxicillin, Chloramphenicol, and Streptomycin. The resistance patterns that were observed in these two studies, were not in line with the present study. However, the three studies indicated high resistance towards the beta-lactam group of antibiotics. Over five years have passed since groundwater was investigated for antibiotic resistance patterns in the NWP, and the environment has changed over time, this could be the reason of the change in resistance patterns.

Antibiotics that had high resistance in the baseline studies such as Chloramphenicol and Streptomycin were either susceptible or intermediate resistant based on the sampling season in this study. *Escherichia coli* was the predominant species that had the most antibiotic resistance and was among the antibiotic resistance organisms in the two previous studies conducted by Carstens (2012) and Ferreira (2011), whereas in this study, *Bacillus* genera were predominant among the HPC group with high antibiotic resistance patterns. A groundwater study conducted in Ireland by O'Dwyer *et al*. (2017) indicated that the isolated bacteria had high resistance towards ampicillin in great numbers. Other antibiotics such as ciprofloxacin, norfloxacin, nitrofurantoin and trimethoprim also displayed high resistance. Similarities resistance patterns were observed in this study with the exception of ciprofloxacin.

According to the findings obtained in this study and previous studies, it is clear that groundwater, like other water bodies, is contaminated by antibiotics and antibiotic resistant bacteria (ARBs). Antibiotics used for human and veterinary purposes have been rapidly identified and described as one of the emerging contaminants in groundwater, surface water as well as recreational water (Ma *et al*., 2015). According to O'Dwyer *et al*. (2018) antibiotic resistant microorganisms and the genes they harbor are currently recognized as important emerging aquatic contaminants that

have the potential to cause human and ecological health impacts. Surface water, drinking water and wastewater are usually the most vulnerable waterbodies with regards to antibiotic resistant microorganism's contamination as they are exposed and easily accessible. However, the presence of antibiotic resistant microorganisms has also been documented in groundwater (Chee-Sanford *et al*., 2001). The North-West Province comprises many rural areas that depend solely on boreholes as their source of water and it would be of great importance to consider treatment procedures be implemented to remove antibiotic resistant bacteria as well as antibiotic residues towards purifying groundwater to ensure safe and clean water for drinking use (Samie *et al*., 2011; Tsholo, 2019).

5.5. *Clostridium*, *Aeromonas* and HPC identification and microbial diversity in groundwater systems

Microbial communities grow vigorously in natural waters as well as deep in the terrestrial subsurface (Fredrickson and Balkwill, 2006). In most cases groundwater does not contain as many nutrients as surface water, wastewater and drinking water that enhance the growth of multiple bacterial communities. However, with the little nutrients that it has, it is able to harbour and promote some bacterial communities. In the present study, HPCs were the predominant group, with vast species throughout the entire study period. Although HPCs levels were different at each site, depending on the season, it was the most observed as compared to *Clostridium* and *Aeromonas* groups. HPCs are important in terms of determining the state of water and studies have indicated that it is a significant microbiological parameter that can be used as a water quality indicator to determine the health impacts it has on humans (Payment *et al*.,1994; Donskey, 2006). This group is an umbrella that has different genera. Some of the genera are of pathogenic origin and others are classified as non-pathogenic. According to Allen *et al*. (2004) there is a lack of evidence that proves that HPCs are considered opportunistic pathogens and can cause gastro-intestinal when ingested via food or water in clinical settings.

Allen (2004) and Allen *et al*. (2004) also indicated that there was not sufficient evidence to suggest that opportunistic pathogenic HPCs can cause gastro-intestinal diseases. The studies that are listed above were conducted more than ten years ago and over time microorganisms acquired certain mechanisms that enable them to prosper in unfavourable conditions. In the present studies that have been conducted, it is clear that opportunistic pathogenic HPCs are becoming a problem in the health sector as many have acquired antibiotic resistance towards widely used antibiotics as well as harbour ARGs. Each water body comprises of different dominant HPC genus. For instance, a study that was conducted by Tsholo (2019) which focused on raw and drinking water,

indicated two main genera that were prevalent, namely *Pseudomonas* and *Bacillus*. This was also observed in groundwater studies conducted by Carstens (2012) and another by Ferreira (2011) whereby the most abundant genus identified was *Pseudomonas*. However, in the present study, this was not the case, the most abundant genera identified obtained was *Bacillus* and *Citrobacter*.

The *Bacillus* genus consisted of a total of 16 different species that were identified, namely *B. aerius, B. cereus, B. cucumis, B. endophyticus, B. kochii, B. megaterium, B. mobilis, B. paramycoides, B. proteolyticus, B. pumilus, B. saferensis, B. simplex, B. subtilis, B. thuringiensis, B. toyonensis, B. tropicus* and *B. wiedmannii*. Another genus that falls under the family *Bacillacea* (same family as *Bacillus*) known as *Vigiobacillus* was observed with only a single species. The citrobacter genus consisted of only a single species. Other HPC genera that were observed in this study included *Escherichia*, *Flavobcterium*, *Paenibacillus*. *Plesiomonas*, *Salmonella*, *Staphylococcus* and *Stenotrophomonas*. The genus *Flavobacterium* was also present in one of the HPC genera in a study done by Carstens (2012). *Clostridium* and *Aeromonas* were observed in relatively low numbers. The presence of these two genera in water bodies is associated with faecal pollution. The presence of *Aeromonas* in groundwater was also observed in Carstens's study (2012). There is a lack of evidence in terms of the presence of *Clostridium* in groundwater in the North West Province and South Africa. However, the prevalence of this microorganism has been investigated in both surface water and wastewater. Fourie (2017) investigated the presence and prevalence of *Clostridium* in surface water and found that there were several pathogenic representatives of this genus. Few pathogenic species that were identified included *Clostridium perfringens*, *Clostridium bifermentans* as well as *Clostridium sordelli*. Two of the *Clostridium* pathogenic strains identified in Fourie's study, were also identified in the present study, namely *Clostridium perfringens*, of which was the most prevalent and *Clostridium sordellii*.

5.6. Phylogenetic tree associations

Phylogenetic trees can be used to describe the evolutionary association of different species as well as their common ancestor (Hall, 2013). In the current study, two phyla (Firmicutes and Proteobacteria) were observed. Phylum Firmicutes was the largest observed as it consisted not only various species of *Bacillus*, but also consisted of three *Clostridium* species. The Proteobacteria phylum consists of bacterial genera such as *Citrobacter*, *Escherichia*, *Staphylococcus* etc. *Bacillus* species are one of the significant bacterial genera that is composed of various heterogeneous species (Liu *et al*., 2013). To date, about 172 *Bacillus* species have been recognized with more than half found in both the terrestrial and aquatic environments. Liu *et al*. (2013) divided different *Bacillus* species strains into five groups solely based on their 16S rRNA gene phylogenetic analysis. The five groups were as follows; *B. cereus, B. megaterium, B. subtilis, B. circulans* and *B. brevis.* Three (*B. cereus, B. megaterium,* and *B. subtilis)* of the five groups were observed in the current study. According to Guinebretiere *et al*. (2013) over 97% 16S rRNA similarities were observed between the *B. cereus* group and other *Bacillus* species such as *B. thuringiensis*. The similarity between the two species was observed in the present study. Berkeley *et al*. (2008) highlighted that *B. pumilus* belongs to the *B. subtilis* group as they both have significant similarities and Liu *et al*. (2013) highlighted that *B. pumilus* shared similarities of over 99.5% in 16s rRNA sequences gene with *B. safensis*. This was also evident in the present study. Generally, *Bacillus* species are related, however they can be distinguished by house-keeping genes such as rpoB (RNA polymerase beta-subunit), gyrB (gyrasre B subunit), 23S tRNA (Tourasse *et al*., 2011; Liu *et al*., 2013). An example stated by Helgason *et al*. (2004) confirmed that indeed the presence of these genes in *Bacillus* species was used to differentiate between *B. cereus* and *B. subtilis*. *Virgibacillus* genus belongs to the phylum Firmicutes as *Bacillus* genus. The two genera are somehow related but there are significant differences that distinguish the two.

Another genus observed in the current study, belonging to phylum Firmicutes was *Clostridium*. This genus only had three species that were observed, namely *C. perfringens*, *C. sordellii* and *C. tepidum*. A study conducted by Scaria *et al*. (2015) stated that *C. sordellii* is more closely related to *C. difficile* than any other Clostridial species as both of these species are considered opportunistic pathogens. In that particular study, a 71% similarity was observed between *C. sordellii* and *C. difficile*, hence suggesting that these two species are sister groups. Kiu and co-workers (2017) observed that *C. perfringens* indicated similarities with *C. sordellii* and *C. difficile*, however the relatedness was not as great as the similarities that can be seen between *C. perfringens*, *C. baratii* as well as *C. sardiniense*. This was observed in this study as well. *C. perfringes* indicated close relatedness with *C. tepidum* (99%) than with *C. sordellii*.

Phylum Proteobacteria that was evident in the current study was dominated by the main representatives of HPCs that are considered as pathogens belonging to the family of *Enterobacteriaceae*. According to Paradis and co-workers (2005) phylogenetic associations observed between *Salmonella*, *Escherichia coli* and *Citrobacter freundii* are not well defined. Furthermore, both 16S and 23S rRNA gene sequences findings collected from Christensen and Olsen (1998) and Sproer *et al*. (1999) revealed that there is a closer association existing between *Salmonella* and *E. coli* than the relationship observed between *Salmonella* and *C. freundii*.

5.7. Pathogenicity: Haemolysin and Extracellular enzymes assays

According to Casadevall and Pirofski (2009) pathogenicity in bacteria can be defined as the microorganism's ability to cause diseases and infections that are mediated by specific virulence factors. There are several studies that have highlighted that the HPC bacteria consist of characteristics that are of virulent nature, that are in connection with opportunistic pathogenicity such as haemolysin and the secretion of extracellular enzymes (Pavlov *et al*., 2004; Horn *et al*., 2016). These extracellular enzymes causes' bacteria to become (i) cytotoxic to cells, (ii) adhere to cells as well as (iii) enable them to survive passage through gastric fluids of stomach (Pavlon *et al*., 2004; Yuk and Marshall, 2004). The previous studies that were conducted by Ferreira (2011) and Carstens (2012) did not investigate the pathogenicity of the microorganisms identified. However, in a study that was conducted by Prinsloo (2014) that focused on untreated borehole used for drinking water highlighted both haemolysin and extracellular enzymes observed in HPCs during their study period. In this work, pathogenicity was investigated in *Clostridium* sp., *Aeromonas* sp., and HPCs.

5.7.1. *Clostridium* sp.

Clostridium sp. had a total of six isolates, and all were beta-hemolytic (100%). According to Berger (2015) the extensive pathogenicity of *Clostridium* species has not been well studied, however it has been associated with a variety of infections and diseases. A variety of *Clostridium* species have been reported as possible pathogens, some of which were observed in this study: *C. perfringens* and *C. sordellii* (Fourie, 2017). The latter mentioned species are able to produce enterotoxins and histotoxins that can cause severe gastrointestinal diseases as well as necrotising gas-gangrene in humans and animals (Stevens and Bryant, 2002). The presence of such *Clostridium* species in groundwater systems is alarming, as it compromises the quality of groundwater and might cause several health implications when consumed prior to treatment.

5.7.2. *Aeromonas* sp.

According to Merino *et al*. (1999) *Aeromonas* genus are able to produce various virulence factors, which include (i) cytotoxic and cytotonic enterotoxins, (ii) aerolysins, (iii) haemolysins, (iv) proteases, (v) haemagglutinin and (vi) lipase. In this study *Aeromonas* genus yielded a total of four isolates (2 *A. hydrophila* and 2 *Aeromonas* species), where only three were beta-hemolytic (75%). Similarly, Bhowmik *et al*. (2009) obtained 71% beta-hemolytic *Aeromonas* in environmental samples. Mulamattathil (2014) also highlighted that the *Aeromonas* species in drinking water tested positive for hemolytic activity (beta-hemolytic). Furthermore Prinsloo (2014) observed *Aeromonas* species of beta-hemolytic activity as well as the production of two other extracellular enzymes. *Aeromonas hydrophila* was responsible for gelatinase production, whereas other *Aeromonas* species obtained, were positive for only hemolytic activity. Production of gelatinase in *Aeromonas hydrophila* was also observed by Bhowmik *et al*. (2009). *Aeromonas* species in this study, were only positive for production of two extracellular enzymes. In both Prinsloo's (2014) and Bhonwmik *et al*. (2000) studies, production of lecithinase, lipase, DNase, Chondroitinase as well as hyaluronidase enzymes were seen in various *Aeromonas* species. However, in this study, production of lecithinase and DNase was investigated but not observed.

Virulent environmental *Aeromonas hydrophila* strains are usually prevalent due to the ability of this strain to produce proteinase, enterotoxins and cytotoxins (Mateos *et al*., 2008). *Aeromonas* species are regarded as opportunistic pathogens that are able to grow at temperatures from 4°C up to 42°C and their hemolytic activity as well as cytotoxic effects have been proved to be most severe at 37°C (Mateos *et al*., 2008; Prinsloo, 2014). The high presence of *Aeromonas* in the environment and humans has long been documented by two authors (Albert *et al*., 2000; Bhowmik *et al*., 2000). Mateos *et al*. (2008) highlighted a few cases of human infections related to high numbers of *A. hydrophila* in water used for drinking purposes. However, the pathogenesis associated with *Aeromonas* infections has been proven complex and multifactorial (Janda and Abbott, 1998; Chopra *et al*., 2000).

5.7.3. HPCs

Out of the 90 HPCs isolates that were isolated from boreholes, 69 (76%) isolates were beta-hemolytic and only 21 (23%) isolates were alpha hemolytic. This was in correspondence with the studies that were conducted by Prinsloo (2014) and Pavlon *et al*. (2004) also observed more beta-hemolytic HPCs in borehole water. HPCs indicated an overall of 55% of DNase production.

a. *Bacillus* sp.

Amongst the HPCs, *Bacillus* species were the most predominant. *Bacillus* species obtained in this study produced beta-hemolytic activity (79%), alpha-hemolytic (21%) and DNase production (67%). In studies that were conducted by Prinsloo (2014), Pavlon *et al*. (2004) and Tsholo (2019) more extracellular enzymes such as proteinase, lecithinase, lipase and gelatinase were investigated, and positive results were observed in *Bacillus* species. In this study, two other extracellular enzymes (lecithinase and gelatinase) were also investigated but were not observed. Previous studies have highlighted *Bacillus* species are known for their ability to produce lecithinase (Molva *et al*., 2009, Cadot *et al*., 2010), however this was not the case in the present study. *B. cereus* and *B. thuringiensis* were the most predominant *Bacillus* species that indicated high prevalence of hemolytic activity and extracellular enzyme production. Overall *B. cereus* had the most of isolates that were beta-hemolytic and produced DNase. This was in correlation with the studies that were done by Molva *et al*. (2009) and Prinsloo (2014) in terms of the presence of DNase only. *Bacillus cereus* has been recognised as an opportunistic pathogen as it is able to cause both gastrointestinal and non-gastrointestinal infections in humans when ingested (Kotiranta *et al*., 2000). The latter described *B cereus* pathogenicity is associated with the secretion of toxins that include, (i) haemolysin, (ii) emesis-inducing compounds, (iii) phospholipids and (iv) non-hemolytic enterotoxins and cytotoxin K (Horn *et al*., 2016).

Bacillus thuringiensis, the second largest group recorded, illustrated 92% beta-hemolytic isolates, while 54% were responsible for DNase production. This was also observed by Molva *et al*. (2009) and Prinsloo (2014). The pathogenicity of *B. thuringiensis* is relatively similar to *B. cereus* pathogenicity (Kotiranta *et al*., 2000), but the two species can be distinguished by the production of the insecticidal crystal protein that is evident in *B. thuringiensis* (Prinsloo, 2014). Since insecticides and pesticides are commonly used for crop production has increased the prevalence of *B. thuringiensis* in water sources as well as in food sources, which when consumed, can be detrimental to human health. Other *Bacillus* species such as *B. megaterium*, *B. mobilis*, *B. paramycoides*, *B. proteolyticus*, *B. pumilus*, *B. subtilis* and *B. wiedmannii* were positive for both haemolysin and DNase production but were not prevalent as the afore mentioned species. Findings of *B. pumilus* and *B. subtilis* were also observed and reported in Prinsloo (2014).

b. *Citrobacter* sp.

Citrobacter freundii, a member of the genus Citrobacter is regarded an opportunistic pathogen as it is of faecal origin. In the present study, *Citrobacter freundii* was the second largest group in

HPCs. According to Hossain *et al*. (2017) *Citrobacter* associated virulence factors include hemolysis, proteolysis as well as biofilm formation. In this work, beta-hemolytic activity was observed in all *C. freundii* and was in correspondence with findings obtained by Hossain *et al*. (2017), however biofilm formation and proteolysis were not investigated. This species is relatively prevalent in both soil and water due to pollution of waste material of humans and animals. The virulence factors that are expressed by this organism are causing agents of diarrhoea in humans (Hossain *et al*., 2017; Liu *et al*., 2018).According to Bai *et al*. (2012) there are several crucial virulence factors found in *C. freundii* that cause diarrhoea in humans and they include Shiga-like toxins, heat stable toxins and a cholera toxin B subunit homolog.

5.8 Detection of ARGs

In most cases microbiological analysis of both groundwater and drinking water are focused on the occurrence of faecal indicators and pathogenic bacteria with the aim of preventing outbreaks of infectious diseases (WHO, 2006; Szekeres *et al*., 2018). High bacterial count of these bacteria poses a serious health risk and their concurrent presence that is associated with traces of antibiotic and antimicrobial resistant microbes levels in groundwater makes it even more alarming in terms of health risks (Xi *et al*., 2009; Chen *et al*., 2017). According to Pazda *et al*. (2019) the WHO and ECDC identified antibiotic resistance as one of the major public health problems of the 21st century and needs to be resolved promptly. Microorganisms have been known to acquire and transfer ARGs via mobile genetic elements such as plasmids, transposons and integrons (Stalder *et al*., 2014; Szekeres *et al*., 2018). According to Gillings, 2017 anthropogenic input such as contaminated water triggers horizontal gene transfer, thereby creating an opportunity for bidirectional gene exchange to take place between environmental and clinical bacteria. Currently Wastewater treatment plants (WWTPs) and Drinking Water Purification Plants (DWPPs) are regarded as hotspots that enhance antibiotic resistant bacteria's (ARBs) ability to share and integrate ARGs into their genome by means of mechanisms such as transformation, transduction (Lu *et al*., 2018; Tehrani and Gillbride, 2017; Tsholo, 2019). According to Bockelmann *et al*. (2008) crucial concerns with regards to the recharge of groundwater aquifers arise from contamination with pathogenic bacteria as well as multi-drug resistant bacteria. When such wastewater is released back into the environment, it may play a significant role in increasing ARBs in surface water, thereby influencing groundwater in the long run. A study that was conducted by Auerbach *et al*. (2007) stated that antibiotics and antimicrobials enter natural environments by the release of treated wastewater effluent and through animal husbandry, especially in agriculture. In the present study, five antibiotic resistant genes derived from commonly resistant antibiotics were investigated in HPCs species.

5.8.1 *ampC* and *bla$_{TEM}$* genes

The ARGs *ampC* and *bla$_{TEM}$* are derived from a family of beta-lactam antibiotics. These group of antibiotics are widely used for various infections and the resistance observed within these antibiotics is a serious threat as they have low toxicity (Zhang *et al*., 2009). The mechanism of resistance that beta-lactams use include inaccessibility of antibiotics to their target enzymes, modifications of target enzymes as well as direct inactivation of antibiotics by beta-lactamases (Li and Zhang, 2010). Various bla genes have been detected and identified in dairy farm water, water or sediments of aquaculture, surface water as well as natural water (Srinivasan *et al*. 2008). This gene is usually found in microbial species such as *Aeromonas* spp. (Jacobs and Chenia, 2007), *Enterobacter* spp. (Volkmann *et al*., 2004), *Salmonella* spp. (Antunes *et al*., 2006), *Staphylococcus* spp. (Schwartz *et al*., 2003) as well as in *Vibrio* spp. (Taviani *et al*., 2008). Among the various bla genes, only one bla gene (*bla$_{TEM}$*) was investigated in this work and was detected in four *Bacillus* species (*B. aerius*, *B. cereus*, *B. simplex* and *B. thuringiensis*) and one *Paenibacillus* species. Previous groundwater studies that have been conducted do not highlight the presence and prevalence of the *bla$_{TEM}$* gene, however other *bla* genes were detected and identified instead. This was highlighted in a study that was conducted by Szekeres *et al*. (2018) in which beta-lactam gene blaSHV was detected in the groundwater systems investigated. Furthermore, this gene was relatively abundant in the systems especially in *Enterococci* species as compared to other genes that were observed.

According to Schwartz *et al*. (2003) and Zhang *et al*. (2009) *ampC* genes has been detected in microbial isolates that are usually obtained from wastewater, surface water as well as in drinking water films. During the period of the study, the *ampC* gene was more prevalent than the *bla$_{TEM}$* gene and it was detected and observed in three *Bacillus* species (*B. aerius*, *B. cereus* and *B. pumilus*), and single species of *Citrobacter* sp., *Escherichia* sp. *Salmonella* sp. and *Stenotrophomonas* sp. Unfortunately, there are insufficient studies that focus on the presence of the *ampC* gene in groundwater. Both the *ampC* and *bla$_{TEM}$* genes are usually found in abundance in wastewater, surface as well as drinking water. The studies that were conducted by Schwartz *et al*. (2003) and another by Obst *et al*. (2006) found *ampC* gene abundantly in both wastewater biofilms and drinking water biofilms. Several studies, including those conducted by Tsholo (2019), Fernando *et al*. (2016) and Shi *et al*. (2013) observed high occurrence of the *ampC* gene in drinking water (treated water and water from distribution systems.

5.8.2 *ermB* and *ermF* genes

According to Weisblum (1998) and Leclercq and Courvalin (1991) the erythromycin ribosome methylation (erm) gene family consists of methyltransferase enzymes that are able to reduce the binding ability of antibiotics within antibiotic families known as MLS that are made up of macrolides, lincosamides and streptogramin B. Currently, there is a significant increase in the resistance of the MLS family due to the overuse of its antibiotic representatives as they are important and widely used in agriculture and human health (Rieke *et al*., 2018). In various studies (drinking water, surface water, wastewater as well as groundwater) this gene has been detected, however in the current study, both *ermB* and *ermF* were detected.

5.8.3 *tetM* gene

A study conducted by Dancer *et al*. (1997) indicated that tetracycline-resistant microorganisms were notable in water environments previously exposed to tetracycline. Tetracycline (tet) consists of over 40 resistant genes that can be characterized in bacteria (Thompson *et al*., 2007). According to Zhang *et al*. (2008) among over 40 *tet* genes that exist, 22 have been detected in bacteria obtained in water environments. The mechanism used by this particular gene is to protect the ribosomal protein (Chen *et al*., 2017; Tehrani and Gilbride, 2017, Zhang *et al*., 2008). In the present study only one tetracycline gene, known as *tetM* gene was investigated and was not detected in any groundwater system during the period of the study. However, tetracycline genes, including *tetM*, encoding for ribosomal protection proteins were identified in microbial communities obtained from WWTPs, hospital or animal production wastewater as well as in natural water environments (Auerbach *et al*., 2007; Zhang *et al*., 2008).

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

In general, groundwater is considered as a natural water purification system but is it equipped to remove advanced pollutants that it is currently exposed to. Over the years groundwater systems have suffered in the hands of pollution due to the extensive use of fertilizers, pesticides, industrial effluents as well as chemicals introduced into the environment (Mohan *et al*, 2013). This however affects the quality and health of groundwater. Anthropogenic activities such as those involved in agriculture affecting surface water will eventually affect groundwater in the long run, thereby compromising both its physio-chemical and microbiological properties negatively (Sasakova *et al*., 2018). The health sector also plays a role in the contamination of groundwater by releasing medical waste (harboring antibiotics /antimicrobials) into the environment. This however plays a vital role in the genetic material alteration of microorganisms, enabling them to gain resistance towards widely used antibiotics and antimicrobials.

6.1. Physico-Chemical parameters

The physico-chemical parameters of selected groundwater systems in the NWP were investigated over a period of three seasons (2018-2019). The findings obtained in the current study indicated that the quality of groundwater systems is questionable due to certain parameters that were of concern. Parameters such as TDS and Salinity were very high, with TDS exceeding the limitations set out by the TWQR standards in all three seasons, with the highest concentrations observed during the summer and autumn seasons. In all of the systems investigated, sites LV, LR and B4 had the highest concentrations of both TDS and Salinity throughout the course of the study. COD also portrayed high concentrations in several sites. However other parameters such as temperature, pH, nitrates and phosphates were within the TWQR standards throughout the course of study. The presence of these physico-chemical parameters played a significant role in the harbouring, growth and maintenance of microorganisms in groundwater systems.

6.2. Microbiological parameters

Microorganisms found in groundwater systems were isolated and characterized. Since the study focus was solely based on groundwater systems, the presence of opportunistic pathogenic microorganisms such as *Clostridium* sp. and *Aeromonas* sp. was not expected because groundwater is limited and protected from pollution exposure. However, the current study

revealed that groundwater systems indeed have the potential to harbour these microorganisms although their presence was only evident in summer season at low levels. In the current study, HPCs were observed throughout the course of the study and the highest levels thereof were evident in the autumn season. HPCs consists of a wide spectrum of microorganisms hence identification was of essence. The predominant species of HPCs included *Bacillus* sp., *Citrobacter* sp., and *Escherichia* sp. Microbial genera such as those observed in the study are considered as opportunistic pathogens and their presence in any water environment raises concerns.

6.3. Pathogenicity detection

Since most of the species obtained in this study were classified as potential pathogens according to literature, the pathogenicity thereof was significant in terms of haemolysin and extracellular enzymes production. About 80% of the species obtained were beta-haemolytic, indicating that they possess the ability to break down and lyse mammalian cells. Furthermore, extracellular enzymes such as DNase, Lecithinase and Gelatinase were investigated but HPCs produced DNase only, *Aeromonas* sp., produced Gelatinase only and Lecithinase production was not observed. According to Prinsloo (2014) the ability of microorganisms to produce more than one pathogenic enzyme, suggests that they are indeed opportunistic pathogens and can be detrimental towards human health.

6.4. Antibiotic resistance

Water is considered a vehicle of antibiotic resistance as it is known to harbour various ARBs (Antibiotic Resistant Bacteria) hence it was of importance to screen for antibiotic resistance profiles of microorganisms obtained against various commonly used antibiotics. During the course of the study, microorganisms indicated high resistance towards three antibiotics namely: Ampicillin, Penicillin-G and Trimethoprim. These are the most common and widely used antibiotics, with Ampicillin and Penicillin-G belonging to one of the oldest class of antibiotics. Supplementary findings observed from MAR indices indicated that the groundwater systems were recently exposed to antibiotics as all the values obtained were greater than 0.2 and the predominant MAR phenotypes were AMP-PEN-G - W.

6.5. ARGs and Virulence genes

Several questions were raised about how microorganisms, especially in groundwater could have gained resistance towards the latter mentioned antibiotics. These microorganisms were screened for five ARGS namely; *ampC*, bla_{TEM}, *ermB*, *ermF* and *tetM* and findings revealed that most of the ARGs *ampC* and bla_{TEM} were present in HPC species. The *ampC* gene was more predominant in *Bacillus* sp., *Citrobacter* sp., and also showed presence in *Escherichia* sp. whereas bla_{TEM} was predominant in *Bacillus* sp. Virulence gene were only investigated in *Aeromonas* sp., but unfortunately none were detected.

In conclusion, with regards to the findings of the current study, it is evident that groundwater can be utilized as an alternative source of water, should drought occur. However, it cannot be used as is without any purification procedures. The quality of groundwater is no longer of good quality as it used to be centuries ago and as seen in this study that opportunistic pathogens are harboured within these systems and can cause health problems to immunocompromised consumers.

6.6. Recommendations

In the coming years, groundwater will be highly dependent on as a sole source of water and the following recommendations were suggested with regards to the current study and they are as follows:

This was a relatively small-scale study that focussed on limited groundwater systems that were not even used for drinking purposes but for irrigation purposes. In order to understand the quality of groundwater in the NWP, more groundwater systems need to be investigated, especially those situated in rural areas and informal settlement. This will give a clear thorough insight in terms of the quality, quantity as well as driving factors that introduce pollution into these systems.

Limited physico-chemical parameters were investigated in this study. Physico-chemical parameters such as sulphates, BOD (Biological Oxygen Demand), ammonia and etc were side-lined and were not investigated. These parameters are important and go hand in hand together with parameters such as TDS, COD and Salinity as they contribute greatly towards determining the levels of nutrients present in water as well as can give more insight about, the type of microbial communities harboured within these systems. In systems that are currently used for drinking

purpose and chlorination is used as a purification measure, testing for free chlorine would be beneficial.

The sample sizes collected could have impacted the results observed in terms of microbiological parameters, more especially the detection of *Clostridium* and *Aeromonas* species. Only one litre bottles of water sample were collected per site, and only about 300 ml was filtered for the detection of *Clostridium* and *Aeromonas* each. For thorough detection of these microorganisms, larger volume of water samples such as 10L should be filtered and eDNA conducted on membrane filters, as this will aid in determining the overall microbial communities in various sites.

Culture based methods are good and have been trusted for many years but with the evolution of science, advanced methods such as eDNA and whole genome sequencing would be very beneficial in studies such as this.

This study only focussed on specific microorganisms, whereas there are vast opportunistic pathogens as well as faecal indicators such as *Pseudomonas* sp., and *Enterococcus* sp. That could have been explored, to determine if the groundwater systems were faecally contaminated.

ARGs were only detected by means of End-Point PCR and were not explored further. The use of methodologies such as qPCR can be used to quantify the ARGs obtained.

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ANNEXURE A

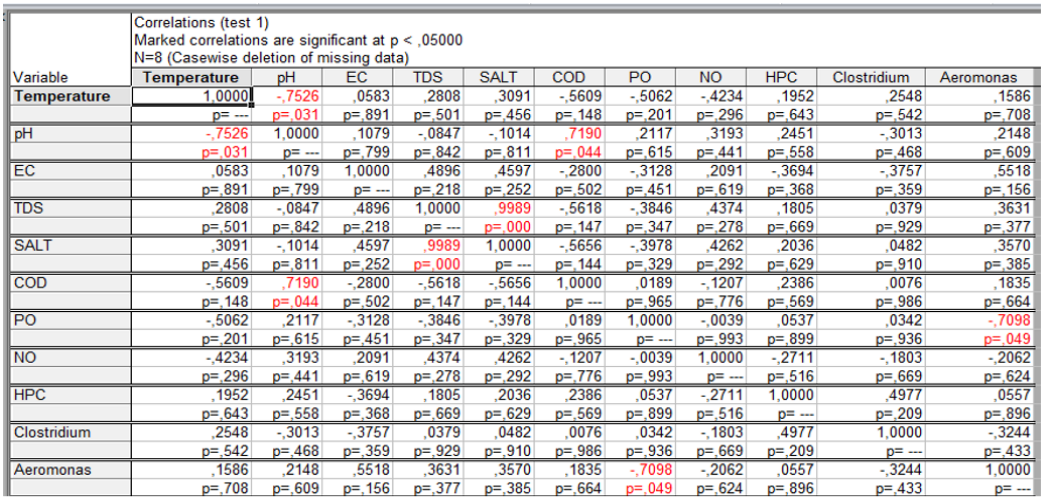
Table A. GPS Co-ordinates of sampling sites.

BOREHOLE	Campus Layout	Description	latitude	longitude
PB1	H4	Draak	26°40'58.59"S	27° 5'38.87"E
PB10	E14	LaVaria	26°41'20.73"S	27° 5'38.77"E
PB14	B4	B4	26°41'40.58"S	27° 5'30.60"E
PB17	K22	Fanie	26°41'41.86"S	27° 5'56.07"E
PB20	O1	Mediclinic	26°41'25.01"S	27° 4'57.38"E
PB22	H4	Botanical Gardens	26°40'53.68"S	27° 5'42.46"E
PR6	A4	Lareus	26°41'44.34"S	27° 5'20.25"E
Gerard minnebron	Off-campus	GM	26.4797° S,	27.1514° E

Correlations (test 1)
Marked correlations are significant at p < ,05000
N=8 (Casewise deletion of missing data)

Variable	Temperature	pH	EC	TDS	SALT	COD	PO	NO	HPCs
Temperature	1,0000	-,4756	-,2755	-,2702	-,4038	,1066	-,0467	,7269	-,0452
	p= ---	p=,234	p=,509	p=,517	p=,321	p=,802	p=,913	p=,041	p=,915
pH	-,4756	1,0000	,1844	,2146	,2305	-,0313	,0481	-,3352	,1350
	p=,234	p= ---	p=,662	p=,610	p=,583	p=,941	p=,910	p=,417	p=,750
EC	-,2755	,1844	1,0000	,9992	,9459	,8016	-,8568	,0981	,7296
	p=,509	p=,662	p= ---	p=,000	p=,000	p=,017	p=,007	p=,817	p=,040
TDS	-,2702	,2146	,9992	1,0000	,9451	,7989	-,8565	,0986	,7317
	p=,517	p=,610	p=,000	p= ---	p=,000	p=,017	p=,007	p=,816	p=,039
SALT	-,4038	,2305	,9459	,9451	1,0000	,8004	-,8755	-,1251	,7276
	p=,321	p=,583	p=,000	p=,000	p= ---	p=,017	p=,004	p=,768	p=,041
COD	,1066	-,0313	,8016	,7989	,8004	1,0000	-,9028	,3384	,8322
	p=,802	p=,941	p=,017	p=,017	p=,017	p= ---	p=,002	p=,412	p=,010
PO	-,0467	,0481	-,8568	-,8565	-,8755	-,9028	1,0000	-,2496	-,8172
	p=,913	p=,910	p=,007	p=,007	p=,004	p=,002	p= ---	p=,551	p=,013
NO	,7269	-,3352	,0981	,0986	-,1251	,3384	-,2496	1,0000	,4171
	p=,041	p=,417	p=,817	p=,816	p=,768	p=,412	p=,551	p= ---	p=,304
HPCs	-,0452	,1350	,7296	,7317	,7276	,8322	-,8172	,4171	1,0000
	p=,915	p=,750	p=,040	p=,039	p=,041	p=,010	p=,013	p=,304	p= ---

Figure A1: Correlations of physico-chemical parameters with p-vlues from winter season.



Variable	Temperature	pH	EC	TDS	SALT	COD	PO	NO	HPC	Clostridium	Aeromonas
Correlations (test 1)											
Marked correlations are significant at p < ,05000											
N=8 (Casewise deletion of missing data)											
Temperature	1,0000	-,7526	,0583	,2808	,3091	-,5609	-,5062	-,4234	,1952	,2548	,1586
	p= ---	p=,031	p=,891	p=,501	p=,456	p=,148	p=,201	p=,296	p=,643	p=,542	p=,708
pH	-,7526	1,0000	,1079	-,0847	-,1014	,7190	,2117	,3193	,2451	-,3013	,2148
	p=,031	p= ---	p=,799	p=,842	p=,811	p=,044	p=,615	p=,441	p=,558	p=,468	p=,609
EC	,0583	,1079	1,0000	,4896	,4597	-,2800	-,3128	,2091	-,3694	-,3757	,5518
	p=,891	p=,799	p= ---	p=,218	p=,252	p=,502	p=,451	p=,619	p=,368	p=,359	p=,156
TDS	,2808	-,0847	,4896	1,0000	,9989	-,5618	-,3846	,4374	,1805	,0379	,3631
	p=,501	p=,842	p=,218	p= ---	p=,000	p=,147	p=,347	p=,278	p=,669	p=,929	p=,377
SALT	,3091	-,1014	,4597	,9989	1,0000	-,5656	-,3978	,4262	,2036	,0482	,3570
	p=,456	p=,811	p=,252	p=,000	p= ---	p=,144	p=,329	p=,292	p=,629	p=,910	p=,385
COD	-,5609	,7190	-,2800	-,5618	-,5656	1,0000	,0189	-,1207	,2386	,0076	,1835
	p=,148	p=,044	p=,502	p=,147	p=,144	p= ---	p=,965	p=,776	p=,569	p=,986	p=,664
PO	-,5062	,2117	-,3128	-,3846	-,3978	,0189	1,0000	-,0039	,0537	,0342	-,7098
	p=,201	p=,615	p=,451	p=,347	p=,329	p=,965	p= ---	p=,993	p=,899	p=,936	p=,049
NO	-,4234	,3193	,2091	,4374	,4262	-,1207	-,0039	1,0000	-,2711	-,1803	-,2062
	p=,296	p=,441	p=,619	p=,278	p=,292	p=,776	p=,993	p= ---	p=,516	p=,669	p=,624
HPC	,1952	,2451	-,3694	,1805	,2036	,2386	,0537	-,2711	1,0000	,4977	,0557
	p=,643	p=,558	p=,368	p=,669	p=,629	p=,569	p=,899	p=,516	p= ---	p=,209	p=,896
Clostridium	,2548	-,3013	-,3757	,0379	,0482	,0076	,0342	-,1803	,4977	1,0000	-,3244
	p=,542	p=,468	p=,359	p=,929	p=,910	p=,986	p=,936	p=,669	p=,209	p= ---	p=,433
Aeromonas	,1586	,2148	,5518	,3631	,3570	,1835	-,7098	-,2062	,0557	-,3244	1,0000
	p=,708	p=,609	p=,156	p=,377	p=,385	p=,664	p=,049	p=,624	p=,896	p=,433	p= ---

Figure A2: Correlations of physico-chemical parameters with p-vlues from summer season.

Variable	Correlations (test 1) Marked correlations are significant at p < ,05000 N=8 (Casewise deletion of missing data) **Temperature**	pH	EC	TDS	SALT	COD	PO	NO	HPC
Temperature	1,0000	-,0460	-,5437	-,5718	-,5419	,7236	,7453	,7640	,0724
	p= ---	p=,914	p=,164	p=,139	p=,165	p=,042	p=,034	p=,027	p=,865
pH	-,0460	1,0000	,1006	,1057	,1150	,0757	-,0985	-,5656	-,0975
	p=,914	p= ---	p=,813	p=,803	p=,786	p=,859	p=,816	p=,144	p=,818
EC	-,5437	,1006	1,0000	,9993	,9987	,1127	-,3978	-,5799	-,6398
	p=,164	p=,813	p= ---	p=,000	p=,000	p=,790	p=,329	p=,132	p=,088
TDS	-,5718	,1057	,9993	1,0000	,9979	,0820	-,4153	-,5983	-,6305
	p=,139	p=,803	p=,000	p= ---	p=,000	p=,847	p=,306	p=,117	p=,094
SALT	-,5419	,1150	,9987	,9979	1,0000	,1100	-,3946	-,5841	-,6355
	p=,165	p=,786	p=,000	p=,000	p= ---	p=,795	p=,333	p=,128	p=,090
COD	,7236	,0757	,1127	,0820	,1100	1,0000	,5298	,4216	-,3360
	p=,042	p=,859	p=,790	p=,847	p=,795	p= ---	p=,177	p=,298	p=,416
PO	,7453	-,0985	-,3978	-,4153	-,3946	,5298	1,0000	,8183	-,3743
	p=,034	p=,816	p=,329	p=,306	p=,333	p=,177	p= ---	p=,013	p=,361
NO	,7640	-,5656	-,5799	-,5983	-,5841	,4216	,8183	1,0000	,0171
	p=,027	p=,144	p=,132	p=,117	p=,128	p=,298	p=,013	p= ---	p=,968
HPC	,0724	-,0975	-,6398	-,6305	-,6355	-,3360	-,3743	,0171	1,0000
	p=,865	p=,818	p=,088	p=,094	p=,090	p=,416	p=,361	p=,968	p= ---

Figure A3: Correlations of physico-chemical parameters with p-vlues from autumn season.

ANNEXURE B

Table B1: Antibiotic resistant patterns observed in winter, 2018.

Isolates	AMP	C	CIP	E	K	N	OT	P	S	W	VA
LV10-2(A)	R	S	S	S	R	S	R	R	S	R	I
WC10-3(A)	R	R	S	I	I	S	R	R	S	R	-
LV10-4(B)	R	S	S	S	I	S	I	R	S	R	I
LV10-2(C)	R	S	S	S	R	S	I	R	S	R	I
LR10-3(D)	R	S	S	S	R	S	R	R	S	R	-
LR10-3(C2)	R	S	I	S	R	S	I	R	S	R	R
LR10-3(-C)	S	I	I	R	S	S	R	R	S	R	I
LR10-3(B)	R	S	S	S	I	S	I	R	S	R	I
DK10-4(D)	R	S	S	S	I	S	R	R	S	R	-
DK10-4(C)	R	S	S	S	S	S	S	R	S	R	-
DK10-4(B2)	R	I	S	S	R	S	R	R	S	R	I
DK10-4(B)	R	S	S	S	S	S	R	R	S	R	S
DK10-4(A)	R	S	S	S	S	S	I	R	S	R	S
DK10-4(A)	R	I	S	S	I	S	R	R	S	R	-
B410-3(-C)	R	S	S	S	S	S	S	R	S	R	S
B410-3(B2)	R	I	S	S	I	S	R	R	S	R	R
B410-3(B)	R	S	S	S	I	S	R	R	S	R	-
B410-3(A)	R	S	S	S	I	S	R	R	S	R	-

Table B2. Antibiotic resistance patterns obtained in summer, 2018.

Isolates	AMP	C	CIP	E	K	N	OT	P	S	W	VA
NON	R	S	S	S	S	S	I	R	S	R	S
BMD10-4(B)	R	S	S	S	S	S	R	R	S	R	R
BMD10-2(C2)	R	S	S	S	S	S	R	-	S	R	-
BMD10-2(C1)	R	I	S	S	S	S	S	-	S	R	-
BMD10-2(B2)	R	S	S	S	R	S	S	R	S	R	R
BMD10-2(B1)	R	R	S	S	S	S	R	R	R	R	S
BLV10-3(A)	R	S	S	S	S	S	R	R	S	R	R
BLV10-2(D2)	R	S	S	S	S	S	R	-	S	R	-
BLV10-2(D1)	S	S	S	S	S	S	R	-	S	R	-
BLV10-2(C2)	R	S	S	S	S	S	R	R	S	R	S
BLV10-2(C1)	R	S	S	S	I	S	R	R	S	R	I
BLV10-2(A3)	R	S	S	S	S	S	S	R	S	R	S
BLR10-2(A)	R	S	S	S	S	S	R	-	S	R	-
BGM10-3(C)	R	S	S	S	S	S	R	-	S	R	-
BGM10-3(B)	R	S	S	S	R	S	S	R	S	R	S
BGM10-3(A)	R	S	S	S	S	S	I	R	S	R	I
BGM10-2(C2)	R	I	R	R	S	S	S	R	S	R	S
BGM10-2(-C)	R	S	S	S	S	S	R	R	S	R	S

BGM10-2(B)	R	S	S	S	S	S	S	R	S	R	S
BGM10-2(A)	R	S	S	S	I	S	I	R	S	R	S
BFA10-4(A2)	R	I	S	S	S	S	R	R	S	R	R
BFA10-4(A1)	R	S	S	S	S	S	R	-	R	R	-
BFA10-4(A)	R	S	S	S	S	S	R	R	S	R	R
BFA10-3(A)	R	I	S	S	S	S	S	-	S	R	-
BFA10-2(A)	R	S	S	S	I	S	R	-	S	R	-
BDK10-3(D1)	R	S	S	S	S	S	R	R	S	R	S
BDK10-3(A2)	R	I	S	S	S	S	I	R	S	R	S
BDK10-2(C21)	R	S	S	S	S	S	R	-	S	R	-
BDK10-2(C2)	R	S	S	S	R	S	R	R	S	R	R
BDK10-2(C1)	R	S	S	S	I	S	R	R	S	R	I
BDK10-2(A)	R	S	S	S	I	S	R	R	S	R	S
BB410-4(D)	R	S	S	S	S	S	R	-	S	R	-

Table B3. Antibiotic resistance obtained from autumn, 2018.

Isolates	AMP	C	CIP	E	K	N	OT	P	S	W	VA
GM10-3A1	R	S	S	S	I	S	I	R	S	R	I
GM10-3B2	R	S	S	S	I	S	S	R	S	R	I
GM10-2C1	R	S	S	S	I	S	S	R	S	R	S
GM10-3C2	R	S	I	S	I	S	I	R	S	R	I
AEROGMA1	R	S	S	S	I	S	S	R	S	R	S
AEROGMA2	R	S	S	S	I	S	I	R	S	R	R
AEROGMA3	R	S	S	S	I	R	I	R	S	R	R
AEROGMA4	R	S	S	S	I	I	I	R	S	R	R
AEROGMA5	R	I	I	I	I	S	I	R	S	R	R
AEROGMA6	R	S	S	R	R	S	I	R	S	R	R
BT10-2A1	R	I	S	S	S	S	I	R	S	R	I
BT10-2A2	R	S	I	S	I	S	I	R	S	R	S
BT10-2A3	R	S	I	S	I	S	I	R	S	R	I
BT10-2A4	R	S	I	S	I	S	I	R	S	R	I
BT10-2A5	R	S	S	S	I	S	I	R	S	R	S
BT10-2A6	R	S	S	S	I	S	I	R	S	R	S
BT10-2C1	R	S	I	S	I	S	I	R	S	R	I
BT10-2C2	R	S	I	S	S	S	R	R	S	R	S
BT10-2C3	R	S	S	S	R	S	R	R	S	R	I
BT10-2C4	R	S	S	S	I	S	S	R	S	R	S
BT10-2C5	R	S	S	S	I	S	S	R	S	R	S
BT10-2C6	R	S	S	S	I	S	S	R	S	R	I
BT10-2B1	R	S	I	S	I	S	I	R	S	R	S
BT10-2B2	R	S	S	I	I	S	I	R	S	R	I
BT10-2B3	R	S	S	S	I	S	S	R	S	R	I

AEROBT1	**R**	**S**	**S**	**R**	**R**	**S**	**I**	**R**	**S**	**R**	**R**
AEROBT2	**R**	**S**	**S**	**R**	**I**	**S**	**I**	**R**	**S**	**R**	**R**
AEROBT3	**R**	**S**	**S**	**R**	**I**	**S**	**R**	**R**	**S**	**R**	**R**
AEROBT4	**R**	**S**	**S**	**R**	**R**	**I**	**R**	**R**	**S**	**R**	**R**
AEROBT5	**R**	**S**	**I**	**R**	**R**	**I**	**R**	**R**	**S**	**R**	**R**
AEROBT6	**R**	**S**	**I**	**S**	**I**	**S**	**R**	**R**	**S**	**R**	**R**
LR10-2A1	**R**	**S**	**I**	**R**	**I**	**S**	**R**	**R**	**S**	**R**	**R**
DK10-3B1	**R**	**S**	**R**	**S**	**I**	**S**	**I**	**R**	**S**	**R**	**I**
DK10-2A1	**R**	**S**	**I**	**S**	**I**	**S**	**I**	**R**	**S**	**R**	**I**
DK10-3A1	**R**	**S**	**S**	**S**	**I**	**S**	**I**	**R**	**S**	**R**	**I**
DK10-3A2	**R**	**S**	**S**	**S**	**R**	**S**	**I**	**R**	**S**	**R**	**S**
DK10-4A1	**R**	**S**	**S**	**S**	**I**	**S**	**I**	**R**	**S**	**R**	**I**
LV10-2A	**R**	**S**	**S**	**S**	**I**	**S**	**R**	**R**	**S**	**R**	**I**
LV10-2B	**R**	**S**	**S**	**S**	**I**	**S**	**R**	**R**	**S**	**R**	**I**
LV10-2C	**R**	**S**	**S**	**S**	**I**	**S**	**R**	**R**	**S**	**R**	**S**