

Identification and characterization of bacteria isolated from selected groundwater systems in the North West Province

N Metsing



orcid.org/0000-0001-9143-1153

Dissertation accepted in fulfilment of the requirements for the
degree *Master of Science in Microbiology* at the North-West
University

Supervisor: Dr LG Molale-Tom

Co-Supervisor: Prof CC Bezuidenhout

Graduation May 2022

25777742

DECLARATION

I Nyakallo Metsing, hereby declare that, this dissertation is my own work in design and execution, and that all materials contained herein have been duly acknowledged. It is submitted for the degree Master of Science in Microbiology at the North-West University and has not been submitted by me for a degree at this or any other University.

Nyakallo Metsing

Date

DEDICATION

I would firstly like to thank God for all the strength he has given me through this whole project. I have learnt so much.

I would also like to thank the following people, without whom I would not have been able to complete this research, and without whom I would not have made it through this degree:

The Microbiology Department staff for allowing me to study further and conduct my research and the support of fellow researchers in the lab. I would like to express my gratitude particularly to Mrs Chenhaka and Mr Mahlatsi for showing me the ropes around the lab and ensuring that I never run out of reagents. Tannie Sarah, for always listening to me and assisting me with clearing up whenever I had a lot of work to do. I truly appreciate the time I got to spend with her. May God continue to bless her kind soul.

Dr Bezuidenhout, for assisting me with Canoco and tricks to excel shortcuts. I would like to extend my gratitude for his time and patience.

Lohan, for helping me construct the GIS map and always availing himself whenever I needed his assistance. Thuto, for assisting me when I needed his assistance.

Dr Didi, for always ensuring that I understand molecular related work whenever I am stuck.

Jaco and Henry, for taking time out of their busy schedules to accompany me to go sample and make sure that I have everything needed. Their efforts and kindness do not go unnoticed. Thank you...

Fifi always took her time to ensure that I understood all the concepts I needed to apply when compiling a thesis. As well as assisting me throughout this degree. I appreciate the time and effort that she has shown me.

Seipati and Boipelo, for always making me laugh and forgetting how stressed I am. I truly appreciate their kindness and support, as well as venting sessions during experiments.

Nolwandle, for proofreading and supporting me when I needed her, as well as the beautiful friendship.

My supervisor Dr Lesego Molale-Tom, whose insight and knowledge into the subject matter steered me through this research. I am grateful that she was patient and resilient towards me. Thank you so much for your guidance, support and time. Thank you to my co-supervisor Prof CC Bezuidenhout for all his insight on this project.

Malome, for always being there for me through thick and thin. Your love, kindness and support mean a lot to me.

Last but certainly not least, my loving family. I am truly blessed to have parents, sisters, brothers and an aunt like them. The support and love they have shown me is out of this world. Thank you ...

ACKNOWLEDGEMENTS

This work is based on the research supported wholly by the National Research Foundation of South Africa (Grant Number TTK170518231376).

ABSTRACT

Sources of water in the North West Province (NWP) include groundwater, surface water, imported water and reusable effluent. Nonetheless, despite developed infrastructure, water in the NWP is a limited resource and the effects of demand exceeding supply come at a great cost. Mining and agriculture primarily contribute to the economy of the region, however, they are among the main contributors to water quality problems that are experienced in the province. In recent times it has been speculated that groundwater systems may contain microorganisms that harbour antibiotic-resistance genes. Considering the various mechanisms by which bacteria can acquire and transfer these genes, the microbial quality of the groundwater is questionable. This study aimed to identify and characterize bacteria isolated from groundwater systems in the North West Province. This was accomplished by determining the physico-chemical as well as microbial quality (coliforms, *Enterococcus* spp. and Heterotrophic Plate Count bacteria) of the groundwater systems of interest. The Kirby-Bauer and extracellular enzyme production tests were performed to determine the antibiotic-resistance profiles of the isolated bacteria as well as their pathogenic potential. Lastly, the presence of antibiotic-resistance as well as virulence genes was determined in the bacteria isolated. Consistently high TDS levels ranging from 201-1857 ppm were observed throughout this study. Moreover, the level of coliforms increased as the temperature increased. The highest levels of coliform contamination were found in areas frequented by cattle. The high use of manure produced by cattle is one of the sources of organic nutrients for agricultural purposes. Furthermore, consistently high levels of enterococci were observed in sites S6, S1 and S5. This is alarming because the presence of enterococci indicates faecal matter in water. Consistently high HPC levels were observed throughout the duration of the study. The high concentration of HPC in water could be because of various reasons such as water temperature or the amount of organic matter. Moreover, 186 isolates were subjected to haemolysin production testing. A sum of 146 isolates were alpha haemolytic, whilst 20 were beta haemolytic and the remaining 20 were gamma haemolytic. These isolates were subjected to the Kirby-Bauer disk diffusion test. The highest antibiotic-resistance was noted for: ampicillin (30µg), ciproflaxacin (5µg), chloramphenicol (30 µg), oxy-tetracycline (30 µg) and trimethoprim (5 µg). Furthermore, coliforms and *Enterococcus* spp. were further tested for the presence of selected virulence genes. In the case of coliforms, all the screened genes (*stx1*, *stx2*, *hlyA* and *eaeA*) were detected in one *Klebsiella grimontii*, one *Enterobacter cloacae* and two *Escherichia fergusonii*. Shiga toxin (*stx*) is among the most efficacious bacterial toxins ever discovered. The presence of shiga toxin in water could cause kidney diseases. Furthermore, in the case of *Enterococcus* spp., the most detected virulence determinants were *hlyA* and *cylA*. Lastly, All the isolates were subjected to antibiotic-resistance gene detection, only 23 of the 186 isolates harboured antibiotic-resistance genes. The most harboured gene was *amp C*. *Bacillus* spp., were the most prominent bacteria that harboured antibiotic-resistance genes. This suggests that the screened groundwater systems harbour

potential pathogenic bacteria and that the water might need to go through purification steps before being consumed by humans and animals.

Keywords: groundwater, antibiotic-resistance, virulence genes, antibiotic-resistance genes

TABLE OF CONTENTS

Declaration.....	i
Dedication.....	iii
Acknowledgements.....	iv
Abstract	v
Table of contents	vii
List of figures	ix
Chapter 1.....	1
INTRODUCTION.....	1
1.1 Introduction and problem statement	1
1.2 Aims and objectives	2
Chapter 2.....	3
Literature review.....	3
2.1 Global water crisis	3
2.2 Water accessibility and use in South Africa	3
2.3 Groundwater systems in South Africa.....	5
2.4 Physico-chemical parameters	6
2.5 Microbial indicators.....	10
2.6 Methods of bacterial characterization	14
CHAPTER 3.....	24
Materials and Methods	24
3.1 Study site	24
3.2 Sample collection	25
3.3 Physico-chemical analyses	25
3.4 Enumeration of bacterial isolates.....	26
3.5 Preliminary identification of isolates.....	27
3.6 Extracellular enzyme	28
CHAPTER 4.....	37
Results	37
Introduction.....	37
4.1 Physico-chemical groundwater quality analysis.....	37
4.2 Microbial groundwater quality analysis	44
4.3 Correlation of the physico-chemical and microbiological parameters.....	50
4.4 Species diversity of bacteria from groundwater systems in the North West Province ..	54

4.5	Haemolysis and Extracellular enzyme production.....	66
4.6	Antibiotic-resistance	Error! Bookmark not defined.
4.7	Characterisation of isolates	79
CHAPTER 5.....		85
Discussion.....		85
Introduction.....		85
5.1	Physico-chemical parameters	85
5.2	Microbial water quality analysis	90
5.3	Correlation of the physico-chemical and microbiological parameters.....	94
5.4	Species diversity and phylogenetic association	95
5.5	Pathogenicity and extracellular enzyme production	101
5.6	Characterization of isolates	104
CHAPTER 6.....		115
Conclusion and Recommendation		115
Introduction.....		115
6.1	Physico-chemical parameters	115
6.2	Microbial parameters and species diversity	115
6.3	Pathogenicity.....	116
6.4	Antibiotic-resistance	Error! Bookmark not defined.
6.5	Antibiotic-resistance genes and virulence genes	116
Recommendations		117
REFERENCE LIST		118
APPENDIX.....		185

LIST OF FIGURES

Figure 3.1: A map depicting the geographical location of groundwater systems of interest (The map was created by Bredenhann, 2021).

Figure 4.1 – Redundancy analysis (RDA) triplot depicting the correlation between varying physico-chemical (TDS, COD, PO_4^{2-} , NO_3^-) and microbiological parameters (Coliforms, *Enterococcus* spp., HPCs) at various sites during the autumn season – May 2019

Figure 4.2 – Redundancy analysis (RDA) triplot depicting the correlation between varying physico-chemical (TDS, COD, PO_4^{2-} , NO_3^-) and microbiological parameters (Coliforms, *Enterococcus*, HPCs) at various sites during the winter season – July 2019.

Figure 4.3 – Redundancy analysis (RDA) triplot depicting the correlation between varying physico-chemical (TDS, COD, PO_4^{2-} , NO_3^-) and microbiological parameters (Coliforms, *Enterococcus*, HPCs) at various sites during the summer season – February 2020.

Figure 4.4 – Redundancy analysis (RDA) triplot representing the relationship between varying physico-chemical (TDS, COD, NO_3^- , PO_4^{2-}) and microbiological parameters (Coliforms, *Enterococcus*, HPCs) at various sites during the spring season – September 2020.

Figure 4.5 – Pie chart illustrating the prevalence of coliforms, *Enterococcus* spp., and HPC isolates identified in groundwater systems of the North West Province.

Figure 4.6 – Pie chart illustrating the diversity and prevalence of coliforms isolated from groundwater systems of interest in the North West Province.

Figure 4.6.1 - Phylogenetic tree (Neighbour joining tree) of coliform isolates derived from 16S rRNA gene sequences. A Neighbour joining tree was constructed using the Jukes Cantor model and 1000 bootstraps.

Figure 4.7 – Diversity and prevalence of *Enterococcus* spp. isolated from groundwater systems of interest in the North West Province.

Figure 4.7.1 – Phylogenetic tree of *Enterococcus* spp. derived from 16S rRNA gene sequences. A Neighbour joining tree was constructed using the Jukes Cantor model and 1000 bootstraps.

Figure 4.7.2 – Phylogenetic tree (Neighbour joining tree) of HPC species derived from 16S rRNA gene sequences. A Neighbour joining tree was constructed using the Jukes Cantor model and 1000 bootstraps.

Figure 4.8 – Pie chart illustrating the overall haemolysis pattern of 186 bacterial isolates comprising Coliforms *Enterococcus* spp. and HPC bacteria isolated from selected groundwater systems in the North West Province.

Figure 4.9. A summary of the overall extracellular enzyme production profile of coliforms isolated from the groundwater systems of interest.

Figure 4.10 – A summary of the extracellular enzyme production profiles of various *Enterococcus* spp. isolated from the groundwater systems of interest

Figure 4.11 – A summary of the extracellular enzyme production profiles of HPC bacteria isolated from groundwater systems of interest

Figure 4.6a - Diversity of coliforms in autumn

Figure 4.6b - Diversity of coliforms in winter

Figure 4.6c - Diversity of coliforms in summer

Figure 4.6d - Diversity of coliforms in spring

Figure 4.7a - Diversity of *Enterococcus* spp. in autumn

Figure 4.7b - Diversity of *Enterococcus* spp. in winter

Figure 4.7c - Diversity of *Enterococcus* spp. in summer

Figure 4.7d - Diversity of *Enterococcus* spp. in spring

Figure C: An image of some of the amplified genes.

CHAPTER 1

INTRODUCTION

1.1 Introduction and problem statement

Accessibility to safe and clean water is still a global issue, particularly in developing continents like Africa and Asia (Boretti and Rosa, 2019). The global use of raw water has increased tremendously due to the population growth in the past twentieth century (du Plessis, 2017). Thus, some regions are unable to provide adequate water services, because most water systems that support ecosystems and nourish rising populations are under stress (du Plessis, 2019). In South Africa, more than three million people lack access to adequate water supply (Stats SA, 2019). Additionally, South Africa is amongst one of the countries that have a high scarcity of water, which is facilitated by the changes in climate that stimulate irregular rainfall (Gbetibouo, 2009; He *et al.*, 2019).

Groundwater is one of the vital water sources in South Africa (Foster *et al.*, 2008; Braune and Xu, 2010; Rohde *et al.*, 2017). The estimated use of groundwater in South Africa is between 2000 and 4000 m³/a (DWA, 2011). Furthermore, 65% of groundwater in South Africa is mainly used for agriculture, with 60% being attributed for irrigation and 5% for livestock watering (DWA, 2012). Whereas, 13% is utilized in the mining industry, 13% is allocated for domestic water supply in cities, whilst 3% is attributed to the industrial areas. The remaining 6% of groundwater is used for rural water supply which includes household use and gardening (DWA, 2013; Hobbs and De Meillon, 2017). According to Stats SA (2018) only 85,2% of the residents in the North West Province (NWP) have access to piped water. While, 12% is reliant on the use of boreholes as their main source of water (StatsSA, 2020).

Water pollution is amongst one of the key environmental problems experienced in South Africa (Herbig, 2019). This is because municipal wastewater treatment plants are not working efficiently (DWA, 2009; Herbig, 2019). Consequently, poor quality effluents are spilled into receiving surface water bodies which ultimately seep into underground aquifers (NWRS, 2013; Herbig, 2019). Inevitably, when water is polluted, it causes waterborne outbreaks, which not only have financial implications, but have a severe impact on the social wellbeing of the population and its health system (Mkwate *et al.*, 2017; Herbig, 2019).

Several studies have reported that the main sources of pollution in the NWP result from industrial and agricultural activities (Ateba and Maribeng, 2011; Montso *et al.*, 2019). According to Talabi and Kayode (2020), the most common pollutants found in groundwater include total dissolved solids,

heavy metals, fluoride, manganese, total hardness, sulphate and nitrate. Moreover, a study by Ferreira (2011) suggests that the groundwater systems in the NWP are susceptible to faecal contamination and harbour opportunistic microorganisms. Moreover, recent studies have also reported the presence of pathogenic *Clostridium*, vancomycin-resistant *Enterococcus* and *Escherichia coli* in groundwater systems of the NWP (Matlou *et al.*, 2019; Mabeo, 2020; Matloko *et al.*, 2021). Nonetheless, pollutants such as antibiotics, pharmaceutical wastes and pesticides are also some of the serious environmental contaminants that present severe health risks to humans globally (Kaushik *et al.*, 2019). In the last decade, water has been recognised as a favourable reservoir for antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) (Savin *et al.*, 2020). Once more, as a result of poorly functioning water treatment facilities, the complete elimination of ARBs and ARGs has proved difficult (Li *et al.*, 2015; Wang *et al.*, 2020). The findings of all these studies are a cause for concern and indicate that the provision of groundwater for agricultural (livestock watering and irrigation), domestic or drinking purposes that are of acceptable microbial quality may present a challenge. This is particularly important when considering that the total amount of stored water is decreasing in South Africa and that our taps are running dry (DWS, 2021). Groundwater may soon be one of the sources of water relied upon heavily in South Africa. So, protection of groundwater resources in South Africa is critical and much needs to be done in terms of groundwater monitoring. The NWRS (2013) stresses that there is to date no verification of existing lawful groundwater use which makes its effective regulation difficult. Nonetheless, when the uses of groundwater are considered, surveillance; resource protection and adequate disinfection need to be implemented in order to ensure protection from emerging waterborne pathogens.

1.2 Aims and objectives

The aim of this study was to identify and characterize bacteria isolated from selected groundwater systems of the North West Province.

The objectives of the study were to:

- I. Isolate and identify coliforms, *Enterococcus* spp., and heterotrophic plate count bacteria from selected groundwater systems;
- II. Determine the ability of the isolated microorganisms to produce extracellular enzymes;
- III. Determine the antibiotic profiles of the isolated microorganisms;
- IV. Determine whether the isolated microorganisms harbour antibiotic-resistance and virulence genes.

CHAPTER 2

Literature review

2.1 Global water crisis

Water is a vital source for life and health and access to it is a fundamental human right (Naidoo *et al.*, 2016). Cosgrove and Loucks (2015) further explain that water is also crucial for the development of a country's economy as well as its surrounding environment. However, due to mismanagement and scarcity of water resources, the abundance of water in many regions is declining (Abott *et al.*, 2019; Lu *et al.*, 2019; Ebadi *et al.*, 2020). du Plessis (2017) explains that the lack of water is due to climate change, urbanization, changes in demographics and population growth. Authors have defined water scarcity as the lack of water due to physical shortages or lack of access due to inadequate infrastructure (Kummu *et al.*, 2016, Lautze and Hanjra, 2014). Furthermore, Bwapwa (2019) states that every other continent has an area affected by drought and climate change that causes scarcity of water. According to the World Health Organization (WHO) (2017), approximately 2.1 billion people lack access to clean water globally. Boretti and Rosa (2019), further explained that the global water demand is rising at about 1% per year, while 4.8 and 5.7 billion people are projected to live in areas that are potentially water-scarce by 2050. Also, it has been noted that countries that still have inadequate access to clean drinking water are primarily those in the Sub-Saharan region (WHO, 2017; Verlicchi and Grillini, 2020). The latter region includes South Africa (Kelbe *et al.*, 2004). This sub-region has a complex climate that ranges from subtropical to humid to arid; whilst it also has several large river basins that are cross-border: Zambezi, Limpopo and Orange (Goulden *et al.*, 2009; Liang *et al.*, 2021).

2.2 Water accessibility and use in South Africa

South Africa is set amongst the countries with the least renewable water resources (UN, 2003). Furthermore, these renewable water resources are currently overexploited (Crookes *et al.*, 2018). According to Matchaya *et al.* (2019), South Africa also faces severe water shortage stresses due to continuous droughts, reduction of surface water supplies and rising water demand from agriculture. Studies have widely discussed and well-documented water shortage in South Africa (Mathebula, 2016, Matchaya *et al.*, 2019, Jordaan and Bezuidenhout, 2016). South Africa is estimated to have a total population of about 58.8 million in 2019, where only 88% of the population has access to safe and clean water

(StatsSA, 2019). According to UKEssays (2018) most of the water used in South Africa comes from surface water runoff which is withdrawn from approximately 320 dams across the country. A report by Crookes *et al.* (2018) states that the water usage per day is approximately 235 liters per capita per day, which is higher than the global average of 175 liters per capita per day and an average of 15 km³ per annum. Water demand is estimated to rise to 17.7 billion m³ by 2030 due to the growth of industry and the population (Steyn *et al.*, 2020). This exceeds by far the possible water allocation limit.

The water resources in South Africa include groundwater, surface water and return flows. Surface water systems are inclusive of rivers, dolomite, wetlands and dams. According to Jaiyeola and Bwapa (2015), 62% of the available water is utilized for irrigation whilst the urban/domestic sector takes up 27%. In addition, industries like mining use 8% which leaves only 3% for the forestry plantation sector. This, has led to a pressurized water system and scarcity of renewable water resources (Hedden and Cilliers, 2014). Groundwater is the only reliable source of fresh water in many semi-arid and arid areas where there is seasonal or consistent lack of surface waters (Taylor *et al.*, 2012).

2.2.1 The water situation in the North West Province

The North West Province (NWP), sometimes referred to as the “Platinum province” is a South African inland province that borders Botswana (NWDACE, 2008). It is situated in the middle of South Africa's northern frontier and shares boundaries with four other South African Provinces: North West Cape, South Free State, East Gauteng and Limpopo (StatsSa, 2019). The backbone of the economy in the province is mining, which creates the greater part of the area's total national output and gives employment to a fourth of its workforce (Ferreira, 2011). The major minerals excavated include platinum, gold, uranium and diamond. The other key industries in the province include agriculture, farming and tourism (NWDACE, 2008). Accounting for 8,7% of South Africa's land area, North West is characterized as the 4th smallest province in the country (NWDACE, 2008). The province houses approximately 3 748 436 people (Stats, 2016). Also, most parts of the NWP are mainly rural, with urban areas such as Rustenburg, Klerksdorp, Potchefstroom and Mafikeng (Mabeo, 2020). A report by NWDC (2014) states that because water is not generally widely distributed in the province, creating opportunities for water recycling and purification investments is imperative. This is done in order to alleviate water pollution induced and naturally.

The water supply from source to tap in the NWP is mainly provided by four water boards: Magalies water, Rand water, Sedibeng water and Botshelo water (NWPG, 2013). Furthermore, surface water, groundwater and dolomite make up the bulk of the water utilized for domestic use. Nonetheless, the NWP is amongst the provinces that trail behind in terms of water infrastructure developments in South Africa (Nnadozie *et al.*, 2011; StatsSa, 2019). This is because the average rainfall in the province ranges between 700 mm on the eastern side to less than 300 mm in the western part of the NWP. Most parts of the NWP experience less precipitation and more evaporation, owing to the aridity of the province (Monapathi and Bezuidenhout, 2017).

2.3 Groundwater systems in South Africa

Groundwater is freshwater contained deep in the soil or pores and creases of rocks (Younger, 2009). This water resource plays a critical role in the hydrological cycle, soil formation and plant growth (D' Odorico *et al.*, 2019). About 30% of the freshwater used globally comes from groundwater (D' Odorico *et al.*, 2019). While approximately half of the world's population depends on groundwater for providing drinking water and more than 40% of the world's irrigation water is deducted from it (Sierbert *et al.*, 2010). Groundwater is important for the functioning of the whole water cycle and plays a vital role in the provision and maintenance of the ecosystem (Braune and Xu, 2010). In addition, groundwater is perceived to be plentiful and the most convenient water source for human use worldwide (Grönwall and Danert, 2020). According to Ferreira (2011), in Africa, groundwater is viewed as a pristine and the cheapest water resource compared to other water sources. This is because precipitation usually restores groundwater systems and, in some cases, surface water runoff can recharge it (Ferreira, 2011). Additionally, groundwater systems also serve as possible drought buffers due to the significant volume of water stored in underground aquifers. Tempelhoff (2014) alludes that the overall use of groundwater in South Africa amounts to 15%, meaning that the available groundwater systems are under exploited.

2.3.1 Groundwater systems in the North West Province

Farmlands are primarily cultivated lands in the North West Province (NWP), where crops are irrigated with groundwater. Research has shown that solid waste dumps, grey water, animal breeding behaviors and pit latrines are significant non-point sources connected to stagnant groundwater quality issues in informal settlements of the NWP (Jordaan and Bezuidenhout, 2016). A study conducted by Mpenyane-Monyatsi and Momba (2012) reported high

concentrations of fluoride and nitrate at reservoirs in the NWP. The concentrations of these compounds, at most places, were above the maximum recommended human consumption concentrations (Mpenyane-Monyatsi and Momba, 2012). A study conducted by Ferreira (2011) illustrated that the quality of the groundwater in the NWP exceeded the standards set by the Department of Water and Sanitation. According to Ferreira (2011), the screened groundwater was contaminated with potentially pathogenic coliforms. Moreover, 10 years since the study of Ferreira (2011), other studies have also reported the presence of potentially pathogenic bacteria that harbor antibiotic-resistance genes in groundwater systems of the NWP (Mabeo, 2020; Matloko *et al.*, 2021). Even so, Mathebula (2016) reported that households in many rural communities of the NWP collect groundwater from communal tanks and taps for domestic purposes.

2.3.2 Groundwater pollution

Contamination of groundwater can occur when man-made products such as petrol, oil, road salts and chemicals seep into the ground (Senapati and Swain, 1991). There are also several microbial contaminants, such as pit latrines, agricultural activities and septic tanks which degrade the quality of groundwater systems (Lapworth *et al.*, 2017). According to Brown *et al.* (2019) the most common pollutants found in groundwater are inclusive of total dissolved solids, fluoride, manganese, total hardness, sulfate, nitrate as well as microbial pollution. Moreover, nitrates and heavy metals are primarily contributed by human farming and industrial production activities, whereas other pollutants are primarily caused by mineral weathering and water-rock interactions (Li *et al.*, 2014). Additionally, groundwater and surface water are intertwined and thus, the water quality of groundwater can also be influenced by the quality of surface water (Phung *et al.*, 2015). Consequently, toxins and contaminants that seep into groundwater supplies may cause poisoning to domestic animals or humans that consume it (Singal *et al.*, 2014).

2.4 Physico-chemical parameters

Water quality is a major problem in most countries and environmental degradation caused by pollution leads to harmful hazards to the environment and health (DWAf, 2012). Anthropogenic activities, surface runoff and wastewater disposal can contribute to groundwater pollution and pose a threat to the public water supply (Sasakova *et al.*, 2018). Water quality is a concept used to describe the appropriateness of water to support different uses or processes (WHO, 1996). The physical, chemical and microbiological properties of

water determine the water quality. Therefore, specific physico-chemical parameters of water must be determined before this resource is used for drinking, domestic, agricultural or industrial purposes. The following are the most important physical and chemical parameters:

2.4.1 Temperature

Water temperature is significant because it affects the physico-chemical properties of water and the biological reactions of organisms (Olaniran *et al.*, 2019). High temperatures allow microorganisms to regrow faster, whereas low temperatures will slow microbial growth (Collins and Moore, 2017). In addition, rising temperature causes the pH of the water to reach neutrality and thus favors the growth of microorganisms (Zamxaka *et al.*, 2004). Water temperature can be measured using a thermometer or multi-probe electronics.

2.4.2 Total dissolved solids

Total Dissolved Solids (TDS) is a term used to describe the inorganic salts and the small quantities of organic matter found in water solution (WHO, 1996). Usually, TDS are released from industrial runoff, municipal wastewater and sewage into the water resources (Osode and Okoh, 2009; Rahmanian *et al.*, 2015). Furthermore, dissolved minerals, gases and organic constituents can produce aesthetically unpleasant water colour, taste and smell (Durmishi *et al.*, 2015). Increased salt concentrations give water an unpleasant taste (Arshad and Shakoor, 2017). Some physiological effects can be directly related to high concentrations of dissolved salts, including the effects of laxatives and some kidney function effects (Rahmanian *et al.*, 2015). Water intake that has a high total dissolved solid levels may cause central nervous system abnormalities, tongue, lip, nose, irritability, and dizziness paralysis (Gupta *et al.*, 2013).

2.4.3 Salinity and Electrical conductivity

Salinity is a measurement of the concentration of dissolved salts in water. The salinity of the water is higher during low tide seasons and it increases as the level of the water decreases (Leidonald *et al.*, 2019). Salinity can either be measured as electrical conductivity or as total dissolved solids (Nthunya *et al.*, 2018).

According to Tutmez *et al* (2006) electricity conductivity is determined by the composition of mineral salts in groundwater and the relationship between mineral salt composition and electric conductivity is important to understand. The presence of charged particles in water increases the conductivity in the groundwater (Jiang *et al.*, 2019). In general, as the concentration of total dissolved solids in the water increases, so does their conductance. Elevated electrical conductivity levels can affect human health by disrupting the salt and water balance in heart patients, people with high blood pressure and infants (Memon *et al.*, 2008).

2.4.4 pH

pH is known as the activity of hydrogen ions and relates to the acidity and alkalinity of the water. For drinking water, the pH is considered as one of the most critical parameters of water quality (Omer, 2019). This is because the hydrogen ion concentration (H^+) influences almost all the chemical and biological processes in water. The pH value is a good indication of whether the water is alkaline or acidic (Trick *et al.*, 2008). While an ideal pH level of drinking water should be between 6-8.5, living organisms are capable of maintaining a constant pH balance and are not impaired by water consumption (Banks *et al.*, 2004; Islam *et al.*, 2017). Water consumption with low pH values may cause gastrointestinal disorders such as ulcers, pain in the stomach, hyper-acidity and creating burning sensations (DWAF, 1996). Unregulated pH can result in drinking water contamination and adverse effects on its taste, appearance and odour.

2.4.5 Chemical oxygen demand (COD)

The first methods for determining COD were established around 150 years ago, and they involved observing the colour changes of a permanganate solution combined with water samples (Geerdink *et al.*, 2017). The levels of dissolved oxygen required in water to oxidize chemical organic matter such as hydrocarbons are referred to as the chemical oxygen demand (Boyles, 1997). COD is a measure of the short-term effect of wastewater effluents on the oxygen concentration of receiving water bodies. It can be used as an indicator of the environmental health of the water.

2.4.6 Phosphates

Phosphates are chemical compounds that contain the phosphorus element and are essential for plant and animal production (Gupta *et al.*, 2017). Phosphorus is a highly reactive chemical, essential to life, forming a variety of compounds in terrestrial and aquatic ecosystems. Phosphorus can be found as the orthophosphate ion (PO_4^{3-}) in water and is also present as an essential component of cellular material in all life forms (Domagalski and Johnson, 2012). In soil, phosphorus is largely retained through an adsorption cycle. Soil has little capacity to store phosphorus and once the soil's capacity to absorb phosphorus is surpassed, the waste dissolves and flows more easily through water, either directly into a stream or down into an aquifer (Domagalski and Johnson, 2012). It has been observed that the availability of phosphorus will control microbial growth in drinking water. This discovery creates new possibilities for controlling microbial growth in water distribution systems through the creation of technologies that effectively extract phosphorus from drinking water (Miettinen *et al.*, 1997). Phosphates do not affect human health unless they are found in very high concentrations (Kumar and Puri, 2012).

2.4.7 Nitrites and nitrates

Nitrate and nitrite are ions that occur naturally and are ubiquitous in the environment. Both are products of nitrogen oxidation, as part of the cycle of development of complex organic molecules such as enzymes and other proteins needed by all living things (WHO, 2016). The abundance of free ammonia reaching the distribution system raises nitrate and nitrite levels in the water, thus contributing to nitrification (WHO, 2011). The most common sources of both nitrate and nitrite in water include farming practices (inorganic fertilizers and manure), wastewater treatment, human nitrogen wastes and industrial process discharges (WHO, 2011).

2.4.8 Turbidity

Turbidity is one of the main criteria for analysis of drinking water; it is an indicator of how pure the water is. Turbidity in water can be induced by particulate matter in the soil near water sources; it also contributes to the aesthetics of the water. (Rahmanian *et al.*, 2015). Turbidity is not always a direct threat to public health; however, it can interfere with the treatment of disinfection or provide a platform for microbial growth by providing nutrients for the growth of microbial populations while decreasing the efficiency of chlorination in

downstream treatment plants (Azhdarpoor *et al.*, 2019). The detection point is important when considering possible impacts (DWAF, 1996). High turbidity levels in source water may indicate in-catchment pollution events (e.g. heavy rain, spills, or groundwater contamination).

2.5 Microbial indicators

One of the key points specifically related to personal and public health, is the study of the microbiological quality of the water (Sogbanmu *et al.*, 2019). The purpose is to protect consumers of water from pathogenic bacteria, viruses and protozoa found in water systems (Wen *et al.*, 2020). Preferably, drinking water should not contain any known pathogenic microorganisms and it should be free from bacteria indicative of pollution with waste (Atanasova *et al.*, 2018). To ensure that a drinking water source meets these bacterial quality guidelines, it is critical that water be regularly examined for contamination indicators (Afnafe *et al.*, 2017). Since it is nearly impossible to regularly test water for all possible pathogens, it has led to the use of microbial indicators such as heterotrophic plate count bacteria, coliforms, faecal streptococci (Some *et al.*, 2021).

2.5.1 Total coliforms

Total coliforms are Gram-negative, non-spore-forming bacilli as well as aerobic or facultative anaerobic rod-shaped bacteria that ferment lactose at 35 °C within 48 hours. They are also able to form gas and β -galactosidase during lactose fermentation (Martin *et al.*, 2016). Total coliforms are a group of bacteria commonly found in soil and vegetation in the aquatic environment and in mammalian intestines, including humans (Al-Bahry *et al.*, 2014). Their presence in water is mostly an indicator of the water's hygienic quality, as well as potential distribution system failures (Snozzi, 2001). In addition, previous studies have shown that the rate at which coliform bacteria decline is closely associated with increased water temperatures, pH and nutrient scarcity. A study by Hong *et al* (2010) found that the physico-chemical properties of water correlated with the viability, degradation and growth rate of coliform bacteria. In addition, total coliforms can also survive and thrive in water supply systems, particularly when biofilms are present. Nonetheless, the adequacy of coliform bacteria in aquatic environments as a measure of faecal contamination has increasingly been the subject of discussion (WHO, 2008; Martin *et al.*, 2016).

2.5.1.1 Faecal coliforms

Faecal coliforms belong to the *Enterobacteriaceae* family which consists of facultative anaerobic, Gram-negative, non-spore forming and naturally rod-shaped bacteria (Figueras and Borrego, 2010). Faecal coliforms are a total coliform subgroup. Their appearance in water samples suggests recent pollution of the faecal or faecal waste after treatment. This group of coliform is inclusive of *Escherichia coli*, *Enterobacter* spp., *Citrobacter* spp. and *Klebsiella* spp., (Gabutti *et al.*, 2000). These bacteria populate the large intestines and play a role in the digestive system of mammals including humans and other warm blooded animals. Faecal coliforms can be identified with inexpensive, reliable and easy to interpret laboratory methods, thereby making them good indicators of pathogenic microorganisms in water (Panigrahi *et al.*, 2021).

2.5.1.2 Clinical relevance

Since *Escherichia coli* was discovered, scientists have a better understanding of phenotypic and genotypic virulence in the various environments, including water quality (Panigrahi *et al.*, 2021). *Escherichia coli* is a prevalent member of faecal coliform found in mammalian faeces and is also used as a faecal indicator to assess faecal contamination of both pathogenic and nonpathogenic bacteria in drinking water (Clarkes *et al.*, 2017). Pathogenic *Escherichia coli* are essentially partitioned into two categories, namely, intestinal and extra-intestinal pathogenic *Escherichia coli*, depending on the point of infection (Rojas-Lopez *et al.*, 2018). The pathogenicity of a given *Escherichia coli* strain is determined primarily by specific virulence factors including adhesives, invasions, haemolysin, toxins, factor erasure, cytotoxic necrotic factors and capsules (Titilawo *et al.*, 2015). There has been an emergence of shiga toxin-producing *E. coli* (STEC) causing bloody diarrhea in hospital patients (Ateba and Bezuidenhout, 2008; Kalule *et al.*, 2018). A specific *Escherichia coli* strain called *E coli* 0157:H7 is known to cause a lot of outbreaks.

2.5.2 Enterococcus spp.

Enterococcus spp. is a genus of Gram-positive, non-catalase producing, facultative anaerobic bacteria that can exist as single cocci or in chains (Fisher and Phillips, 2009). *Enterococcus* spp. has a high tolerance to salt and are known as lactic acid bacteria that produce bacteriocins (Altheus, 1982). *Enterococcus* spp. is part of the gastrointestinal flora of humans and animals and is therefore highly present in faeces and can be found in food

and water (Cabral, 2010; Molale, 2012). Enterococci isolates are used as an indicator of faecal contamination in water because they are rapidly released by human and animal excretions into the environment (Rincé *et al.*, 2003; Algebeleye and Sant'Ana, 2020). This group's survival was documented to be prolonged than that of faecal coliforms, although they showed similar patterns of persistence to those of pathogenic bacteria transmitted by water (Dionisio and Borrego, 1995). Examples of *Enterococcus* spp. known to date include: *Enterococcus casseliflavus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gallinarum*, *Enterococcus avium*, *Enterococcus villorum*, *Enterococcus durans*, *Enterococcus cecorum*, *Enterococcus hirae*, *Enterococcus mundtii*, *Enterococcus dispar* and *Enterococcus saccharolyticus*. Some of these enterococci like *Enterococcus faecium* and *Enterococcus faecalis* have clinical relevance and have been known to cause infections. *Enterococcus faecium*, *Enterococcus faecalis* and *Enterococcus casseliflavus* are commonly found in production animals, human gastrointestinal tract and plants (Klein, 2003; Zaheer *et al.*, 2020; Holman *et al.*, 2021). Thus infections related to the latter are associated with the source to which it is commonly found.

2.5.2.1 Clinical relevance

Clinical enterococci are less diverse than those acquired from the environment or other human sources, with *Enterococcus faecalis* being the most common (Fisher and Phillips, 2009). Urinary tract infections, abnormal liver septicaemia, endocarditis, post-operative infection, bacteraemia, and neonatal sepsis are all examples of enterococcal infections (Muller *et al.*, 2017). For a long time, infection with *Enterococcus* spp. has been thought to be harmless to people in Europe (Ramo *et al.*, 2020). However, enterococci have been mentioned as the second leading cause of lesion and urinary tract infection, as well as the third leading cause of bloodstream infections in the last decade (Molechan *et al.*, 2019). Since they are widely distributed in both humans and animals, *Enterococcus* spp. are frequently used as bio-indicators for the monitoring of ABR in food animals (Rebelo *et al.*, 2021). Despite being thought of as commensals, *Enterococcus* spp. have emerged as one of the primary causes of bloodstream infections (Raza *et al.*, 2018). According to WHO (2017), vancomycin resistant enterococci are added to the list of antibiotic-resistant bacteria for further studies as well as for the development of new drugs. Enterococci immune to vancomycin are ever more important reasons for morbidity and mortality in developed countries (Raza *et al.*, 2018). While VRE is an important cause of nosocomial sepsis in these countries, there is limited data on the part that this pathogen plays in South Africa (Mahabeer *et al.*, 2016).

2.5.3 Heterotrophic plate count bacteria

The definition "heterotrophic bacteria" (hpc) comprises all bacteria that utilize organic nutrients for growth. These bacteria are uniformly present in all kinds of water, food, soil, vegetation and air (Allen *et al.*, 2004). These species are naturally found in the ecosystem and can be found in soils, sediments, food, water, human and animal faeces (Pepper *et al.*, 2018). While generally considered harmless, certain heterotrophic microorganisms are sometimes opportunistic pathogens, with virulence factors that could affect the health of consumers with suppressed immune systems (Burgalassi *et al.*, 2021). For more than 100 years, the heterotrophic plate count method, developed by Robert Koch in 1881, has been used as one of the first drinking water analysis techniques (Allen *et al.*, 2004). Often, heterotrophic plate count bacterial numbers are used to track treatment performance and disinfection processes. Higher levels of HPC bacteria in treated water suggest a decline in the quality of microbiological water, bacterial regrowth, potential stagnation and biofilm formation (Xi *et al.*, 2009).

2.5.3.1 Clinical relevance of heterotrophic plate count bacteria

There are numerous heterotrophic bacteria that are still not well characterized in water. These bacteria have different growth requirements and need some media for cultivation (Pavlov *et al.*, 2004). Heterotrophic plate count bacteria are generally believed not to be harmful to healthy individuals, but there is increasing knowledge that some of the bacteria may be pathogenic and have the ability to cause adverse health effects to individuals with poor health (Kalpoe *et al.*, 2008; Keynan *et al.*, 2007). The majority of the drinking-water heterotrophic bacteria are not human pathogens (Rusen *et al.*, 1997). *Pseudomonas aeruginosa*, *Acinetobacter* and *Stenotrophomonas maltophilia* are leading causes of high death rates in hospital-acquired infections. Furthermore, *Pseudomonas aeruginosa* is a common cause of infection among patients who are hospitalized. While, *Klebsiella pneumoniae* is a pathogenic Gram-negative bacterium. It has a mucoid phenotype on agar plates, which is conferred by the polysaccharide capsule attached to the outer bacterial membrane and fermented lactose (Schreiner, 2013). The first Shiga bacillus infection was detected in South Africa in 1997 (Pillay *et al.*, 1997). Due to shiga bacillus's number of resistance mechanisms, it has acquired resistance to certain antibiotics. Treatment is further limited by the organism's ability to rapidly establish additional resistance during treatment (Mudau *et al.*, 2013).

2.6 Methods of bacterial characterization

2.6.1 Extracellular enzymes

Pathogenicity refers to the capability of a microorganism to cause disease and an opportunistic pathogen refers to an organism that can infect a host, particularly hosts with weak immune systems (Wiley *et al.*, 2017; Prinsloo, 2014). The bacteria that have the ability to cause infections have the following virulence characteristics:

- They can secrete extracellular enzymes
- Cytotoxicity of cells and adherence to cells occurs
- They can survive passing through the gastric fluids of the stomach.

Additionally, the bacteria also have the ability to secrete extracellular enzymes that act as toxins and these toxins are for pathogenicity and can cause diseases in a host (Khashid *et al.*, 2010). There are a few extracellular enzymes that could be used as markers of possible pathogenicity (Fiorino *et al.*, 2014). Such enzymes are haemolysin, DNase, proteinase and lipase.

2.6.1.1 Haemolysin

Haemolysin is responsible for the lysis of erythrocytes (De Croos *et al.*, 2004). Furthermore, hemolysis analysis is conducted on a medium consisting mainly of soy trypticase and agar in sheep blood (Ebenebe *et al.*, 2014; Yeh *et al.*, 2015). Trypticase soy enriches the nutrition of the medium to create a favorable condition for bacterial growth. Supplementing the medium with 5% sheep blood is essential to determine and identify the haemolytic patterns of bacteria (Ebenebe *et al.*, 2014; Yeh *et al.*, 2015). Studies have illustrated that bacteria that are β or α haemolytic on 5% sheep blood have to be subjected to extracellular enzymes tests (Yeh *et al.*, 2015; Pavlov *et al.*, 2002). According to Prinsloo (2014), if a bacterium produces two or more extracellular enzymes, it is considered a potential pathogen.

2.6.1.2 Deoxyribose nuclease (DNase)

DNase is a group of enzymes that are responsible for the breakdown of the nucleic acid into oligonucleotides (Inagaki *et al.*, 2018). This leads to the weakening of the host immune response by allowing the liberation of nucleotides that are responsible for growth (Palmer *et al.*, 2014; Sumby *et al.*, 2005). Furthermore, DNase minimizes the production of purulent

exudate that forms part of the host cell immune response, responsible for the inhibition of the spread of bacteria to vital tissues (Jubb *et al.*, 2012).

2.6.1.3 Proteinase

Proteinases are a group of enzymes that catalyze the hydrolysis of proteins into short peptides and amino acids (Sharma *et al.*, 2017). Studies have illustrated that bacterial species produce proteinases to infect the host and cause disease (Culp and Wright, 2017; Yang *et al.*, 2007). Furthermore, proteinase producing bacteria have the ability to weaken the immune system of humans by inactivating immunoglobulin A (IgA) and subsequently making the host cell susceptible to more pathogens in the environment (Collin and Olsén, 2003).

2.6.1.4 Lipases

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

2.6.1.5 Gelatinase

Gelatinase is an enzyme that catalyses the hydrolysis of gelatin and other compounds such as pheromone, collagen, casein and fibrinogen (Balan, 2021). Furthermore, gelatinase hydrolyzes gelatin only while extracellular endopeptidases hydrolyze gelatin and other compounds (Asitok and Ekpenyong, 2019; Balan, 2019). According to Park *et al.* (2007), gelatinase production in water environments is associated with the development of biofilms and promotes the translocation of bacteria across the intestinal cell layer while in the host.

2.6.1.6 Lecithinase

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

2.6.2 Antibiotic-resistance profiling of bacteria

2.6.2.1 The development of antibiotic-resistance

The world is facing a major public health problem with antibiotic-resistant bacteria appearing in drinking water and their related genes. As the use of antimicrobial drugs continues to increase, the resistance to antimicrobials often increases in bacteria (Rubin & Samore, 2002). The discovery of antibiotics has triggered a huge shift in medicine by defending lives from pathogenic microorganisms, thus increasing human and animal life spans (Davies and Davies, 2010; Chropra, 2012). There are four main genetic reactors in which antibiotic-resistance evolves, namely: human and animal microbiota, hospital and farm aquaculture, wastewater effluent and surface water, and lastly, surface and groundwater systems (Baquero *et al.*, 2008). The primary reactor is a human and animal microbiome that is affected by therapeutic and prophylactic antibiotics (Rochegüe *et al.*, 2021). The secondary reactor includes hospitals, long-term care facilities, farms or any other location where vulnerable individuals are crowded and exposed to bacterial transmission (Langdon, 2016). The tertiary reactor is a wastewater reactor, like all other locations where biological contaminants occur in the secondary reactor. Types include lagoons, wastewater treatment plants and other places where bacterial populations of various individuals have the ability to communicate genetically (Baquero *et al.*, 2008). Horizontal gene transfer occurs when microorganisms obtain foreign DNA material and is regarded as one of the most significant drivers of microorganism evolution and are generally responsible for the production of antibiotic /antimicrobial resistance (Zhang *et al.*, 2020).

Additionally, the world has entered a post-antibiotic age where resistance to antibiotics is expected to intensify, making antibiotic therapy complicated and costly (WHO, 2018). The prevalence of antibiotic-resistance of bacteria has affected and threatened antibiotic prevention and treatment which results in high levels of morbidity and mortality (Singh *et al.*, 2016). The Kirby Bauer method is used to test for the susceptibility of microorganisms to antibiotics. Antibiotics used for humans can be divided into the following groups according to

Gibbs *et al.* (2004): β -lactams, aminoglycosides, quinolones, tetracyclines, tigecycline, macrolides, ketolides, clindamycin, chloramphenicol, glycopeptides, oxazolidinones, fosfomycin, cotrimoxazole and polymixins, as outlined in *Table 2.1* below.

Table 2.1: Classes of antibiotics

Class of Antibiotic:	Characteristics:
1. β -lactam	• Includes highly reactive 3-carbon and one nitrogen ring (Pandey and Cascella, 2020). • Interfere with proteins that are responsible for cell wall synthesis by killing or inhibiting growth (Naclerio and Sintim, 2020). • > Beta lactams can bind to penicillin-binding protein (PBP), thereby interfering with the synthesis of the peptidoglycan, leading to the cell lysing or dying (Etebu and Ariekpar, 2016).
2. Cephalosporins	• Consists of 7-aminocephalosporanic (Tan <i>et al.</i>, 2018) • acid nucleus and a side chain containing 3,6-dihydro-2H-1,3-thiazane rings (Etebu and Ariekpar, 2016). • Used to treat bacterial infections and diseases arising from penicillin-producing as well as methicillin-susceptible staphylococci and streptococci (Healy, 2007). • > Subdivided into five generations according to their target organisms
3. Monobactam	• 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). • > Ability to inhibit aerobic Gram-negative bacteria and have been proven to be ineffective against Gram-positive bacteria (Liu <i>et al.</i>, 2021).

4. Macrolides	• Categorized by the 14-, 15-, or 16- membered macrocyclic lactose rings with unusual deoxy sugars L-cladinose and D-desoamine attached. • > Able to kill or inhibit microbes by successfully inhibiting bacterial protein synthesis (Baquero <i>et al.</i>, 2008).
5. Tetracycline	• Consists of four hydrocarbon rings and they are known as by the suffix 'cycline'. • Targets the ribosome of bacteria as their mode of action (antimicrobial activity). They disrupt the addition of amino acids to polypeptide chains during (Wilson, 2014). • protein synthesis within bacterial cells (Baquero <i>et al.</i>, 2008).
6. Carbapenems	• 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.

2.6.2.2 Antibiotic-resistance genes

Antibiotic-resistance genes are DNA sequences that impart and administer antibiotic-resistance to bacteria (Vaz-moreira *et al.*, 2014). There are different ways in which these antibiotic-resistance genes play a role in resistance. Intracellular antibiotic-resistance genes are harbored within the genomes of bacterial cells and are subject to and bound by the basic laws of biology (Xiang *et al.*, 2019). Extracellular antibiotic-resistance genes are DNA fragments that are not present inside bacterial cells at a particular time (Yu *et al.*, 2021). Consequently, these antibiotic-resistance genes are freely suspended in their atmosphere. Bacteria have developed antibiotic-resistance in a variety of ways.

2.6.2.2.1 Macrolide related resistance genes

Macrolides have been acknowledged for years, and a multitude of these molecules have been developed for clinical use since the introduction of erythromycin into therapy (Yao *et al.*, 2020). Streptomyces with a 14-, 15-, or 16-member lactose ring is used to make macrolide antibiotics (Zin *et al.*, 2020). Macrolide resistance is aided by one of two

pathways. Firstly, by target alteration prompted by ribosomal methylase encoded by *erm B* gene (Zin *et al.*, 2020). This gene affords a high level of resistance to lincosamides, streptogramins and macrolides. Macrolides prevent the 23S subunit of the bacterial ribosome from synthesizing proteins, causing premature peptide release during translation (Denis *et al.*, 1999). Erythromycin is effective against infections of the respiratory, gastrointestinal, and genital tracts, as well as tonsillopharyngitis, otitis media, acute bronchitis, primary atypical pneumonia, and skin and soft tissue infections (Zin *et al.*, 2020). The first erythromycin-resistant streptococci strains were identified in the United Kingdom in 1959 and in North America in 1967 (Lowbury and Hurst, 1959; Leclercq, 2002). Thus far, ribosomal methylation has been identified as the most common mechanism of resistance to macrolides and lincosamides. The *erm* proteins in pathogenic bacteria dimethylate a single adenine in nascent 23S rRNA, which is part of the large (50S) ribosomal subunit (Weisblum, 1995). Nearly 40 *erm* genes have been identified. These virulence factors are mostly carried by self-transferable plasmids and transposons in pathogenic bacteria (Leclercq, 2002). Bacterial pathogens have four major classes: *ermA*, *ermB*, *ermC*, and *ermF*. Staphylococcal gene classes *ermA* and *ermC* are common. Streptococci and enterococci have the most *ermB* class genes, while *Bacteroides* species and other anaerobes have the most *ermF* class genes (George, 2002).

2.6.2.2.2 Tetracycline related resistance genes

Tetracycline is the most commonly used antibiotic in the treatment and management of various poultry diseases (Schnappinger and Hillen, 1996). Tetracycline can be taken orally and has a very low risk of serious side effects. Tetracycline antibiotics are designed particularly to inhibit bacterial protein synthesis by preventing aminoacyl-tRNA from interacting with the ribosomal receptor (A) site (Chopra, 2013). Because of the multiple advantages of tetracyclines, such as their widespread availability, low cost and lack of side effects, the use of such antibiotics for the treatment of animal and human infections has increased in recent years (Martínez-García and Lorenzo, 2011). As a result, tetracycline-resistant bacteria have emerged, limiting the use of these antibiotics. Tetracycline resistance genes are typically encoded in plasmids as well as transposons and transmitted via conjugation (Guillaume *et al.*, 2012). However, in some isolates, the relevant genes are also found on the chromosome. Tetracycline resistance is achieved primarily through the acquirement of *tet* genes, which include efflux pumps, ribosomal protection and enzymatic discontinuation. Antibiotic-resistance is also influenced by mutations (Koo and Woo, 2011). *Tet* genes found in Gram-negative bacteria are associated with efflux pumps, which are encoded by the *tetA*, *tetB*, *tetC*, *tetD* and *tetG* genes (Skockova *et al.*, 2015). Tetracycline resistance could be used to assess antibiotic-resistance genes. Furthermore,

epidemiological-molecular analysis of resistant strains to these compounds can broaden knowledge of effective treatments and prevent capital loss (Gao *et al.*, 2012).

2.6.2.2.3 Ampicillin related resistance genes

Since the late 1970s, *ampC*-lactamases have been studied. The majority of these enzymes are cephalosporinases, but they can hydrolyze all β -lactams to some extent (Sanders, 1987). Ampicillin antibiotics work by inhibiting membrane-bound enzymes involved in bacterial cell wall synthesis. Ampicillins are frequently used to treat enteric fever, respiratory infections, urinary tract infections, skin and soft tissue infections, and other infections (Kaushik *et al.*, 2019). Organisms that overexpress *ampC* -lactamases are a major clinical concern because they are typically resistant to all β -lactam drugs except cefepime, ceftazidime, and carbapenems (Girlich *et al.*, 2000). The widespread use of ampicillin has resulted in the development of β -lactamase enzymes (Soeung *et al.*, 2020). These enzymes catalyze the hydrolysis of the amide bond of a four-membered β -lactam ring, conferring resistance to cell wall destruction (Labbate *et al.*, 2009). There are four types of β -lactamases: A, B, C, and D. Serine enzymes are classified into classes A, C, and D, whereas Zn-metalloenzymes are classified into class B. The most common mechanism of resistance displayed by most bacterial species is Class A (Barlow *et al.*, 2004).

2.6.2.2.4 Class 1 integron related resistance genes

Genes from class 1 integrons have been linked to the capture and spread of antibiotic-resistance genes in the environment (González-Plaza *et al.*, 2019). They are distinguished by three characteristics: an integron-integrase gene (*int1*), a recombinant site (*att1*), and a promoter (PC) (Gillings *et al.*, 2015; Li *et al.*, 2021). Class 1 integrons have been well identified and documented in Gram-negative microorganisms, and their role in the distribution and spread of antimicrobial resistance has also been validated and identified (Lina *et al.*, 2007). They are linked to a variety of resistance gene cassettes, but the majority of integrons contain an *aadA* resistance determinant that encodes streptomycin-spectinomycin resistance (Fluit and Schmitz, 2004). Trimethoprim resistance determinants are also repeatedly detected. These gene cassettes impart resistance to a variety of antibiotics, including aminoglycosides, β -lactams, chloramphenicol, and others (Doherty *et al.*, 2000). Class 1 integrons present a hazard in water environments because they are not

removed by traditional water purification processes, increasing their likelihood of being present in drinking water (Giillings *et al.*, 2015).

2.6.2.3 Kirby Bauer method

In the 1950s the Kirby Bauer disk diffusion test for susceptibility testing was developed (Hudzicki, 2009). There were no criteria at the time the approach was adopted that led to a lot of inconsistencies (Hudzicki, 2009). As a result, the World Health Organization (WHO) developed the process in 1961 (Gautam *et al.*, 2013). This approach is highly recommended by the Clinical and Laboratory Standards Institute (CLSI) to assess the efficacy of antimicrobials against a pathogenic aerobic or facultative anaerobic bacterium; it decides whether a bacterium is susceptible or intermediate to a specific antimicrobial (Vineetha *et al.*, 2015). Testing how microorganisms react to antibiotics is important for medicinal prescriptions. This test is cost-effective and can be carried out with ease in contrast with other methods of dilution (Gautam *et al.*, 2013).

2.6.3 Bacterial Virulence

The ability of a microorganism to cause a disease in the host is referred to as virulence (Jajere, 2019). Microorganisms have a variety of virulence mechanisms that allow them to evade host defenses, enter host cells and disable or lyse host cells (Sarowska *et al.*, 2019). Virulence mechanisms are constantly evolving and being passed down between microbes via a process known as lateral or horizontal gene transfer (Emamalipour *et al.*, 2020). According to Jajere, (2019) virulence genes could be used to assess risk and develop intervention techniques. This is so that the discovery of virulence genes may lead to the development of rapid screening tests to expedite effective isolation measures in hospitalized patients or to postpone hospitalization after carriage decontamination (Green *et al.*, 2019). Comparative sequence analyses shed light on bacterial pathogenic mechanisms, allowing the detection of known virulence proteins with conserved sequences or motifs, as well as new virulence proteins (Fournier and Raoult, 2017).

2.6.3.1 Virulence factors

Virulence factors are described as constituents that increase a pathogen's ability to cause a disease (Pontes *et al.*, 2020). Several of these virulence factors are known to be produced

only by particular bacterial strains. Furthermore, these factors can be cytosolic or membrane-associated (Sharma *et al.*, 2017). The cytosolic factors allow the pathogen to make rapid biochemical, sensory and structural changes (Zhang and Rock, 2008). Whilst the membrane associated virulence factors assist the pathogen in cell attachment and evasion of the host organism (Cossart and Lebreton, 2014). Secretory factors are critical components of the bacterial arsenal that aid the pathogen in navigating the host's innate immunity (Patel *et al.*, 2017). Also, secretory virulence factors in extracellular pathogens work together to destroy healthy cells (Kholdi *et al.*, 2021).

2.6.3.1.1 Shiga and verotoxin

Shiga toxins (Stxs), are a highly toxic class II ribosome-inactivating protein. These toxins are virulence factors that cause major clinical symptoms of Stx-mediated pathogenicities, such as gastrointestinal symptoms, haemolyticuremic syndrome (HUS) and neurological dysfunction (Detzer *et al.*, 2020). The shiga toxin, first isolated in *Shigella dysenteriae*, is expressed by two molecular different genes: *stx1* and *stx2* (Zhang *et al.*, 2008). Interestingly, shiga toxins in dairy cattle animals are assumed to be important environmental reservoirs for Stx-producing *Escherichia coli* (STEC). However, other developing or emerging reservoirs of the toxin-producing bacteria have not been fully studied (Hwang *et al.*, 2021). Another known group of toxins the verotoxins share similar characteristics with shiga toxins, hence, they were named shiga-like toxins (Hwang *et al.*, 2021). These verotoxins were first observed in Canada from several *E. coli* isolates during the 1970's (Karmali *et al.*, 2018). In recent times, Pal and Ayele (2017) reported on the ability of Verotoxins to cause gastrointestinal diseases and haemorrhagic colitis.

2.6.3.1.2 Intimin

Intimin as well as its transposons intimin receptor (Tir) are microbial proteins that facilitate binding between human cells and pathogenic organisms that attach and efface (A/E) (Yang *et al.*, 2018; Bhatt, 2020). It is usually located and reported present in Enteropathogenic *Escherichia coli* (EPEC) and Enterohaemorrhagic *Escherichia coli* (EHEC) (Hartland *et al.*, 2000). This virulence gene is expressed by the gene *eaeA* and produces A/E lesions which cause enteropathogenic and enterohaemorrhagic related diarrhea (Hosein *et al.*, 2019).

2.6.3.1.3 Cytolysin, aggregation substance, extracellular surface protein

Todd's 1934 study of cytolysin or hemolysin was the first to characterize enterococcal virulence factors in depth (Coburn *et al.*, 2003). Cytolysin is an *Enterococcus faecalis* toxin that displays β -haemolytic characteristics and has the ability to deteriorate the immune systems of humans (Laverde *et al.*, 2011). The five expressed cytolysin genes *cylA*, *clyS*, *clyB*, *cylM*, *cylL* are located on the cytolysin operon (Ali *et al.*, 2021). This operon encompasses two promoters, namely, the structural gene promoter P_L and the regulator gene promoter P_R (Sellek *et al.*, 2013). The expression of the cytosin genes has been involved in numerous known enterococci related diseases (Lang *et al.*, 2020).

Aggregation substance expressed by the *asa* gene is a virulence factor in *Enterococcus* spp., that facilitates efficacious attachment between bacteria. In so doing, plasmid exchange occurs as part of the bacterial sex pheromone system (Jett *et al.*, 1994). Aggregation substance also plays a critical role in adhesion, which facilitates cell-to-cell communication between donor and inheritor strain (Hällgren *et al.*, 2009).

Extracellular surface protein, a cell wall-linked protein, is known to play a critical role in the antibiotic-resistance of *Enterococcus* spp. (Sellek *et al.*, 2013). This is achieved by promoting the adhesion, incursion and colonization of *Enterococcus* spp. on the immune system of the host (Kafil *et al.*, 2013). It is expressed by the *esp* gene, which consists of 5622 bp and is known to promote biofilm formation in *Enterococcus* spp. (Sakuma *et al.*, 2009).

CHAPTER 3

Materials and Methods

3.1 Study site

Groundwater samples were collected from 15 boreholes in Potchefstroom and surrounding areas. Four sites were located at the North West University Potchefstroom campus, whilst three sites were located close to the university's premises. METSI (a sampling site previously known as Gerhard minnebron) was selected as the study reference site as it lies 20 km's out of Potchefstroom. Additionally, six sampling sites were located in private boreholes surrounding the Loopspruit River. The Loopspruit River falls under the eastern reach of the three sub-catchment areas of the Mooi River. The Mooi River is the main supply of water in Potchefstroom. The coordinates of each sample site were generated using Global Positioning Systems (GPS). The map shown in *Figure 3.1* was generated using ArcMAP (10.6) which was developed by ESRI (Environmental Systems Research Institute (www.esri.com)). The use of the respective groundwater is presented in *Table 3.1* below.

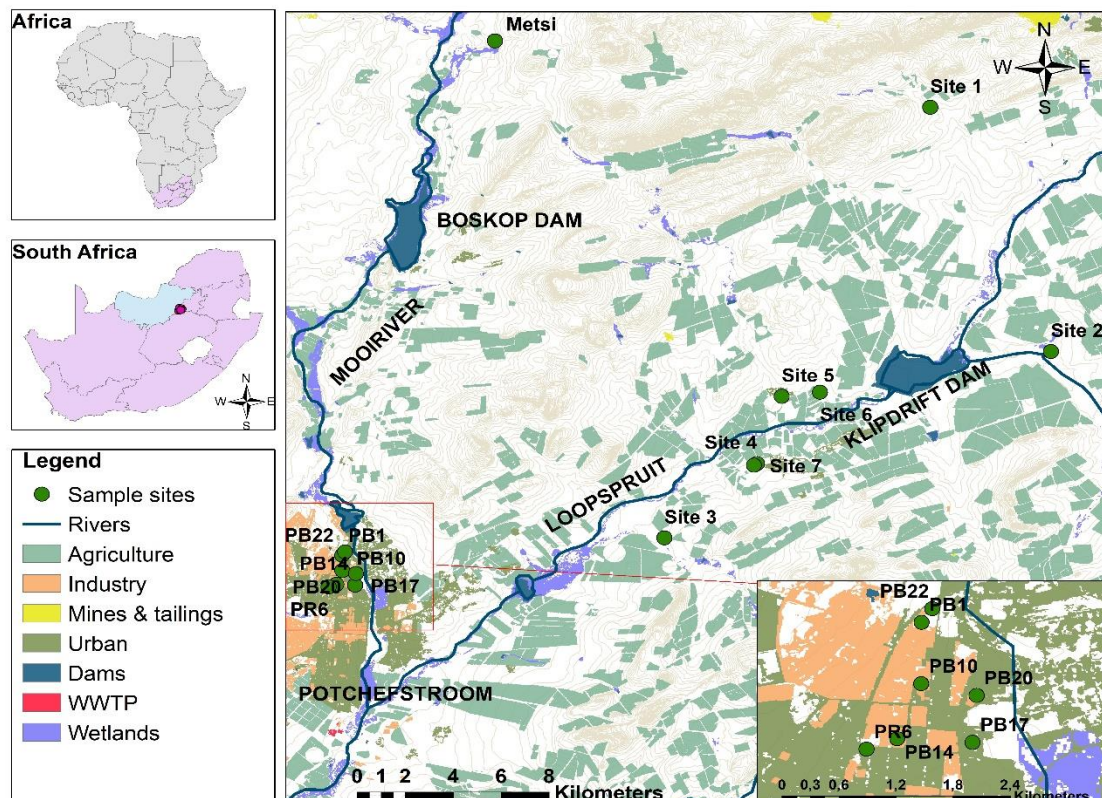


Figure 3.1. A map depicting the geographical location of groundwater systems of interest (The map was created by Bredenhann, 2021).

Table 3.1 – The various use of groundwater from all sample sites.

Site	Groundwater use
S1	Drinking, livestock watering and irrigation
S2	Drinking, livestock watering and irrigation
S3	Drinking, livestock watering and irrigation
S4	Drinking, livestock watering and irrigation
S5	Drinking, livestock watering and irrigation
S6	Drinking, livestock watering and irrigation
S7	Drinking, livestock watering and irrigation
DK	Irrigation
BG	Irrigation
LS	Irrigation
LV	Irrigation
H	Irrigation
FD	Irrigation
SG	Irrigation
GM	Irrigation

DK - Draak, **BG** - Botanical garden, **LS** - Laureus, **LV** - Lavaria, **H** - Hombre, **FD** - Fannie du Toit, **SG** - Sportsground, **GM** – METSI

3.2 Sample collection

Samples were collected in May 2019, July 2019, February 2020 and September 2020. The sample collection technique was determined by the physical setting of the sampling sites (US EPA, 2006). Samples were collected using either the pipe or the tap outlet, which was then sprayed with ethanol for sterilization. Furthermore, the taps or pipes were purged for approximately 5 minutes prior to sampling to prevent collection of stagnant water in the pipelines (Molale, 2012). Approximately 1 l groundwater samples were collected aseptically, using gloves in labelled sterilized glass bottles (sterilized prior to sampling) and thereafter capped immediately. All groundwater samples were kept on ice in a sealed cooler box and analyzed within 12 hours of sampling (DWAF, 2000).

3.3 Physico-chemical analyses

The physical analysis of the water samples was performed onsite using a calibrated Oakton testerTM multi-parameter probe (Thermo Fisher scientific, US). The physical parameters measured in this study were pH, temperature, salinity and total dissolved solids.

A sterile glass beaker was used for the measurement of all physical parameters and measurements were conducted in triplicates. The multi-parameter probe was rinsed with distilled water prior and after each physical parameter was measured (Molale, 2012). This was done in order to minimize the possibility of contamination. The chemical parameters were measured within 12 hours of sampling. The chemical parameters such as phosphate (cat.2106069), nitrate (cat.2106169), and chemical oxygen demand (cat. 2125815) were measured in milligrams per liter (mg/L) using the HACH DR 2800™ (HACH, US). The physico-chemical parameters obtained was compared to the South African Target Water Quality Range (DWAF, 1996).

3.4 Enumeration of bacterial isolates

3.4.1 Coliforms and *Enterococcus* spp. enumeration

The membrane filtration technique was used to enumerate coliforms and *Enterococcus* spp. isolates from water samples. The filtration system was sterilized with 70% ethanol and thereafter washed with sterile distilled water. Triplicates of 100 ml water samples were filtered through a 0.45 µm diameter, 47 mm grid PALL Corporation sterilized filter membranes (PALL Life Sciences, Mexico) and placed on MLG agar to select for coliforms (Oxoid, RSA). The MLG agar plates were incubated for 24 hours at 37 °C. The green colonies (representing coliforms) on the filter membranes were counted and recorded as colony forming units per 100 ml. Thereafter, these green colonies were aseptically cultured at least 3 times on nutrient agar using the streak plate technique for isolation and purification purposes, and incubated at 37 °C for 24 hours. Triplicates of 100 ml water samples were filtered through a 0.45 µm diameter, 47 mm grid PALL Corporation sterilized filter membranes was placed on KF-Streptococcus agar supplemented with 1ml 2,3,5-Triphenyltetrazolium chloride (TTC) for every 500 ml to select for *Enterococcus* spp. (Oxoid, RSA) (Molale, 2012). The dark purple colonies of interest on the filter membranes were counted and recorded as colony forming units per 100 ml. Selected dark purple colonies (representing *Enterococcus* spp.) were aseptically cultured at least 3 times on nutrient agar using the streak plate technique for isolation and purification purposes, and incubated at 37 °C for 24 hours.

3.4.2 Heterotrophic plate count bacteria enumeration

A serial dilution series ranging from (10^{-1} to 10^{-5} ml) was prepared on the collected groundwater. For each diluent (10^{-1} to 10^{-5} ml), 100 μ l was spread plated on solidified R2A agar (Merck, US) (Zhou *et al.*, 2010). All plates were incubated for 4-7 days at room temperature. Post incubation, colonies were counted and recorded as colony forming units per 100 ml. Random colonies were selected and subjected to purification using the streak plate method and incubated for 4-7 days (Mabeo, 2020). This purification step was conducted thrice. After the third purification step, the morphology of all colonies, in terms of shape; colour and size of the bacterium was noted.

3.5 Preliminary identification of isolates

3.5.1 Burke's Gram reaction

The purity of the isolates selected was confirmed with Gram staining. The bacterial smears were prepared from cultures grown in nutrient agar (coliforms and *Enterococcus* spp.) and R2A agar (HPCs) overnight at 37 °C for 24 hours. The slides were stained with 1% Gram's crystal violet for 1 minute, rinsed, stained with Gram's iodine for 1 minute, decolourized with acetone alcohol and counter-stained with Gram's safranin for 1 minute (Pandolfi & Pons, 2004). The slides were inspected at 1 000x magnification under the oil immersion objective of a light microscope (Nikon E200, China). Purple and red stained organisms were regarded as Gram-positive and Gram-negative microorganisms, respectively. Presumptive *Escherichia coli* stained pink and were deemed to be Gram-negative, whilst, presumptive *Enterococcus* spp. stained purple and thus were deemed Gram-positive. The HPC isolates were grouped according to their reaction to Gram staining, where HPC bacterial cells that stained purple were deemed Gram-positive and the ones that stained pink were deemed Gram-negative (Tsholo, 2019).

3.5.2 Identification of presumptive coliforms

All Gram-negative isolates were subjected to the triple sugar iron (TSI) agar test to validate the presumptive coliform isolates. Presumed coliforms isolates were inoculated using a zig-zag streak and stab technique on TSI agar slants (Merck, US) using an inoculation needle (Molale, 2012). The slants were incubated for 18 hours at 37 °C. A positive TSI agar reaction test was confirmed when an air bubble formed in the agar and the colour of the agar colour changed from red to yellow with the absence of a black precipitate indicating Hydrogen sulfide production.

3.5.3 Identification of presumptive *Enterococcus* spp.

The catalase activity of all presumed *Enterococcus* isolates was evaluated using the agar slant method defined by the Health Protection Agency (Parshionikar *et al.*, 2009). An isolate was deemed catalase-negative if no gas bubbles were produced when treated with 3% hydrogen peroxide H₂O₂. *Enterococcus* spp. are known to be catalase negative.

3.6 Extracellular enzyme

3.6.1 Haemolysis

The haemolysin protein is responsible for the lysis of red blood cells, thus, the production of haemolysin serves as a virulence characteristic of microorganisms (Payment *et al.*, 1994). Briefly, pure coliform, *Enterococcus* spp. and HPC isolates were streaked on 5% sheep blood agar (BioMérieux, SA) and incubated at 37 °C for 24 hours (Xiao *et al.*, 2009). Thereafter, the bacteria were classified as either alpha (α), beta (β) or gamma (γ) haemolytic (Xia *et al.*, 2009). The presence of a distinct greenish-black ring around the colony was noted as α haemolysis (Şeker, 2010). A zone of clearing where inoculation occurred was noted as β-haemolysis (Xiao *et al.*, 2009; Hoeffler, 1977). Whilst, the absence of haemolysin production (only bacterial growth) was noted as γ-haemolysis (Tetlock *et al.*, 2012; Şeker, 2010). Isolates that produced α- and β-haemolysin were selected for further testing of extracellular enzyme production (Upadhyaya *et al.*, 2010).

3.6.2 DNase

Ten grams of DNase agar (Oxoid, RSA) was used and prepared according to the manufacturer's instructions. Additionally, 0.025 g of toluidine blue (Sigma, Germany), an indicator agent, was added to the DNase agar. Toluidine blue binds to an organisms hydrolyzed DNA resulting in bright zones of clearing where inoculation occurred (Sen *et al.*, 2010). The bacterial smear was spot inoculated on DNase agar and incubated for 48 hours at 37 °C. After incubation, the plates were flooded with 1 mol HCl (Merck, Germany) and screened for DNase activity (Pavlon *et al.*, 2004).

3.6.3 Lipase

Tryptone soy agar (Merck, Germany) was prepared following the manufacturer's suggestions. Thereafter, 2 ml of Tween80 (Sigma Aldrich, US) was added to Tryptone soy agar. Pure coliform, *Enterococcus* spp. and HPC isolates were spot inoculated on the agar and incubated at 37 °C for 48 hours. A halo around the inoculation spot was noted as a positive result (Mzimkhulu, 2018).

3.6.4 Proteinase

Pure coliform, *Enterococcus* spp. and HPC Isolates were screened for proteolytic enzyme production on skimmed milk agar plates (Sen *et al.*, 2010; Janda & Bottone, 1981). The following were prepared and autoclaved separately: 1,5 g of skimmed milk (Oxoid, England) in 50 ml distilled water, 1,9 g of Brain Heart Infusion Broth (Merck, Germany) in 50 ml of distilled water and 100 ml of nutrient agar (Merck, Germany). The ingredients were mixed together post autoclaving. The mixture was poured into sterile petri dishes and left to solidify (Saran *et al.*, 2007). Isolates were inoculated and the plate incubated at 37 °C for 48 hours. The development of transparent zones around the colonies indicated proteolytic activity (Boominadhan *et al.*, 2009).

3.6.5 Gelatinase

The production of gelatinase in pure coliform, *Enterococcus* spp. and HPC isolates was determined by using gelatine powder as described by (Sen *et al.*, 2010). Purified 24 hour cultures were stab inoculated into nutrient agar slants (Merck, Germany) supplemented with 12% gelatin (Oxoid, UK). After incubation at 28 °C for seven days, the tubes were chilled for 30 minutes in cold conditions (5-10 °C). Cultures that remained liquefied were considered positive for gelatin hydrolysis (dela Cruz and Torres, 2012).

3.7 Kirby Bauer disk diffusion test - Antibiotic susceptibility testing

The Kirby-Bauer disk diffusion method (developed by Kirby and co-workers, 1966) was used to determine the antibiotic susceptibility of coliform, *Enterococcus* and HPC bacteria. 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 (Mabeo, 2020). 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).

3.8 Molecular identification of bacteria

3.8.1 Coliform and *Enterococcus* spp.

Pure coliform and *Enterococcus* spp. isolates were cultured overnight at 37 °C in Brain Heart Infusion broth and then harvested by centrifugation. A genomic DNA isolation kit was used to extract total DNA. The DNA was extracted following the manufacturer's instruction (Macherey-Nagel, Germany). Overnight bacterial broth was prepared and 2 ml of the bacteria was centrifuged for 3 minutes at 13 000 rpm prior to the DNA extraction. The resultant DNA's quality and concentration were determined using the Nanodrop™ 1000 Spectrophotometer v.3.5.2 (Thermo Scientific, US). The isolates were also subjected to gel electrophoresis using 1.5% agarose gel in 1x TAE buffer [20 Mm Acetic acid, 40 mM Tris and 1mM EDTA at pH 8.0]. The gel contained 10 µl of ethidium bromide and it was run at 80V for 45 minutes.

3.8.2 DNA extraction of HPC bacteria

The DNA of pure heterotrophic plate count bacteria was subjected to extraction using a Chemagic DNA isolation kit (PerkinElmer, Germany). The instructions were followed as recommended by the manufacturer. Overnight bacterial broth was prepared and 2 ml of the bacteria was centrifuged for 3 minutes at 13 000 rpm prior to the DNA extraction. The concentration and quality of the resultant DNA was measured using a Nanodrop™ 1000 Spectrophotometer v.3.5.2 (Thermo Scientific, US). The isolates were also subjected to gel electrophoresis using 1.5% agarose gel in 1x TAE buffer [20 Mm Acetic acid, 40 mM Tris

and 1mM EDTA at pH 8.0]. The gel contained 10 µl of ethidium bromide and it was run at 80V for 45 minutes (Mabeo, 2020).

3.8.3 Polymerase Chain Reaction

All the PCR reactions were performed using a 1000Cycler (Biorad, UK) thermocycler. The PCR reactions amounted to a volume of 25 µl and contained 2 µl of DNA template (20-40 ng/µl), 12,5 µl of DreamTaq mastermix (2mM MgCl₂, 0.2 mM of each dNTP, 0.025 µl Taq-polymerase) (Thermo Scientific, US); 1 µl (10µm/pmol) of forward primer and 1 µl (10µm/pmol) of reverse primer. The remaining volume was made up with nuclease free water. The PCR products were confirmed using gel electrophoresis.

3.8.3.1 Confirmation of coliform bacteria (*E. coli*)

A duplex PCR reaction was used to detect coliform bacteria (*E. coli*) in groundwater samples (Ferreira, 2011). The duplex PCR reaction contained *lacZ* and *mdh* primers (Table 3.2). Each PCR reaction contained 2 µl of DNA (20-40 ng/µl), 12,5 µl of DreamTaq mastermix (2mM MgCl₂, 0.2 mM of each dNTP, 0.025 µl Taq-polymerase) (Thermo Scientific, US); 0.5 µl (5µm/pmol) *lacZ* forward primer and 0.5 µl (5µm/pmol) of *lac Z* reverse primer; 0.5 µl (5µm/pmol) of *mdh* forward primer and 0.5 µl (5µm/pmol) of *mdh* reverse primer. The rest of the 25 µl volume was made up of nuclease free water. Cycle conditions consisted of initial denaturation at 95°C for 300 seconds, 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 60 seconds. The final extension was at 72°C for 300 seconds. PCR reactions were processed in an ICycler (Bio-Rad, UK).

Table 3.2: Primer sets used for duplex PCR

Primer	Specificity	Sequence (5'....3')	Size (bp)	Reference
<i>mdh</i>	<i>E. coli</i>	FW: GGTATGGATCGTTCCGACCT	301	Tarr <i>et al.</i> , 2004
		RV: GGCAGAATGGTAACACCAGAGT		
<i>lac Z</i>	<i>E. coli</i>	FW: CTGGCGTAATAGCGAAGAGG	228	Ram and Shanker, 2005
		RV: GGATTGACCGTAATGGGATATG		

3.8.4 Gel electrophoresis

PCR amplification products were visualized using ethidium bromide staining agarose gel electrophoresis. Electrophoresis was conducted using 1.5% (w/v) Seakem agarose gels in 1x TAE buffer [20 mM Acetic acid, 40 mM Tris and 1mM EDTA at pH 8.0]. The gel contained 10 µl of ethidium bromide and it was run at 80V for 45 minutes. 2 µl of PCR was mixed with 5 µl 6 x Orange loading dye (Fermentas Life Sciences, US) and loaded into the gel. Each gel contained a 1kbp molecular marker (O'GeneRuler, Fermentas Life Sciences, US) to indicate the molecular weight of the product (Ferreira, 2011).

3.8.5 16S rRNA amplification

All the presumptive coliform, *Enterococcus* spp. and HPC isolates were subjected to 16S rRNA amplification. The PCR steps in **section 3.8.3** were used. The thermal cycling conditions included an: initial denaturation at 95°C for 120 seconds; 30 cycles of denaturation at 95°C for 30 seconds; annealing at 54°C for 30 seconds; extension at 72°C for 60 seconds; and the final extension at 72°C for 300 seconds. The primer sequences are presented in *Table 3.5*. The product was subjected to gel electrophoresis, as described in **section 3.8.4**.

3.9 Virulence detection

3.9.1 Coliform

Total bacterial DNA was prepared as described by Blanco *et al.* (2013). The base sequences and predicted sizes of the amplified products for the specific oligonucleotide primers used in this study are shown in *Table 3.3 below*. The amplification conditions for *stx1* and *stx2* were as follows: initial denaturation at 96 °C for 3 minutes; 25 cycles of a secondary denaturation at 96 °C for 60 seconds; and annealing at 59 °C for 45 seconds; extension at 72 °C for 90 seconds. The amplification conditions for *hlyA*, and *eae A* were the same as *stx1*, except, the annealing temperature was 53 °C for 45 seconds PCR amplification was done using the 1000C™ (BioRad, UK) thermal cycler.

Table 3.3: PCR primer sequence for determination of coliforms virulence genes

Virulence factor	Target gene	Primer sequences	Product size (bp)
Shiga toxin	<i>stx 1</i>	F- ACACTGGATGATCTCAGTGG- R- CTGAATCCCCCTCCATTATG	614
Shiga toxin	<i>stx 2</i>	F- CCATGACAACGGACAGCAGTT- R- CCTGTCAACTGAGCAGCACTTTG	779
Hyaluronidase	<i>hylA</i>	F- ACGATGTGGTTTATTCTGGA R- CTTACAGTGCCATACATAT	165
Intimin	<i>eae A</i>	F- GACCCGGCAACAAGCATAAGC R- CCACCTGCAGCAACAAGAGG	384

3.9.2 *Enterococcus* spp.

The detection of virulence genes was performed in separate PCR reactions for *asa1* and *gelE*, and for *cylA*, *esp*, and *hyl*. The PCR reaction was prepared as described in **section 3.8.3**. Samples were amplified by denaturing at 95 °C for 3 minutes, followed by 30 cycles of 95 °C for 30 seconds, annealing at 56 °C for 30 seconds and 72 °C for 1 minute. This was followed by a final step of 72 °C for 5 minutes (Molale, 2016). The sequences and the amplicon sizes of the primers are summarized in *Table 3.4* below. The PCRmax Alpha Cyclor thermal cycler was used to amplify the DNA sequences.

Table 3.4: PCR primer sequence for determination of virulence gene for *Enterococcus* spp.

Virulence factor	Target Gene	Oligonucleotide sequence (5'-3')	Product size (bp)
Aggregation substance	<i>asa1</i>	ASA 11F - GCACGCTATTACGAACTATGA ASA 12R -TAAGAAAGAACATCACCACGA	375
Cytolysin	<i>cylA</i>	CYT IF -ACTCGGGGATTGATAGGC CYT IIbR - GCTGCTAAAGCTGCGCTT	688
Enterococcal surface protein	<i>esp</i>	ESP 14F -AGATTTTCATCTTTGATTCTTGG ESP12R - AATTGATTCTTTAGCATCTGG	510
Gelatinase	<i>gelE</i>	GEL11F - TATGACAATGCTTTTTGGGAT GEL 12R - AGATGCACCCGAAATAATATA	210
Hyaluronidase	<i>hyl</i>	HYL n1F – ACAGAAGAGCTGCAGGAAATG HYL n2R - GACTGACGTCCAAGTTTCCAA	276

3.10 Detection of Antibiotic-resistance genes

All PCR reactions were prepared as described in **section 3.8.3**. The sequences and amplicon sizes of all the antibiotic-resistance primers are summarized in Table 3.5 below. All the PCR products were subjected to gel electrophoresis as described in **section 3.8.4**, in order to see whether the target genes were amplified.

3.10.1 *ermF* and *ermB*

The following conditions for the thermal cycling were used as described by (Fourie, 2017): initial denaturation at 95 °C for 300 seconds, 35 cycles of denaturation at 95 °C for 30 seconds, annealing (*ermF* at 50 °C for 30 seconds and *ermB* at 48 °C for 60 seconds), elongation at 72 °C for 120 seconds and final extension at 72 °C for 300 seconds.

3.10.2 *ampC*

For amplification of the *ampC* gene the following thermal cycling conditions were used: initial denaturation at 94 °C for 5 minutes, 33 cycles of denaturation at 94 °C for 30 seconds, annealing at 49 °C for 30 seconds, elongation at 72 °C for 60 seconds and final extension at 72 for 7 minutes (Coertze and Bezuidenhout, 2018).

Table 3.5: Oligonucleotide antibiotic-resistance gene primers for PCR amplification

Target gene	Sequence (5' - 3')	Size (bp)	Reference
<i>bla_{TEM}</i>	TEM F - ATT CTT GAA GAC GAA AGG GC TEM R - ACG CTC AGT GGA ACG AAA AC	1 150	Costa <i>et al.</i> , 2007
<i>ermF</i>	ermF-F - CGG GTC AGC ACT TTA CTA TTG ermF-R - GGA CCT ACC TCA TAG ACA AG	466	Fourie, 2017
<i>ermB</i>	ermB-F - GAA AAG GTA CTC AAC CAA ATA ermB-R -AGT AAC GGT ACT TAA ATT GTT TAC	638	Fourie, 2017
<i>Int1</i>	HS463A F - CTG GAT TTC GAT CAC GGC ACG HS464 R - ACA TGC GTG TAA ATC ATC GTC G	473	Labbate <i>et al.</i> , 2008
<i>ampC</i>	ampC-F - TTC TAT CAA MAC TGG CAR CC ampC-R - CCY TTT TAT GTA CCC AYG A	550	Coertze and Bezuidenhout, 2018
<i>tetM</i>	tetM-FW - ACA GAA AGC TTA TTA TAT AAC tetM-RV - TGG CGT GTG TCT ATT GAT GTT CAC	171	Aminov <i>et al.</i> , 2001
16S rRNA	F - AGA GTT TGA TCM TGG CGC AG R - GG TTA CCT TGT TAT GAT TT	1465	Jhang <i>et al.</i> , 2006

3.10.3 *Int1*

The *intl* gene has previously been amplified by (Ozgumus *et al.*, 2007; Coertze and Bezuidenhout, 2018). The thermal cycling conditions were as follows: initial denaturation at 95 °C for 5min; 30 cycles of denaturation at 94 °C for 30 seconds; annealing at 64 °C for 30 seconds; extension at 72 °C for 60 seconds; and the final extension at 72 °C for 5min.

3.10.4 *bla*_{TEM}

The PCR conditions for detection of *bla*_{TEM} genes were as follows: initial denaturation at 95 °C for 5 minutes, 30 cycles of denaturation at 95 °C for 60 seconds, annealing at 60 °C for 60 seconds, elongation at 72 °C for 60 seconds and final extension at 72 °C for 5 minutes (Costa *et al.*, 2007).

3.10.5 *tetM*

For detection of *tetM* genes the PCR protocol was used as described by Aminov *et al.*, (2001). Briefly, PCR conditions were as follows: initial denaturation at 95 °C for 300 seconds, 35 cycles of denaturation at 95 °C for 45 seconds, annealing at 55 °C for 45 seconds, elongation at 72 °C for 45 seconds and final extension at 72 °C for 420 seconds.

3.11 Statistical analysis

Microsoft Office Excel 2019 was used to determine all statistical analyses relating to the physico-chemical and microbiological parameters, such as averages, standard deviation, minimum and maximum values. Microsoft Excel was also used to generate all pie charts and bar graphs. Canoco 4.5 (Canoco for Windows Version 4.0, GLW-CPRO ©) for windows was used to construct redundancy analysis biplots (RDA) in order to determine the correlation between the physico-chemical and microbiological data (Ter Braak, 1990). The statistical correlation of the physico-chemical and microbiological data was determined using Statistica 14.0 and correlations were marked significant at $P < 0.05$. Mega 7.0 was used to construct all phylogenetic trees.

CHAPTER 4

Results

Introduction

This chapter highlights the results and provides a summary of the overall quality of the groundwater systems of interest. It comprises different segments: physical and chemical parameters, microbial parameters, correlation between microbial and physico-chemical parameters, species diversity, haemolysis and extracellular enzyme production, as well as antibiotic susceptibility testing and gene detection. Groundwater was collected from 15 boreholes in autumn (May 2019), winter (July 2019), summer (February 2020) and spring (September 2020). This was done in order to assess the quality of the groundwater and observe the variation between the different seasons. The groundwater sites of interest were located at the North West University, as well as private farms surrounding the Loopspruit River. The North West University sites included: GM (METSI), SG (Sportsground), LS (Laureus), BG (Botanical garden), DK (Draak), LV (Lavaria), FD (Fannie du Toit) and H (Hombre). Groundwater samples were not collected at site S3 during summer and spring due to the COVID lockdown restrictions and the availability of the farmer. Lastly, water samples were not collected at site GM during spring due to technical related issues.

4.1 Physico-chemical groundwater quality analysis

The water samples collected from groundwater systems of interest were subjected to physical and chemical quality testing. The overall measured physico-chemical parameters are presented in *Table 4.1*.

4.1.1 Temperature

Temperature is a physical parameter that indicates how hot or cold something is (Ali and Ali, 2018). The measured temperature of groundwater samples is presented in *Table 4.1* below. During autumn, the highest groundwater temperature was observed at sites H and GM (26.3 °C) whilst the lowest groundwater temperature was observed at site S3 (8.8 °C). Overall, the trend in autumn showed relatively high water temperatures for a season that is expected to have much cooler water temperatures, with an overall average of 20.95 ± 5.20 °C. The measured highest and lowest groundwater temperatures in winter (21.5 °C and 7.6 °C) were

observed at sites S6 and S3, respectively. Furthermore, during summer, the highest groundwater temperature was measured at site S6 (26.9 °C) whilst the lowest groundwater temperature was measured at sites S5 and LV (20.9 °C). During spring, the highest and lowest groundwater temperatures were measured at sites S5 (20.4 °C) and SG (34.2 °C), respectively. Overall, the highest (26.48 ± 4.31 °C) and lowest (14.90 ± 3.55 °C) average groundwater temperatures were observed during spring and winter, respectively. Noticeably, the measured groundwater temperatures at all sampled sites were over 20 °C during summer and spring. Additionally, the measured groundwater temperature at some sites during spring were above 30 °C [S4 (33.4 °C), DK (30.1 °C), H (30 °C) and SG (34.2 °C)].

4.1.2 Total Dissolved Solids (TDS)

Total dissolved solids (TDS) are inorganic salts and small amounts of organic matter that are present in a water solution (Chang, 2005). During autumn the highest and lowest TDS values were measured at sites S6 (1482.37 ppm) and S4 (201 ppm), respectively. The TDS values of groundwater sampled during winter were also elevated with the highest and lowest values observed at sites S5 (1856 ppm) and BG (413.33 ppm), respectively. However, TDS values at sites BG, LS and LV were within the TWQR guideline. Additionally, the highest TDS value measured during summer was observed at site S6 (1399 ppm) whilst the lowest value was measured at site GM (121.3 ppm). Similar to the results observed in summer, the highest TDS values observed during spring were measured at site S6 (1302 ppm). Nonetheless, the lowest TDS value during summer was measured at site S1 (340 ppm). Thus, the measured groundwater TDS values were generally high during this study and exceeded the TWQR standards (0-450 ppm) (DWAF, 1996), *Table 4.1*.

4.1.3 Salinity

The amount of salt in water is measured as salinity. High salt levels in water interfere with the binding of dissolved oxygen consequently, reducing the amount of dissolved oxygen in the water. There are no TWQR guidelines available for salinity of groundwater. Site S4 (854 ppm) had the highest level of salinity during autumn, whilst site S7 (199 ppm) had the lowest salinity level in autumn. In winter, the highest and lowest nitrate levels were measured at sites S6 (855 ppm) and LS (213 ppm). Similar to the observations of winter, the highest salinity level measured during summer was observed at site S6 (700 ppm). The lowest salinity level during summer was observed at site GM (61.2 ppm). Lastly, groundwater from site S7 had the lowest measured salinity level (202 ppm) during spring. Overall, as depicted

in *Table 4.1*, site S6 had the highest salinity level in winter, summer and spring. The trend witnessed during this study showed that the salt content was higher in colder seasons as compared to the warmer seasons.

4.1.4 pH

pH is a measure used to determine the acidity or basicity of an aqueous solution (Nola *et al.*, 2002). All measured pH levels are presented in *Table 4.1*. Groundwater assessed at site S3 (8.2), during autumn, displayed the highest pH level whilst the lowest pH level was measured at site LV (6.7). Furthermore, the pH of groundwater during winter was highest and lowest at sites GM (7.89) and BG (6.5). These results were consistent during summer as both the highest and lowest pH levels were observed at sites GM (7.89) and BG (6.5) with the same pH measurements. In general, the pH measurements of groundwater collected during winter and summer, at the various sites, were in close proximity. Lastly, during spring, the highest and lowest groundwater pH levels were measured at sites S6 (7.43) and SG (6.58). Overall, the measured pH levels of all sampled groundwater, across the four seasons, were within the recommended TWQR standards (5.0 - 9.5) (DWAF, 1996). Also, there was no evident difference in the pH values when comparing the warmer (summer and spring) and colder seasons (autumn and winter).

4.1.5 Chemical Oxygen Demand (COD)

The amount of dissolved oxygen required in water to oxidize chemical organic materials is known as the chemical oxygen demand (COD) (Christensen *et al.*, 1992). The COD levels for the duration of the study are summarized in *Table 4.1*. During autumn, the highest COD level was observed at site DK (16.45 mg/L) whilst the lowest COD level was measured at site S6 (0.76 mg/L). In winter, the highest and lowest COD levels were measured at sites S7 (72 mg/L) and LV (0.33 mg/L). Similar to the observations of winter, the highest COD level measured during summer was observed at site S7 (111 mg/L). The lowest COD level during summer was observed at site H (1.07 mg/L). Similarly, groundwater from this site (H) also had the lowest measured COD level (0.33 mg/L) during spring. Lastly, the highest COD level during spring was measured at site S6 (111 mg/L). The COD levels of the groundwater systems of interest fluctuated for most sites throughout the duration of the study. However, elevated COD levels were more prevalent during the warmer seasons (summer and spring) as compared to colder seasons (autumn and winter). No TWQR standards are available for COD levels in groundwater (DWAF, 1996).

4.1.6 Phosphates (PO_4^{2-})

Phosphates are chemicals that comprise the element phosphorus and have an impact on water quality by producing excessive algae growth (Carpenter *et al.*, 1998). The phosphate levels recorded for the duration of this study are documented in *Table 4.1*. During autumn, the highest phosphate level was observed at site S5 (4.3 mg/L) whilst the lowest phosphate level was measured at site S3 (0.01 mg/L). In winter, the highest and lowest phosphate levels were measured at sites S6 (1.02 mg/L) and S7 (0.03 mg/L). Whilst, the highest phosphate levels were observed at site S7 (0.96 mg/L), during summer. The lowest phosphate level measured during summer were observed at site LV (0.01 mg/L). This is similar to the results observed in spring where the lowest phosphate levels were also measured at site LV (0.1 mg/L). Lastly, the highest phosphate level measured during spring was measured at site S6 (2.9 mg/L). There are currently no TWQR standards for acceptable phosphate levels in groundwater. Nonetheless, groundwater phosphate levels measured during this study were below 5 mg/L.

4.1.7 Nitrate (NO_3^-)

Nitrate is a type of dissolved nitrogen that naturally occurs in soil and water. It is the principal source of nutrition for plants which can be used as fertilizer (Sasakova *et al.*, 2018). All nitrate levels measured in this study are summarized in *Table 4.1*. Site S6 (20.23 mg/L) had the highest level of nitrates during autumn, whilst site S1 (0.12 mg/L) had the lowest nitrate level in autumn. In winter, the highest and lowest nitrate levels were measured at sites DK (4 mg/L) and S1 (1.01 mg/L). Similar to the observations of autumn, the highest nitrate level measured during summer was observed at site S6 (4.1 mg/L). The lowest nitrate level during summer was observed at site H (0.67 mg/L). Lastly, groundwater from sites FD and LS had the lowest measured nitrate level (0.89 mg/L) during spring. Overall, as depicted in *Table 4.1*, the lowest levels of nitrates were measured in site S1 (0.12 mg/L) and the highest levels of nitrates were observed at site S6 (20.23 mg/L) respectively. In general, the measured nitrate levels in the sampled groundwater were below the TWQR (<10 mg/L) and suitable for irrigation purposes. This is with the except for nitrate levels measured at site S6 during autumn. This phosphate level exceeded the current TWQR (DWAf, 1996).

Table 4.1. – The physico-chemical groundwater quality analysis of samples collected throughout the four seasons in May 2019, July 2019, February 2020 and September 2020.

SEASON	SITES	TEMPERATURE °C	TDS ppm	SALINITY ppm	pH	COD mg/L	PO ₄ ²⁻ mg/L	NO ₃ ⁻ mg/L
TWQR		N/A	0-450	N/A	5-9.5	N/A	N/A	<10
AUTUMN MAY 2019	S1	18.5	518	332	7.8	5.62	0.06	0.12
	S2	13.5	497.68	330	7.21	3.45	0.51	0.24
	S3	8.8	854.67	555.7	8.2	8	0.01	5.51
	S4	21.5	201	854	7.02	2.67	0.06	5.52
	S5	14.6	798	421.33	7.5	2.67	4.3	3.37
	S6	19.3	1482.37	593	7.31	0.76	3.29	20.23
	S7	21	503	199	6.82	5.6	0.51	3.7
	DK	25	722	451	7.87	16.45	0.88	0.22
	BG	22.4	854	459.67	7.26	7.76	2.1	0.36
	LS	22.5	701.67	322	6.74	3.59	0.1	0.85
	LV	25.2	632.3	325.9	6.7	2.6	0.142	2.1
	H	26.3	864.3	472.67	6.74	9	1.63	0.98
	FD	23.66	856	601	7.04	12.18	4	0.2
	SG	25.7	854	540	6.87	12.2	2.1	0.44
	GM	26.3	753.8	394.72	7.48	1.43	0.13	0.2
	AVG	20.95 ± 5.20	739.52 ± 279.13	456.80 ± 158.71	7.24 ± 0.46	6.27 ± 4.595	1.32 ± 1.51	2.94 ± 5.16
WINTER JULY 2019	S1	18	506	243	6.75	12	0.17	2
	S2	14.7	495	233	7.312	2	0.01	2.2
	S3	7.6	925	435	7.24	11	0.17	3.9
	S4	13.1	814	390	6.8	22	0.031	1.36

	S5	18.22	1857	658	6.86	7	0.58	1.01
	S6	21.5	1700	855	6.86	11	1.02	2.4
	S7	20.1	798	237	7	72	0.03	2.4
	DK	13.2	513	312	6.73	12.55	0.58	4
	BG	12.2	413.33	248.66	6.5	8.37	0.96	2.03
	LS	14	350.66	213	7.12	2.33	0.99	2.65
	LV	14.2	378.66	231.33	7.1	0.33	0.954	2.8
	H	12.3	898.66	445.66	6.85	6.13	0.74	2.8
	FD	12.3	813.33	397.66	7.36	17.8	0.85	2.9
	SG	15.3	849.66	415.66	6.7	11.33	0.3	1.89
	GM	16.8	628.66	415.66	7.89	1.33	0.96	1.77
	AVG	14.90 ± 3.55	796.06 ± 445.24	382.042 ± 178.45	7.00 ± 0.35	13.14 ± 17.39	0.56 ± 0.40	2.41 ± 0.82
SUMMER FEBRUARY 2020	S1	24.8	487	235	6.75	2	0.94	4
	S2	23.8	463	223	7.13	32	0.05	1.01
	S3	-	-	-	-	-	-	-
	S4	24.9	774	380	7.23	9	0.51	2
	S5	20.9	1260	630	6.96	22	0.02	2
	S6	26.9	1399	700	6.88	7	0.3	4.1
	S7	25.9	518	247	6.96	111	0.96	2.4
	DK	22.9	673	328	6.65	3.5	0.067	1.02
	BG	21.5	553	268	6.5	3.99	0.45	3.6
	LS	21.4	1066	525	7.14	4.22	0.02	1.22
	LV	20.9	685	356	7.2	3	0.01	0.8

	H	21.9	933	459	6.85	1.07	0.01	0.67
	FD	23.1	841	412	7.47	42.3	0.59	0.88
	SG	22.8	604	294	6.74	10	0.58	0.79
	GM	24.3	121.3	61.2	7.89	2.44	0.21	1.02
	AVG	23.29 ± 1.89	732.57 ± 357.06	365.59 ± 170.6	7.03 ± 0.36	18.11 ± 29.51	0.34 ± 0.34	1.82 ± 1.24
SPRING SEPTEMBER 2020	S1	23.4	340	231	7.36	2	0.16	2.8
	S2	21.5	338	289	7.18	1.21	0.51	1.09
	S3	-	-	-	-	-	-	-
	S4	33.4	574	385	7.2	9	0.81	1.01
	S5	20.4	1067	232	7.2	72	0.22	1.04
	S6	23.6	1302	489	7.43	111	2.9	1.4
	S7	25.6	899	202	6.92	36	0.59	3.3
	DK	30.1	760	283.96	7	12.5	0.76	1.2
	BG	26.7	645	488	7.01	1.02	0.48	1.65
	LS	23.5	522	396.8	6.98	1.22	2	0.89
	LV	25.6	670	245.8	6.78	2.3	0.1	1.8
	H	30	546	366.9	6.99	0.33	0.22	1.9
	FD	26.3	566	400	7	4.5	0.37	0.89
	SG	34.2	675	309.66	6.58	2	1.45	1.17
	GM	-	-	-	-	-	-	-
	AVG	26.48 ± 4.31	684.92 ± 272.61	332.24 ± 96.19	7.05 ± 0.23	19.62 ± 34.24	0.81 ± 0.83	1.55 ± 0.75

Values of levels at all sites, as well as average values of levels of all sampling sites per groundwater are presented and ± standard deviation values. **TDS** - Total Dissolved Solids; **NO₃⁻** - Nitrate, **PO₄²⁻** - Phosphate, **COD** - Chemical Oxygen Demand. **BG** - Botanical gardens, **SG** - Sportsground, **LV** - LaVaria, **LS** -Lareus, **GM** - METSI, **DK** - Draak, **FD** - Fanie; **H** - Hombre. **TWQR** - Target Water Quality Range; **AVG** – Average.

4.2 Microbial groundwater quality analysis

This section highlights the microbial quality of the 15 groundwater systems of interest. The average bacterial levels of coliforms, *Enterococcus* spp. and HPC's were recorded during autumn (May 2019), winter (July 2019), summer (February 2020) and spring (September 2020).

4.2.1 Coliform bacteria

Coliforms were isolated, enumerated and documented in cfu/ml as displayed in *Table 4.2* below. Throughout the study, coliforms were identified at nine sampled sites (S3, S6, GM, S4, S5, DK, H, BG and FD). During autumn, coliforms were identified at three sites (S3, S6 and GM); with the highest and lowest coliform levels measured at sites S3 (53 cfu/ml) and GM (27 cfu/ml), respectively. In winter, coliforms were present in four sites (S4, S5, DK and H). Furthermore, the highest and lowest coliform levels were measured at sites H (238 cfu/ml) and DK (39 cfu/ml), respectively. Similarly, in summer, coliforms were identified at four sites (S5, S6, BG and FD). The highest and lowest coliform levels in summer were measured at sites S5 (165 cfu/ml) and BG (39 cfu/ml), respectively. In autumn and winter, coliforms were not detected at site BG. Additionally, coliforms were detected in three sites (S5, S6 and BG) during spring. The highest coliforms levels during spring were measured at site S5 (538 cfu/ml) whilst the lowest coliform levels were measured at site BG (134 cfu/ml).

Overall, throughout the seasons, the average faecal coliform levels measured ranged from 53 to 538 cfu/ml. The highest coliform levels were measured at site S5 (538 cfu/ml) during spring, whilst the lowest coliform levels were measured at site GM (53 cfu/ml) during autumn. The average level of coliforms measured was 15.93 ± 36.46 cfu/ml in autumn, 44.47 ± 87.73 cfu/ml in winter, 72.93 ± 128.87 cfu/ml in summer and 72.92 ± 161.88 cfu/ml in spring. Thus, as the temperatures of the seasons increased, the levels of coliforms also increased.

4.2.2 *Enterococcus* spp.

The levels of *Enterococcus* spp. in the groundwater systems of interest were recorded and documented in *Table 4.2* below. Five sampled sites (S1, S4, S5, S6 and S7) were recorded to have the presence of *Enterococcus* isolates in autumn. Also, during autumn, the highest level of *Enterococcus* isolates was observed at site S1 (746 cfu/ml) with the lowest level recorded at site S7 (86 cfu/ml). In winter, *Enterococcus* isolates were found present at 10

sites. The lowest *Enterococcus* levels were witnessed at sites S3 (53 cfu/ml) and GM (53 cfu/ml). The highest level of *Enterococcus* isolates in winter was observed at site S1 (532 cfu/ml). In summer, *Enterococcus* isolates were present at nine sites (S1, S4, S5, S6, S7, BG, LV, H and GM). Out of the nine sites, site H had the lowest *Enterococcus* levels (134 cfu/ml), whilst site S4 had the highest *Enterococcus* levels (539 cfu/ml). The *Enterococcus* levels in spring, as presented in Table 4.2, were relatively high. The highest *Enterococcus* levels were witnessed at site S7 (866 cfu/ml), and the lowest at site LV (76 cfu/ml).

Overall, the presence of *Enterococcus* isolates was consistent at sites S4 and S5, throughout the study. Furthermore, *Enterococcus* isolates were detected at only 13 sites (S1, S3, S4, S5, S6, S7, DK, LS, LV, SG, GM, H, and FD). Thus *Enterococcus* isolates were not detected at sites BG and S2. The average *Enterococcus* levels within all groundwater systems ranged from 53 to 866 cfu/ml as seen in Table 4.2. The lowest overall average *Enterococcus* levels (86.8 ± 193.61 cfu/ml) were measured in autumn and the highest overall average were measured in September 2020 (232.38 ± 305.47 cfu/ml). Also, the overall trend throughout this study, showed higher levels of *Enterococcus* isolates during the warmer seasons (summer and spring) as compared to the colder seasons (autumn and winter).

4.2.3 Heterotrophic plate count bacteria (HPC)

Heterotrophic plate count bacteria were detected in all sampled sites, as depicted in Table 4.2, detection levels varied between the different sites and ranged from 0 to 718666.87 cfu/ml. During autumn, HPC bacteria were identified at 11 sites (S1, S2, S3, S4, S5, S6, S7, DK, BG, FD and GM). Additionally, Heterotrophic plate count bacteria were identified in winter, summer and spring at all sampled sites. The HPC levels in autumn were generally high, with the highest and lowest HPC levels witnessed at sites S3 (718666.67 cfu/100ml) and BG (39966 cfu/100ml), respectively. In winter, the highest and lowest observed HPC level was at sites GM (536000 cfu/ml) and LV (174333.3 cfu/ml). In summer, the HPC levels ranged from 174333.3 cfu/ml to 536000 cfu/ml. The highest observed HPC level in summer was witnessed at site S1 (536000 cfu/ml). Lastly, the highest and lowest observed HPC level in spring was observed at sites S5 (532333 cfu/ml) SG (167668 cfu/ml). HPC bacteria levels in groundwater increased as the seasons got warmer. During winter, summer and summer, HPCs were identified at all sites with varying HPC levels between sites.

HPC bacterial levels in groundwater increased as the seasons became warmer. Furthermore, during autumn, summer and spring, HPCs were identified at all sites with varying HPC levels between sites. The lowest levels of HPCs obtained during autumn at site BG (39966 cfu/ml). Autumn results indicated eleven sites having the presence of HPCs,

whilst the remaining four sites (LS, LV, H, SG) showed no trace of HPCs. However, HPC bacteria were successfully isolated in all water samples from autumn. The level of HPCs in the groundwater systems across the North West Province is extremely high compared to the other two groups.

Table 4.2 – The Microbial groundwater quality analysis of samples collected throughout the four seasons in May 2019, July 2019, February 2020 and September 2020.

SEASON	SITES	Coliforms cfu/100ml	<i>Enterococcus spp.</i> cfu/100ml	HPCs cfu/100ml
TWQR		N/A	N/A	N/A
AUTUMN MAY 2019	S1	0	746	345333.33
	S2	0	0	672000
	S3	59	0	718666.67
	S4	0	169	413333.3
	S5	0	152	394000
	S6	127	149	397333.3
	S7	0	86	404000
	DK	0	0	408333
	BG	0	0	39966
	LS	0	0	0
	LV	0	0	0
	H	0	0	0
	FD	0	0	699668
	SG	0	0	0
	GM	53	0	696333.3
	AVG	15.93 ± 36.46	86.8 ± 193.61	345931.13 ± 278030.62
WINTER JULY 2019	S1	0	532	536000
	S2	0	0	532666
	S3	0	53	532333
	S4	155	159	232333
	S5	235	229	213333

	S6	0	172	196000
	S7	0	0	362665
	DK	39	359	399666.67
	BG	0	0	426333.3
	LS	0	395	376666.67
	LV	0	134	174333.3
	H	238	0	236667
	FD	0	0	346200
	SG	0	198	392667
	GM	0	53	536000
	AVG	44.47± 87.73	152.27± 166.96	366257.60± 131687.42
SUMMER FEBRUARY 2020	S1	0	532	536000
	S2	0	0	532666.67
	S3	-	-	-
	S4	0	539	532362
	S5	165	159	232333.3
	S6	298	229	213333.3
	S7	0	172	196000
	DK	0	0	362666.67
	BG	393	359	399666.67
	LS	0	0	426333.3
	LV	0	395	376645
	H	0	134	174333.3

	FD	238	0	236689
	SG	0	0	346200
	GM	0	198	392669
	AVG	72.93± 128.87	194.07± 195.26	354135.59± 127676.28
SPRING SEPTEMBER 2020	S1	0	746	786000
	S2	0	0	532200
	S3	-	-	-
	S4	0	0	532200
	S5	538	169	532333
	S6	276	523	232333.3
	S7	0	866	256235
	DK	0	0	176000
	BG	134	0	362666.67
	LS	0	0	199666.67
	LV	0	76	426398
	H	0	289	376200
	FD	0	352	176333.3
	SG	0	0	167668
	GM	-	-	-
	AVG	72.92± 161.88	232.38± 305.47	365864.15± 189751.01

Values of levels at all sites, as well as average values of levels of all sampling sites per groundwater are presented and ± standard deviation values. **BG** - Botanical gardens, **SG** - Sportsground, **LV** - LaVaria, **LS** - Lareus, **GM** - METSI, **DK** - Draak, **FD** - Fanie; **H** - Hombre. **HPCs** - Heterotrophic plate count bacteria, **TWQR** - Target Water Quality Range; **AVG** – Average.

4.3 Correlation of the physico-chemical and microbiological parameters

The correlation of all microbial and physico-chemical parameters per season was determined by using redundancy analysis (RDA) plots. Correlations in the RDAs were determined by the size of the angles between the plots. The smaller the angle between the parameters, the closer the correlation. Additionally, the statistical association of all measured physico-chemical and microbial parameters was determined using basic correlation matrices with all significant correlations at $p < 0.05$ using Statistica 14.0 (StatSoft, US). The p-values per season are summarized in *Table A2-A5* in **Appendix A**.

4.3.1 Autumn

Figure 4.1 below is a redundancy analysis (RDA) plot indicating the correlation between the measured physico-chemical and microbial parameters in autumn. As depicted in *Figure 4.1*, the temperature had a positive correlation with pH, $p = 0.028$ during autumn. Also, a statistically significant positive correlation was observed between TDS and both phosphates as well as nitrates, $p = 0.019$ and $p = 0.020$, respectively. Additionally, coliforms had a positive correlation with TDS and nitrates, with p-values of $p = 0.003$ and $p = 0.000$, respectively. A positive correlation was also observed between TDS and nitrates $p = 0.020$. Site S6 was highly associated with nitrates, TDS and coliforms. No positive correlations were observed between *Enterococcus* spp. and HPCs to all measured physico-chemical parameters.

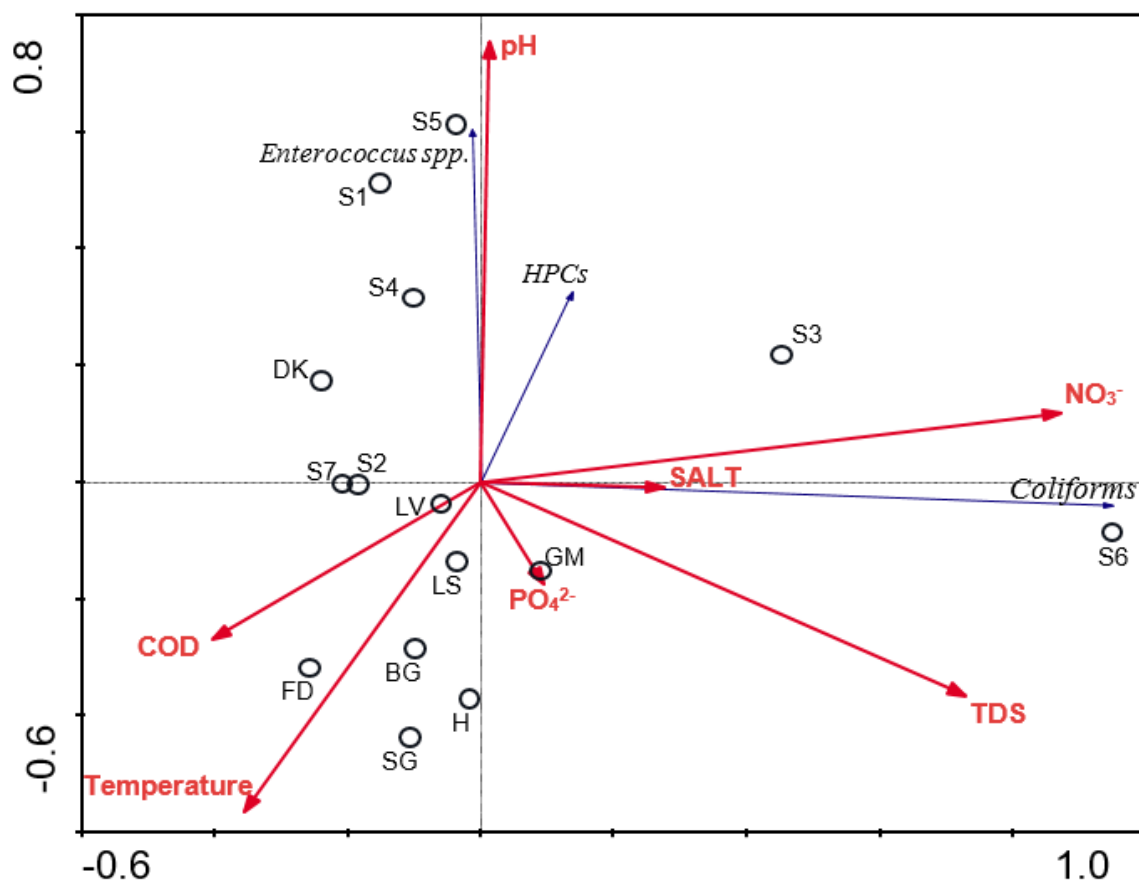


Figure 4.1 – Redundancy analysis (RDA) triplot depicting the correlation between varying physico-chemical (TDS, COD, PO_4^{2-} , NO_3^-) and microbiological parameters (Coliforms, *Enterococcus* spp., HPCs) at various sites during the autumn season – May 2019. The red arrows represent the physico-chemical parameters, and the blue arrows represent the microbiological parameters. All circles are indicative of the various sampling sites. **TDS** - Total Dissolved Solids, **NO_3^-** - Nitrate, **PO_4^{2-}** - Phosphate, **COD** – Chemical Oxygen Demand, **DK** -Draak, **BG** - Botanical gardens, **LS** - Laureus **LV** - LaVaria, **H** – Hombre, **FD** - Fanie du Toit, **SG** - Sportsground, **GM** – METSI.

4.3.2 Winter

The redundancy analysis (RDA) plot depicting the correlation between physico-chemical and microbial parameters in winter is presented in *Figure 4.2* below. The sites that were mostly influenced by the positive association between HPCs and coliforms are sites GM, S3, BG, S4, FD, H and S5. The association is backed by statistics, as HPC had a positive correlation with coliforms ($p = 0.041$). *Figure 4.2* further showed a close association between salt and TDS, $p = 0.00$. Although *Figure 4.2* shows a close association between *Enterococcus* spp. and temperature, the p-value was $p = 0.390$, thus there is no statistical significance between the two parameters. Just as TDS was a prominent parameter in autumn, it remained a

dominant parameter in winter. Although correlations were observed for most parameters, pH, COD and HPCs yielded no correlation with other parameters.

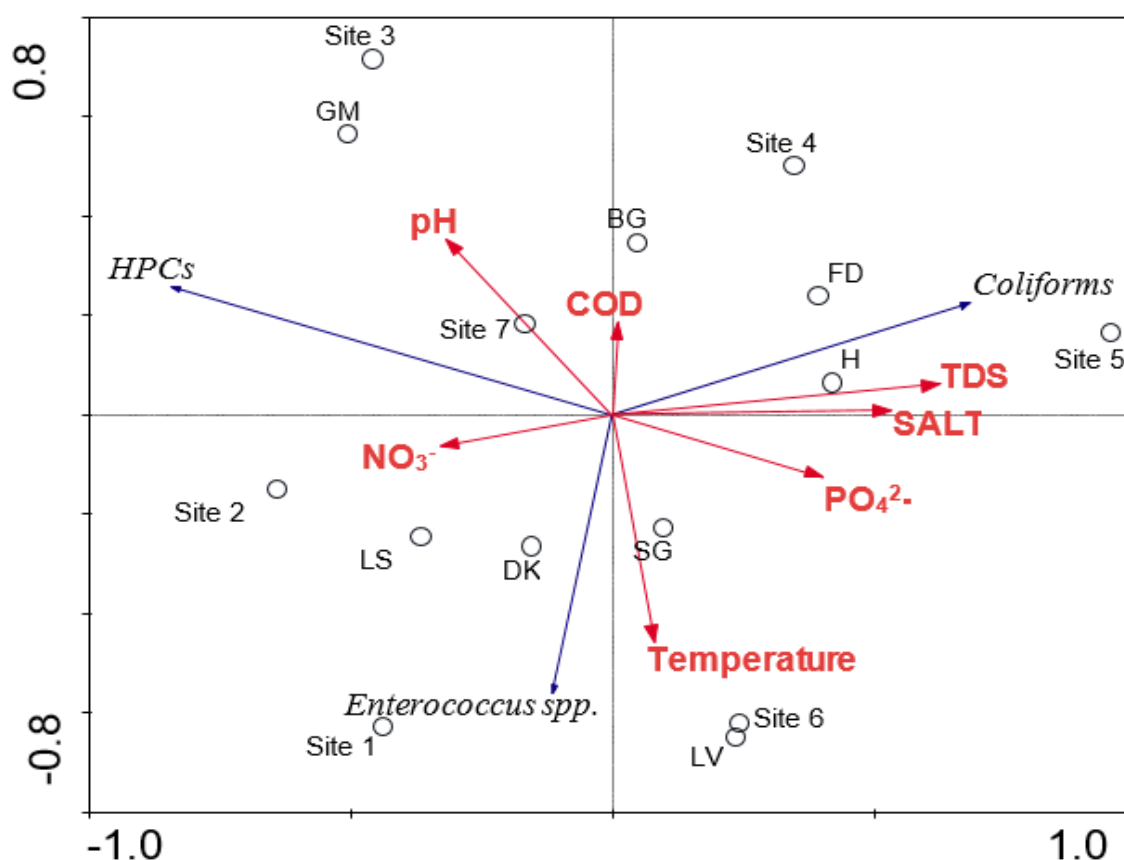


Figure 4.2 – Redundancy analysis (RDA) triplot depicting the correlation between varying physico-chemical (TDS, COD, PO_4^{2-} , NO_3^-) and microbiological parameters (Coliforms, *Enterococcus*, HPCs) at various sites during the winter season – July 2019. The red arrows represent the physico-chemical parameters, and the blue arrows represent the microbiological parameters. All circles are indicative of the various sampling sites. **TDS** - Total Dissolved Solids, **NO_3^-** - Nitrate, **PO_4^{2-}** - Phosphate, **COD** – Chemical Oxygen Demand, **DK** -Draak, **BG** - Botanical gardens, **LS** - Laureus **LV** - LaVaria, **H** – Hombre, **FD** - Fanie du Toit, **SG** - Sportsground, **GM** – METSI.

4.3.3 Summer

Figure 4.3 below is a redundancy analysis (RDA) plot indicating the correlation between the physico-chemical and microbial parameters measured in summer. A statistically significant correlation was observed between nitrates and *Enterococcus* spp., $p = 0.030$. Furthermore, temperature and TDS had a statistically significant correlation, $p = 0.004$. Site S1 showed close association with *Enterococcus* spp., Figure 4.3. Additionally, phosphates had a positive correlation with temperature, $p = 0.035$. Nonetheless, no significant correlations

were observed for COD, coliforms, HPCs and pH with other measured parameters in summer.

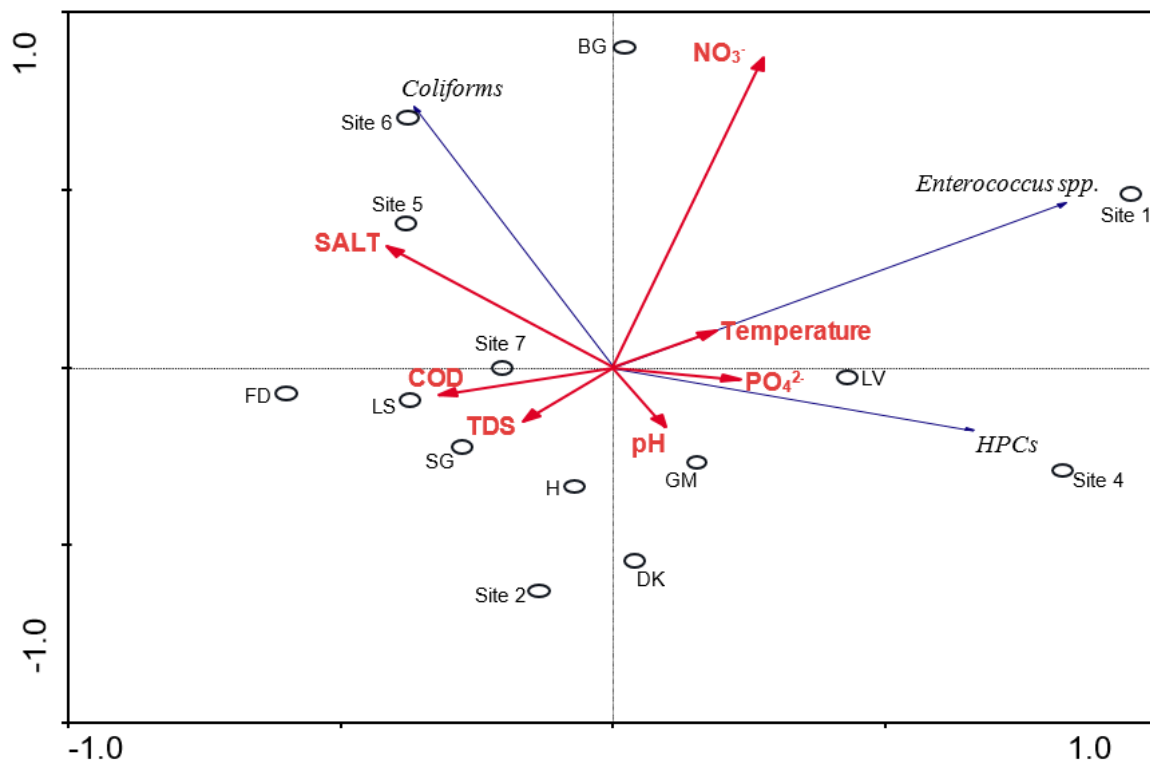


Figure 4.3 – Redundancy analysis (RDA) triplot depicting the correlation between varying physico-chemical (TDS, COD, PO_4^{2-} , NO_3^-) and microbiological parameters (Coliforms, *Enterococcus*, HPCs) at various sites during the summer season – February 2020. The red arrows represent the physico-chemical parameters, and the blue arrows represent the microbiological parameters. All circles are indicative of the various sampling sites. **TDS** - Total Dissolved Solids, **NO_3^-** - Nitrate, **PO_4^{2-}** - Phosphate, **COD** – Chemical Oxygen Demand, **DK** - Draak, **BG** - Botanical gardens, **LS** - Laureus **LV** - LaVaria, **H** – Hombre, **FD** - Fanie du Toit, **SG** - Sportsground, **GM** – METSI.

4.3.4 Spring

The redundancy analysis (RDA) plot depicting the correlation between physico-chemical and microbial parameters measured during spring is presented in *Figure 4.4* below. Temperature, salt, pH, phosphate and HPCs illustrated no positive correlation with the other measured parameters. However, a positive correlation was observed between COD and TDS, $p = 0.000$. In addition, coliforms had a statistically significant correlation with TDS and COD, with p -values of $p = 0.008$ and $p = 0.001$, respectively. Additionally, similar to the statistical significant observed during summer, nitrates and *Enterococcus* spp. had a significant correlation, with a $p = 0.001$.

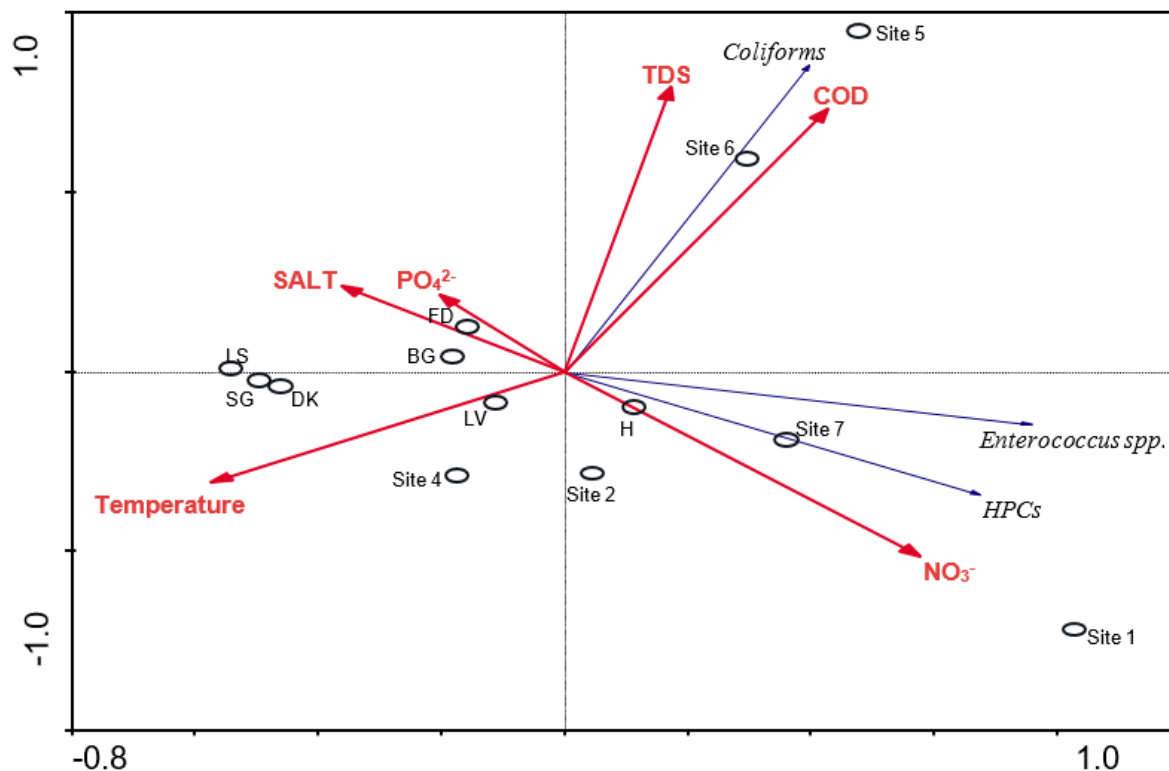


Figure 4.4 – Redundancy analysis (RDA) triplot representing the relationship between varying physico-chemical (TDS, COD, NO_3^- , PO_4^{2-}) and microbiological parameters (Coliforms, *Enterococcus*, HPCs) at various sites during the spring season – September 2020. The red arrows represent the physico-chemical parameters, and the blue arrows represent the microbiological parameters. **TDS** - Total Dissolved Solids, **NO_3^-** - Nitrate, **PO_4^{2-}** - Phosphate, **COD** - Chemical Oxygen Demand **DK**-Draak, **BG**- Botanical gardens, **LS**- Laureus **LV**- LaVaria, **H** – Hombre, **FD**- Fanie du Toit, **SG** - Sportsground, **GM** - METSI.

4.4 Species diversity of bacteria from groundwater systems in the North West Province

A total of 188 isolates were identified post 16S rRNA gene sequencing. *Figure 4.5* below is a pie chart illustrating the presence (in percentages) of the three bacterial groups of interest. Of all identified 188 bacterial isolates, 26 (14%) belonged to the coliform bacterial group, 44 (23%) belonged to the *Enterococcus* bacterial group, and lastly, 118 (63%) belonged to the HPC bacterial group. The accession numbers of the bacterial isolates identified in this study are presented in *Annexure B*.

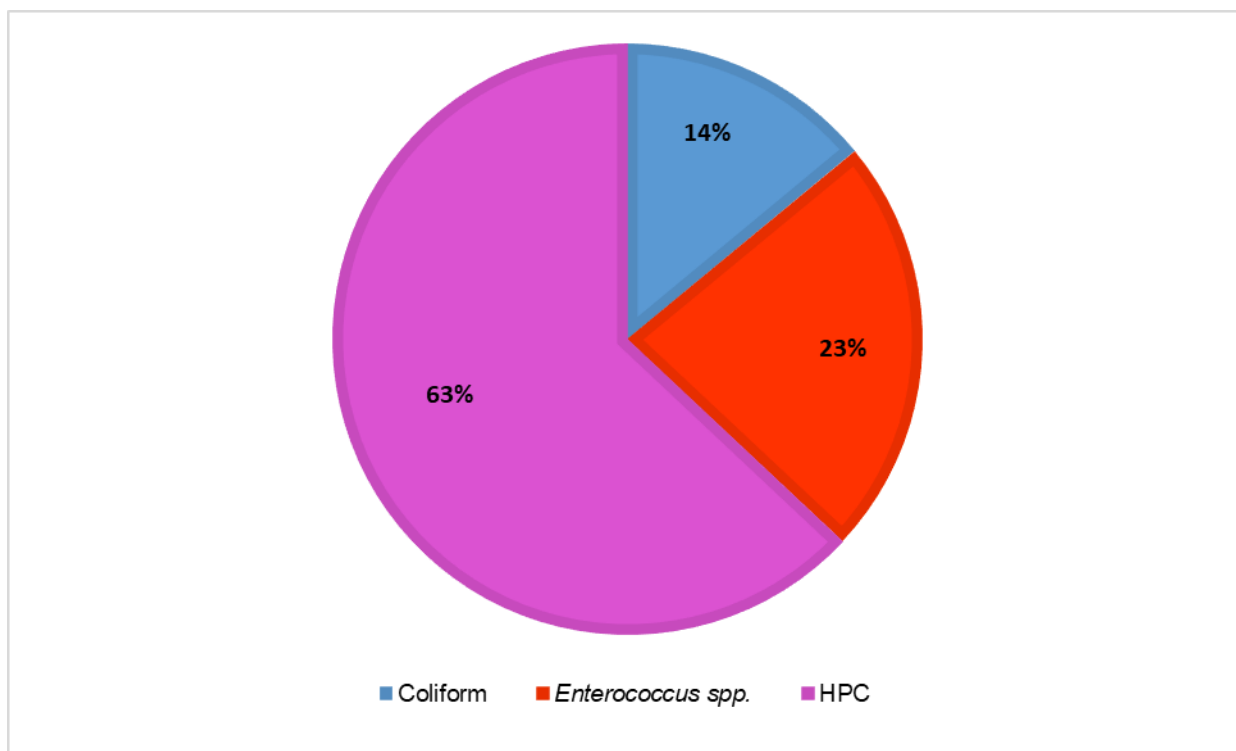


Figure 4.5 – Pie chart illustrating the prevalence of coliforms, *Enterococcus* spp., and HPC isolates identified in groundwater systems of the North West Province.

4.4.1 Diversity of the coliform bacterial group

Figure 4.6 is a pie chart illustrating the diversity and prevalence of coliforms in the groundwater systems of interest. Sub pie charts (Figure 4.6a - 4.6d) representative of each season can be found in the Appendix A. A total of six species were identified within the isolated coliform bacterial group: *Escherichia fergusonii*, *Citrobacter freundii*, *Enterobacter cloacea*, *Klebsiella grimontii*, *Citrobacter braaki* and *Buttiauxella agrestis*. *Escherichia fergusonii* was the most dominant species, making up 54% (14) of the overall population of coliforms identified. Two *Citrobacter* species were identified during the study, *Citrobacter braaki* and *Citrobacter freundii*, 11% (2) and 15% (3), respectively. In autumn and winter, (Figure 4.6a and Figure 4.6d Appendix B), the only coliform species identified was *Escherichia fergusonii* accounting for 100% in autumn and 100 % in winter. In summer, a total of five coliform species were identified, the most prevalent being *Escherichia fergusonii*, comprising 8 (58%) isolates (Figure 4.6c – Appendix B). The remaining four coliform species identified during summer included *Citrobacter freundii* 2 (14%), *Citrobacter braaki* 2 (14%), *Klebsiella grimontii* 1 (7%) and *Enterobacter cloacea* 1 (7%). A total of six coliform species were identified in spring (Figure 4.6d Appendix B). *Escherichia fergusonii* and *Citrobacter freundii* were the most prevalent species in spring, accounting for 50% of the overall species

diversity in spring. The least detected species in spring were *Klebsiella grimontii* 1 (12%) and *Enterobacter cloacea* 1 (12%).

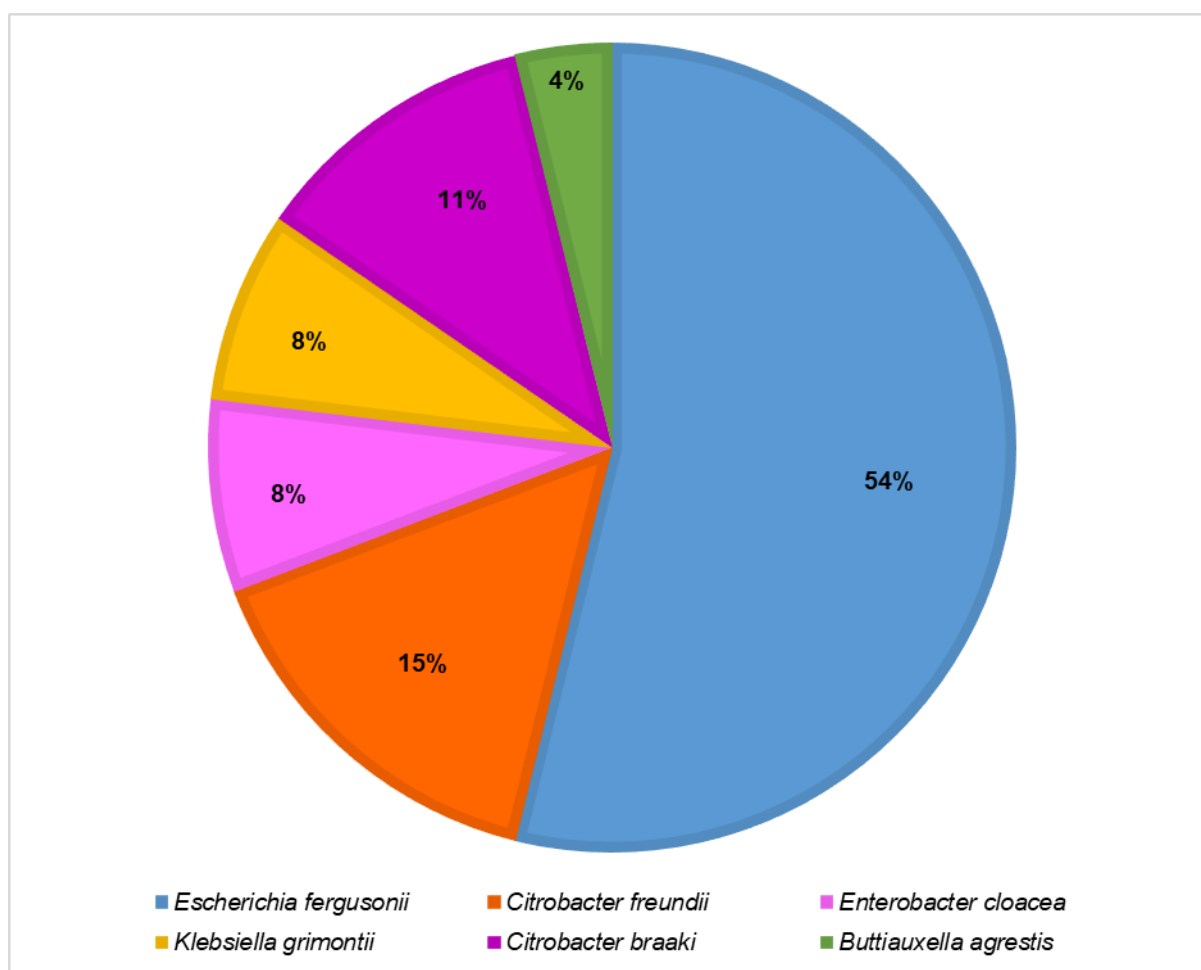


Figure 4.6 – Pie chart illustrating the diversity and prevalence of coliforms isolated from groundwater systems of interest in the North West Province.

4.4.1.1 Phylogenetic analysis of the coliform bacterial group

Figure 4.6.1 presented below is a phylogenetic tree derived from 16S rRNA gene sequences. Two representatives of each species were used to construct the tree. The outgroup used for this section was *Candida albicans* (AF134720.1). There were two clusters evident in this section, namely cluster A and cluster B. Under cluster A there were three subgroups, A1, A2.1 and A2.2. There was only one sub-group under cluster B. The only species under cluster B was *Buttiauxella agrestis* (OKO30977.1). The bootstrap value between cluster A and cluster B was 99%. Furthermore, *Enterobacter cloacea* (OKO30980.1) was the only species under cluster A1. *Enterobacter cloacea* was also under

cluster A2.2, with 70% similarity to *Citrobacter braaki* species. Sub-cluster A2.1 only consisted of two *Escherichia fergusonii* species, which had a 95% similarity. Under sub-cluster A2.2, there were three different species, namely, *Citrobacter braakii*, *Citrobacter freundii*, and *Enterobacter cloacea*. *Citrobacter freundii* (OKO30979.1) and *Citrobacter freundii* (OKO30984.1) had 35% similarity.

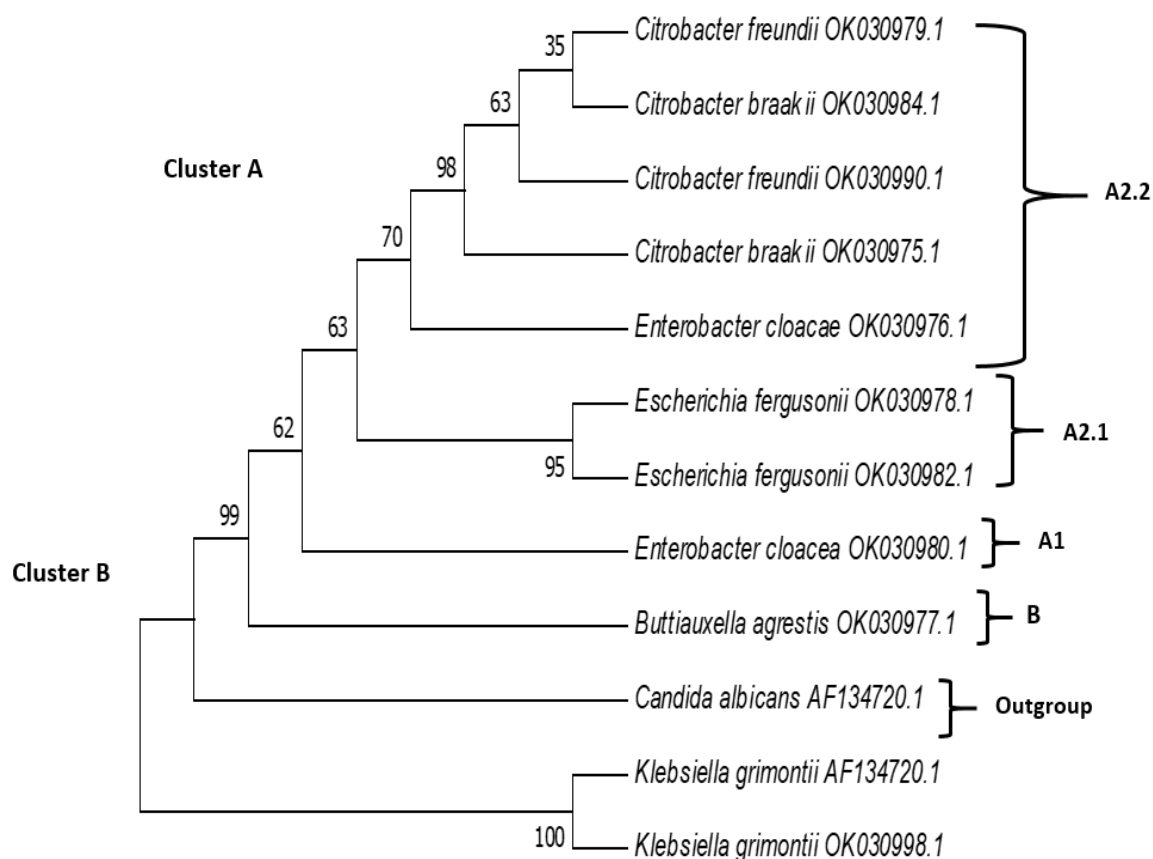


Figure 4.6.1 - Phylogenetic tree (Neighbour joining tree) of coliform isolates derived from 16S rRNA gene sequences. A Neighbour joining tree was constructed using the Jukes Cantor model and 1000 bootstraps.

4.4.2 Diversity of the *Enterococcus* spp. group

The species diversity and prevalence of *Enterococcus* species identified during this study are displayed in Figure 4.7 below. The sub-pie charts (Figure 4.7a-4.7d) for each season are presented in Appendix B. A total of nine *Enterococcus* species were identified during this study: *Enterococcus casseliflavus*, *Enterococcus faecalis*, *Enterococcus gallinarum*, *Enterococcus dispar*, *Enterococcus saigonensis*, *Enterococcus saccharolyticus*, *Enterococcus mundtii*, *Enterococcus villorum* and *Enterococcus saccharolyticus*.

Enterococcus casseliflavus 19 (43%) was the most predominant species observed overall in the *Enterococcus* spp. group. The second most prevalent species observed were *Enterococcus faecalis* 9 (21%) followed by *Enterococcus gallinarum* 7 (16%). Furthermore, *Enterococcus dispar*, *Enterococcus saigonensis*, *Enterococcus saccharolyticus* and *Enterococcus mundtii* were the least detected species, all amounting to 2% each. Compared to the coliform group, the *Enterococcus* group had more species diversity.

In autumn 2019, only three *Enterococcus* spp. were identified (Figure 4.7a AppendixB): *Enterococcus gallinarum*, *Enterococcus casseliflavus* and *Enterococcus faecalis*. The most prevalent species identified in autumn was *Enterococcus faecalis*, comprising 6 (46%) isolates. *Enterococcus gallinarum* and *Enterococcus casseliflavus* accounted for 3 (23%) and 4 (31%) of the total *Enterococcus* spp. identified during autumn. Additionally, *Enterococcus* spp. that were present in groundwater during autumn, were also detected during winter. *Enterococcus faecalis* accounted for 2 (50%) of the total diversity of *Enterococcus* spp. group identified during winter (Figure 4.7b AppendixB). Whilst, 1 (25%) *Enterococcus gallinarum* and 1 (25%) *Enterococcus casseliflavus* were also detected in winter. A total of five *Enterococcus* spp. were identified in groundwater during summer (Figure 4.7d AppendixB). The least detected species were *Enterococcus faecalis* 1 (6%) and *Enterococcus hirae* 1 (6%). In summer, *Enterococcus gallinarum* 2 (13%) and *Enterococcus villorum*. 2 (13%) were detected although in low levels. Furthermore, a total of seven *Enterococcus* species were identified in spring 2020: 1 (9%) *Enterococcus mundtii*, 4 (37%) *Enterococcus casseliflavus*, 2 (18%) *Enterococcus hirae*, 1 (9%) *Enterococcus gallinarum*, 1 (9%) *Enterococcus dispar*, 1 (9%) *Enterococcus saigonensis* and 1 (9%) *Enterococcus saccharolyticus*.

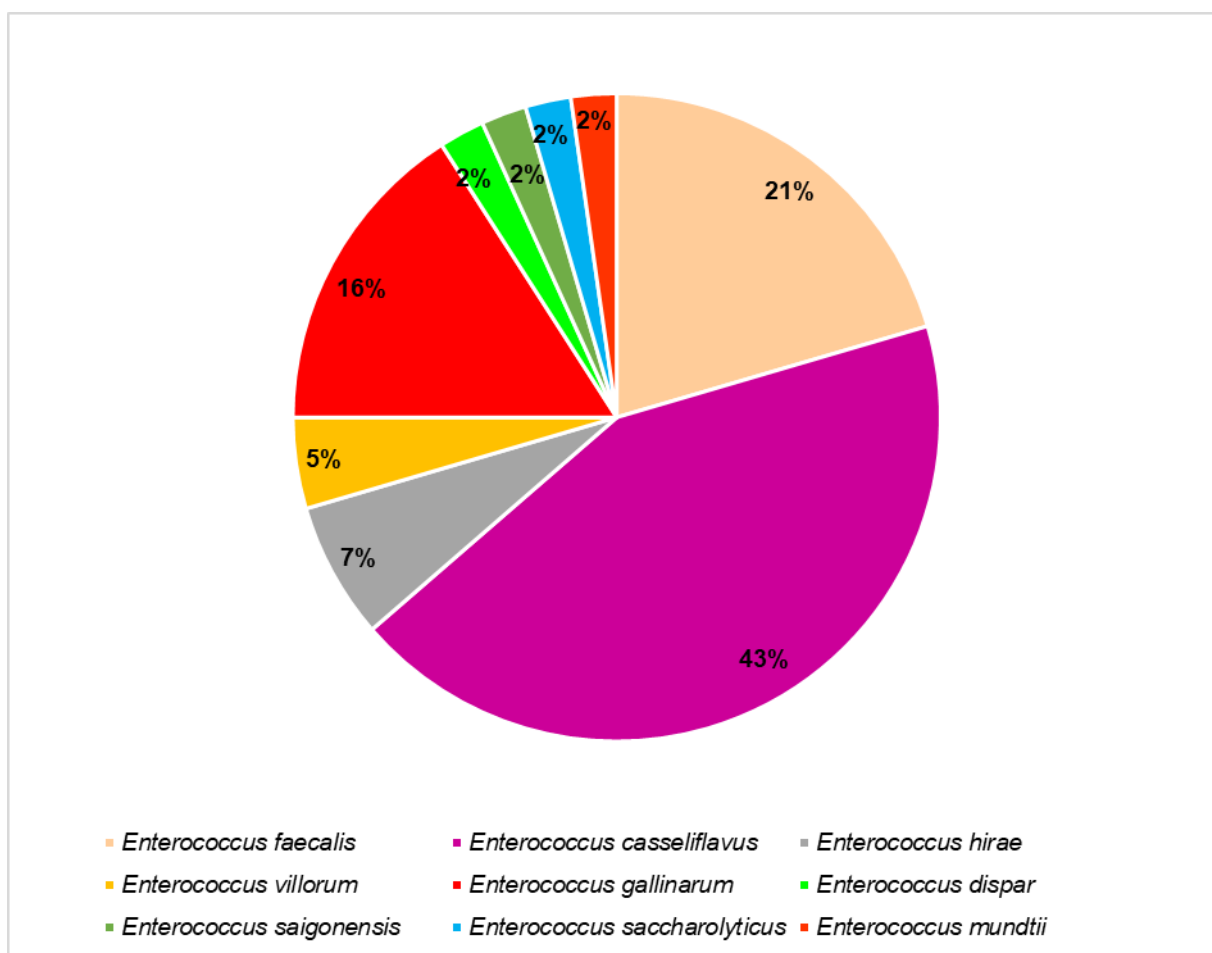


Figure 4.7 – Diversity and prevalence of *Enterococcus* species isolated from groundwater systems of interest in the North West Province.

4.4.2.1 Phylogenetic analysis of *Enterococcus* spp.

Figure 4.7.1 below presents a neighbour joining phylogenetic tree derived from partial 16S rRNA gene sequences. Two representatives of each species was used to construct the tree. The outgroup used for this tree was *Penicillium chrysogenum* (M55628.1). There were a total of two clusters, namely, Cluster A (A1 and A2) and Cluster B. Under cluster B, two *Enterococcus* species were evident, namely, *Enterococcus mundtii* (MZ680544.1) and *Enterococcus dispar* (MZ680535.1). Cluster A was divided into two subgroups (A1 and A2). In sub-group A1, there was a 77% similarity between cluster A1.1 and cluster A1.2. The species under sub-cluster A1.1 was *Enterococcus hirae*, and the species under A1.2 was *Enterococcus villorum* and *Enterococcus casseliflavus*. Within cluster A1.2, there was a 78% similarity between *Enterococcus villorum* and *Enterococcus casseliflavus*. Under cluster A2.1, there was a total of 10 *Enterococcus faecalis* species. Under cluster A2.2, two different *Enterococcus* species were present, namely *Enterococcus casseliflavus*, *Enterococcus*

gallinarum, *Enterococcus saccharolyticus* and *Enterococcus saigonensis*. *Enterococcus casseliflavus* was under both cluster A1.1 and cluster A2.2.

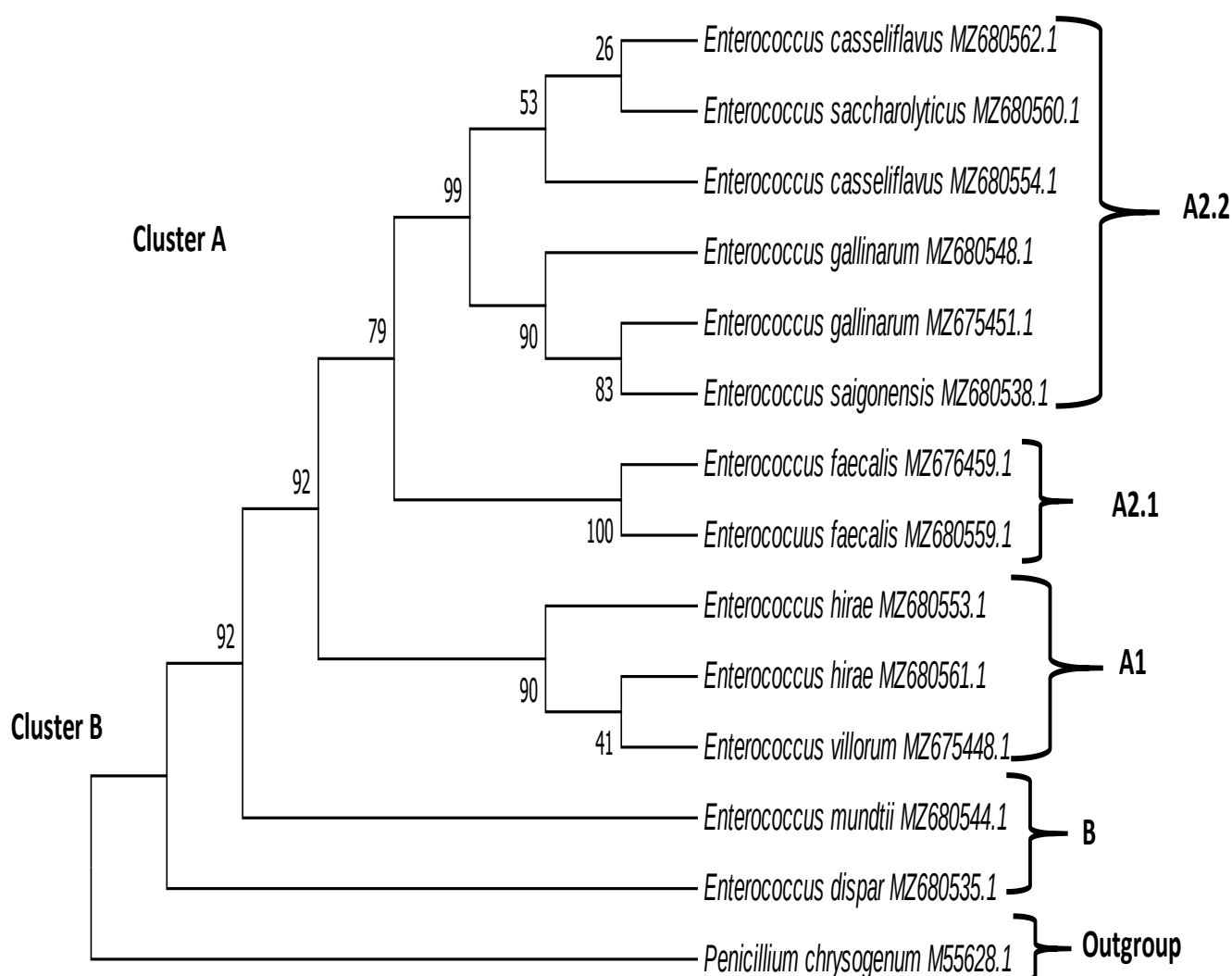


Figure 4.7.1 – Phylogenetic tree of *Enterococcus* species derived from 16S rRNA gene sequences. A Neighbour joining tree was constructed using the Jukes Cantor model and 1000 bootstraps.

4.4.3 HPC bacterial group diversity

The HPC group diversity for each season is presented in *Table 4.3* below. A total of 27 HPC genera were identified in this study. These genera are: *Bacillus*, *Pseudomonas*, *Brevundimonas*, *Sphingomonas*, *Microbacterium*, *Tahibacter*, *Fictibacillus*, *Stenotrophomonas*, *Sphingopyxis*, *Exiguobacterium*, *Paenibacillus*, *Xanthomonas*, *Lysobacter*, *Acinetobacter*, *Vogesella*, *Elstera*, *Ensifer*, *Caulobacter*, *Chryseobacter*, *Flavobacterium*, *Sphingobium*, *Brevibacterium*, *Hydrogenophaga*, *Nevskia*, *Lysinibacillus*,

Aeromonas, and *Chitinimonas*. The most prevalent HPC genera in this study was *Bacillus*, accounting for eight (19%) species. The most prevalent species within the *Bacillus* genus was *Bacillus cereus* 10 (9%) and the most infrequent *Bacillus* species were *Bacillus niacini* 1 (4%) and *Bacillus simplex* 1 (4%). *Bacillus cereus* was more dominant in autumn and spring. The second most prevalent HPC genera in this study was *Stenotrophomonas* 17 (14%), followed by *Pseudomonas* 14 (12%). There was only one species found under the *Stenotrophomonas* genus, namely *Stenotrophomonas maltophilia*. A total of 17 *Stenotrophomonas maltophilia* isolates were identified during this study. Furthermore, 8 (12%) *Stenotrophomonas maltophilia* isolates were identified in autumn, 5 (7%) in winter, 2 (8%) in summer and 2 (8%) in spring. Under the *Pseudomonas* genus, a total of eight species were identified in this study. *Pseudomonas songnenensis* was the most prominent species within the genus. Most of the *Pseudomonas* species were identified in winter. The *Aeromonas*, *Lysinibacillus*, *Nevskia*, *Brevibacterium*, *Flavobacterium* and *Caulobacter* genera were the least dominant genera, amounting to one (0.85%) isolate each.

4.4.3.1 Phylogenetic analysis of HPC bacteria

Figure 4.7.2 below is a neighbour joining tree of HPC bacteria. Two representatives of each species are represented in the phylogenetic tree. The tree is divided into two clusters Cluster A and Cluster B. Cluster B comprises of two sub-groups, B1 and B2. Cluster A is also divided into sub-groups, A1 and A2. Cluster B1 was mainly dominated by *Bacillus* spp. and *Paenibacillus* spp. The other genera under cluster B1 are *Fictibacillus*, *Brevibacillus*, *Exiguobacterium*, *Lysinibacillus*. *Bacillus simplex* (OKO65838) and *Brevibacterium frigoritolerans* (OKO87650) had a 100% bootstrap value. Furthermore, cluster B2 consisted of 4 *Microbacterium* species, namely, *Microbacterium chocolatum* (OKO65641), *Microbacterium aurum* (OKO65645), *Microbacterium zeae* (OKO65642) and *Microbacterium* (OKO65832). Cluster A2 is further differentiated into two sub-clusters. These sub-clusters have a bootstrap value of 92%.

Table 4.3 – Species diversity of HPC isolates identified from the selected groundwater systems.

Genera	Species	Autumn	Winter	Summer	Spring
<i>Bacillus</i>	<i>Bacillus simplex</i>	1	-	-	-
	<i>Bacillus tropicus</i>	-	1	-	1
	<i>Bacillus cereus</i>	4	1	1	4
	<i>Bacillus piscis</i>	-	-	-	2
	<i>Bacillus safensis</i>	-	-	1	1
	<i>Bacillus pumilus</i>	2	-	-	1
	<i>Bacillus halotolerans</i>	-	-	-	2
	<i>Bacillus niacin</i>	-	-	-	1
<i>Pseudomonas</i>	<i>Pseudomonas taiwanensis</i>	1	-	-	-
	<i>Pseudomonas benzenivorans</i>	1	-	-	-
	<i>Pseudomonas hibiscicola</i>	-	1	-	-
	<i>Pseudomonas aeruginosa</i>	-	1	-	1
	<i>Pseudomonas geniculate</i>	-	1	-	1
	<i>Pseudomonas monteilii</i>	-	1	1	-
	<i>Pseudomonas songnenensis</i>	1	1	2	-
	<i>Pseudomonas fulva</i>	-	-	1	-
<i>Brevundimonas</i>	<i>Brevundimonas albigilva</i>	1	2	-	-
	<i>Brevundimonas aurantiaca</i>	1	2	1	1
	<i>Brevundimonas denitrificans</i>	-	1	-	-
	<i>Brevundimonas nasdae</i>	1	1	-	1

<i>Sphingomonas</i>	<i>Sphingomonas trueperi</i>	1	-	-	-
	<i>Sphingomonas ginsenosidimutans</i>	-	-	1	-
	<i>Sphingomonas panacis</i>	-	-	1	-
	<i>Sphingomonas ursincola</i>	-	-	-	1
<i>Microbacterium</i>	<i>Microbacterium saccharophilum</i>	-	-	1	-
	<i>Microbacterium chocolatum</i>	-	1	-	-
	<i>Microbacterium zeae</i>	1	2	-	1
	<i>Microbacterium aurum</i>	-	-	-	1
<i>Tahibacter</i>	<i>Tahibacter aquaticus</i>	-	-	-	1
<i>Fictibacillus</i>	<i>Fictibacillus phosphorivorans</i>	3	1	1	-
	<i>Fictibacillus aquaticus</i>	-	-	-	2
<i>Stenotrophomonas</i>	<i>Stenotrophomonas maltophilia</i>	8	5	2	2
<i>Sphingopyxis</i>	<i>Sphingopyxis soli</i>	1	-	1	-
	<i>Sphingopyxis bauzanensis</i>	-	-	-	-
<i>Exiguobacterium</i>	<i>Exiguobacterium mexicanum</i>	2	-	-	-
<i>Paenibacillus</i>	<i>Paenibacillus lautus</i>	-	-	1	-
	<i>Paenibacillus yonginensis</i>	-	-	-	-
	<i>Paenibacillus glucanolyticus</i>	-	1	-	-
<i>Xanthomonas</i>	<i>Xanthomonas floridensis</i>	-	-	-	1
<i>Lysobacter</i>	<i>Lysobacter hankyongensis</i>	-	-	-	1
<i>Acinetobacter</i>	<i>Acinetobacter kookii</i>	2	-	-	2
	<i>Acinetobacter johnsonii</i>	1	1	-	-

	<i>Acinetobacter lwoffii</i>	-	1	-	-
<i>Vogesella</i>	<i>Vogesella facilis</i>	1	1	1	-
	<i>Vogesella perlucida</i>	-	-	-	1
<i>Elstera</i>	<i>Elstera litoralis</i>	-	1	-	-
<i>Ensifer</i>	<i>Ensifer adhaerens</i>	-	1	-	1
<i>Caulobacter</i>	<i>Caulobacter segnis</i>	-	-	-	1
<i>Chryseobacter</i>	<i>Chryseobacterium phosphatilyticum</i>	1	-	-	-
	<i>Chryseobacterium cucumeris</i>	-	-	-	1
<i>Flavobacterium</i>	<i>Flavobacterium aquidurens</i>	-	-	1	-
<i>Sphingobium</i>	<i>Sphingobium yanoikuyae</i>	1	1	3	1
<i>Brevibacterium</i>	<i>Brevibacterium frigoritolerans</i>	-	-	1	-
<i>Hydrogenophaga</i>	<i>Hydrogenophaga caeni</i>	-	-	-	2
<i>Nevskia</i>	<i>Nevskia aquatilis</i>	-	1	-	-
<i>Lysinibacillus</i>	<i>Lysinibacillus sinduriensis</i>	-	-	-	1
<i>Aeromonas</i>	<i>Aeromonas molluscorum</i>	-	1	-	-
<i>Chitinimonas</i>	<i>Chitinimonas viridis</i>	1	-	-	1
GRAND TOTAL		34	31	21	32

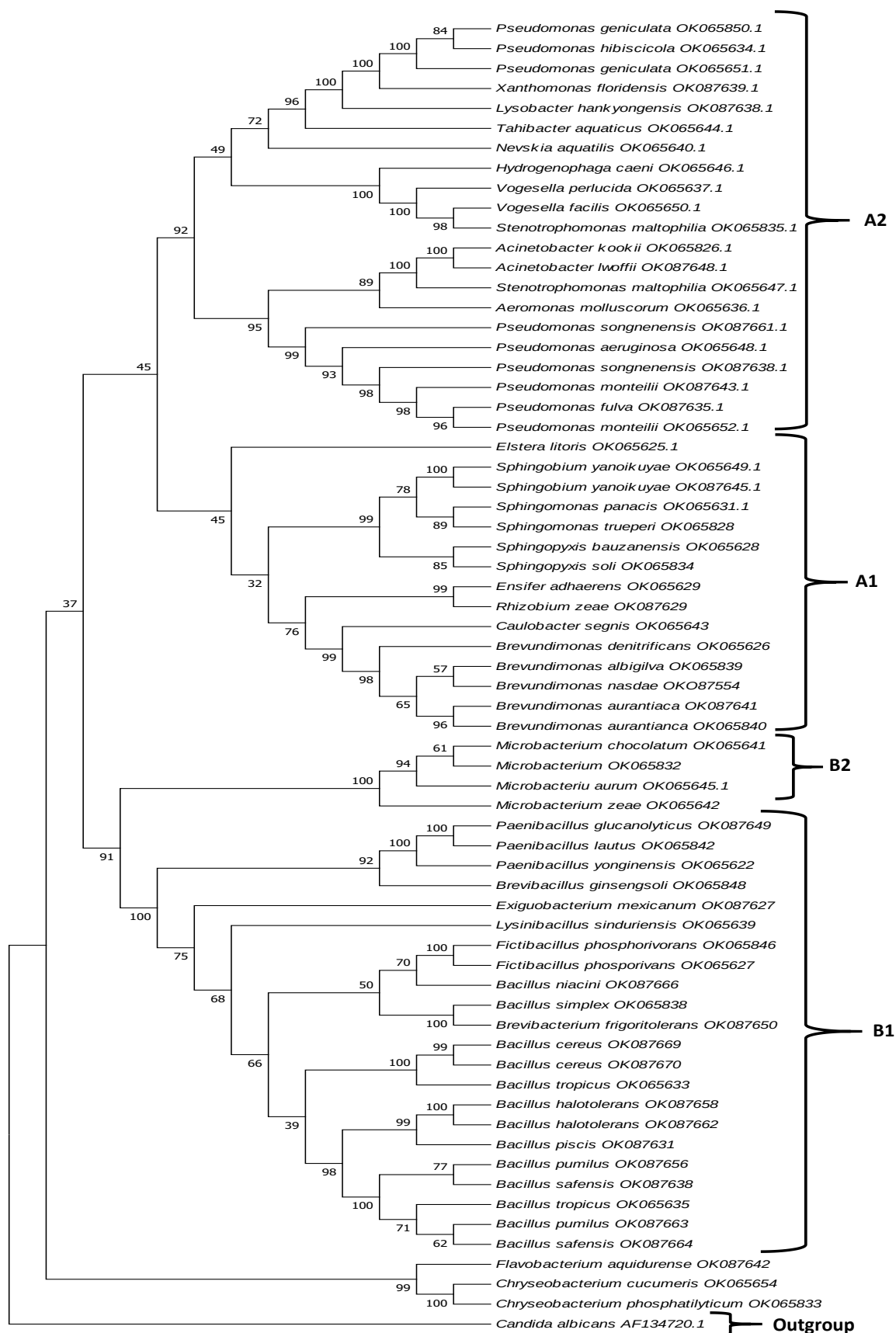


Figure 4.7.2 – Phylogenetic tree (Neighbour joining tree) of HPC species derived from 16S rRNA gene sequences. A Neighbour joining tree was constructed using the Jukes Cantor model and 1000 bootstraps.

4.5 Haemolysis and Extracellular enzyme production

4.5.1 Overall haemolysin patterns of coliforms, *Enterococcus* spp. and HPC bacteria

A total of 188 isolates comprising coliforms, *Enterococcus* spp. and HPC bacteria were subjected to haemolysis testing. The results displayed in *Figure 4.8* and *Table A6 (Appendix C)*, are an illustration of the haemolysis patterns observed in all bacterial isolates obtained during this study. A total of 146 (78%) isolates were alpha haemolytic. Whilst, 20 (11%) isolates were beta haemolytic and the remaining 20 (11%) were gamma haemolytic. In autumn, a total of 42 isolates were either beta or alpha haemolytic. Of these, two (5%) were coliforms, 13 (31%) were *Enterococcus* spp., and 27 (64%) were HPC isolates. Isolates showing the lowest beta and alpha haemolysis patterns were isolates collected during summer.

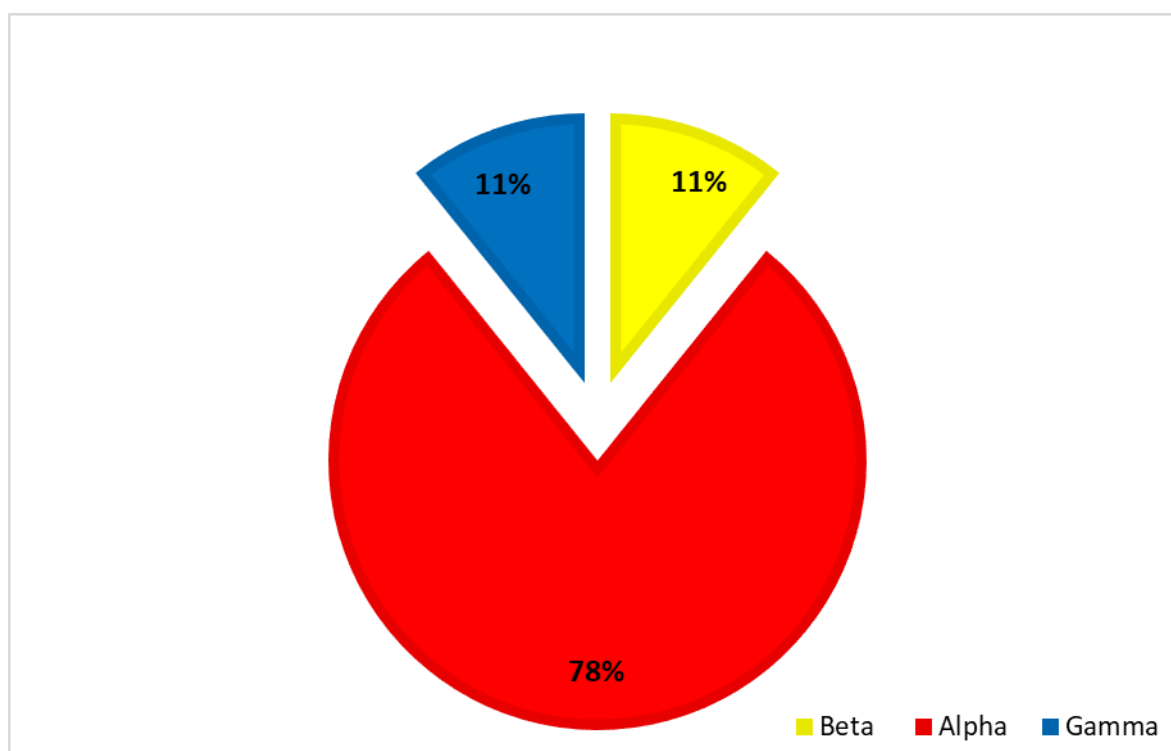


Figure 4.8 – Pie chart illustrating the overall haemolysis pattern of 186 bacterial isolates comprising Coliforms *Enterococcus* spp. and HPC bacteria isolated from selected groundwater systems in the North West Province.

4.5.2 Extracellular enzyme production

A total of 146 isolates were subjected to extracellular enzyme production testing, 20 coliforms, 39 *Enterococcus* spp., and 87 HPC bacteria, *Table A6* (Appendix C). Four extracellular enzymes were screened, namely: proteinase, lipase, gelatinase and DNase. The results per bacterial group are presented in *Figures 4.9, 4.10 and 4.11*.

4.5.2.1 Extracellular enzyme production of coliforms

Figure 4.9 presented below, outlines the extracellular enzyme production observed amongst the coliform group. The overall percentages of extracellular production of coliforms are presented in *Table A7* (Appendix C). A total of 20 coliforms which were alpha/beta haemolytic were further screened for enzymatic activity. Of these 20, only six isolates displayed enzyme production, *Escherichia fergusonii*, *Citrobacter freundii*, and *Citrobacter braakii*. Proteinase and DNase were the enzymes predominantly produced by the coliform group. Proteinase production was observed in 4 (66%) *Escherichia fergusonii* isolates and 1 (17%) *Citrobacter freundii* isolate. Additionally, as depicted in *Figure 4.9*, none of the coliforms screened during this study produced lipase. Nonetheless, DNase and gelatinase production were observed in 1 (17%) *Citrobacter freundii* spp. and 1 (17%) *Escherichia fergusonii* spp., respectively. Overall, *Escherichia fergusonii* was the coliform group which displayed the most extracellular enzyme production.

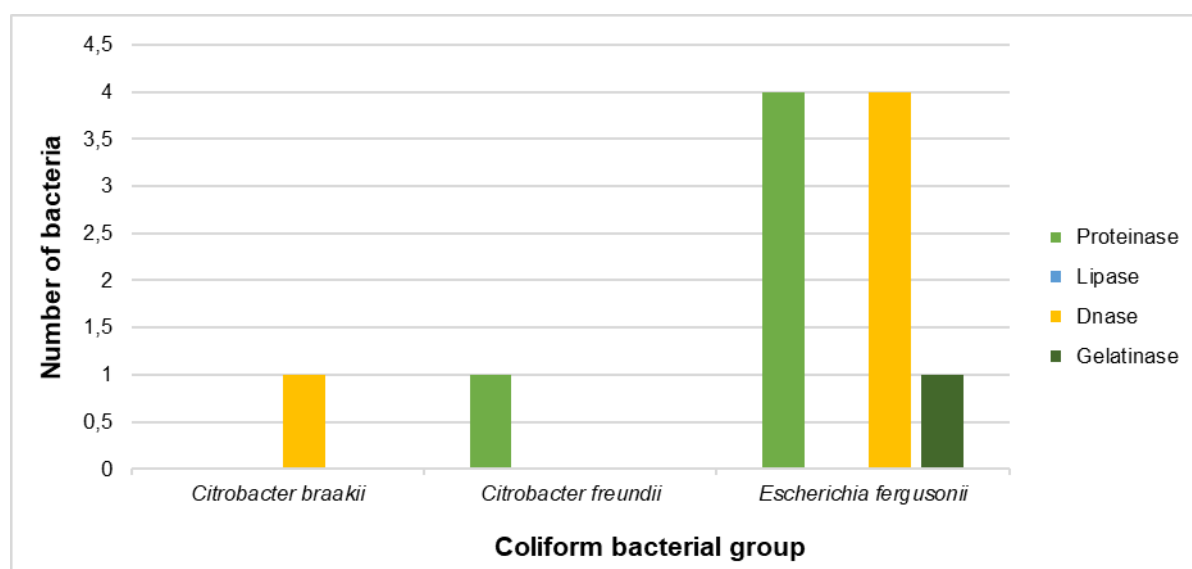


Figure 4.9. A summary of the overall extracellular enzyme production profile of coliforms isolated from the groundwater systems of interest.

4.5.2.2 Extracellular enzyme production of *Enterococcus* spp.

The overall percentages of extracellular production of *Enterococcus* spp. are presented in Table A8 (Appendix C). Figure 4.10 is a graph presenting the extracellular production profile of 39 *Enterococcus* spp. in groundwater systems across the North West Province. Of the 39 *Enterococcus* spp. screened, 35 displayed extracellular enzyme activity (Figure 4.10). Also, all 35 *Enterococcus* isolates that displayed enzyme isolates produced proteinase. Furthermore, proteinase production was observed in 15 (42%) *Enterococcus casseliflavus* isolates, 1 (3%) *Enterococcus dispar*, 6 (17%) *Enterococcus faecalis*, 6 (17%) *Enterococcus gallinarum*, 3 (9%) *Enterococcus hirae*, 1 (3%) *Enterococcus mundtii*, 1 (3%) *Enterococcus saccharolyticus*, 1 (3%) *Enterococcus saigonensis* and 1 (3%) *Enterococcus villorum*. Gelatinase was the second most produced extracellular enzyme among the *Enterococcus* bacterial group. A total of 5 (14%) isolates produced gelatinase, with 1 (2.8%) being *Enterococcus casseliflavus*, 1 (2.8%) *Enterococcus faecalis*, 1 (2.8%) *Enterococcus gallinarum*, 1 (2.8%) *Enterococcus hirae* and 1 (2.8%) *Enterococcus saigonensis*. Lipase was the least produced enzyme and produced by 1 (3%) *Enterococcus casseliflavus* isolate, 1 (3%) *Enterococcus gallinarum* isolate and 1 (3%) *Enterococcus hirae* isolate. DNase was also produced less by the *Enterococcus* spp. group and these isolates included 1 (3%) *Enterococcus casseliflavus*, 1 (3%) *Enterococcus faecalis*, 1 (3%) *Enterococcus gallinarum* and 1 (3%) *Enterococcus saigonensis* (Figure 4.10). Overall, *Enterococcus casseliflavus* and *Enterococcus gallinarum* were the two *Enterococcus* spp. which produced all the extracellular enzymes that were screened in this study.

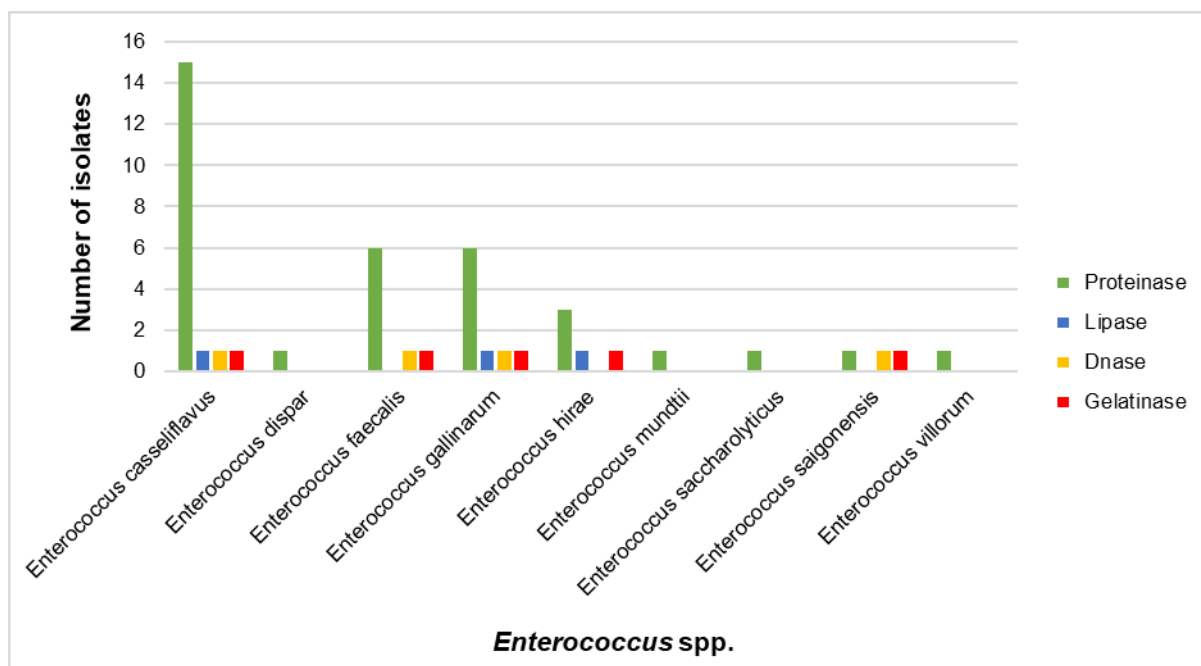


Figure 4.10 – A summary of the extracellular enzyme production profiles of various *Enterococcus* spp. isolated from the groundwater systems of interest.

4.5.2.3 Extracellular enzyme production of HPC isolates

The overall percentages of extracellular production of HPC bacteria are presented in *Table A9* (Appendix C). Extracellular enzyme production testing was performed on the 87 alpha/beta haemolytic HPC bacteria and the results are presented in *Figure 4.11* below. Of the 87 alpha/beta haemolytic HPC bacteria, 52 produced extracellular enzymes. Protease production was predominantly observed in *Stenotrophomonas maltophilia*. A total of 6 (16%) of the 37 protease active isolates were *Stenotrophomonas maltophilia* isolates. The second highest HPC protease producing species was *Fictibacillus phosphorivans* 3 (8%). *Bacillus pumilus*, *Brevundimonas aurantiaca*, *Pseudomonas geniculata*, *Sphingopyxis soli* and *Pseudomonas taiwanensis* did not produce proteinase, however, they produced other extracellular enzymes. Fifteen HPC isolates produced lipase: 1 (7%) *Acinetobacter johnsonii*, 1 (7%) *Acinetobacter kookii*, 2 (14%) *Bacillus cereus*, 1 (7%) *Bacillus safensis*, 1 (7%) *Fictibacillus phosphorivorans*, 1 (7%) *Lysobacter hankyongensis*, 1 (7%) *Paenibacillus lautus*, 1 (7%) *Pseudomonas geniculata* and five (36%) *Stenotrophomonas maltophilia*. Furthermore, *Stenotrophomonas maltophilia* were the highest DNase producing species among the HPC group. The second highest DNase producing HPC species was *Fictibacillus phosphorivorans*, *Figure 4.11*. A total of three HPC isolates produced gelatinase in this study. These isolates are *Brevundimonas aurantiaca* one (33.33%), *Chryseobacterium phosphatilyticum* one (33.33%) and *Vogesella facilis* one (33.33%).

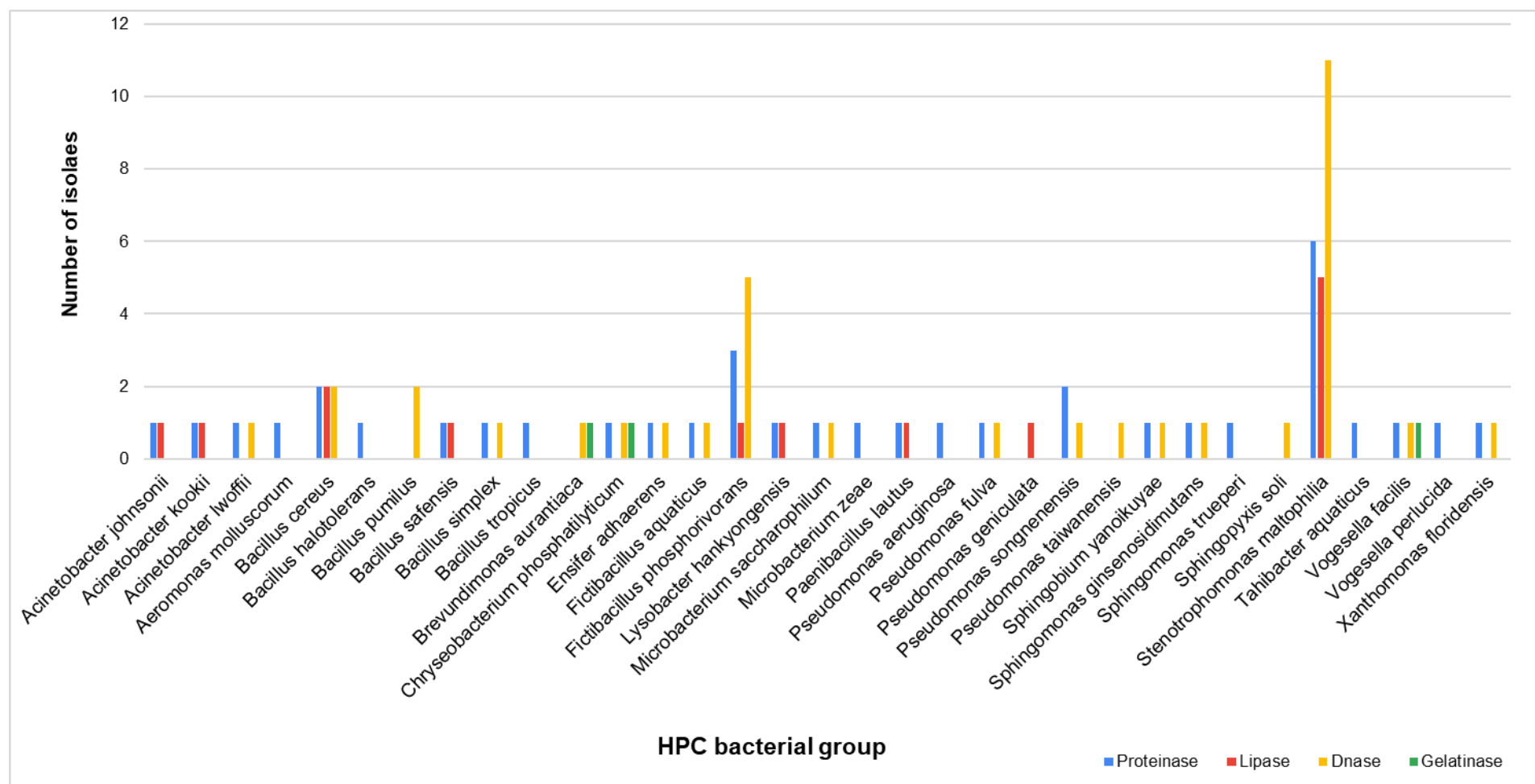


Figure 4.11 – A summary of the extracellular enzyme production profiles of HPC bacteria isolated from groundwater systems of interest.

4.6 Antibiotic-resistance

The antibiotic-resistance profiles of 188 of isolates comprising coliforms, *Enterococcus* spp. and HPCs was determined. Penicillin G (10 µg) and Vancomycin (30 µg) were only screened on Gram-positive isolates. This is because Gram-negative bacteria have a cell wall (peptidoglycan layer) that is surrounded by protein layers and lipopolysaccharides (Chow, 2021). These protein layers inhibit the mode of action of these two antibiotics. Thus, 11 antibiotics were screened on all Gram-positive bacteria and 9 antibiotics were screened on all Gram-negative bacteria.

4.6.2 Antibiotic-resistance profiles of coliforms

The overall antibiotic-resistance of 26 coliforms was determined and the results are presented in Table 4.4. All screened isolates were resistant to ampicillin (100%) and chloramphenicol (100%). The latter isolates comprised 14 *Escherichia fergusonii*, 4 *Citrobacter freundii*, 3 *Citrobacter braaki*, 2 *Enterobacter cloacea*, 2 *Klebsiella grimontii* and 1 *Buttiarella agrestis*. Furthermore, a total of 18 (69%) coliforms were resistant to erythromycin and trimethoprim. *Escherichia fergusonii* (86%) showed the highest resistance to erythromycin, whereas *Buttiarella agrestis* was susceptible to erythromycin. Amongst the 18 trimethoprim resistant coliforms, *Escherichia fergusonii* (86%) showed the highest resistance, followed by *Citrobacter braakii* (67%). Furthermore, *Citrobacter freundii*, *Enterobacter cloacea* and *Klebsiella grimontii* each showed 50% resistance to trimethoprim. A total of 16 (62%) coliforms were resistant to oxy-tetracycline, amoxicillin and neomycin. Additionally, between 50 and 67% of *Citrobacter braakii*, *Escherichia fergusonii*, *Citrobacter cloacea*, *Klebsiella grimontii* and *Citrobacter freundii* showed resistance to ciprofloxacin. Also, 12 (46%) coliforms were resistant to streptomycin with *Escherichia fergusonii* accounting for 71% of the streptomycin resistant isolates. Nonetheless, streptomycin resistance was also observed in the *Citrobacter* spp.

Table 4.4 – Antibiotic-resistance profiles of coliforms isolated from groundwater systems of interest.

Species	No. of isolates (N)	Antibiotics								
		AMP	E	OT	CIP	C	AMO	NE	W	S
		n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*
<i>Escherichia fergusonii</i>	14	14 (100)	12 (86)	10 (71)	9 (64)	14 (100)	11 (79)	9 (64)	12 (86)	10 (71)
<i>Citrobacter freundii</i>	4	4 (100)	2 (50)	2 (50)	2 (50)	4 (100)	2 (50)	2 (50)	2 (50)	1 (25)
<i>Enterobacter cloacea</i>	2	2 (100)	1 (50)	1 (50)	1 (50)	2 (100)	1 (50)	1 (50)	1 (50)	0
<i>Klebsiella grimontii</i>	2	2 (100)	1 (50)	1 (50)	1 (50)	2 (100)	1 (50)	1 (50)	1 (50)	0
<i>Citrobacter braakii</i>	3	3 (100)	2 (67)	2 (67)	2 (67)	3 (100)	1 (33)	2 (67)	2 (67)	1(33)
<i>Buttiauxella agrestis</i>	1	1 (100)	0	0	0	1 (100)	0	1 (100)	0	0
Total	26	26 (100)	18 (69)	16 (62)	15 (58)	26 (100)	16 (62)	16 (62)	18 (69)	12 (46)

Amp- Ampicillin (10 µg), **C** – Chloramphenicol (30 µg), **CIP** – Ciprofloxacin (5 µg), **E** – Erythromycin (15 µg), **NE** – Neomycin (30 µg), **OT** - Oxy-tetracycline (30 µg), **S** – Streptomycin (25 µg), **W** – Trimethoprim (5 µg), **Amo** – Amoxicillin (30 µg), **VA**-Vancomycin (30 µg), **PEN**-Penicillin (10 µg) *Percentage (%) was determined as a function of the number of isolates of the coliform spp., thus (n/(N) x 100).

Table 4.5 – Antibiotic-resistance profiles of *Enterococcus* spp. isolated from groundwater systems of interest.

Species	No. of isolates	Antibiotics										
		PEN	VAN	AMP	E	OT	CIP	C	AMO	NE	W	S
		(N)	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*
<i>Enterococcus faecalis</i>	9	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	7 (78)	9 (100)	4 (44)
<i>Enterococcus casseliflavus</i>	19	19 (100)	19 (100)	16 (84)	12 (63)	15 (79)	16 (84)	19 (100)	19 (100)	19 (100)	14 (74)	12 (63)
<i>Enterococcus hirae</i>	3	3 (100)	3 (100)	1 (33)	2 (67)	1 (33)	1 (33)	1 (33)	1 (33)	1 (33)	1 (33)	1 (33)
<i>Enterococcus villorum</i>	2	2 (100)	2 (100)	1 (50)	1 (50)	0	0	0	0	0	0	0
<i>Enterococcus gallinarum</i>	7	7 (100)	7 (100)	5 (71)	4 (57)	5 (71)	3 (43)	1 (14)	7 (100)	6 (86)	1 (14)	1 (14)
<i>Enterococcus dispar</i>	1	1 (100)	1 (100)	0	0	0	0	0	0	0	0	0
<i>Enterococcus saigonensis</i>	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
<i>Enterococcus saccharolyticus</i>	1	1 (100)	1 (100)	0	0	0	0	0	0	0	0	0
<i>Enterococcus mundtii</i>	1	1 (100)	1 (100)	0	0	0	0	0	0	0	0	0
Total	44 (100)	44 (100)	44 (100)	33 (75)	29 (66)	31 (70)	30 (68)	31 (70)	37 (84)	34 (77)	26 (59)	19 (43)

Amp- Ampicillin (10 µg), **C** – Chloramphenicol (30 µg), **CIP** – Ciprofloxacin (5 µg), **E** – Erythromycin (15 µg), **NE** – Neomycin (30 µg), **OT** - Oxy-tetracycline (30 µg), **S** – Streptomycin (25 µg), **W** – Trimethoprim (5 µg), **Amo** – Amoxicillin (30 µg), **VA**-Vancomycin (30 µg), **PEN**-Penicillin (10 µg) *Percentage (%) was determined as a function of the number of isolates of the coliform spp., thus (n/(N) x 100).

4.6.3 Antibiotic-resistance profiles of *Enterococcus* spp.

The overall antibiotic-resistance of 44 *Enterococcus* spp. was determined and the results are presented in *Table 4.5* above. All the *Enterococcus* spp. isolated in this study were resistant to penicillin (100%) and vancomycin (100%). Between 75% and 84% were resistant to amoxicillin, neomycin and ampicillin. All *Enterococcus casseliflavus* screened (19) as well as 6 (86%) *E. mundtii* showed high resistance to neomycin. However, three *Enterococcus* species groups (*E. dispar*, *E. saccharolyticus*, *E. mundtii*) were susceptible to ampicillin. Of the 26 (59%) *Enterococcus* spp. resistant to trimethoprim, all 9 *E. faecalis* spp. screened were resistant. A sum of 31 (70%) *Enterococcus* spp. were resistant to oxy-tetracycline and chloramphenicol. While only 19 (43%) *Enterococcus* spp. were resistant to streptomycin. *E. saccharolyticus* and *E. mundtii* were only resistant to penicillin and ampicillin.

4.6.4 Antibiotic-resistance profiles of HPC bacteria

The antibiotic-resistance profiles of 118 HPC bacteria are presented in *Table 4.6* below. Most HPC bacteria illustrated resistance to the screened antibiotics. *Bacillus simplex* showed resistance to all 11 antibiotics screened. Amongst the *Bacillus* spp., *B. tropicus* and *B. niacini* showed the least resistance to the screened antibiotics. *Elstera litoralis* also showed resistance to oxytetracycline and trimethoprim. The highest resistance was to oxy-tetracycline (81%) and chloramphenicol (77%). *Stenotrophomonas maltophilia* isolates showed 100% resistance to ampicillin, erythromycin, oxy-tetracycline, ciproflaxacin, chloramphenicol, neomycin and amoxicillin. *Fictibacillus phosphorivans* isolates expressed 100% resistance to penicillin, vancomycin, ampicillin, erythromycin, ciprofloxacin, chloramphenicol, streptomycin, neomycin and amoxicillin. A total of 84 (71%) HPC bacteria were resistant to erythromycin. Amongst the three bacterial groups of interest in this study, HPC bacteria showed the highest resistance to ampicillin. A total of 18 HPC species were susceptible to erythromycin. Similar to the coliforms and *Enterococcus* spp., HPC bacteria also showed minimal resistance to streptomycin.

Table 4.6 – Antibiotic-resistance profiles of HPC bacteria isolated from groundwater systems of interest.

Species	No. of isolates (N)	Antibiotics										
		PEN	VAN	AMP	E	OT	CIP	C	AMO	NE	W	S
		n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*
<i>Bacillus simplex</i>	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1(100)
<i>Bacillus tropicus</i>	2	2 (100)	2 (100)	0	0	0	0	0	0	0	0	1(50)
<i>Bacillus cereus</i>	10	10 (100)	10 (100)	8 (80)	8 (80)	5 (50)	8 (80)	10 (100)	8 (80)	6 (60)	10(100)	3(30)
<i>Bacillus piscis</i>	2	2 (100)	2 (100)	1 (50)	2 (100)	2 (100)	1 (50)	0	2 (100)	1 (50)	0	1(50)
<i>Bacillus safensis</i>	2	2 (100)	2 (100)	1 (50)	1 (50)	1 (50)	1 (50)	2 (50)	1 (50)	1 (50)	1(50)	1(50)
<i>Bacillus pumilus</i>	3	3 (100)	3 (100)	1 (33)	2 (67)	2 (67)	1 (33)	3 (100)	2 (67)	1 (33)	1(33)	1(33)
<i>Bacillus halotolerans</i>	2	2 (100)	2 (100)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)	0	1(50)
<i>Bacillus niacini</i>	1	1 (100)	1 (100)	0	0	0	0	0	0	0	0	1(100)
<i>Pseudomonas taiwanensis</i>	1	–	–	1 (100)	1 (100)	1 (100)	1 (100)	0	1 (100)	1 (100)	0	0
<i>Pseudomonas benzenivorans</i>	1	–	–	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0
<i>Pseudomonas hibiscicola</i>	1	–	–	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0
<i>Pseudomonas aeruginosa</i>	2	–	–	2 (100)	2 (100)	2 (100)	2 (100)	1 (50)	1 (50)	2 (100)	1 (50)	0

<i>Pseudomonas geniculata</i>	2	–	–	2 (100)	2 (100)	2 (100)	2 (100)	1 (50)	1 (50)	2 (100)	1 (50)	0
<i>Pseudomonas monteilii</i>	2	–	–	2 (100)	2 (100)	2 (100)	2 (100)	1 (50)	1 (50)	2 (100)	1 (50)	0
<i>Pseudomonas songnenensis</i>	4	–	–	2 (50)	2 (50)	4 (100)	2 (50)	1 (25)	2 (50)	2 (50)	3(75)	0
<i>Pseudomonas fulva</i>	1	–	–	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	0
<i>Brevundimonas albigilva</i>	3	–	–	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	2(67)	0
<i>Brevundimonas aurantiaca</i>	4	–	–	2 (50)	4 (100)	4 (100)	2 (50)	4 (100)	4 (100)	2 (50)	0	0
<i>Brevundimonas denitrificans</i>	1	–	–	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	0	0
<i>Brevundimonas nasdae</i>	1	–	–	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	0	0
<i>Sphingomonas trueperi</i>	1	–	–	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	1 (100)	0
<i>Sphingomonas ginsenosidimutans</i>	1	–	–	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	1 (100)	0
<i>Sphingomonas panacis</i>	1	–	–	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	1 (100)	0
<i>Sphingomonas ursincola</i>	1	–	–	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	1 (100)	0
<i>Microbacterium saccharophilum</i>	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	1 (100)	1(100)
<i>Microbacterium chocolatum</i>	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	1 (100)	1(100)

<i>Microbacterium zeae</i>	4	4 (100)	4 (100)	2 (50)	3 (75)	4 (100)	2 (50)	4 (100)	3 (75)	2 (50)	1(25)	2(50)
<i>Microbacterium aurum</i>	1	1 (100)	1(100)	0	1 (100)	0	0	0	1 (100)	0	0	0
<i>Tahibacter aquaticus</i>	1	–	–	1 (100)	0	1 (100)	0	0	1 (100)	0	0	1(100)
<i>Fictibacillus phosphorivorans</i>	5	5 (100)	5 (100)	5 (100)	5 (100)	4 (80)	5 (100)	5 (100)	5 (100)	5 (100)	0	5(100)
<i>Fictibacillus aquaticus</i>	2	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	0	2(100)
<i>Stenotrophomonas maltophilia</i>	17	–	–	17 (100)	17 (100)	17 (100)	17 (100)	17 (100)	17 (100)	15 (88)	17(100)	10(59)
<i>Sphingopyxis soli</i>	2	–	–	1 (50)	1 (50)	2 (100)	1 (50)	1 (50)	1 (50)	0	1 (50)	0
<i>Sphingopyxis bauzanensis</i>	1	–	–	1 (100)	0	1 (100)	0	0	1 (100)	1 (100)	1 (100)	0
<i>Exiguobacterium mexicanum</i>	2	–	–	1 (50)	0	0	0	0	1 (50)	1 (50)	1 (50)	0
<i>Paenibacillus lautus</i>	1	1(100)	1 (100)	0	1 (100)	0	1 (100)	0	1 (100)	1 (100)	1 (100)	1(100)
<i>Paenibacillus yonginensis</i>	1	1 (100)	1 (100)	1 (100)	0	1 (100)	0	1 (100)	0	1 (100)	1 (100)	1(100)
<i>Paenibacillus glucanolyticus</i>	1	1 (100)	1 (100)	1 (100)	0	0	1 (100)	1 (100)	0	0	0	1(100)
<i>Xanthomonas floridensis</i>	1	–	–	0	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	0
<i>Lysobacter hankyongensis</i>	1	–	–	1 (100)	0	1 (100)	0	1 (100)	1 (100)	1 (100)	0	0
<i>Acinetobacter kookii</i>	4	–	–	2 (50)	4 (100)	4 (100)	2 (50)	4 (100)	2 (50)	2 (50)	2(50)	0
<i>Acinetobacter johnsonii</i>	2	–	–	1 (50)	0	1 (50)	0	4 (100)	0	2 (100)	0	0
<i>Acinetobacter lwoffii</i>	1	–	–	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	0

<i>Vogesella facilis</i>	3	—	—	2 (67)	3 (100)	2 (67)	2 (67)	3 (100)	3 (100)	2 (67)	0	0
<i>Vogesella perlucida</i>	1	—	—	1 (100)	1 (100)	0	1 (100)	0	1 (100)	0	0	0
<i>Elstera litoralis</i>	1	—	—	0	0	1 (100)	0	0	0	0	1(100)	0
<i>Ensifer adhaerens</i>	2	—	—	0	1 (50)	1 (50)	0	0	1 (50)	0	1(50)	0
<i>Caulobacter segnis</i>	1	—	—	0	0	1 (100)	0	1 (100)	0	0	1(100)	0
<i>Chryseobacterium phosphatilyticum</i>	1	—	—	1 (100)	0	1 (100)	0	1 (100)	0	0	1(100)	0
<i>Chryseobacterium cucumeris</i>	1	—	—	0	1 (100)	1 (100)	0	1 (100)	0	0	1(100)	0
<i>Flavobacterium aquidurens</i>	1	—	—	0	1 (100)	1 (100)	0	1 (100)	0	0	1(100)	0
<i>Sphingobium yanoikuya</i>	1	—	—	0	0	1 (100)	0	1 (100)	0	0	1(100)	0
<i>Brevibacterium frigoritolerans</i>	1	—	—	0	0	1 (100)	0	1 (100)	0	0	1(100)	0
<i>Hydrogenophaga caeni</i>	1	—	—	0	0	1 (100)	0	1 (100)	0	0	1(100)	0
<i>Nevskia aquatilis</i>	1	—	—	0	0	1 (100)	0	1 (100)	0	0	1(100)	0
<i>Lysinibacillus sinduriensis</i>	1	1 (100)	1 (100)	0	0	1 (100)	0	1 (100)	0	0	1(100)	0
<i>Aeromonas molluscorum</i>	1	—	—	0	0	1 (100)	0	1 (100)	0	0	1(100)	0
<i>Chitinimonas viridis</i>	2	—	—	1(50)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)	1(50)	1(50)
Total	118 (100)	41 (35)	41 (35)	79 (64)	84 (71)	95 (81)	74 (63)	91 (77)	80 (68)	64 (54)	64 (54)	36 (31)

Amp- Ampicillin (10 µg), **C** – Chloramphenicol (30 µg), **CIP** – Ciprofloxacin (5 µg), **E** – Erythromycin (15 µg), **NE** – Neomycin (30 µg), **OT** - Oxy-tetracycline (30 µg), **S** – Streptomycin (25 µg), **W** – Trimethoprim (5 µg), **Amo** – Amoxicillin (30 µg), **VA**-Vancomycin (30 µg), **PEN**-Penicillin (10 µg) *Percentage (%) was determined as a function of the number of isolates of the coliform spp., thus (n/(N) x 100).

4.7 Characterisation of isolates

4.7.2 Multiple antibiotic-resistance phenotype, antibiotic-resistance genes and virulence genes of the coliforms identified in this study

The phenotypic and genotypic characteristics of coliforms are summarized in *Table 4.7* below. All 26 coliforms were subjected to antibiotic susceptibility testing as well as detection of ARG's and virulence genes. The MAR phenotypes of isolates resistant to three or more antibiotics were noted. A total of 7 MAR phenotypes were observed in the screened coliform isolates. One hundred percent (7/7) of the MAR phenotypes observed in the coliform bacterial group comprised ampicillin and oxy-tetracycline. Additionally, 86% (6/7) comprised amoxicillin and chloramphenicol while 71% (5/7) comprised ciprofloxacin. Two *Escherichia fergusonii* isolates were resistant to eight antibiotics and harboured four virulence genes (*stx1*, *stx2*, *hlyA* and *eaeA*). One of these isolates (OKO30978.1) harboured the *ermB* resistance gene. Only one *Citrobacter freundii* isolate was resistant to three or more antibiotics (AMP-C-CIP-OT-W). One *Enterobacter cloacae* (OK030995.1) showed resistance to six antibiotics (AMO-AMP-C-CIP-OT-W) and harboured three virulence genes (*stx1*, *stx2* and *eaeA*). Whereas another *E. cloacae* isolate was resistant to five antibiotics (AMO-AMP-C-CIP-OT) and harboured the *bla*_{TEM}, *ampC* resistance genes. One *Klebsiella grimontii* also harboured the *ampC* and *bla*_{TEM} gene but also the *int1* gene.

4.7.3 Multiple antibiotic-resistance phenotype, antibiotic-resistance genes and virulence genes of the *Enterococcus* spp. identified in this study

The phenotypic and genotypic characteristics of *Enterococcus* spp. are summarized in *Table 4.8* below. All 44 *Enterococcus* spp. were subjected to antibiotic susceptibility testing as well as detection of ARG's and virulence genes. A total of 8 MAR phenotypes were observed in the screened *Enterococcus* spp. group. One hundred percent (8/8) of the MAR phenotypes observed in *Enterococcus* spp. bacterial group comprised of vancomycin and penicillin. Additionally, 88% (7/8) comprised chloramphenicol and ampicillin, while 50% (4/8) comprised ciprofloxacin. In total, seven isolates were resistant to all 11 antibiotics. These isolates included one *Enterococcus saigonensis*, two *Enterococcus hirae*, three *Enterococcus gallinarum* and one *Enterococcus faecalis*. Furthermore, five *Enterococcus* spp. harboured the *ampC* gene: three *Enterococcus casseliflavus* isolates, one *Enterococcus hirae* and one *Enterococcus saigonensis*. The *Enterococcus casseliflavus* isolate (MZ675459.1) which harboured the *ampC* gene, also harboured *gelE* and *asa1*

virulence gene. One *Enterococcus hirae* (MZ680553.1) which showed resistance to all 11 antibiotics harboured four antibiotic-resistance genes, *bla*_{TEM}, *int1*, *ampC* and *ermB*. Whilst a *Enterococcus saigonensis* isolate also showed resistance to 11 antibiotics and harboured three antibiotic-resistance genes *Int1*, *ampC*, *tetM*. Lastly, a *Enterococcus gallinarum* (MZ675447.1) isolate and one *Enterococcus hirae* (MZ675547.1) harboured the *hly* and *cylA* virulence genes.

Table 4.7: Phenotypic and genotypic characteristics of coliforms isolated from groundwater systems of interest

Species	Antibiotic-resistance phenotype	Isolate accession number	Antibiotic-resistance genes	Virulence genes
<i>Escherichia fergusonii</i>	AMO-AMP-C-CIP-E-OT	OK030997.1		<i>stx1, stx2, hlyA, eaeA</i>
	AMO-AMP-C-E-NE-OT-W-S	OK030988.1		<i>stx1, stx2, hlyA, eaeA</i>
		OK030978.1	<i>ermB</i>	
	AMO-AMP-C-CIP-E-OT-W	OK030994.1		
		OK030991.1		
	AMO-AMP-C-CIP-OT	OK030989.1		
<i>Citrobacter freundii</i>	AMP-C-CIP-OT-W	OK030996.1		
<i>Enterobacter cloacae</i>	AMO-AMP-C-CIP-OT-W	OK030995.1		<i>stx1, stx2, eaeA</i>
	AMO-AMP-C-CIP-OT	OK030980.1	<i>bla_{TEM}, ampC</i>	
<i>Klebsiella grimontii</i>	AMO-AMP-OT-W	OK030998.1		<i>stx1, hlyA, eaeA</i>
	AMO-AMP-C-CIP-OT	OK030999.1	<i>bla_{TEM}, int1, ampC</i>	

Amp – Ampicillin (10 µg), **C** – Chloramphenicol (30 µg), **CIP** – Ciprofloxacin (5 µg), **E** – Erythromycin (15 µg), **NE** – Neomycin (30 µg), **OT** – Oxy-tetracycline (30 µg), **S** – Streptomycin (25 µg), **W** – Trimethoprim (5 µg), **Amo** – Amoxicillin (30 µg), **VA** – Vancomycin (30 µg), **PEN** – Penicillin (10 µg).

Table 4.8 – Phenotypic and genotypic characteristics of *Enterococcus* spp. isolated from groundwater systems of interest.

Species	Antibiotic-resistance phenotype	Isolate accession number	Antibiotic-resistance genes	Virulence genes
<i>Enterococcus faecalis</i>	AMO-AMP-C-CIP-OT-PEN-S-VA-W	MZ675458.1		
		MZ675449.1		
		MZ675451.1		
		MZ676459.1	<i>ermB</i> , <i>tetM</i>	
	AMO-AMP-C-CIP-E-NE-OT-PEN-VA-W	MZ675445.1		
		MZ675454.1		
	AMO-AMP-C-CIP-E-NE-OT-PEN-S-VA-W	MZ680541.1		
	AMO-AMP-C-OT-PEN-S-VA-W	MZ680532.1		
<i>Enterococcus casseliflavus</i>	AMO-AMP-C-E-NE-OT-PEN-VA-W	MZ675446.1		
		MZ680533.1		
		MZ680554.1		
		MZ680551.1		
		MZ675451.1	<i>ampC</i>	
		MZ675459.1	<i>ampC</i>	<i>gelE</i> , <i>asa1</i>
	AMP-C-CIP-E-OT-PEN-VA-W	MZ675457.1		
	AMO-AMP-C-CIP-E-NE-OT-PEN-VAN-W	MZ680534.1		
		MZ675450.1	<i>ampC</i>	
	AMO-AMP-E-NE-OT-PEN-W	MZ680543.1		
<i>Enterococcus gallinarum</i>	AMO-AMP-C-CIP-E-NE-OT-PEN-S-VA-W	MZ675452.1		
		MZ675456.1		
		MZ680537.1		
	AMO-AMP-C-E-NE-OT-PEN-S-VA-W	MZ675447.1		<i>hly</i> , <i>cylA</i>
<i>Enterococcus hirae</i>	AMO-AMP-C-CIP-E-NE-OT-PEN-S-VA-W	MZ680553.1	<i>bla_{TEM}</i> , <i>int1</i> , <i>ampC</i> , <i>ermB</i>	
		MZ675547.1		<i>hly</i> , <i>cylA</i>
<i>Enterococcus saigonensis</i>	AMO-AMP-C-CIP-E-NE-OT-PEN-S-VA-W	MZ680538.1	<i>Int1</i> , <i>ampC</i> , <i>tetM</i>	

Amp- Ampicillin (10 µg), **C** – Chloramphenicol (30 µg), **CIP** – Ciprofloxacin (5 µg), **E** – Erythromycin (15 µg), **NE** – Neomycin (30 µg), **OT** - Oxy-tetracycline (30 µg), **S** – Streptomycin (25 µg), **W** – Trimethoprim (5 µg), **Amo** – Amoxicillin (30 µg), **VA**-Vancomycin (30 µg), **PEN**-Penicillin (10 µg).

4.7.4 Multiple antibiotic-resistance phenotype, antibiotic-resistance genes of the HPC isolates identified in this study

The phenotypic and genotypic characteristics of HPC bacteria are summarized in *Table 4.9* below. All 118 HPC bacteria were subjected to antibiotic susceptibility testing as well as detection of ARG's. In totality, 11 MAR phenotypes were observed in the screened HPC bacterial group. Additionally, 91% (10/11) of the observed phenotypes comprised ampicillin whilst 82% (9/11) comprised amoxicillin and chloramphenicol. In total, five isolates were resistant to all 11 antibiotics. These isolates included three *Fictibacillus phosphorivans*, one *Bacillus simplex*, and one *Bacillus safensis*. Furthermore, the following 11 MAR HPC isolates harboured the *ampC* antibiotic-resistance gene: two *Stenotrophomonas maltophilia*, one *Bacillus simplex*, one *Bacillus halotolerans*, one *Bacillus safensis*, two *Bacillus cereus*, one *Fictibacillus phosphorivans*, one *Chitinimonas viridis*, one *Vogesella facilis* as well as one *Pseudomonas songnenensis*. Two *Fictibacillus phosphorivans* isolates were resistant to 11 antibiotics (AMO-AMP-C-CIP-E-NE-OT-PEN-S-VA-W) and harboured the *ermF* antibiotic-resistance gene. Additionally, one of the latter isolates (OK065623.1) harboured the *ampC* resistance gene. Furthermore, *Bacillus simplex* displaying a MAR phenotype was resistant to 11 antibiotics and harboured three antibiotic-resistance genes: *bla_{TEM}*, *ampC*, *tetM*. Lastly, one *Stenotrophomonas maltophilia* (OK065840.1) and one *Acinetobacter kookii* (OK065826.1) harboured the *int1* antibiotic-resistance gene.

Table 4.9 – Phenotypic and genotypic characteristics of HPC bacteria isolated from groundwater systems of interest.

Species	Antibiotic-resistance phenotype	Isolate accession number	Antibiotic-resistance genes
<i>Bacillus simplex</i>	AMO-AMP-C-CIP-E-NE-OT-PEN-S-VA-W	OK065837.1	<i>bla_{TEM}</i> , <i>ampC</i> , <i>tetM</i>
<i>Bacillus halotolerans</i>	AMP-C-CIP-E-OT-PEN-VA-W	OK087658.1	<i>ampC</i>
<i>Bacillus safensis</i>	AMO-AMP-C-CIP-E-NE-OT-PEN-S-VA-W	OK087638.1	<i>ampC</i>
<i>Bacillus cereus</i>	AMO-AMP-C-E-NE-OT-PEN-S-VA-W	OK087670.1	<i>ampC</i>
		OK087655.1	<i>bla_{TEM}</i> , <i>ampC</i> , <i>tetM</i>
<i>Acinetobater kookii</i>	AMO-AMP-C-CIP-E-OT-S-W	OK065826.1	<i>int1</i>
<i>Acinetobacter lwoffii</i>	AMO-AMP-C-CIP-E-NE-OT-S-W	OK065647.1	<i>ermB</i>
<i>Stenotrophomonas maltophilia</i>	AMO-AMP-C-E-NE-OT-S-W	OK065835.1	
		OK065836.1	
		OK065842.1	
	AMO-AMP-C-CIP-E-NE-OT-S-W	OK065650.1	
		OK065843.1	
		OK065830.1	<i>ampC</i> , <i>tetM</i> , <i>ermF</i>
		OK065840.1	<i>int1</i> , <i>tetM</i>
		OK065827.1	<i>ampC</i> , <i>ermB</i> , <i>tetM</i> , <i>ermF</i>
		OK065825.1	
		OK065629.1	
		OK065622.1	
		OK087634.1	
		OK087662.1	
		OK065653.1	
<i>Fictibacillus phosphorivans</i>	AMO-AMP-C-CIP-E-NE-OT-PEN-S-VA-W	OK065844.1	
		OK065621.1	<i>ermF</i>
		OK065623.1	<i>ampC</i> , <i>ermF</i>
<i>Chitinimonas viridis</i>	AMO-AMP-C-CIP-E-OT-S-W	OK065836.1	<i>ampC</i> , <i>ermF</i>
<i>Sphingopyxis soli</i>	AMO-C-CIP-E-OT-W	OK065834.1	
<i>Brevundimonas albigilva</i>	AMO-AMP-C-CIP-E-NE-OT-W	OK065838.1	
<i>Brevundimonas aurantiaca</i>	AMO-AMP-C-CIP-E-NE-OT-S-W	OK065652.1	
<i>Vogesella facilis</i>	AMP-CIP-E-OT-S-W	OK065650.1	<i>ampC</i>
<i>Pseudomonas geniculata</i>	AMO-AMP-OT-S-W	OK065648.1	
<i>Pseudomonas songnenensis</i>	AMO-AMP-C-CIP-E-OT-S-W	OK087538.1	<i>bla_{TEM}</i> , <i>ampC</i>

Amp- Ampicillin (10 µg), **C** – Chloramphenicol (30 µg), **CIP** – Ciprofloxacin (5 µg), **E** – Erythromycin (15 µg), **NE** – Neomycin (30 µg), **OT** - Oxy-tetracycline (30 µg), **S** – Streptomycin (25 µg), **W** – Trimethoprim (5 µg), **Amo** – Amoxicillin (30 µg), **VA**-Vancomycin (30 µg), **PEN**-Penicillin (10 µg).

CHAPTER 5

Discussion

Introduction

The Department of Water and Sanitation has recently reported that the dam levels in South Africa are decreasing, thus the dependence on other sources of water such as groundwater is increasing (DWS, 2021). Groundwater is an important source of freshwater particularly in developing countries such as Malawi, Nigeria, Lesotho, Mozambique and South Africa (Adeogun *et al.*, 2015; Aladejana *et al.*, 2020). To guarantee safe consumption, this source of water must meet a certain standard of quality based on the physico-chemical and microbial composition (Ferreira, 2011; Aladejana *et al.*, 2020; Nwankwo *et al.*, 2020). The results obtained in the previous chapter will be discussed in order to ascertain if the water quality from the evaluated groundwater systems is safe for use for various purposes. The quality of the physico-chemical and microbial parameters of groundwater will be linked to several activities done in the area. The health risk to humans and animals utilising and being exposed to these groundwater systems will be addressed, taking into consideration the existence of possible pathogens and observed species with potentially virulent characteristics.

In this study, the groundwater at several sites (DK, BF, LS, LV, H, FD, SG and GM) is used for irrigation; while the groundwater at the private farms (S1, S2, S3, S4, S5, S6 and S7) is used for irrigation, livestock watering and drinking purposes.

5.1 Physico-chemical parameters

5.1.1 Temperature

One of the most important characteristics of groundwater is temperature which influences its ecological processes (Ali and Ali, 2018). Several studies have stated that groundwater temperature varies from time, space and depth (Horton *et al.*, 2001; Tague *et al.*, 2007; Bethke and Johnson, 2008). Furthermore, groundwater temperature changes which occur over time can highlight the potential aftereffects of climate change and urbanisation (Green, 2016). To date there is no guideline for the suitable temperature for groundwater. Nonetheless, the groundwater temperature of the systems of interest increased as the seasons got warmer. A total of five sampled sites had temperatures greater than 20 °C in

autumn. Similar observations were reported in several studies across the world (Gunarathna *et al.*, 2016, Vadde *et al.*, 2018 Graff and Neidell, 2020; Mabeo, 2020). According to Aladejana *et al.* (2020) higher groundwater temperatures during the dry season may be attributed to evaporation and transpiration. This is because climate change can impact soil infiltration thus resulting in deeper percolation which facilitates groundwater recharge from the hydrological cycle (Green, 2016). Allen *et al.* (2004a) further explains that climate change not only affects the quantity but also the quality of groundwater. As sea levels rise, salt water may infiltrate coastal aquifers, influencing groundwater quality and pollute drinking water sources (Allen *et al.*, 2004b).

The groundwater temperature during winter was cold, with the lowest temperature at 7.6 °C. Whilst, groundwater temperatures increased in summer and spring, reaching a maximum of 26.3 °C and 34.2 °C, respectively. The cold temperatures frequently observed in groundwater systems during the winter are affected by both air and ambient temperature (Schneider, 1961). According to Chen *et al.* (1999) cold irrigation water slows down the growth of plants. Drosch (2013) further explained that although crops have varying degrees of resiliency, if the temperature is too low, it reduces the plants enzyme activity and changes the fluidity of cellular membranes. Nonetheless, studies by Dong *et al.* (2016) and Fahlquist (2003) investigated the ideal water temperature for irrigation purposes and it was found to be between 17 °C - 25 °C. Thus, the groundwater temperature of most sites during autumn, summer and spring was suitable for irrigation and allowed for optimum crop yield. However, the temperature has been identified as a significant factor influencing bacterial pathogen emergence (Lammel *et al.*, 2018).

5.1.2 Total Dissolved Solids (TDS)

Total dissolved solids are used to assess the quantity of solid materials dissolved in water (Sluiter *et al.*, 2008). In this study, high TDS levels (> 450 ppm) were observed at most sites where water is used for drinking purposes, livestock watering and irrigation throughout the four seasons. A study by Cui *et al.* (2019) reported similar observations in selected groundwater systems of China. The high levels of TDS in the groundwater systems of interest may be a result of the continued use of fertilizers, presence of animal faeces, leaching of salts from the soil and possibly, discharge of domestic waste. Atherholt *et al.* (2017) explain that dissolved solids are a result of water's contact with its surrounding during its movement from source to tap, or through incidental sources. Furthermore, Morris *et al.* (2003) explained that organic substances can come from local or agricultural runoff,

industrial effluent, sewage, chemical products used in water purification, carbonate or salt deposits, seawater intrusion, storm-water runoff, or even the water pipes that run beneath towns.

According to DWAF (1996) water with TDS levels greater than 1000 ppm/L, does not have any adverse short-term effects on the consumer. However, Chang (2005) explained that high TDS levels have a negative impact on the central nervous system of humans by causing irritability, dizziness as well as paralysis of the tongue, lips and face. In addition, Increased TDS levels cause toxicity by increasing the salinity of the water body, increasing ionic composition, and increasing the toxicity of individual ions (Cimanga, 2017). According to the WHO (2016), when TDS exceeds 3000 ppm/L corrosion and scaling occurs on water pipes as well as kitchen utensils, which can result in extremely salty and bitter tasting water. Furthermore, the recognized acceptable TDS TWQR for water used for livestock watering and irrigation, is 0-2000 ppm/L and 0-3000 ppm/L, respectively (DWAF, 1996). Thus, the TDS results obtained in this study show that the groundwater is suitable for livestock watering and irrigation. Nonetheless, cautionary measures such as filtration systems should be put into place where the groundwater is used for consumption and domestic purposes.

5.1.3 Salinity

Salinity is a global issue, but it is especially severe in water-stressed arid and semi-arid regions where groundwater is the primary source of water (Hussain *et al.*, 2019). In arid and semi-arid climates, the materials of mineral and rock erosion accumulate in the soil, resulting in the development of high saline soils (Leidonald *et al.*, 2019). The salinity results in this study ranged from 61.2 – 855 ppm. Overall, the high salinity levels were observed in autumn and winter. Salinity levels within similar ranges were reported by Mabeo (2020) and Yan *et al.* (2015). According to Van Weert *et al.* (2009), evaporation does not occur optimally during colder seasons thus, groundwater becomes enriched with minerals that result in increased salinity. Banks *et al.* (2004) further stated that anthropogenic pollutants such as fertilizers, agricultural and industrial effluent are some of the factors leading to high salt content in groundwater systems.

Extremely high salinity levels (reaching 855 ppm) were measured in groundwater used for irrigation and livestock watering. According to Dunlop *et al.* (2005), the salinization of water has a negative impact on the quality of drinking and irrigation water, with significant economic, social and environmental implications for both rural and urban communities. Furthermore, high salt concentrations may have an impact on the taste of drinking water

(Irvine and Doughton, 2001). Whilst, sodium and magnesium sulfate levels in drinking water can have a laxative effect and make the water supply unsuitable for grazing livestock. Thus, the use of groundwater, particularly in sites where high levels were measured, should be used with caution as the water can contain toxins harmful to animal health and even render milk or meat unsuitable for human consumption (Mokhtar *et al.*, 2014). In these cases, supplying a substitute high-quality water supply could provide an alternative measure.

5.1.4 pH

The pH of a groundwater system can be a beneficial indicator of water quality because it specifies the viability of water for various purposes (Nqowana *et al.*, 2016). Furthermore, the pH of water becomes more apparent when water tastes soapy due to it being alkaline or bitter as a result of its acidity (Banks, 2004). According to Lammel *et al.* (2018) pH has a direct influence on the microbial community in soil and water. The data obtained in the latter study revealed that the higher pH levels result in more diverse microbial communities. Also, pH is a significant parameter because it can be used to determine whether water has been contaminated by chemicals (Jähne *et al.* 1987). Furthermore, the pH of water can be used to establish the absorption and biological nutrient as well as heavy metals found in the environment (Stewart *et al.*, 2019). In most cases, the emission of pollutants changes the pH of groundwater (Patni *et al.*, 1998; Martins *et al.*, 2004; Gunarathna *et al.*, 2016).

The known pH range of groundwater is 6.5 - 8.5, according to the various standards proposed by the WHO (WHO, 2007). In this study, the pH of all the groundwater systems of interest, across all seasons, were within the TWQR guidelines (5.0 - 9.5) and suitable for irrigation (6.5 - 8.5). These results were similar to those observed by Ferreira (2011). The pH results obtained in this study were also within the SANS:241 standards for drinking water (5.0-9.7). Despite pH having no direct impact on water consumers, it is one of the most significant operational water quality indices and should be monitored regularly in sites where water is used for drinking purposes. This is particularly important as the pH levels in drinking water should always remain between 6.5-8.5 so as to prevent disturbances that may occur during the production of vitamins and minerals in the human body (Gupta *et al.*, 2017). Thus, monitoring of water sources used for domestic purposes requires adequate water monitoring, disinfection and quality control (WHO, 2007).

5.1.5 Chemical oxygen demand

Chemical Oxygen Demand relates to the amount of oxygen required for the chemical oxidation of organic material in water (Christensen *et al.*, 1992). Furthermore, the COD levels of water also highlight the strength of pollutants in water and could be utilized as an organic pollution marker (Koda *et al.*, 2017). The levels of COD alternated throughout all the sites, whilst an increase in COD levels was observed with the warming of temperatures. The COD levels ranged between 0.33 mg/L - 111 mg/L. This is in agreement with the findings of Kritzinger (2019) and El-Salam *et al.* (2015). According to Wu *et al.* (2011) high COD levels can be positively correlated to contaminated water. Furthermore, a study by El-Salam *et al.* (2015), suggested that landfill leachates are the primary contaminant influencing CODs in groundwater systems. Thus, when considering the physical location of all sampled groundwater sites, the COD observations in this current study could be attributed to high rainfall levels which occurred during the warmer seasons resulting in the transport and leaching of pollutants from urban watersheds and agricultural fields into the groundwater systems.

5.1.6 Phosphates

Phosphates are nutrients found in water that are necessary for the growth of both plants and microorganisms (Douterelo *et al.*, 2020). Phosphorus is a strong oxidizing agent that is required for life and forms part of a wide range of compounds in both aquatic and terrestrial ecosystems (Tao *et al.*, 2020). Furthermore, phosphorus can be found in water as an orthophosphate ion; and it can also be found in all living things as an essential component of cellular material (Holman *et al.*, 2008). All measured phosphate levels in this research study were less than 5 mg/L, in all seasons. A study by Fadiran *et al.* (2008) reported similar phosphate levels in both surface water and groundwater. The presence of phosphates in the assessed groundwater systems can be attributed to the agricultural activities that occur in the area. According to Sharpley *et al.* (2003), numerous fertilizers used in modern agriculture contain high levels of plant nutrients such as nitrogen and phosphorus, which help to increase crop yield. Also, fertilizers and pesticides do not remain static in the environment where they are applied; instead, runoff and infiltration transport these pollutants into nearby streams, rivers, and groundwater (Giri, 2018). Additionally, Domagalski and Johnson (2012) explain that phosphorus can also be sourced from the decay of animals and plant tissues in natural ecosystems

5.1.7 Nitrates

Nitrate is one of the most prevalent groundwater pollutants in rural areas (Yu *et al.*, 2020). The groundwater nitrate levels in this study ranged from 0.12 mg/L - 20.23 mg/L. Furthermore, generally low nitrate values were observed, except at site S6 during autumn, 20.23 mg/L. The nitrate levels at this site exceeded the TWQR, <10 mg/L. Also, moderately elevated nitrate levels were observed in private farms where small scale farming occurs. According to Yu *et al.* (2020), agricultural activities, particularly those involving fertilizers and animal waste deposited in the soil, have an impact on nitrate levels in groundwater. This explains the high nitrate value at site S6, because it is a farm area with considerable activities and agricultural as well as domestic animals. Additionally, the majority of the groundwater systems were beneath small scale farmlands that are constantly fertilized. Furthermore, Sasakova *et al.* (2018), the other sources of nitrate in groundwater include sewer tanks and manure storage, as well as anthropogenic activities. Under oxidizing conditions, nitrate predominates in surface water and groundwater, while ammonium ions predominate under reducing conditions (Abiriga *et al.*, 2020). When groundwater or surface water comes into close interaction with wastewater, septic tanks, or sewage systems, this is common (Aladejana *et al.*, 2020). Farming leachates from fertilizer application and animal waste are also sources of nitrate in groundwater systems (Patni *et al.*, 1998; White *et al.*, 2012; Zhang *et al.*, 2014).

Jordaan and Bezuidenhout (2016) conducted a study for the Water Research Commission that documented the effects of physico-chemical parameters on the water systems in the North West Province. The mentioned study revealed that the high nitrate and nitrite levels in the province are caused by the leaching of livestock manure, pesticide residues, herbicides, remnant manure, and other agricultural residues into groundwater. Another study by Ntshangase (2019) recorded nitrate levels greater than 20 mg/L in water samples in the North West Province. High nitrite and nitrate levels are also associated with numerous health risks in humans, most notably certain cancers. Therefore, it is important for chemical levels in groundwater to be continually assessed particularly where water is used for consumption purposes.

5.2 Microbial water quality analysis

Microbiological water assessment is a technique for examining water in order to estimate and identify the number of bacteria present in water (Sandal, 2016). The microbial analysis of water is important in order to control the growth of microorganisms (Hou *et al.*, 2017).

Furthermore, microbial quality is surveilled in both drinking and recreational waters (Pandey *et al.*, 2018). Indicator microbes such as coliforms, faecal streptococci, enterococci, clostridia, HPC bacteria and bacteriophages are some of the indicators of microbial quality used worldwide (WHO, 2013; WHO, 2016). A microbiological water quality indicator is typically one particular species or a group of microorganisms that can gain entry into water via faeces but are easier to assess than the entire array of microorganisms that present a hazard to the public health (Wen *et al.*, 2020). Environmental pollution and municipal wastes have emerged as major sources of pollution in surface and groundwater (Patil *et al.*, 2012). Consistent faecal contamination in source water is a worldwide problem that is especially crippling in rural communities that rely on untreated source water for all of their domestic and other needs (Bezuidenhout *et al.*, 2002; Edokpayi *et al.*, 2018; Odonkor and Ampofo, 2020). Therefore, in this study, selected groundwater systems were surveyed for the presence of coliforms, enterococci and HPC bacteria to determine its suitability for use.

5.2.1 Coliforms

Total and faecal coliform bacteria are widely utilized as indicators of contamination in water quality assessments across the globe (Boehm and Sassoubre, 2014; Bigham *et al.*, 2019). Additionally, coliforms were selected as one of the microbial indicator organisms in this study since they are, to date, the only water quality microbial indicator organism monitored in South Africa (Bezuidenhout and Adeleke, 2011; Luyt *et al.*, 2012). The present study assessed the microbial quality of groundwater used for irrigation, livestock watering and domestic purposes. The target water quality range for the acceptable level of coliform for irrigation and livestock watering (<1 cfu/100ml) and (<200 cfu/100ml) respectively. The guideline for the suitable number of coliforms in water used for human consumption is between 0-5 cfu/100ml, anything greater than 5 cfu/100ml is indicative of insufficient treatment, post-treatment pollutants, or distribution system growth (WHO, 1996). The SANS 241 (2015) standard for drinking water is 0 cfu/100 mL and all groundwater sites that harboured coliforms in the present study did not adhere to this standard. Sites S3, S4, S5, S6, GM, DK, H, BG and FD had coliform levels that exceeded the acceptable coliform level for irrigation. Additionally, the coliform level at sites S5 and S6 exceeded the acceptable coliform level for livestock watering.

In this study, the most frequent coliform contamination was mostly identified in areas frequented by cattle (S3, S4, S5, S6). The microbial community in water sources such as groundwater can be influenced by biological contaminants such as manure and septic

systems (Burri *et al.*, 2019). Furthermore, Pokhrel (2020) explains that excreta from livestock contains significant amounts of nutrients, pathogens and antibiotics. According to Cabral (2010), total coliforms are bacteria found in soil and groundwater which has been impacted by surface water and human or animal waste, whilst faecal coliform bacteria are found in the intestines of homothermic animals (Rochelle-Newall, 2015). Thus, similar to the findings of other studies, the presence of coliforms in groundwater illustrates contamination could have resulted from faecal matter pollution (Entry and Farmer, 2001; Odonkor, 2013, Scharping and Garey, 2021). This is also supported by results observed during this study where coliform levels increased and were the highest during the warm rainy seasons. As a result, leaching could have resulted in a higher load of microorganisms in groundwater during rainfall events.

Water temperature of above 15 °C is ideal for the growth of coliforms (LeChevallier, 2003). The seasonal observations of higher coliform levels during the warm rainy seasons (summer and spring) are similar to the findings of several other studies (Chigbu *et al.*, 2005; Hong *et al.*, 2010; Atherholt *et al.*, 2017). Also, the low levels of coliforms during the colder months could be a result of the microorganisms entering the viable but non-culturable state (Atherholt *et al.*, 2017). Nonetheless, the presence of coliforms in groundwater systems used for domestic purposes is a cause for concern.

5.2.2 *Enterococcus* spp.

Enterococcus species are Gram-positive, facultative anaerobic cocci that look like streptococci when Gram stained (Faklam and Collins, 1989). Enterococci have been accepted as a measure of human faecal contamination of water as a result of their prevalence in human faeces as well as persistence in the environment (Byappanahalli *et al.*, 2012; Boehm and Sassoubre, 2014).

Consistently high levels of enterococci were observed at sites located at private farms near the Loopspruit River (S1, S5 and S6). Also, the highest *Enterococcus* spp. levels were witnessed at a site located upstream from the Loopspruit River where there is a cattle farm. These high *Enterococcus* spp. levels could be a result of the presence of agricultural activities and the presence of animals such as sheep and cattle. Numerous studies have reported the normal habitat of *Enterococcus* spp. to be the gastrointestinal tract of humans and other mammals (Cetinkaya *et al.*, 2000; Lebreton *et al.*, 2014). In addition, several studies in Brazil (Lebreton *et al.*, 2018), United Kingdom (Bradley *et al.*, 2018) and the United States (Kaur *et al.*, 2021) have reported high levels of enterococci in water systems close to

cattle farms. Lastly, the *Enterococcus* spp. levels measured in this study were highest during spring. Similar results were observed by Solaiman *et al.* (2020).

Another study has reported the presence of *Enterococcus* spp. from groundwater systems in the North West Province (Ateba and Maribeng, 2011). Also, high levels of enterococci have been isolated from other water sources in South Africa, such as wastewater (Iweriebor *et al.*, 2015; Molale-Tom and Bezuidenhout, 2020; Mbanga *et al.*, 2021). Although not determined in this study, the microbial quality of the Loopspruit River could also have an influence on the quality of groundwater in the surrounding aquifers. The physical interaction and exchange of groundwater and surface water at the groundwater/surface water interface is an essential component of the hydrological cycle (Conant *et al.*, 2019). Thus, the interconnectedness of the surface water in the Loopspruit River cannot be ignored when the groundwater in the assessed groundwater systems is considered. This is because some studies have reported the presence of *Enterococcus* spp. from surface water systems in the North West Province and internationally (Chigor *et al.*, 2013; Cho *et al.*, 2020; Molale-Tom and Bezuidenhout, 2020; Verlicchi and Grillini, 2020).

The presence of *Enterococcus* species in groundwater systems used for drinking purposes and livestock watering is worrisome when considering that *Enterococcus* spp. are responsible for numerous infections and diseases whilst some species are known to be resistant to multiple antibiotics (Molale and Bezuidenhout, 2020).

5.2.3 HPC bacteria

All bacteria that use organic nutrients for growth are classified as heterotrophic bacteria (Allen *et al.*, 2004). Relatively high heterotrophic plate count bacteria levels were consistently detected throughout this study and no evident seasonal difference was observed. This finding is similar to the findings of Sasakova *et al* (2018) and Nqowana *et al* (2017), who also reported high HPC bacterial levels in groundwater systems. Studies, similar to the current study, have reported the presence of pathogenic HPC bacteria isolated from South African water supplies (Pavlov *et al* 2004; Pal *et al.*, 2014). Ferreira (2011) proposed that HPC bacteria in drinking water may present a risk to consumers due to their obvious potentially pathogenic properties. The presence of HPC bacteria in the water is not indicative of pathogenic conditions, however, opportunistic pathogenic HPC infectious to humans like *Pseudomonas* and *Aeromonas* are a cause for concern (Amanidaz *et al.*, 2015). Numerous HPC bacteria found in water are currently unculturable; thus in order to obtain HPC results from a given water sample, the culture medium, temperature, and incubation time must be

carefully selected (Pavlov *et al.*, 2004). Furthermore, Huo *et al.* (2021), cautions that high HPC levels in water are a cause for concern as they indicate a higher rate of biofilm formation in the water system.

5.3 Correlation of the physico-chemical and microbiological parameters

The physico-chemical parameters that prevail within water sources play an important role in the maintenance and determination of the bacterial community in water (Rahmanian *et al.*, 2015). One of the factors used to determine microbial composition and distribution are physico-chemical parameters. Redundancy analysis plots were generated in this study to determine the relationship between the physico-chemical and microbiological parameters in groundwater systems of interest per season.

In the present study, there was no correlation between temperature and microbial levels, however, statistically significant correlations were observed in autumn between temperature and other physico-chemical parameters (TDS and pH). In summer, a significant correlation was noted between temperature and phosphates. These results are in accordance with those of Mihiu-Pintilie *et al.* (2014) and Shinde *et al.* (2012) where positive correlations were observed between various physico-chemical parameters. According to Amanidaz *et al.* (2015), temperature has an influence on the pH, TDS, nitrogen and phosphate levels of a water body. Also, pH is a significant parameter because it can be used to determine whether or not water has been contaminated by chemicals (Palamuleni *et al.*, 2015). Bacteria can grow under different pH conditions and most bacteria grow best at a pH of 6–7.5 (Dauqan *et al.*, 2011). The latter study stated that pathogenic bacteria can withstand extremely low and extremely high pH levels (Dauqan *et al.*, 2011). Nonetheless, no significant correlation was observed between pH and the microbial parameters identified in the current study.

A significant correlation was observed between coliform bacteria and TDS levels in autumn. According to Patel and Parikh, (2013), high TDS levels in water have the ability to degrade the quality of water by propagating potentially pathogenic bacteria. Thus, the positive correlation observed between coliform bacteria and TDS levels could be a result of favorable growth conditions.

In winter, a significant correlation was observed between coliform bacteria and nitrates. In addition, a positive correlation was also observed between HPC bacteria and coliforms. According to Caltagirone *et al.* (2017), coliforms such as *Klebsiella* have previously been identified in water, soil and plants. Additionally, several species within the *Klebsiella* genus

are well known nitrogen fixers (Lin *et al.*, 2019). Similarly, it is well documented that HPC bacteria such as *Bacillus* are nitrogen fixers (Yousuf *et al.*, 2017). Also, a similar significant correlation was observed between *Enterococcus* spp. and nitrates in summer and spring. Thus, although not determined in this study, the significant correlation observed between the coliform bacterial group to HPC's and nitrates as well as *Enterococcus* spp. and nitrates could be an indicator of potential nitrogen fixation and nitrogen metabolic process occurring in the groundwater systems.

Lastly, in summer, a significant correlation was observed between coliforms and COD as well as TDS. According to Seo *et al.* (2019), the presence of numerous bacteria from water with high COD suggests that the bacteria can thrive under stressful conditions. Chemical oxygen demand is an effective water quality indicator as it provides a metric for determining the impact of surrounding activities such as effluent on receiving bodies (Varol and Balci, 2020).

Although phosphate is not toxic to humans, high levels in freshwater environments can enhance microbial and algae growth, leading to algal blooms (Douterelo *et al.*, 2020). Several studies have revealed that phosphate in water can stimulate bacterial growth (Miettinen *et al.*, 1997; Jang *et al.*, 2012; Mei *et al.*, 2021). In this study, the correlation between phosphate and microbial levels was not witnessed. This is in agreement with studies by Kroll and Von Appen (2001) and Qaiser *et al.* (2018) where no correlation was observed between microbial and phosphate levels. Additionally, a study by Benson *et al.* (2011) observed higher levels of bacteria in lower temperatures as compared to higher temperatures. However, such correlations were not noted in the current study. Nonetheless, temperature is important for microbial enzymatic activities to occur, thus it is an essential parameter for microbial growth (Liu *et al.*, 2015).

5.4 Species diversity and phylogenetic association

The investigation of the evolutionary history of a species, a group of organisms, or a specific characteristic of an organism is known as phylogenetic analysis (Brinkman and Leipe, 2001). A phylogenetic tree was established using the 16S rRNA gene sequence data obtained from the study as well as reference species.

5.4.1 Coliform bacterial group

In autumn and winter, the only coliform that was identified was *Escherichia fergusonii* and it remained the most prominent coliform species throughout this study. According to Gaastra *et*

al. (2014), *Escherichia fergusonii* has optimum growth temperatures of 21°C – 45 °C under aerobic conditions. Gaastra et al. (2014) further explain that *Escherichia fergusonii* have illustrated the ability to grow at temperatures lower than 21°C but will not be able to replicate at temperatures below 11°C. This explains why *Escherichia fergusonii* species identified in this study were consistently identified throughout the four seasons.

According to Huys et al. (2003), *Escherichia fergusonii* is one of the seven species within the genus *Escherichia*. Several studies have revealed that *Escherichia fergusonii* is closely related to *Escherichia coli* and *Shigella spp.* (Adnan et al., 2013; Olowe et al., 2017). This species has also been identified from drinking water systems in Nigeria as well as intestines of warm-blooded animals (Freney et al., 1987; Savini et al., 2008; Olowe et al., 2017). According to Gaastra et al. (2014) and Parin et al. (2018) *Escherichia fergusonii* is an emerging opportunistic pathogen. According to the literature review conducted as part of this study, the current study is the first to report the isolation of *Escherichia fergusonii* from groundwater systems in South Africa. Thus, the presence of *Escherichia fergusonii* in water used for livestock watering and domestic purposes suggests that the water is not fit for use as this organism is a causal agent of diarrhea in animals and urinary tract infections in humans (Glover et al., 2017).

Four other coliform species were identified in addition to *Escherichia fergusonii*, these include: *Citrobacter freundii*, *Klebsiella grimontii*, *Citrobacter braaki* and *Enterobacter cloacea*. The increase in the diversity of coliforms from the colder seasons to the warmer seasons was also observed by Soon et al. (2014). A study by Schwab et al (2014) reported increased levels of Gram-negative bacteria during summer from clinical samples. The latter study identified *Escherichia coli*, *Enterobacter cloacea*, and *Citrobacter spp.* and concluded that these bacteria are most likely to occur during warmer seasons. A study by Waideman et al. (2020) linked the presence of *Citrobacter freundii* in water to public health concerns as it is a potential pathogen known to cause animal and human diarrhea. Thus the presence of these coliforms, particularly *Citrobacter freundii* in water used for domestic purposes and livestock watering is a cause for concern.

Additionally, the other identified coliforms, *Klebsiella spp.* and *Enterobacter spp.* are also known to be opportunistic pathogens and are usually associated with human infections (Passet and Brisse, 2018). Furthermore, *Klebsiella grimontii* has previously been reported in human blood samples of patients from France, Germany and South Africa (Liu et al., 2018). According to Hubbard et al. (2020), *Klebsiella grimontii* was previously thought to be a phylogroup of *Klebsiella oxytoca*, but it was discovered to be a unique pathogenic species of *Klebsiella* which is distinguished by the existence of the mobile genetic elements located on

the beta-lactamase gene. This explains why the two *Klebsiella grimontii* isolates included on the phylogenetic tree clustered together and were closest to the outgroup instead of the other coliforms. Hubbard *et al.* (2020) further reported that *Klebsiella grimontii* has the ability to form biofilms and is a species resistant to a wide array of antibiotics (Hubbard *et al.*, 2020).

In spring, a new coliform species, *Buttiarella agrestis*, was identified and this species is a Gram-negative, rod-shaped bacteria that can be found in insects, fish, soil and water (Antonello *et al.*, 2014). A study by Ntuli *et al.* (2016) reported the presence of *Buttiarella agrestis* from milk in South Africa whilst a study by Aijuka *et al.* (2015) reported cases of *Buttiarella agrestis* in irrigation water at very low levels. The presence of *Buttiarella agrestis* in groundwater systems used for drinking, irrigation and livestock watering is a cause for concern as this species is known to cause wound infections and inflammation in the appendix (Ssepuuya *et al.*, 2019). Furthermore, the presence of all coliforms identified in groundwater systems of interest proves that groundwater systems are not as pristine as they are presumed to be.

5.4.1.1 Phylogenetic association of coliforms

The phylogenetic relationship of partial 16S rRNA sequences revealed that the species presented in this study share a high degree of similarity. Two clusters were observed in this study (Cluster A and B). *Citrobacter* spp. and *Enterobacter cloacea* were under the same cluster with a bootstrap of 70%. These findings are similar to those reported by Zhang *et al.* (2015) and Iversen *et al.* (2020) where *Citrobacter* and *Enterobacter* from water samples were under the same cluster group and strongly related. Mandal *et al.* (2013) explains that *Citrobacter* and *Enterobacter* are categorized under the *Enterobacteriaceae* family and share phenotypic characteristics such as citrate utilization, oxidase negativity, and facultative anaerobic growth (Mandal *et al.* (2013). Interestingly, *Citrobacter freundii* and *Citrobacter braakii* only showed 35% relatedness. The latter results were different from the findings of Ture and Kutlu (2018) where *Citrobacter freundii* and *Citrobacter braaki* were closely related. Nonetheless, Wertz *et al.* (2003) illustrated that the nucleotide diversity of *Citrobacter freundii* is considerably high. This explains the low relatedness of the *Citrobacter freundii* and *Citrobacter braaki* isolates isolated in the current study.

5.4.2 *Enterococcus* spp.

Nine *Enterococcus* species were identified in this study: *Enterococcus casseliflavus*, *Enterococcus faecalis*, *Enterococcus gallinarum*, *Enterococcus villorum*, *Enterococcus hirae*, *Enterococcus mundtii*, *Enterococcus saigonensis*, *Enterococcus dispar* and *Enterococcus saccharolyticus*. The highest *Enterococcus* species diversity was observed in Spring. This could be attributed to warmer temperatures which allow for optimal growth. Nonetheless, some species were identified during the colder temperatures. *Enterococcus* spp. can withstand a variety of stressful environments, even those with extreme temperatures ranging between 5 to 65 °C (Fisher and Phillips, 2009). They can also survive in highly saline environments.

The most predominant *Enterococcus* spp. in the present study was *Enterococcus casseliflavus*. Sukhwani *et al* (2017) also reported *Enterococcus casseliflavus* in groundwater. There have been reports across the world of Enterococcal meningitis caused by invasion of *Enterococcus casseliflavus* in humans (Iaria *et al.*, 2005; Sukhwani *et al.*, 2017; Khan, 2021). This was a disease previously known to be caused by *Enterococcus faecium* and *Enterococcus faecalis*, however, more *Enterococcus* spp. are known to cause this disease to date (Khanum *et al.*, 2019). Additionally, *Enterococcus casseliflavus* has been found to cause bacteraemia in chronic renal disease patients (Vasilakopoulou *et al.*, 2020).

The second most prevalent species was *Enterococcus faecalis*. These findings are in conjunction with the findings of Ateba and Maribeng (2011). *Enterococcus faecalis* has a diverse host range and is the most broadly spread enterococcal species because it has been isolated from humans, animals, and the environment (Cho *et al.*, 2020). *Enterococcus faecalis* isolates in the present study were mostly identified in boreholes close to livestock. This suggests that the source of *Enterococcus faecalis* could be due to faecal matter from animals. The presence of enterococci, particularly *Enterococcus faecalis* in aquatic environments continues to pose a hazard to public health and should be carefully monitored. This is because *Enterococcus faecalis* and *Enterococcus faecium* are known to cause clinical infections (Adenjii *et al.*, 2021). The presence of *Enterococcus faecalis* in groundwater has been identified in South Africa (Ateba and Maribeng, 2011; Montwedi *et al.*, 2018). The latter studies have revealed that *Enterococcus faecalis* was resistant to a large array of antibiotics. Considering the mentioned, the presence of this bacteria is alarming.

Research suggests that *Enterococcus casseliflavus*, *Enterococcus gallinarum*, *Enterococcus hirae* and *Enterococcus mundtii* are some of the bacteria associated with human infections (Park *et al.*, 2018). Some of the human infections associated with *Enterococcus casseliflavus*

and *Enterococcus gallinarum* include abdominal infections, urinary tract infection and pneumonia (Reid *et al.*, 2001; Monticelli *et al.*, 2018). There have been reports of patients dying from *Enterococcus casseliflavus* bacteremia and *Enterococcus gallinarum* bacteremia related infections (Reid *et al.*, 2001; Choi *et al.*, 2004, Ryu *et al.*, 2019). Moreover, *Enterococcus hirae* has been found in a variety of animals and plants, it has also been widely linked to cattle faeces, commercial poultry, and related production systems (Mbanga *et al.*, 2021). The *Enterococcus hirae* identified in the present was from an area that is frequented by cattle. Some of the infections caused by *Enterococcus hirae* include wound infection, bacteremia, cholangitis and gastritis (Bilek *et al.*, 2020).

Enterococci are thought to be a marker of faecal contamination of environmental water sources (Motina and Rao, 2021). As markers of the hygienic safety of water supplies, enterococci have some benefits over coliform bacteria. They are less vulnerable to environmental stressors (Alipour *et al.*, 2014). The possibility of enterococci found in human and animal waste contaminating water in nearby wells requires special consideration. Agricultural runoff from agricultural and grazing lands, as well as urban areas, is a significant cause of contamination of surface and ground waters (Stańczyk-Mazanek and Stępnia, 2021). Faecal bacteria such as enterococci enter surface water through direct faeces deposit and overland runoff. The activity of animal wastes into surface waters could be a significant contributor to enterococci contamination in the groundwater systems of interest (Papadaki *et al.*, 2020). Therefore, the presence of enterococci in groundwater systems is very alarming.

5.4.2.1 Phylogenetic association of *Enterococcus* spp.

The analysis of the 16S rRNA gene sequence revealed that *Enterococcus gallinarum* and *Enterococcus saigonensis* are closely related. *Enterococcus saigonensis* was recently identified in retail chicken and very limited data is available on this bacterium. A study by Li and Gu (2019) showed that *Enterococcus saigonensis* isolated from pickle juice was 55 % related to *Enterococcus dispar* and *Enterococcus canintestini*. These results differ from those of the current study where *Enterococcus saigonensis* and *Enterococcus dispar* were distantly related. Furthermore, in this study, *Enterococcus casseliflavus* and *Enterococcus saccharolyticus* were under the same cluster but the bootstrap value was 26%. Takakura *et al.* (2019) reported a close association between *Enterococcus casseliflavus*, *Enterococcus gallinarum* and *Enterococcus saccharolyticus* in stool samples. Considering the clustering of isolates in cluster A2.2, the findings of this study are similar to those of Takakura *et al.* (2019).

5.4.3 HPC bacteria

Heterotrophic bacteria are ubiquitous in nature and can be found in soil, air, water, and food, and they get their energy from organic nutrients (LeChevallier, 2003) In this study 27 genera were identified, namely: *Bacillus*, *Pseudomonas*, *Brevundimonas*, *Sphingomonas*, *Microbacterium*, *Tahibacter*, *Fictibacillus*, *Stenotrophomonas*, *Sphingopyxis*, *Exiguobacterium*, *Paenibacillus*, *Xanthomonas*, *Lysobacter*, *Acinetobacter*, *Vogesella*, *Elstera*, *Ensifer*, *Caulobacter*, *Chryseobacter*, *Flavobacterium*, *Sphingobium*, *Brevibacterium*, *Hydrogenophaga*, *Nevskia*, *Lysinibacillus*, *Aeromonas*, and *Chitinimonas*. The most prevalent genera were *Bacillus* spp. and the most infrequent genera were *Caulobacter*, *Nevskia*, *Chitinimonas*, *Aeromonas* and *Lysinibacillus*.

The presence of *Bacillus* in the groundwater is mainly caused by irrigation and other agricultural activities. *Bacillus* spp. is widely spread in soil particles, run-off from soil, and decaying matter, contaminants that typically end up in irrigation water sources as a result of environmental, human, and/or animal contamination (Xiao *et al.*, 2013). Most of the *Bacillus* spp. were identified in the spring season. A high prevalence of *Bacillus* spp. in sampled Skeerpoort river water was observed by Aijuka *et al* (2015) in summer and spring. The increased prevalence of *Bacillus* spp. during the summer as compared to the winter could be attributed to warmer temperatures that promote spore germination into vegetative cells and thus proliferation (Ells and Hansen, 2006). *Bacillus* spp. may eventually return to spores during the winter as a means of protection against adverse conditions. Some *Bacillus* spp. like *Bacillus cereus* are known to cause diarrhoea and non-gastrointestinal infections in humans (Granum *et al.*, 2012).

Some of the identified isolates in the present study are known to cause opportunistic infections. These include *Brevundimonas* spp., *Stenotrophomonas* spp., *Sphingomonas* spp., *Acinetobacter* spp., *Pseudomonas* spp., and *Aeromonas* spp. (Ryan and Pembroke, 2018). These last-mentioned isolates are known to survive numerous environments and also are also resistant to a broad spectrum of antibiotics (Kulakov *et al.*, 2002; Falcone-Dias *et al.*, 2012; Handschuh *et al.*, 2017). Bacteria like these have the potential to infect individuals suffering from underlying medical diseases (Flores-Treviño *et al.*, 2014). Various studies revealed a variety of infections caused by *Brevundimonas* spp. such as urinary tract infections and leg ulcers (Han and Andrade, 2005; Almuzara *et al.*, 2012; Ryan and Pembroke, 2018). This suggests that the genus is a more prevalent pathogen than previously thought, with *Brevundimonas* spp. infections being severe.

Stenotrophomonas maltophilia is a common pathogen that is commonly found in wet environments and it causes opportunistic infections (Brook, 2012). The immunocompromised host experiences a rise in the incidence and severity of illness, particularly opportunistic septicemia and pneumonitis syndromes (Abott *et al.*, 2011). Extensive studies have revealed that *Stenotrophomonas maltophilia* is a persistent and resilient pathogen due to its ability to survive in biofilms and adapt to environmental stressors (Hošťacká *et al.*, 2010). Biofilm production is linked to tolerance to environmental factors by promoting intimate adhesion to surfaces, resistance to inflammatory processes, protection from antimicrobial activity, and enhanced spread across surfaces via bacterial motility (Imfran *et al.*, 2015; Abott *et al.*, 2011). The presence of *Stenotrophomonas maltophilia* and *Brevundimonas* spp. in water could be indicative of nosocomial contamination (Brooke, 2012).

5.4.3.1 Phylogenetic association of HPC bacteria

In this study, two clusters were observed for the HPC group. Cluster B (B1) was mostly dominated by the phylum Firmicutes, such as *Bacillus*. Firmicutes are microbes that exist in the human digestive tract. Numerous Firmicutes phylum members produce butyrate, an essential constituent that retains the health of the colon (Seong *et al.*, 2018). The human gut microflora is made up of trillions of microbes, the vast majority of which are bacteria. Firmicutes are particularly popular among scientists because many of them aid in the maintenance of metabolic and immune wellbeing (Gomes *et al.*, 2018). The phylum includes Bacilli, Clostridia, Erysipelotrichales, Mollicutes and Veillonellaceae (Rowin *et al.*, 2017). *Bacillus* was the most prominent species within the HPC group. Atnafu *et al.* (2021) found that phylum Firmicute was more dominant than other phylum in drinking water.

5.5 Pathogenicity and extracellular enzyme production

Water-borne pathogen pollutants in water, as well as associated diseases, are major water quality concerns around the world (Park and Oh, 2012). Pathogen contamination is a major problem for almost all types of ambient water bodies, therefore recognizing and comprehending it is critical (Pandey *et al.*, 2014). Groundwater is widely utilized as the predominant source of domestic drinking water supply across the world, and polluted groundwater is set to increase the risk of public health (Alley *et al.*, 1999; John and rose, 2005; Pandey *et al.*, 2014). In South Africa, groundwater is predominantly used for irrigated agriculture, mining, livestock, aquaculture, industrial purposes and domestic water supply

(Mvandaba *et al.*, 2019). Pathogens in groundwater have caused numerous outbreaks in the United States, resulting in 2739 cases of illnesses (John and Rose, 2005). Mpenyana-Monyatsi *et al.* (2012) reported on the groundwater outbreaks in rural South Africa. The latter study reported that the contaminated groundwater caused cholera, diarrhoea and typhoid infections in humans. Given the reported cases of outbreaks, it is imperative to assess the pathogenicity of the bacteria isolated from groundwater.

5.5.1 Coliform bacterial group

Only six haemolytic coliform isolates comprising three species produced extracellular enzymes: *Escherichia fergusonii*, *Citrobacter freundii* and *Citrobacter braakii*. According to Walen *et al.* (2007) *Citrobacter freundii* is described as an opportunistic pathogen. The latter study revealed that *Citrobacter freundii* is known to attack patients with weak immune systems. Other studies have reported some *Citrobacter freundii* isolates to be capable of conferring virulence and inducing foodborne diseases or gastroenteritis in humans (Bai *et al.*, 2012; Liu *et al.*, 2017). In the current study, the isolated *Citrobacter freundii* produced proteinase but did not hydrolyze gelatinase. This is contrary to the findings of Sharma and Sharma (2017). Nonetheless, Mahmood and Atyah (2021) state that virulence factors of *Citrobacter* spp. include hemolysis, proteolysis and biofilm formation. Furthermore, β -haemolytic activity was observed in all *Citrobacter freundii* isolates screened in this study. This is consistent with the findings of Hossain *et al.* (2017). Contamination with haemolytic *Citrobacter* spp., has been found to cause gastroenteritis and haemolytic uraemic syndrome in humans (Hidayatullah *et al.*, 2020).

Additionally, proteinase activity was also observed in *Escherichia fergusonii* isolates. Proteinase enables microorganisms the ability to bypass a host's defense mechanisms by dissolving peptide bonds that connect different amino acids (Willet *et al.*, 2008; Pavlon *et al.*, 2002). Thus, the presence of proteinase producing isolates in groundwater systems used for domestic and livestock watering is worrisome. *Escherichia fergusonii* is an emerging pathogen originating from human blood and urine (Mahapatra *et al.*, 2005). The production of gelatinase in *Escherichia fergusonii* causes an impairment in the epithelial barrier of the gut in patients suffering from hand, mouth, foot disease (Li *et al.*, 2018).

5.5.2 *Enterococcus* spp.

In this study, the pathogenicity of *Enterococcus* spp. isolated from groundwater systems in the North West Province were extensively studied. *Enterococcus casseliflavus*, *Enterococcus faecalis*, *Enterococcus hirae* and *Enterococcus saigonensis* are amongst the most pathogenic isolates identified in this study. These isolates were α/β -haemolytic and tested positive for three or more extracellular enzymes. Several studies have reported the presence of β -haemolytic *Enterococcus* spp. in surface water (Molale, 2012; Bezuidenhout, 2013; Olaniran *et al.*, 2015; Molale and Bezuidenhout, 2016) and wastewater (Olaniran *et al.*, 2015; Madu, 2019; Mbanga *et al.*, 2020; Mbanga *et al.*, 2021) in South Africa.

The pathogenicity of *Enterococcus* spp. is mediated by a cluster of hydrolases that includes hyaluronidases, gelatinase, and protease. Both gelatinase and protease are thought to play important roles in the pathogenicity of *Enterococcus* spp. by supplying nutrition to the bacteria. This occurs by *Enterococcus* spp. damaging the host tissue. Additionally, the production of gelatinase, protease and DNase in bacteria plays a critical role in their ability to form a biofilm (Ramaraj *et al.*, 2020). The production of DNase and gelatinase is mostly observed in *Enterococcus faecalis* (Kishi *et al.*, 2001). There was very low production of gelatinase and lipase in the *Enterococcus* spp. of interest. This is in accordance with three studies that investigated the yield of gelatinase positive *Enterococcus* spp. in India (Kiruthiga *et al.*, 2020; Ravichandran *et al.*, 2016; Padmasini *et al.*, 2014). The secretion of gelatinase was associated with the *gel E* virulence gene in *Enterococcus faecalis* in Egypt (Sonbol *et al.*, 2013). The study further alludes to the fact that the production of gelatinase plays a huge role in the pathogenicity of *Enterococcus* spp.

5.5.3 HPC bacterial group

Extensive research has been done on the pathogenicity of HPC in South Africa, particularly ones that were isolated from water sources (Pavlov *et al.*, 2002; Venter, 2010; Ferreira, 2011; Prinsloo, 2014; Horn *et al.*, 2016; Kritzing, 2019; Tsholo, 2019). The studies highlighted the pathogenicity of HPC bacteria, particularly those that are α - and β - haemolytic. In this study, autumn had the most haemolytic HPC bacteria. Most of these HPC bacteria were isolated from site S6. The extracellular enzyme profile of the HPC bacteria isolated from site S6 suggests that the site harbours bacteria that are pathogenic. Edberg *et al.* (1996) indicated that a microorganism could only be deemed pathogenic when it has two or more extracellular enzymes.

The most commonly produced extracellular enzymes by HPC bacteria were DNase and proteinase. The production of proteinase was predominantly observed in *Stenotrophomonas maltophilia*. These enzymes have been shown to degrade cell components like nucleic acids and proteins (Pavlov *et al.*, 2002). DNase causes the breakdown of cell components such as DNA (Pavlov *et al.*, 2004). According to MacFaddin (1985), pathogens use the degraded DNA as an energy source. Because DNA is found in humans and almost all other organisms (except RNA viruses), it is cause for concern when DNase is one of the most prevalent enzymes produced by HPC isolates obtained from drinking water sources.

In this study, *Bacillus* and *Fictibacillus* were the second predominant enzyme active genera. This is in accordance with the study reported by Horn *et al* (2016) where *Bacillus* spp. from water samples produced lipase, gelatinase and DNase. *Bacillus cereus* has been linked to diarrhoea and emesis in humans, as well as some non-gastrointestinal infections (Venter, 2010). Fresh and saltwater, decaying organic matter, soil, vegetables, and invertebrate intestinal tracts are all part of this organism's natural environment (Pavlon *et al.*, 2004; Martin *et al.*, 2018). The presence of pathogenic bacteria is a health hazard because groundwater serves as a vehicle for the spread of antibiotic-resistant microorganisms, which can propagate even after water treatment (Mulamattathil *et al.*, 2014). Thus, the presence of these virulence bacteria in groundwater is very alarming.

5.6 Characterization of isolates

Antibiotic-resistant bacteria and antibiotic-resistance genes are emerging environmental contaminants. They are primarily from the hospital and public wastewater, as well as livestock farming, agricultural lands, and wastewater treatment plants (Liaqat *et al.*, 2017). Antibiotic-resistance determinants in the water system may spread horizontally and be transmitted to humans and animals (Agoba *et al.*, 2017). The presence and distribution of antibiotic and virulence genes were studied in order to gain insight into the role of the emergence of antibiotic-resistance in natural aquatic environments.

Antibiotics are emitted into the environment through a variety of sources (Polianciuc *et al.*, 2020). For instance, massive amounts of antibiotics are used in animal production to boost growth or help animals thrive in crowded, strenuous, and unhygienic environments (Imran *et al.*, 2019). Antibiotics used by humans can also be emitted into the environment via septic tank spills (Wu *et al.*, 2020). Antibiotic byproducts can be observed at higher concentrations in wastewater, surface waters, agricultural runoff, and aquaculture water (Serwecińska, 2020). Also, Pruden *et al.* (2013) revealed that antibiotics enter the groundwater system

through urine and faeces excreted by livestock, especially during the rainy season. Eight of the selected sites were located in areas where farming is the main activity.

In recent times, the occurrence of antibiotic-resistance genes and virulence genes in groundwater systems have been documented. Several studies have reported the presence of antibiotic-resistance genes (Chen *et al.*, 2017; Wu *et al.*, 2020) and virulence genes (Loesch *et al.*, 2015; Matlou *et al.*, 2019; Sidhu *et al.*, 2020), particularly in groundwater. In South Africa, the emergence of bacteria that carry antibiotic-resistance genes in groundwater are also studied (Matlou *et al.*, 2019; Ekwanzala *et al.*, 2018; Szekeres *et al.*, 2018; Zainab *et al.*, 2020). Considering these facts, it is critical to detect any resistance in bacteria isolated from groundwater.

5.6.1 Multiple antibiotic-resistance phenotype, antibiotic-resistance genes and virulence of the coliform isolates identified in this study

5.6.1.1 Antibiotic-resistance profiles of the coliform bacterial group coliform

The highest resistance amongst coliform bacteria in the present study was to ampicillin, oxytetracycline, amoxicillin and chloramphenicol. Ampicillin is a wide range of penicillin that have been used for a long time and is active against a wide range of bacteria (Khatoon *et al.*, 2019). As a result, most bacteria have developed resistance to this drug, which has been associated with the production of beta-lactamases (Annavajhala *et al.*, 2019). The latter are enzymes that bacteria secrete and are responsible for breaking down the drug. This could explain why the majority of coliform bacteria in this study were ampicillin resistant. A study by Adinortey *et al* (2020) reported 98.4% resistance to ampicillin amongst coliforms. Coliforms are Gram-negative bacteria, which means that they have a slimy layer on the outer membrane, which prevents beta-lactam bacteria from penetration (Breijyeh *et al.*, 2020). Also, Furthermore, antibiotics such as oxy-tetracycline and neomycin are used to treat infections in cattle (Adesiyun *et al.*, 2020). Thus, antibiotic residues could have made their way into the water system through animal waste, thus increasing the possibility of resistance. These byproducts may have a variety of side effects, including the transmission of antibiotic-resistant bacteria to humans, immunologically mediated effects, allergy, and genotoxicity (Bacalni and Başaran, 2019).

5.6.1.2 Antibiotic-resistance genes the coliform bacterial group

Prevalent faecal contamination in water systems may pose a significant health risk as well as a significant pathway for the spread of antibiotic-resistant bacteria (Stange *et al.*, 2016). The coliforms that harboured antibiotic-resistance genes in this study included *Escherichia fergusonii* (OKO30978.1), *Klebsiella grimontii* (OKO30999.1) and *Enterobacter cloacea* (OKO30980.1).

Klebsiella grimontii (OKO30998.1) harboured the most genes (*bla_{TEM}*, *int1* and *ampC*) in the coliform group. Hubbard *et al* (2020) revealed that *Klebsiella grimontii* isolated from bottled water harboured five antibiotic-resistance genes. Among the five genes detected, *bla_{TEM}* was one of them. Amongst the coliform group, *Klebsiella grimontii* (OKO30998.1) was the only isolate that harbored the *int1* gene. The presence of *int1* gene suggests resistance to antibiotics and disinfectant (Gilling *et al.*, 2008). Integron class 1 plays an important role in the development and spread of antibiotic-resistance, and its prevalence is a warning sign for bacterial infections (Koczura *et al.*, 2016). The presence of *int1* gene was reported by Thongkao and Sudjaroen (2019) in *Klebsiella pneumoniae* from Tilapia fishes. Another study by Lina *et al* (2007) reported the presence of *int1* and *int2* genes in *Klebsiella pneumoniae* from UTI patients. Moreover, *Klebsiella grimontii* (OKO30998.1) harbored *ampC* and *bla_{TEM}* gene. *Klebsiella* spp. have been reported to harbour *ampC* and *bla_{TEM}* gene (Yu *et al.*, 2020; Shibu, 2019).

In this present study, *Enterobacter cloacea* harbored *bla_{TEM}* and *ampC* genes. This is in conjunction with a study conducted by Uzunović *et al* (2018), which revealed that *Enterobacter cloacea* harbored a wide array of beta-lactam associated genes. Ampicillin resistance is encoded by the *bla_{TEM}*. Ampicillin and cephalothin are beta-lactam antibiotics with low toxicity, which has contributed to the excessive usage of these drugs in the medical profession (Olaniran *et al.*, 2009; Aijuka *et al.*, 2015). Furthermore, resistance to cephalosporins and cephamycins is conferred by the *ampC* gene. This gene is linked to clinical settings, as a result, detecting this gene is critical in the surveillance and management of antibiotic-resistance activity in order to prevent the rate of infections (Shanthi *et al.*, 2012).

None of the coliforms identified in this study harbored the *tetM* and *ermF* gene. The resistance to *tetM* is facilitated by obtaining high mobility of conjugative transposons (Doherty *et al.*, 2000; Moreno and Araque, 2018). Recent studies have reported *tetM* harboring coliform from environmental water sources (Tsunoda *et al.*, 2021) and raw cow milk (Godziszewska *et al.*, 2018). Different types of bacteria produce different types of

erythromycin resistance methylases, which are identified by 12 classes of *erm* genes (Weisblum, 1995). The *ermB* and *ermF* genes express resistance to a wide range of antibiotics, including macrolides and lincosamides (Petinaki and Papagiannitsis, 2018). In the present study, *Escherichia fergusonii* harbored the *ermB* gene. Sahulka *et al*, (2021) reported *Escherichia fergusonii* from agricultural samples that harbored the *ermB* gene. These genes attain antibiotic-resistance via methyltransferase, which is associated with the alteration of the bacterial ribosome's 23S rRNA (Osterman *et al.*, 2020).

5.6.1.3 Virulence genes in the coliform bacterial group

In this study, the presence of four virulence genes, *stx1*, *stx2*, *eaeA*, *hlyA*, were investigated in 26 coliform isolates. Furthermore, virulence genes were identified in *Klebsiella grimontii*, *Enterobacter cloacae* and *Escherichia fergusonii*. The genes, *stx1* and *eae A* were harboured by the latter three species. Shiga toxin (Stx) is amongst the most efficacious bacterial toxins ever discovered (Fuller *et al.*, 2011). The *stx1* gene has been reported in *Shigella dysenteriae* as well as some *Escherichia coli* serogroups (*E. coli* 0157, 0103, 026, 091 and 0145) (Hua *et al.*, 2020). Several *Escherichia coli* strains were subsequently found to produce *stx2*, a toxin that is immunologically unique but has the same mechanism of action as *stx1* (Werber *et al.*, 2008). Shiga toxin is the prototypical member of the Shiga toxin family, a group of structurally and functionally related proteins (Aijuka *et al.*, 2015; Melton-Celsa *et al.*, 2011; Melton-Celsa *et al.*, 2014; Bergan *et al.*, 2012). The latter's term is derived from its origin, the chronic diarrhea causing bacillus *Shigella dysenteriae* (Bergan *et al.*, 2012). The Shiga toxin is usually present in *Shigella* and *Escherichia coli*, however in this study, the *stx1* and *stx2* gene were present in *Klebsiella grimontii*, *Enterobacter cloacae* and *Escherichia fergusonii*. Several studies have reported the presence of shiga toxin in *Enterobacter cloacea* from clinical samples (Paton and Paton, 1997; Khalil *et al.*, 2016; Carter *et al.*, 2017). The *Escherichia coli* edition of the shiga toxin was labelled "verotoxin" due to its ability to kill Vero cells in culture. Furthermore, because of its resemblances to shiga toxin, the verotoxin was quickly labelled as shiga-like toxin (Mashak, 2018). Verotoxins are bacterial protein toxins of the AB5 family, protein sequences of shiga toxin from *Shigella dysenteriae*, that stop protein production in cells by cleaving a specific adenosine residue from the 60S ribosomal subunit's 28S RNA (Johnson *et al.*, 1996; Mainil and Daube, 2005; Widgren *et al.*, 2018). Verotoxins are most commonly found in *E. coli*, however in this study, *Escherichia fergusonii* and *Klebsiella grimontii* harboured the *hlyA* gene. A similar observation was documented in a study by Kwak *et al* (2015) where *Shigella* and *Escherichia fergusonii* were found to harbour the shiga-like toxin gene.

The *eaeA* gene was identified in all four coliform species groups identified in this study. This is worrying when considering the role of *eaeA* during diarrheal episodes. Heydari *et al.* (2020) reported the presence of *eaeA*, in *Escherichia coli* (that harboured the *stx 1*, *stx2*, *eaeA* and *hlyA* genes) isolated from stools of acute diarrheal patients. Despite *Escherichia coli* not being identified in this study, virulence genes known to be harboured by *Escherichia coli* were identified in the coliforms isolated from the groundwater systems of interest. This phenomenon is not unusual as studies have suggested and illustrated that several processes allow for the spread of bacterial virulence genes (Hacker *et al.*, 1997; Penadès *et al.*, 2015; Sarowska *et al.*, 2019). According to Sarowska *et al.*, (2019), *Escherichia coli* have mobile genetic elements that allow for the horizontal exchange and transduction of bacterial genetic elements between related as well as unrelated bacterial families. Furthermore, Hacker *et al.* (1997) further explain that horizontal gene transfer is responsible for the transfer of pathogenicity associated islands in bacteria. Lastly, Penadès *et al.* (2015) reported that bacteriophages can also facilitate the transfer of phage encoded virulence genes between different bacterial species and families.

Judging from these results, the quality of the identified groundwater is concerning as it is used for multiple purposes which include drinking, irrigation and livestock watering. The presence of antibiotic-resistance genes as well as virulence genes implies that some of the bacteria isolated from the groundwater systems are pathogens.

5.6.2 Multiple antibiotic-resistance phenotype, antibiotic-resistance genes and virulence genes of the *Enterococcus* spp. identified in this study

5.6.2.1 Antibiotic-resistance profiles of *Enterococcus* spp.

Some *Enterococcus saigonensis*, *Enterococcus hirae*, *Enterococcus gallinarum* and *Enterococcus faecalis* isolates were resistant to all 11 antibiotics. In South Africa there is limited reported data on the occurrence of pathogenic *Enterococcus* spp. from groundwater. Nonetheless, the studies that have been conducted thus far have revealed that *Enterococcus* spp. found in groundwater systems are resistant to antibiotics and show signs of being pathogenic (Obi *et al.*, 2002, Ateba and Maribeng, 2011; Montwedi, 2013; Montwedi *et al.*, 2018; Shobo *et al.*, 2021).

Resistance to vancomycin and penicillin was observed in all *Enterococcus* spp. during this study. The resistance to vancomycin in *Enterococcus* spp. is widely reported in studies worldwide (Harwood *et al.*, 2001; Roberts *et al.*, 2009; Ekwanzala *et al.*, 2020). Antibiotics

like penicillin are prescribed for cardiovascular conditions in South Africa (NdoH, 2020). Vancomycin is a glycopeptide antibiotic obtained from the *Amycolatopsis orientalis* microorganism (Young *et al.*, 2019). Vancomycin was observed in 1953 by Edmund Kornfield and authorized by the United States Food and Drug Administration in 1958 due to the fast expansion of penicillin resistance by staphylococci (Zaffiri *et al.*, 2013). Antibiotics could have been used in the monitoring of farm animals, particularly in those sites which are frequented by cattle. Antibiotics, such as avoparcin, have been linked to high levels of vancomycin resistant enterococci (VRE) in farm animals (Hamiwa *et al.*, 2019). One *Enterococcus hirae* harbored four antibiotic-resistance genes, *bla_{TEM}*, *int1*, *ampC* and *ermB*. The latter isolate was resistant to all subjected antibiotics including trimethoprim and streptomycin. Resistance to trimethoprim is also unsurprising because it is used to cure urinary tract infections (Crellin *et al.*, 2018). The *Enterococcus* spp. that harboured antibiotic genes were from the METSI, S1 and S3, where farms are nearby. Therefore, these antibiotic-resistant bacteria might originate from the animals.

5.6.2.2 Antibiotic-resistance genes in *Enterococcus* spp.

Due to their resistance to many antibiotics and ability to obtain and propagate antibiotic-resistance determinants, enterococci have emerged as a major cause of nosocomial pathogens (Cho *et al.*, 2020). In the *Enterococcus* spp. group, most of the isolates that harboured antibiotic-resistance genes were from a site located close to a mine and alone upstream from the Loopspruit River. It is also located close to a cattle farm. Antibiotic-resistance genes were identified and harboured by various *Enterococcus* isolates: *Enterococcus faecalis*, *Enterococcus casseliflavus*, *Enterococcus casseliflavus*, *Enterococcus hirae* and *Enterococcus saigonensis*. There is a widespread problem concerning bacteria in recreational waters, groundwater, and surface water harbouring antibiotic-resistance and virulence genes (Rivera *et al.*, 1988; Devriese *et al.*, 1992). One of the primary reasons is that it is uncertain whether microorganisms carrying these genes pose a risk to bathers.

Tetracyclines are commonly used to treat infections caused by opportunistic pathogenic *Enterococcus* spp. (Santiago-Rodriguez *et al.*, 2013). The resistance to tetracycline is encoded by the *tet* gene (Stange *et al.*, 2018). In the present study, the *tetM* gene was harboured by *Enterococcus faecalis* and *Enterococcus saigonensis*. A study by Hamiwa *et al.* (2019) reported *tetM* and *tetL* carrying *Enterococcus* spp. including *Enterococcus faecalis* from aquatic systems in Gauteng. Another study by Novais *et al.* (2018) identified

Enterococcus faecalis and *Enterococcus faecium* that harbored the *tetM* gene in aquaculture of rainbow trout. The propagation of *tetM* in water systems was found to correlate with the amount of COD in the water (Zhang *et al.*, 2017). The latter study explained that the higher the organic level in water, the higher the propagation of antibiotic-resistance genes will be in the water system.

Enterococci are resistant to a wide array of antibiotics such as beta-lactams, macrolides, glycopeptides and tetracyclines (Roy *et al.*, 2021). The beta-lactam resistance in enterococci is achieved through penicillin-binding protein mutations and beta-lactamase development (King *et al.*, 2017). Genes associated with beta-lactam resistance include *bla_{TEM}*, which is known to be associated with bloodstream, urine and saliva (da Silver *et al.*, 2019). In this study *bla_{TEM}* was harbored in *Enterococcus hirae* from site GM. The presence of *bla_{TEM}* harboring *Enterococcus* spp. were reported in urine samples from horses (Isgren, 2021). There are horses that frequented site GM, that could be the reason why the gene is present in the groundwater system. Additionally, the *amp C* gene was present in *Enterococcus casseliflavus*, *Enterococcus hirae* and *Enterococcus saigonensis*. Contrary to this study, Stoll *et al.* (2012) did not identify the *ampC* gene in *Enterococcus* spp., but in coliforms.

Integrans have been considered as a major cause of antimicrobial resistance propagation and preservation in foodborne pathogens and microbes from various animal origins (Tu *et al.*, 2019). The *int1* gene is widely used as a generic marker of anthropogenic influenced pollution (Gilling *et al.*, 2015). Some of the advantages of using *int1* as a marker is because of the abundance and presence of the gene in animals and humans (Marano *et al.*, 2017). High presence of *int1* has been reported in regions where manure is used (Cheng *et al.*, 2013). In this present study, *int1* gene was present in *Enterococcus hirae* and *Enterococcus saigonensis* from site GM. This site is close to private farms where manure is used or the presence of faeces is prevalent.

In this study, the presence of *ermB* in *Enterococcus faecalis* and *Enterococcus hirae* was observed. This is similar to the findings by Jensen *et al.* (1999) and Hao *et al.* (2019). The latter studies isolated the *Enterococcus* spp. from animal and human samples. Enterococci were found in a large number of samples, implying that many people in the North West Province are consuming water that does not meet national quality requirements. This global health threat is made even more concerning by the existence of isolates resistant to numerous antibiotic groups.

Bacteria may acquire antimicrobial resistance or develop resistance through random mutation or the procurement of resistant genes (Deng *et al.*, 2019). The procurement of a

resistant gene through horizontal gene transfer is by far the most popular and easy way for bacteria to establish antibiotic-resistance in the environment and in a host (Pei *et al.*, 2007). Numerous bacteria emit antibiotic-resistance genes, but because of their widespread distribution in aquatic ecosystems, these antibiotic-resistance genes have recently been proposed as emerging contaminants (Imran *et al.*, 2019; Pal *et al.*, 2017).

5.6.2.3 Virulence genes in *Enterococcus* spp.

According to Kiruthiga *et al* (2020) the pathogenicity of enterococci is highly associated with a variety of virulence factors such as aggregation substance, gelatinase, cytolysin, enterococcal surface protein, and hyaluronidase. In the current study the virulence genes associated with the latter virulence factors were investigated. In this study, *hyl* and *cytA* were the virulence genes most identified and harboured by the *Enterococcus* spp. A study by Matlou *et al*, (2019) in the North West Province, observed the presence of *gel*, *asa1*, *hyl* and *esp* in *Enterococcus* spp. from surface and groundwater. However, the latter study did not detect the presence of *cytA*. Hyaluronidase is an enzyme that degrades hyaluronic acid, has long been used to increase drug penetration into tissue and to decrease tissue damage in cases of drug extravasation (Zarei *et al.*, 2019). The *hyl* gene encodes a protein that is found in significant organs such as the liver, kidney, spleen, and heart, as well as in serum and urine. It is a major hyaluronidase in plasma that is activated by an acidic pH (Weber *et al.*, 2019).

In this study, only three *Enterococcus* species harboured virulence genes. The *gel E* and *asa1* genes were only detected in one *Enterococcus casseliflavus* isolate. The presence of *gelE* and *asa1* gene in *Enterococcus casseliflavus* and *Enterococcus faecalis* was detected in a study conducted by Sidhu *et al.* (2014) in fresh water. The presence (gelatinase (*gelE*) was predominately witnessed in *Enterococcus faecium* and *Enterococcus faecalis* species (Gutschik *et al.*, 1979; Chow *et al.*, 1993). Studies by Macedo *et al.* (2011) and Wei *et al.* (2017) have reported the presence of *gelE* in *Enterococcus faecalis*. However, recent studies revealed that more *Enterococcus* spp. harbour this gene (Molale, 2016; Huff *et al.*, 2020). Gelatinase causes tissue damage in the host, enabling bacterial mobility and spread, invasion, and persistence via biofilm production (Kiruthiga *et al.*, 2020).

5.6.3 Multiple antibiotic-resistance phenotype, antibiotic-resistance genes of the HPC isolates identified in this study

5.6.3.1 Antibiotic-resistance profiles of HPC bacteria

Antibiotic-resistance is undoubtedly a significant worldwide public health concern, particularly in the water environment (Saga and Yamaguchi, 2009; Coertzee and Bezuidenhout, 2018; Maje *et al.*, 2020). High antibiotic-resistance was observed most in *Bacillus* spp., *Fictibacillus phosphorivans*, *Stenotrophomonas maltophilia*. *Bacillus cereus* is usually resistant to beta-lactam antibiotics like ampicillin, but it could also advance in its resistance to other commonly used antimicrobials like ciprofloxacin, erythromycin, streptomycin, tetracycline (Fiedler *et al.*, 2019). The antibiotic-resistance of the HPC bacteria in the current study differed. High resistance to chloramphenicol, oxy-tetracycline, ciprofloxacin, ampicillin, amoxicillin and erythromycin was observed. This is in accordance with a study conducted by Horn *et al* (2016) where resistance to ampicillin and amoxicillin amongst HPC bacteria. In both humans and animals, chloramphenicol is an effective antibiotic against a wide variety of Gram-positive and Gram-negative bacteria (Gottlieb and Legator, 1953). The use of chloramphenicol has been minimized particularly for food animals in the United States as well as South Africa (Gottlieb and Legator, Eager *et al.*, 2012). This is because it was said to be carcinogenic (Yao and Yao, 2017). Chloramphenicol is seamlessly excreted or launched into natural water, thus, the existence of its byproducts in aquatic environments has exacerbated environmental and ecological issues (Heberer, 2002; Erikson, 2002; Yao, 2017). The presence of chloramphenicol residues in water systems is indicative of toxins that might affect aquatic microorganisms (Daughton, 2004). The presence of chloramphenicol in groundwater used for farming animals was detected in the groundwater used for food animals in the United States (Kivits *et al.*, 2018). This is alarming, due to the fact that chloramphenicol is banned. In South Africa, small amounts (1%) of chloramphenicol is added in eye ointments used to treat conjunctivitis, however, it is banned for use in livestock (Perumal-Pillay and Suleman, 2017). The presence of chloramphenicol in groundwater could be due to the land being fertilized with faeces from non-food animals (Kivits *et al.*, 2018).

The use of antibiotics like ampicillin, penicillin, gentamicin and ciproflaxacin are widely used in South Africa (Farley *et al.*, 2018). This explains the high resistance to ampicillin among the HPC bacteria. Furthermore, antibiotic-resistance is presently a very major problem that has gotten a lot of attention from the scientific community because of the impact it has on both hospital and community settings. A number of studies have been done on antibiotic-resistant

bacteria in drinking water (Armstrong *et al.*, 1981; Armstrong *et al.*, 1982; Pathak and Gopal, 2008; Xi *et al.*, 2009). Additionally, oxy-tetracycline is known to be used for the treatment of bovine respiratory disease in cattle (Ilemobade *et al.*, 1979; Abasi *et al.*, 2011; Kimera *et al.*, 2015;). The sampled sites with the most resistance were located in farm areas where there are cattle present. Since Oxy-tetracycline is used extensively to treat diseases in cattle, the antibiotic-resistant microorganisms could have made their way into the groundwater system through faecal contamination (Kimera *et al.*, 2015).

5.6.3.2 Antibiotic-resistance genes of HPC bacteria

The *ampC* gene was the most dominantly occurring antibiotic-resistance gene in the present study in all three bacterial groups. HPC bacteria that harbored the *ampC* gene included *Fictibacillus phosphorivorans*, *Stenotrophomonas maltophilia*, *Chitimonas virdis*, *Vogesella facilis*, *Brevundimonas albigilva*, *Klebsiella grimontii*, *Bacillus cereus*, *Bacillus safensis*, *Bacillus halotolerans* and *Pseudomonas songnensis*. *Stenotrophomonas maltophilia* (OK065653) from site GM harboured four antibiotic-resistance genes and *Stenotrophomonas maltophilia* (OK087634) from site S1 harboured three antibiotic-resistance genes. A recent review on the latter species suggested that the microorganism's lowered susceptibility to antimicrobials is largely a consequence of genes encoding efflux pumps and antibiotic suppressing enzymes on its chromosome (Gil-Gil *et al.*, 2020). As a result, treatment options for *Stenotrophomonas maltophilia* infections are limited. This is alarming seeing that this organism poses traits of a potentially hazardous pathogen.

Several studies in South Africa have also reported the presence of antibiotic-resistance genes in HPC bacteria in water (Mann *et al.*, 2019; Plaatjie, 2019; Mudau, 2021). The latter studies have also confirmed high resistance to ampicillin amongst the HPC bacteria. This is also in agreement with a study by Montso *et al.* (2019) where high resistance to ampicillin was observed amongst HPC bacteria. A study by Liyanage *et al.* (2021) has also reported *ampC* and *bla_{TEM}* in bacteria from surface and groundwater. The *ampC* gene is most commonly found in pathogens and opportunistic pathogens (Matlou *et al.*, 2019). AmpC beta-lactamases are cephalosporinases which are mostly encoded on the chromosomal sequences of several *Enterobacteriaceae* (Matloko *et al.*, 2021). They facilitate resistance to cephalothin, cefazolin, cefoxitin, and most penicillins, as well as increasing the production of β -lactamase inhibitors (Correa-Martínez *et al.*, 2019). Antibiotic-resistance genes, particularly AmpC-lactamase genes, pose ongoing risks in environmental and clinical setups (Coertze and Bezuidenhout, 2018). AmpC enzymes are heritable in many bacteria and can be

conveyed at high levels through mutation. Increased levels result in resistance to broad-spectrum cephalosporins such as cefotaxime, ceftazidime, and ceftriaxone, which is a concern (Tamma *et al.*, 2019).

The presence of *tetM*, *ermF* and *ermB* genes has been reported in livestock wastes (Koike *et al.*, 2010; Monteiro, 2020). Farm animal waste from constricted animal feeding operations is a significant source of macrolide antimicrobials and antimicrobial resistance genes in the environment. Tetracyclines are most commonly used in plants and animals (Khachatourians, 1998; Jayalakshmi *et al.*, 2017). A study by Zhang and Dick (2014) screened the presence of antibiotic-resistance in soil samples of waste compost from farms. The latter's results revealed a presence of nine antibiotic-resistance genes. The existence of antibiotic-resistance genes in various soils suggests that soils are an underrated reservoir of multiple antibiotic-resistance, and there are striking similarities among both antibiotic-resistance factors carried by soil originating microbes and human pathogens (Forsberg *et al.*, 2012). These contaminated soils, particularly in farms could make their way into the groundwater through agricultural runoff (Mateo-Sagasta *et al.*, 2017). According to Eagar *et al.* (2012), 234 authorized antibiotics were obtainable for food animals in South Africa between 2002 and 2004. This means that animals are exposed to a wider variety of antibiotics. This is alarming because the overuse of antibiotics aids in higher resistance to antibiotics. Antibiotic-resistance is presently a very major problem that has gotten a lot of attention from the scientific community because of the impact it has on both hospital and community settings

Conclusion and Recommendation

Introduction

Groundwater in South Africa is mainly used for mining, domestic water supply, livestock and irrigated agriculture. It is generally a natural water purification system, but it also lacks the ability to remove enhanced contaminants to which it is exposed. Since the investigated groundwater in this study is used for numerous activities such as drinking, irrigation, and livestock watering, it was important to evaluate its quality.

6.1 Physico-chemical parameters

The physico-chemical parameters were examined in autumn, winter, summer and spring (2019-2021). Physical parameters of the groundwater of interest were all within the set guidelines, except for TDS. The observed TDS levels of the water were consistently high throughout the study. Elevated TDS levels were witnessed more in warmer seasons. Elevated TDS level in water causes the plants to stop taking up nutrients (Hoko and Totgo, 2020). This is concerning particularly for the consumers of these nutrient deprived plants. Moreover, the chemical parameters of the groundwater were within the TWQR guidelines. However, the COD levels fluctuated from season to season. It is important to take note of any changes in physico-chemical parameters particularly in ungoverned waters like groundwater. Once the levels of physico-chemical parameters exceed the stipulated guidelines, it creates a harbour for pathogens to thrive. The constant regulation and monitoring of physico-chemical parameters is highly advisable.

6.2 Microbial parameters and species diversity

The presence of faecal indicators as well as HPC bacteria was successfully determined. Coliforms were only identified in nine sites (S3, S6, GM, S4, S5, DK, H, BG and FD). Higher coliform levels were mostly in areas where livestock frequented. A total of six coliform species were identified and some of these coliforms are opportunistic pathogens. This means that the groundwater quality at these sites is affected by faecal contamination. This is alarming because groundwater is presumed to be the safest water to consume without treatment. Among all the *Enterococcus* spp., that were identified, *Enterococcus faecalis*, a well-known human pathogen was identified. This particular species is known to cause nosocomial infections and dental diseases. Therefore, the presence of this particular species in groundwater is a cause for concern, particularly because it is known to transfer resistance via horizontal gene transfer (Anderson *et al.*, 2016). The level of HPC bacteria

was alarmingly high. Amongst the identified species, opportunistic pathogen *Stenotrophomonas maltophilia* was identified. Other HPC bacteria such as *Bacillus* spp., and *Pseudomonas* spp. were identified. These bacteria have been widely studied and known to cause waterborne diseases to consumers. Gathering all the identified species in this study, the quality of groundwater is deteriorating and this is a cause of concern.

6.3 Pathogenicity

The coliforms in this study accounted for 14% of the total haemolytic bacteria in the present study. *Escherichia fergusonii* was the only coliform that tested positive for more than one extracellular enzyme. HPC bacteria are frequently thought to pose no health risks in water sources. However, HPC accounted for 59 % of the overall haemolytic bacteria in this study. Some of the HPC bacteria even produced extracellular enzymes. It is imperative to take note that these HPC bacteria may be intrusive and enter healthy host cells using these extracellular enzymes. This in turn, allows for the HPC to infect host cells. Thus, the presence of pathogenic HPC bacteria in water, particularly one that is used for drinking and irrigation is alarming.

6.4 Antibiotic-resistance

According to the findings of this study, antibiotic-resistant bacteria are prevalent in the examined groundwater. Most bacteria were resistant to chloramphenicol, ampicillin, amoxicillin, oxy-tetracycline and ciprofloxacin. The number of multiple antibiotic-resistant bacteria was quite high in the present study. The most prevalent MAR phenotype in all bacteria in this study was AMO-AMP-C-E-OT. Multiple antibiotic-resistant bacteria are a source of concern due to the possibility of invasion of the intestinal system of the consumer of the contaminated water. Multiple antibiotic bacteria can transfer their resistance to microflora of the healthy host via various mechanisms. Conjugal transfer of antibiotic-resistance to the microflora, as well as resulting shedding of even more MAR organisms, which serves to increase the number of MAR organisms launched into aquatic and other environments (McKeon *et al.*, 1995).

6.5 Antibiotic-resistance genes and virulence genes

The detection of virulence and antibiotic-resistance genes was successful. All the subjected genes were found in the identified bacteria. The most prevalent antibiotic-resistance gene (ARG) amongst the bacteria was *ampC*. The widespread use of human and veterinary antibiotics, as well as hospital wastewater and livestock manure, are thought to be the primary sources of ARGs in the environment. ARGs can enter aquatic environments via direct release of untreated wastewater or via wastewater plants. Additionally, ARGs can be introduced into soils by modifying cropland with

livestock manure and processed biosludge, where they can seep into groundwater or be carried to surface water by runoff and soil erosion. Therefore, awareness in the proper monitoring of emerging contaminants and groundwater preservation are critical, particularly in countries such as South Africa, where a growing portion of the population is reliant on groundwater resources.

Recommendations

The results of several studies across South Africa have proved that groundwater is not as pristine as it is presumed to be, thus, monitoring systems need to be put in place to regularly investigate the state of groundwater. More physico-chemical parameters like turbidity, hardness, total suspended solids, sulphates, dissolved oxygen and biological oxygen demand should be monitored. This would give a detailed state of South Africa's groundwater systems. Since the presence of faecal bacteria and HPC bacteria was successfully identified in groundwater, it would be advisable to test for presence of anaerobic bacteria such as clostridia that have well known clinical significance.

The use of more selective media to detect *Escherichia coli* would be advisable. Furthermore, the use of more specific PCR primers to identify *E. coli* would also be advisable. This is because some of the isolated coliforms in this study had similar characteristics to *E. coli*. Since some of these coliforms have virulent characteristics, the presence of *E. coli* 0157 in groundwater systems should also be tested. Particularly because some of the coliforms harboured the *stx1* and *stx2* gene. Additionally, the use of for extracellular enzymes like lecithinase would be advisable.

In this study, *Enterococcus faecalis* was detected. All the *Enterococcus* spp. in this study were resistant to vancomycin and penicillin. Therefore, it would be advisable to detect whether the genes associated with these antibiotics are harboured in the enterococci from groundwater. These antibiotic genes include *vanA*, *vanB*, *vanC* (Matlou *et al.*, 2019). As seen in the results presented in this study, some bacteria extracted from groundwater of various sites harbour antibiotic-resistance genes, as well as virulence genes. It is therefore recommended that tests should be utilised for the presence of more genes. It is also recommended that these genes be quantified, as it is important to know how much of the genes are expressed.

With this large amounts of data accumulated from the present study, there is a need for whole-genome sequencing, that would not only provide an understanding of the nature of these identified bacterial species, but will also be able to track down their provenance and potentially find ways to combat the present water quality issues.

REFERENCE LIST

- Abbott, I.J., Slavin, M.A., Turnidge, J.D., Thursky, K.A. and Worth, L.J., 2011. *Stenotrophomonas maltophilia*: emerging disease patterns and challenges for treatment. *Expert review of anti-infective therapy*, 9(4):471-488.
- Abbott, B.W., Bishop, K., Zarnetske, J.P., Minaudo, C., Chapin, F.S., Krause, S., Hannah, D.M., Conner, L., Ellison, D., Godsey, S.E. and Plont, S., 2019. Human domination of the global water cycle absent from depictions and perceptions. *Nature Geoscience*, 12(7):533-540.
- Abiriga, D., Vestgarden, L.S. and Klempe, H., 2020. Long-term redox conditions in landfill-leachate-contaminated groundwater. *Science of The Total Environment*, 755:143-725.
- Adeniji, O.O., Sibanda, T. and Okoh, A.I., 2021. Molecular detection of antibiotic-resistance and virulence gene determinants of Enterococcus species isolated from coastal water in the Eastern Cape Province, South Africa. *International Journal of Environmental Studies*, 78(2):208-227.
- Adeogun, A.O., Ibor, O.R., Omiwole, R.A., Hassan, T., Adegbola, R.A., Adewuyi, G.O. and Arukwe, A., 2015. Occurrence, species, and organ differences in bioaccumulation patterns of phthalate esters in municipal domestic water supply lakes in Ibadan, Nigeria. *Journal of Toxicology and Environmental Health, Part A*, 78(12):761-777.
- Adesiyun, A.A., Nkuna, C., Mokgoatlheng-Mamogobo, M., Malepe, K. and Simanda, L., 2020. Food safety risk posed to consumers of table eggs from layer farms in Gauteng Province, South Africa: Prevalence of *Salmonella* species and *Escherichia coli*, antimicrobial residues, and antimicrobial resistant bacteria. *Journal of Food Safety*, 40(3):12783.
- Adnan, M., Khan, H., Kashif, J., Ahmad, S., Gohar, A., Ali, A., Khan, M.A., Shah, S.S.A., Hassan, M.F., Irshad, M. and Khan, N.A., 2017. Clonal expansion of sulfonamide resistant *Escherichia coli* isolates recovered from diarrheic calves. *Pakistan Veterinary Journal*, 37(2):230-232.
- Aijuka, M., Charimba, G., Hugo, C.J. and Buys, E.M., 2015. Characterization of bacterial pathogens in rural and urban irrigation water. *Journal of water and health*, 13(1):103-117.

Aladejana, J.A., Kalin, R.M., Sentenac, P. and Hassan, I., 2020. Assessing the impact of climate change on groundwater quality of the shallow coastal aquifer of eastern Dahomey basin, southwestern Nigeria. *Water*, 12(1):224.

Al-Bahry, S.N., Mahmoud, I.Y. and Al-Musharafi, S.K., 2014. The effect of physical factors on faecal coliform viability rate in sewage sludge. *Journal of Geoscience and Environment Protection*, 2(2):9-13.

Alegbeleye, O.O. and Sant'Ana, A.S., 2020. Manure-borne pathogens as an important source of water contamination: An update on the dynamics of pathogen survival/transport as well as practical risk mitigation strategies. *International journal of hygiene and environmental health*, 227:113524.

Ali, A. and Ali, M.K., 2018. Effect of pyrolysis temperature on chemical form, behavior and environmental risk of Zn, Pb and Cd in biochar produced from phytoremediation residue. *Bioresource technology*, 249:487-493.

Ali, S., Khan, A.S. and Malik, A.U., 2016. Postharvest L-cysteine application delayed pericarp browning, suppressed lipid peroxidation and maintained antioxidative activities of litchi fruit. *Postharvest Biology and Technology*, 121:135-142.

Ali, L., Mustafa, M., Xiao, Z.R., Islam, W., Ara, U., Ajmal, M., Khan, K.A., Ghramh, H.A. and Yu, D., 2021. Responses of *Enterococcus faecalis* resistance and cytolysin up-regulation to nutrients in constructed mesocosms. *Journal of King Saud University-Science*, 15(9):101680.

Alipour, M., Hajiesmaili, R., Talebjannat, M. and Yahyapour, Y., 2014. Identification and antimicrobial resistance of *Enterococcus* spp. isolated from the river and coastal waters in northern Iran. *The Scientific World Journal*, 2014.

Allen, D.M., Mackie, D.C. and Wei, M.J.H.J., 2004a. Groundwater and climate change: a sensitivity analysis for the Grand Forks aquifer, southern British Columbia, Canada. *Hydrogeology journal*, 12(3):270-290.

Allen, M.J., Edberg, S.C. and Reasoner, D.J., 2004b. Heterotrophic plate count bacteria-what is their significance in drinking water ?. *International journal of food microbiology*, 92(3):265-274.

Alley, W.M., Reilly, T.E. and Franke, O.L., 1999. *Sustainability of ground-water resources*. US Department of the Interior, US Geological Survey.

Almuzara, M.N., Barberis, C.M., Rodríguez, C.H., Famiglietti, A.M., Ramirez, M.S. and Vay, C.A., 2012. First report of an extensively drug-resistant VIM-2 metallo- β -lactamase-producing *Brevundimonas diminuta* clinical isolate. *Journal of clinical microbiology*, 50(8):2830-2832.

Althaus, H., Dott, W., Havemeister, G., Müller, H.E. and Sacré, C., 1982. Faecal streptococci as indicator organisms of drinking water. *International Journal of Microbiology and hygiene*, 252(2):154-165.

Amanidaz, N., Zafarzadeh, A. and Mahvi, A.H., 2015. The interaction between heterotrophic bacteria and coliform, faecal coliform, faecal Streptococci bacteria in the water supply networks. *Iranian journal of public health*, 44(12):16-85.

Amarasiri, M., Sano, D. and Suzuki, S., 2020. Understanding human health risks caused by antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARG) in water environments: Current knowledge and questions to be answered. *Critical Reviews in Environmental Science and Technology*, 50(19):2016-2059.

Antonello, V.S., Dallé, J., Domingues, G.C., Ferreira, J.A.S., Fontoura, M.D.C.Q. and Knapp, F.B., 2014. Post-cesarean surgical site infection due to *Buttiauxella agrestis*. *International Journal of Infectious Diseases*, 22(3):65-66.

Appenzeller, B.M.R., Batté, M., Mathieu, L., Block, J.C., Lahoussine, V., Cavard, J. and Gatel, D.J.W.R., 2001. Effect of adding phosphate to drinking water on bacterial growth in slightly and highly corroded pipes. *Water research*, 35(4):1100-1105.

Arshad, M. and Shakoor, A., 2017. Irrigation water quality. *Pakistan Journal of Agricultural Research*, 31(2):102-123.

Armstrong, J.L., Calomiris, J.J. and Seidler, R.J., 1982. Selection of antibiotic-resistant standard plate count bacteria during water treatment. *Applied and Environmental Microbiology*, 44(2):308-316.

Armstrong, J.L., Shigeno, D.S., Calomiris, J.J. and Seidler, R.J., 1981. Antibiotic-resistant bacteria in drinking water. *Applied and Environmental Microbiology*, 42(2):277-283.

Asitok, A.D. and Ekpenyong, M.G., 2019. Statistical distribution of halotolerant/halophilic biosurfactant-producing yeasts in Qua Iboe estuarine sediments. *International Journal of Science*, 8(5).

Atanasova, P., Barron-Cedeno, A., Elsayed, T., Suwaileh, R., Zaghouani, W., Kyuchukov, S., Martino, G.D.S. and Nakov, P., 2018. Overview of the CLEF-2018 CheckThat! lab on automatic identification and verification of political claims. Task 1: Check-worthiness. *arXiv preprint arXiv 156:1808.05542*.

Ateba, C.N. and Bezuidenhout, C.C., 2008. Characterisation of *Escherichia coli* O157 strains from humans, cattle and pigs in the North-West Province, South Africa. *International Journal of Food Microbiology*, 128(2):181-188.

Ateba, C.N. and Maribeng, M.D., 2011. Detection of *Enterococcus* species in groundwater from some rural communities in the Mmabatho area, South Africa: A risk analysis. *African Journal of Microbiology Research*, 5(23):3930-3935.

Atherholt, T.B., Bousenberry, R.T., Carter, G.P., Korn, L.R., Louis, J.B., Serfes, M.E. and Waller, D.A., 2013. Coliform bacteria in New Jersey domestic wells: influence of geology, laboratory, and method. *Groundwater*, 51(4):562-574.

Atherholt, T.B., Procopio, N.A. and Goodrow, S.M., 2017. Seasonality of coliform bacteria detection rates in New Jersey domestic wells. *Groundwater*, 55(3):346-361.

Atnafu, B., Desta, A. and Assefa, F., 2021. Microbial Community Structure and Diversity in Drinking Water Supply, Distribution Systems as well as Household Point of Use Sites in Addis Ababa City, Ethiopia. *Microbial ecology*, 23:1-17.

Ayinla, H.A., Abdullah, W.H., Makeen, Y.M., Abubakar, M.B., Jauro, A., Yandoka, B.M.S. and Abidin, N.S.Z., 2017. Petrographic and geochemical characterization of the Upper Cretaceous coal and mudstones of Gombe Formation, Gongola sub-basin, northern Benue trough Nigeria: Implication for organic matter preservation, paleodepositional environment and tectonic settings. *International Journal of Coal Geology*, 180:67-82.

Azzam, M.I., Ezzat, S., Othman, B.A & El-Douhdoug, K.A. 2017. Antibiotics resistance phenomenon and virulence ability in bacteria from the water environment. *Water science*, 31(2):109-121.

Azhardpoor, A., Salehi, N., Heidari, H., Sarmadipour, M. and Mahmoudian, H., 2019. Relationship between turbidity and microbial load of water in salman Farsi Dam Reservoir. *J. Environ. Pollut. Manage*, 2:201.

Bacanli, M. and Başaran, N., 2019. Importance of antibiotic residues in animal food. *Food and Chemical Toxicology*, 125:462-466.

Bahirathan, M., Puente, L. and Seyfried, P., 1998. Use of yellow-pigmented enterococci as a specific indicator of human and nonhuman sources of faecal pollution. *Canadian journal of microbiology*, 44(11):1066-1071.

Banks, D., Markland, H., Smith, P.V., Mendez, C., Rodriguez, J., Huerta, A. and Sæther, O.M., 2004. Distribution, salinity and pH dependence of elements in surface waters of the catchment areas of the Salars of Coipasa and Uyuni, Bolivian Altiplano. *Journal of Geochemical Exploration*, 84(3):141-166.

Bai, L., Xia, S., Lan, R., Liu, L., Ye, C., Wang, Y., Jin, D., Cui, Z., Jing, H., Xiong, Y. and Bai, X., 2012. Isolation and characterization of cytotoxic, aggregative *Citrobacter freundii*. *PLoS One*, 7(3):33054.

Balan, G., 2021. Association between antibiotic-resistance and virulence factors in *Klebsiella pneumoniae* strains. *InterConf*, 12(2):34-57.

Bhatt, S., 2020. Atypical enteropathogenic *Escherichia coli*: From kittens to humans and beyond!. *Infection and Immunity*, 89(3):e00752-20.

Baquero, F., Martínez, J.L. and Cantón, R., 2008. Antibiotics and antibiotic-resistance in water environments. *Current opinion in biotechnology*, 19(3):260-265.

Barlow, D.H., 2004. Psychological treatments. *American psychologist*, 59(9):869.

Benson, C.A., Bizzoco, R.W., Lipson, D.A. and Kelley, S.T., 2011. Microbial diversity in nonsulfur, sulfur and iron geothermal steam vents. *FEMS microbiology ecology*, 76(1):74-88.

Bergan, J., Lingelem, A.B.D., Simm, R., Skotland, T. and Sandvig, K., 2012. Shiga toxins. *Toxicon*, 60(6):1085-1107.

Bethke, C.M. and Johnson, T.M., 2008. Groundwater age and groundwater age dating. *Annual Rev. Earth Planet. Science*, 36(1):121-152.

Bezuidenhout, C.C., Mthembu, N., Puckree, T. and Lin, J., 2002. Microbiological evaluation of the Mhlathuze river, KwaZulu-natal (RSA). *Water SA*, 28(3):281-286.

Bezuidenhout, C. C.& Adeleke, R. A. 2011. Integrated water resources management (IWRM) in South Africa-challenges of microbiology quality of water resources. *African Journal of Science, Technology, Innovation and Development* 3(4):55–64.

Bigham, T., Dooley, J.S., Ternan, N.G., Snelling, W.J., Castelán, M.H. and Davis, J., 2019. Assessing microbial water quality: electroanalytical approaches to the detection of coliforms. *TrAC Trends in Analytical Chemistry*, 121:115-670.

Boehm, A.B. and Sassoubre, L.M., 2014. *Enterococci* as indicators of environmental faecal contamination.

Boretti, A. and Rosa, L., 2019. Reassessing the projections of the world water development report. *NPJ Clean Water*, 2(1):1-6.

Boyles, W., 1997. Chemical oxygen demand. *Technical information series*, (9):24.

Bradford, S.A. and Harvey, R.W., 2017. Future research needs involving pathogens in groundwater. *Hydrogeology Journal*, 25(4):931-938.

Bradley, A.J., Leach, K.A., Green, M.J., Gibbons, J., Ohnstad, I.C., Black, D.H., Payne, B., Prout, V.E. and Breen, J.E., 2018. The impact of dairy cows' bedding material and its microbial content on the quality and safety of milk—A cross sectional study of UK farms. *International journal of food microbiology*, 269:36-45.

Braune, E. and Xu, Y., 2010. The role of groundwater in Sub-Saharan Africa. *Groundwater*, 48(2):229-238.

Brinkman, F.S. and Leipe, D.D., 2001. Phylogenetic analysis. *Bioinformatics: a practical guide to the analysis of genes and proteins*, 2:349.

Brooke, J.S., 2012. *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. *Clinical microbiology reviews*, 25(1):2-41.

Brown, C.J., Barlow, J.R., Cravotta III, C.A. and Lindsey, B.D., 2019. Factors affecting the occurrence of lead and manganese in untreated drinking water from Atlantic and Gulf Coastal Plain aquifers, eastern United States—Dissolved oxygen and pH framework for evaluating risk of elevated concentrations. *Applied Geochemistry*, 101:88-102.

Burgalassi, S., Ceccanti, S., Vecchiani, S., Leonangeli, G., Federigi, I., Carducci, A. and Verani, M., 2021. Objectionable microorganisms in pharmaceutical production: Validation of a decision tree. *European Journal of Pharmaceutical Sciences*, 166:105984.

Burri, N.M., Weatherl, R., Moeck, C. and Schirmer, M., 2019. A review of threats to groundwater quality in the anthropocene. *Science of the Total Environment*, 684(1):136-154.

Bwapwa, J.K., 2019. Analysis on Industrial and Domestic Wastewater in South Africa as a Water-Scarce Country. *International Journal of Applied Engineering Research*, 14(7):1474-1483.

Byappanahalli, M.N., Nevers, M.B., Korajkic, A., Staley, Z.R. and Harwood, V.J., 2012. Enterococci in the environment. *Microbiology and Molecular Biology Reviews*, 76(4):685-706.

Cabral, J.P., 2010. Water microbiology. Bacterial pathogens and water. *International journal of environmental research and public health*, 7(10):3657-3703.

Caltagirone, M., Nucleo, E., Spalla, M., Zara, F., Novazzi, F., Marchetti, V.M., Piazza, A., Bitar, I., De Cicco, M., Paolucci, S. and Pilla, G., 2017. Occurrence of extended spectrum β -lactamases, KPC-type, and MCR-1.2-producing *Enterobacteriaceae* from wells, river water, and wastewater treatment plants in Oltrepò Pavese area, Northern Italy. *Frontiers in microbiology*, 8:2232.

Carter, M.Q., Pham, A., Huynh, S. and He, X., 2017. Complete genome sequence of a Shiga toxin-producing *Enterobacter cloacae* clinical isolate. *Genome announcements*, 5(37):e00883-17.

Cetinkaya, Y., Falk, P. and Mayhall, C.G., 2000. Vancomycin-resistant enterococci. *Clinical microbiology reviews*, 13(4):686-707.

Chakraborty, R., Khan, K.M., Dibaba, D.T., Khan, M.A., Ahmed, A. and Islam, M.Z., 2019. Health implications of drinking water salinity in coastal areas of Bangladesh. *International journal of environmental research and public health*, 16(19):37-46.

Chang, H., 2005. Spatial and temporal variations of water quality in the Han River and its tributaries, Seoul, Korea, 1993–2002. *Water, Air, and Soil Pollution*, 161(1):267-284.

Chen, J.J., Sun, Y.W. and Sheen, T.F., 1999. Use of cold water for irrigation reduces stem elongation of plug-grown tomato and cabbage seedlings. *HortScience*, 34(5):852-854.

Cheng, W., Chen, H., Su, C. and Yan, S., 2013. Abundance and persistence of antibiotic-resistance genes in livestock farms: a comprehensive investigation in eastern China. *Environment international*, 61:1-7.

Chidamba, L. and Korsten, L., 2015. A scoping study on the prevalence of *Escherichia coli* and *Enterococcus* species in harvested rainwater stored in tanks. *Water SA*, 41(4):501-508.

Chigbu, P., Gordon, S. and Tchounwou, P.B., 2005. The seasonality of faecal coliform bacteria pollution and its influence on closures of shellfish harvesting areas in Mississippi Sound. *International journal of environmental research and public health*, 2(2):362-373.

Chigor, V.N., Sibanda, T. and Okoh, A.I., 2013. Studies on the bacteriological qualities of the Buffalo River and three source water dams along its course in the Eastern Cape Province of South Africa. *Environmental Science and Pollution Research*, 20(6):4125-4136.

Cho, J.C., Cho, H.B. and Kim, S.J., 2000. Heavy contamination of a subsurface aquifer and a stream by livestock wastewater in a stock farming area, Wonju, Korea. *Environmental Pollution*, 109(1):137-146.

Cho, S., Jackson, C.R. and Frye, J.G., 2020. The prevalence and antimicrobial resistance phenotypes of *Salmonella*, *Escherichia coli* and *Enterococcus* spp. in surface water. *Letters in applied microbiology*, 71(1):3-25.

Choi, S.H., Lee, S.O., Kim, T.H., Chung, J.W., Choo, E.J., Kwak, Y.G., Kim, M.N., Kim, Y.S., Woo, J.H., Ryu, J. and Kim, N.J., 2004. Clinical features and outcomes of bacteremia caused by *Enterococcus casseliflavus* and *Enterococcus gallinarum*: analysis of 56 cases. *Clinical infectious diseases*, 38(1):53-61.

Chomba, M.J., Hill, T., Nkhata, B.A. and Förster, J.J., 2017. Paradigms for water allocation in river basins: a society-science-practice perspective from Southern Africa. *Water Policy*, 19(4):637-649.

Chopra, I., 2013. The 2012 Garrod lecture: discovery of antibacterial drugs in the 21st century. *Journal of Antimicrobial Chemotherapy*, 68(3):496-505.

Chow, J.W., Thal, L.A., Perri, M.B., Vazquez, J.A., Donabedian, S.M., Clewell, D.B. and Zervos, M.J., 1993. Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. *Antimicrobial agents and chemotherapy*, 37(11):2474-2477.

Christensen, T.H., Cossu, R. and Stegmann, R., 1992. *Landfilling of waste: leachate*. CRC Press.

Cimanga, L., 2017. Evaluation of the water quality of Lake Nsezi in Richards Bay (Doctoral dissertation).

Clarke, J., Hall, S., Jefferson, T. and Roberts, B., 2017. *Subcultures, cultures and class*, 5:47-116.

Clarridge III, J.E., 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical microbiology reviews*, 17(4):840-862.

Coburn, P.S. and Gilmore, M.S., 2003. The *Enterococcus faecalis* cytolysin: a novel toxin active against eukaryotic and prokaryotic cells. *Cellular microbiology*, 5(10):661-669.

Coertze, R.D. and Bezuidenhout, C.C., 2018. The prevalence and diversity of AmpC β -lactamase genes in plasmids from aquatic systems. *Water Science and Technology*, 2017(2):603-611.

Collin, M. and Olsén, A., 2003. Extracellular enzymes with immunomodulating activities: variations on a theme in *Streptococcus pyogenes*. *Infection and Immunity*, 71(6):2983-2992.

Collins, M. and Moore, K.J., 2017. Preservation of forage as hay and silage. *Forages*, 1:321.

Conant Jr, B., Robinson, C.E., Hinton, M.J. and Russell, H.A., 2019. A framework for conceptualizing groundwater-surface water interactions and identifying potential impacts on water quality, water quantity, and ecosystems. *Journal of Hydrology*, 574:609-627.

Conedera, G., Chapman, P.A., Marangon, S., Tisato, E., Dalvit, P. and Zuin, A., 2001. A field survey of *Escherichia coli* O157 ecology on a cattle farm in Italy. *International journal of food microbiology*, 66(1-2):85-93.

Conrad, J.E., Colvin, C., Sililo, O., Gorgens, A., Weaver, J. and Reinhardt, C., 1999. Assessment of the impact of agricultural practices on the quality of groundwater resources in South Africa. *Water Research Commission Report*, 641(1):99.

Correa-Martínez, C.L., Idelevich, E.A., Sparbier, K., Kostrzewa, M. and Becker, K., 2019. Rapid detection of extended-spectrum β -lactamases (ESBL) and AmpC β -lactamases in Enterobacterales: development of a screening panel using the MALDI-TOF MS-based direct-on-target microdroplet growth assay. *Frontiers in microbiology*, 10:13.

Cosgrove, W.J. and Loucks, D.P., 2015. Water management: Current and future challenges and research directions. *Water Resources Research*, 51(6):4823-4839.

Cossart, P. and Lebreton, A., 2014. A trip in the “New Microbiology” with the bacterial pathogen *Listeria monocytogenes*. *FEBS letters*, 588(15):2437-2445.

Cossart, P., Vicente, M.F., Mengaud, J., Baquero, F., Perez-Diaz, J.C & Berche P. 1989 Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. *Infection Immunology*, 57:3629–3636.

Crookes, C., Hedden, S. and Donnenfeld, Z., 2018. A delicate balance: Water scarcity in South Africa. *ISS Southern Africa Report*, 2018(13):1-24.

Cui, G., Lu, Y., Zheng, C., Liu, Z. and Sai, J., 2019. Relationship between soil salinization and groundwater hydration in Yaoba Oasis, Northwest China. *Water*, 11(1):175.

da Silva, Y., Ferrari, R., Marin, V.A. and Junior, C.A.C., 2019. A Global Overview of β -lactam Resistance Genes in. *The Open Infectious Diseases Journal*, 11(1).

Culp, E. and Wright, G.D., 2017. Bacterial proteases, untapped antimicrobial drug targets. *The Journal of antibiotics*, 70(4):366-377.

Davies, J., 1994. Inactivation of antibiotics and the dissemination of resistance genes. *Science*, 264(5157):375-382.

Davies, J. and Davies, D., 2010. Origins and evolution of antibiotic-resistance. *Microbiology and molecular biology reviews*, 74(3):417-433.

De Croos, J.N.A., Dhaliwal, S.S., Grynpas, M.D., Pilliar, R.M. and Kandel, R.A., 2006. Cyclic compressive mechanical stimulation induces sequential catabolic and anabolic gene changes in chondrocytes resulting in increased extracellular matrix accumulation. *Matrix Biology*, 25(6):323-331.

Denis, A., Agouridas, C., Auger, J.M., Benedetti, Y., Bonnefoy, A., Bretin, F., Chantot, J.F., Dussarat, A., Fromentin, C., D'Ambrières, S.G. and Lachaud, S., 1999. Synthesis and antibacterial activity of HMR 3647 a new ketolide highly potent against erythromycin-resistant and susceptible pathogens. *Bioorganic & medicinal chemistry letters*, 9(21):3075-3080.

Deschenes, G., Casenave, C., Grimont, F., Desenclos, J.C., Benoit, S., Collin, M., Baron, S., Mariani, P., Grimont, P.A. and Nivet, H., 1996. Cluster of cases of haemolytic uraemic syndrome due to unpasteurised cheese. *Pediatric Nephrology*, 10(2):203-205.

Detzer, J., Loikith, P.C., Pampuch, L.A., Mechoso, C.R., Barkhordarian, A. and Lee, H., 2020. Characterizing monthly temperature variability states and associated meteorology across southern South America. *International Journal of Climatology*, 40(1):492-508.

Devriese, L.A., Laurier, L., Herdt, P.D. and Haesebrouck, F., 1992. Enterococcal and streptococcal species isolated from faeces of calves, young cattle and dairy cows. *Journal of Applied Bacteriology*, 72(1):29-31.

Dionisio, L.P.C. and Borrego, J.J., 1995. Evaluation of media for the enumeration of faecal streptococci from natural water samples. *Journal of microbiological methods*, 23(2):183-203.

Dionisio, L.P.C., Joao, M., Ferreira, V.S., Fidalgo, M.L., Rosado, M.E.G. and Borrego, J.J., 2000. Occurrence of *Salmonella* spp in estuarine and coastal waters of Portugal. *Antonie Van Leeuwenhoek*, 78(1):99-106.

D'Odorico, P., Carr, J., Dalin, C., Dell'Angelo, J., Konar, M., Laio, F., Ridolfi, L., Rosa, L., Suweis, S., Tamea, S. and Tuninetti, M., 2019. Global virtual water trade and the hydrological cycle: patterns, drivers, and socio-environmental impacts. *Environmental Research Letters*, 14(5):053001.

Doherty, N., Trzcinski, K., Pickerill, P., Zawadzki, P. and Dowson, C.G., 2000. Genetic diversity of the tet (M) gene in tetracycline-resistant clonal lineages of *Streptococcus pneumoniae*. *Antimicrobial agents and chemotherapy*, 44(11):2979-2984.

Domagalski, J.L. and Johnson, H., 2012. Phosphorus and groundwater: establishing links between agricultural use and transport to streams. *US Geological Survey Fact Sheet*, 3004:2012.

Dong, X., Xu, W., Zhang, Y. and Leskovar, D.I., 2016. Effect of irrigation timing on root zone soil temperature, root growth and grain yield and chemical composition in corn. *Agronomy*, 6(2):34.

Douterelo, I., Dutilh, B.E., Calero, C., Rosales, E., Martin, K. and Husband, S., 2020. Impact of phosphate dosing on the microbial ecology of drinking water distribution systems: Fieldwork studies in chlorinated networks. *Water Research*, 187:116416.

Drosg, B., 2013. *Process monitoring in biogas plants*. Paris, France: IEA bioenergy.

du Plessis, A., 2017. Global water availability, distribution and use. In Freshwater Challenges of South Africa and its Upper Vaal River. *Springer Cham*, 35:3-11.

du Plessis, A., 2019. Current and future water scarcity and stress in Water as an Inescapable Risk. *Springer Cham*, 34:35-48.

Durand, J.F., 2012. The impact of gold mining on the Witwatersrand on the rivers and karst system of Gauteng and North West Province, South Africa. *Journal of African Earth Sciences*, 68:24-43.

Durmishi, B.H., Reka, A.A., Gjuladin-Hellon, T., Ismaili, M., Srbinovski, M. and Shabani, A., 2015. Disinfection of drinking water and trihalomethanes: a review. *International Journal of Advanced Research in Chemical Science (IJARCS)*, 2(11):45-56.

Dunlop, J., McGregor, G. and Horrigan, N., 2005. Potential impacts of salinity and turbidity in riverine ecosystems. *National Action Plan for Salinity and Water Quality. State of Queensland*.

DWAF: Department of Water Affairs and Forestry. 1996. South African water quality guidelines, Vol.1: Domestic water use, 2nd Ed. Pretoria, South-Africa: Government Printer.

DWAF: Department of Water Affairs and Forestry. 2000. Quality of domestic water. Pretoria, South Africa: Government Printer.

DWA: Department of Water Affairs. 2010. Groundwater Strategy 2011: Water Resources Planning Systems. Department of Water Affairs, Pretoria.

DWA: Department of Water Affairs, South Africa. 2011. Green Drop report 2009 version 1: South African wastewater quality management performance. Web: https://www.dws.gov.za/Documents/GreenDropReport2009_ver1_web.pdf (Accessed 1/12/2021).

DWAF: Department of Water Affairs and Forestry. 2011. Annual report. Pretoria, South Africa: Government Printer.

DWAF: Department of Water Affairs and Forestry. 2012. Annual report. Pretoria, South Africa: Government Printer.

DWS: Department of Water and Sanitation. 2021. Water levels. Annual report. Web:[https://nationalgovernment.co.za/departments/annual/349/2020-department-of-water-and-sanitation-\(dws\)-annual-report.pdf](https://nationalgovernment.co.za/departments/annual/349/2020-department-of-water-and-sanitation-(dws)-annual-report.pdf) (accessed 30 October 2021).

DWS: Department of Water and Sanitation. Annual National State of the water resource. Web:https://www.dws.gov.za/Groundwater/documents/Annual%20National%20State%20Water%20Report%20for%20Hydrological%20Year%202012-13_Final.pdf (accessed 10 September 2019).

Eagar, H., Swan, G. and Van Vuuren, M., 2012. A survey of antimicrobial usage in animals in South Africa with specific reference to food animals. *Journal of the South African Veterinary Association*, 83(1):L1-8.

Ebadi, A.G., Toughani, M., Najafi, A. and Babaei, M., 2020. A brief overview on current environmental issues in Iran. *Central Asian Journal of Environmental Science and Technology Innovation*, 1(1):1-11.2019

Ebenebe, J.C., Emeka-Okafor, K.M., Akujobi, C.N., Ezeanya, C.C., Agbakoba, N.R. and Egwuatu, C.C., 2014. Plasmid profile of uropathogens among children. *Journal of Advances in Medicine and Medical Research*, 14:1195-1203.

Edokpayi, J.N., Rogawski, E.T., Kahler, D.M., Hill, C.L., Reynolds, C., Nyathi, E., Smith, J.A., Odiyo, J.O., Samie, A., Bessong, P. and Dillingham, R., 2018. Challenges to sustainable safe drinking water: a case study of water quality and use across seasons in rural communities in Limpopo province, South Africa. *Water*, 10(2):159.

Ekwanzala, M.D., Dewar, J.B., Kamika, I. and Momba, M.N.B., 2018. Systematic review in South Africa reveals antibiotic-resistance genes shared between clinical and environmental settings. *Infection and drug resistance*, 11:1907.

Ekwanzala, M.D., Dewar, J.B., Kamika, I. and Momba, M.N.B., 2020. Comparative genomics of vancomycin-resistant *Enterococcus* spp. revealed common resistome determinants from hospital wastewater to aquatic environments. *Science of The Total Environment*, 719:137275.

Ells, T.C. and Hansen, L.T., 2006. Strain and growth temperature influence *Listeria* spp. attachment to intact and cut cabbage. *International journal of food microbiology*, 111(1):34-42.

El-Salam, Abd M.M. and Abu-Zuid, G.I., 2015. Impact of landfill leachate on the groundwater quality: A case study in Egypt. *Journal of advanced research*, 6(4):579-586.

Emamalipour, M., Seidi, K., Zununi Vahed, S., Jahanban-Esfahlan, A., Jaymand, M., Majdi, H., Amoozgar, Z., Chitkushev, L.T., Javaheri, T., Jahanban-Esfahlan, R. and Zare, P., 2020. Horizontal gene transfer: from evolutionary flexibility to disease progression. *Frontiers in Cell and Developmental Biology*, 8:229.

Entry, J.A. and Farmer, N., 2001. Movement of coliform bacteria and nutrients in ground water flowing through basalt and sand aquifers. *Journal of Environmental quality*, 30:1533-1539.

Erickson, B.E., 2002. Analyzing the ignored environmental contaminants. *Environmental science & technology*, 36(7):140A-145A.

Etebu, E. and Ariekpar, I., 2016. Antibiotics: Classification and mechanisms of action with emphasis on molecular perspectives. *International Journal of Applied Microbiology and Biotechnology Res*, 4(2016):90-101.

Facklam, R.R. and Collins, M.D., 1989. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. *Journal of clinical microbiology*, 27(4):731-734.

Fadiran, A.O., Dlamini, S.C. and Mavuso, A., 2008. A comparative study of the phosphate levels in some surface and groundwater bodies of Swaziland. *Bulletin of the Chemical Society of Ethiopia*, 22(2).

Fahlquist, L., 2003. Ground-water quality of the southern High Plains aquifer, Texas and New Mexico. *US Geological Survey*, 2001(345):2003-2078.

Facklam, R.R. and Collins, M.D., 1989. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. *Journal of clinical microbiology*, 27(4):731-734.

Falcone-Dias, M.F., Vaz-Moreira, I. and Manaia, C.M., 2012. Bottled mineral water as a potential source of antibiotic-resistant bacteria. *Water research*, 46(11):3612-3622.

Farley, E., Stewart, A., Davies, M.A., Govind, M., Van den Bergh, D. and Boyles, T.H., 2018. Antibiotic use and resistance: Knowledge, attitudes and perceptions among primary care prescribers in South Africa. *South African medical journal*, 108(9).

Farmer 3rd, J.J., Fanning, G.R., Davis, B.R., O'hara, C.M., Riddle, C., Hickman-Brenner, F.W., Asbury, M.A., Lowery 3rd, V.A. and Brenner, D.J., 1985. *Escherichia fergusonii* and *Enterobacter taylorae*, two new species of Enterobacteriaceae isolated from clinical specimens. *Journal of clinical microbiology*, 21(1):77-81.

Ferguson, D.M., Moore, D.F., Getrich, M.A. and Zhouwandai, M.H., 2005. Enumeration and speciation of enterococci found in marine and intertidal sediments and coastal water in southern California. *Journal of Applied Microbiology*, 99(3):598-608.

Ferreira, S.L., 2011. Microbial and physico-chemical quality of groundwater in the North-West Province, South Africa (Doctoral dissertation, North-West University (South Africa)).

Fiedler, G., Schneider, C., Igbinosa, E.O., Kabisch, J., Brinks, E., Becker, B., Stoll, D.A., Cho, G.S., Huch, M. and Franz, C.M., 2019. Antibiotics resistance and toxin profiles of *Bacillus cereus*-group isolates from fresh vegetables from German retail markets. *BMC microbiology*, 19(1):1-13.

Figueras, M. and Borrego, J.J., 2010. New perspectives in monitoring drinking water microbial quality. *International journal of environmental research and public health*, 7(12);4179-4202.

Fiorino, S., Bacchi-Reggiani, L., Pontoriero, L., Gallo, C., Chili, E., Masetti, M., Zanini, N., Grondona, A., Silvestri, T., Deleonardi, G. and Fornelli, A., 2014. Tensegrity model hypothesis: may this paradigm be useful to explain hepatic and pancreatic carcinogenesis in patients with persistent hepatitis B or hepatitis C virus infection?. *JOP. Journal of the Pancreas*, 15(2):151-164.

Fisher, K. and Phillips, C., 2009. The ecology, epidemiology and virulence of *Enterococcus*. *Microbiology*, 155(6):1749-1757.

Flores-Treviño, S., Gutierrez-Ferman, J.L., Morfín-Otero, R., Rodríguez-Noriega, E., Estrada-Rivadeneyra, D., Rivas-Morales, C., Llaca-Díaz, J.M., Camacho-Ortíz, A., Mendoza-Olazarán, S. and Garza-Gonzalez, E., 2014. *Stenotrophomonas maltophilia* in Mexico: antimicrobial resistance, biofilm formation and clonal diversity. *Journal of medical microbiology*, 63(11):524-1530.

Fluit, A.C. and Schmitz, F.J., 2004. Resistance integrons and super-integrons. *Clinical microbiology and infection*, 10(4):272-288.

Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O. and Dantas, G., 2012. The shared antibiotic resistome of soil bacteria and human pathogens. *science*, 337(6098), pp.1107-1111.

Foster, S.S.D., Tuinhof, A. and Garduño, H., 2008. Groundwater in Sub-Saharan Africa: A strategic overview of developmental issues. *Applied groundwater studies in Africa*, 20:19-34.

Fournier, P.E., Gouriet, F., Casalta, J.P., Lepidi, H., Chaudet, H., Thuny, F., Collart, F., Habib, G. and Raoult, D., 2017. Blood culture-negative endocarditis: improving the diagnostic yield using new diagnostic tools. *Medicine*, 96(47).

Freney, J., Gavini, F., Ploton, C., Leclerc, H. and Fleurette, J., 1987. Isolation of *Escherichia fergusonii* from a patient with septicemia in France. *European journal of clinical microbiology*, 6(1):78-78.

Fridrich, B., Krčmar, D., Dalmacija, B., Molnar, J., Pešić, V., Kragulj, M. and Varga, N., 2014. Impact of wastewater from pig farm lagoons on the quality of local groundwater. *Agricultural Water Management*, 135:40-53.

Fuller, C.A., Pellino, C.A., Flagler, M.J., Strasser, J.E. and Weiss, A.A., 2011. Shiga toxin subtypes display dramatic differences in potency. *Infection and immunity*, 79(3), pp.1329-1337.

Gaastra, W., Kusters, J.G., Van Duijkeren, E. and Lipman, L.J.A., 2014. *Escherichia fergusonii*. *Veterinary microbiology*, 172(1-2):7-12.

Gabutti, G., De Donno, A., Bagordo, F. and Montagna, M.T., 2000. Comparative survival of faecal and human contaminants and use of *Staphylococcus aureus* as an effective indicator of human pollution. *Marine Pollution Bulletin*, 40(8):697-700.

Gao, J., Yu, F.Q., Luo, L.P., He, J.Z., Hou, R.G., Zhang, H.Q., Li, S.M., Su, J.L. and Han, B., 2012. Antibiotic-resistance of *Streptococcus agalactiae* from cows with mastitis. *The Veterinary Journal*, 194(3):423-424.

Gautam, S., Dinda, A.K. and Mishra, N.C., 2013. Fabrication and characterization of PCL/gelatin composite nanofibrous scaffold for tissue engineering applications by electrospinning method. *Materials Science and Engineering: C*, 33(3):1228-1235.

Gbetibouo, G.A., 2009. Understanding farmers' perceptions and adaptations to climate change and variability: The case of the Limpopo Basin, South Africa. *International Food Policy Res Institute*, 849.

Geerdink, R.B., van den Hurk, R.S. and Epema, O.J., 2017. Chemical oxygen demand: Historical perspectives and future challenges. *Analytica Chimica Acta*, 96(1):1-11.

George, J.F., 2002. Influences on the intent to make Internet purchases. *Internet Research*.

Gibbs, S.G., Green, C.F., Tarwater, P.M. and Scarpino, P.V., 2004. Airborne antibiotic-resistant and nonresistant bacteria and fungi recovered from two swine herd confined animal feeding operations. *Journal of occupational and environmental hygiene*, 1(11);699-706.

Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M. and Zhu, Y.G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *The ISME journal*, 9(6):1269-1279.

Giordano, M., 2006. Agricultural groundwater use and rural livelihoods in sub-Saharan Africa: A first-cut assessment. *Hydrogeology Journal*, 14(3):310-318.

Giri, J., Bhosale, R., Huang, G., Pandey, B.K., Parker, H., Zappala, S., Yang, J., Dievart, A., Bureau, C., Ljung, K. and Price, A., 2018. Rice auxin influx carrier OsAUX1 facilitates root hair elongation in response to low external phosphate. *Nature communications*, 9(1):1-7.

Girlich, D., Naas, T., Bellais, S., Poirel, L., Karim, A. and Nordmann, P., 2000. Biochemical-genetic characterization and regulation of expression of an ACC-1-like chromosome-borne cephalosporinase from *Hafnia alvei*. *Antimicrobial Agents and Chemotherapy*, 44(6):1470-1478.

Glover, B., Wentzel, J., Jenkins, A. and Van Vuuren, M., 2017. The first report of *Escherichia fergusonii* isolated from non-human primates, in Africa. *One Health*, 3:70-75.

González-Plaza, Juan José, Khald Blau, Milena Milaković, Tamara Jurina, Kornelia Smalla, and Nikolina Udiković-Kolić. "Antibiotic-manufacturing sites are hot-spots for the release and spread of antibiotic-resistance genes and mobile genetic elements in receiving aquatic environments." *Environment international* 130 (2019):104735.

Gottlieb, D. and Legator, M., 1953. The growth and metabolic behavior of *Streptomyces venezuelae* in liquid culture. *Mycologia*, 45(4):507-515.

Goulden, M., Conway, D. and Persechino, A., 2009. Adaptation to climate change in international river basins in Africa: a review/Adaptation au changement climatique dans les bassins fluviaux internationaux en Afrique: une revue. *Hydrological Sciences Journal*, 54(5):805-828.

Graff Zivin, J. and Neidell, M., 2014. Temperature and the allocation of time: Implications for climate change. *Journal of Labor Economics*, 32(1):1-26.

Granum, P.E. and Lindbäck, T., 2012. *Bacillus cereus*. *Food microbiology: Fundamentals and frontiers*, 491-502.

Green, M.S., LeDuc, J., Cohen, D. and Franz, D.R., 2019. Confronting the threat of bioterrorism: realities, challenges, and defensive strategies. *The Lancet Infectious Diseases*, 19(1):e2-e13.

Green, R., 2016. Threshold-voltage bias-temperature instability in commercially-available SiC MOSFETs. *Japanese Journal of Applied Physics*, 55(4S):04EA03.

Grönwall, J. and Danert, K., 2020. Regarding groundwater and drinking water access through a human rights lens: Self-Supply as a norm. *Water*, 12(2):419.

Guillaume, O., Garric, X., Lavigne, J.P., Van Den Berghe, H. and Coudane, J., 2012. Multilayer, degradable coating as a carrier for the sustained release of antibiotics: preparation and antimicrobial efficacy in vitro. *Journal of controlled release*, 162(3):492-501.

Gunarathna, M.H.J.P., Kumari, M.K.N. and Nirmanee, K.G.S., 2016. Evaluation of interpolation methods for mapping pH of groundwater. *International journal of latest technology in engineering, management & applied science*, 3:1-5.

Gupta, N., Pandey, P. and Hussain, J., 2017. Effect of physicochemical and biological parameters on the quality of river water of Narmada, Madhya Pradesh, India. *Water Science*, 31(1):11-23.

Gupta, V.K., Ali, I., Saleh, T.A., Siddiqui, M.N. and Agarwal, S., 2013. Chromium removal from water by activated carbon developed from waste rubber tires. *Environmental Science and Pollution Research*, 20(3):1261-1268.

Gutschik, E., Møller, S. and Christensen, N., 1979. Experimental Endocarditis in Rabbits: 3. Significance of the Proteolytic Capacity of the Infecting Strains of *Streptococcus Faecalis*. *Acta Pathologica Microbiologica Scandinavica Section B Microbiology*, 87(1-6):353-362.

Gwimbi, P., George, M. and Ramphalile, M., 2019. Bacterial contamination of drinking water sources in rural villages of Mohale Basin, Lesotho: exposures through neighbourhood sanitation and hygiene practices. *Environmental health and preventive medicine*, 24(1):1-7.

Gyles, C.L., 2007. Shiga toxin-producing *Escherichia coli*: an overview. *Journal of animal science*, 85(13):E45-E62.

Hacker, J., Blum-Oehler, G., Mühldorfer, I. and Tschäpe, H., 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Molecular microbiology*, 23(6):1089-1097.

Hällgren, A., Claesson, C., Saeedi, B., Monstein, H.J., Hanberger, H. and Nilsson, L.E., 2009. Molecular detection of aggregation substance, enterococcal surface protein, and cytolysin genes and in vitro adhesion to urinary catheters of *Enterococcus faecalis* and *E. faecium* of clinical origin. *International Journal of Medical Microbiology*, 299(5):323-332.

Hamiwe, T., Kock, M.M., Magwira, C.A., Antiabong, J.F. and Ehlers, M.M., 2019. Occurrence of enterococci harbouring clinically important antibiotic-resistance genes in the aquatic environment in Gauteng, South Africa. *Environmental Pollution*, 245:1041-1049.

Han, X.Y. and Andrade, R.A., 2005. *Brevundimonas diminuta* infections and its resistance to fluoroquinolones. *Journal of Antimicrobial Chemotherapy*, 55(6):853-859.

Hancock, D.D., Besser, T.E., Kinsel, M.L., Tarr, P.I., Rice, D.H. and Paros, M.G., 1994. The prevalence of *Escherichia coli* O157. H7 in dairy and beef cattle in Washington State. *Epidemiology & Infection*, 113(2):199-207.

Handschuh, H., Ryan, M.P., O'Dwyer, J. and Adley, C.C., 2017. Assessment of the bacterial diversity of aircraft water: identification of the frequent fliers. *PLoS One*, 12(1):e0170567.

Harada, T., Nguyen, D.P., Nguyen, T.A.D., Sakamoto, M., Ohkuma, M., Motooka, D., Nakamura, S., Uchida, K., Jinnai, M., Yonogi, S. and Kawahara, R., 2016. *Enterococcus saigonensis* sp. nov., isolated from retail chicken meat and liver. *International journal of systematic and evolutionary microbiology*, 66(10):3779-3785.

Harada, T., Nguyen, D.P., Nguyen, T.A.D., Sakamoto, M., Ohkuma, M., Motooka, D., Nakamura, S., Uchida, K., Jinnai, M., Yonogi, S. and Kawahara, R., 2016. *Enterococcus saigonensis* sp. nov., isolated from retail chicken meat and liver. *International journal of systematic and evolutionary microbiology*, 66(10):3779-3785.

Harnisz, M & Korzeniewska, E. 2018. The prevalence of multidrug-resistant *Aeromonas* spp. In the municipal wastewater system and their dissemination in the environment. *Science of the Total Environment*, 626(2018):377-383.

Hartland, E.L., Huter, V., Higgins, L.M., Goncalves, N.S., Dougan, G., Phillips, A.D., MacDonald, T.T. and Frankel, G., 2000. Expression of intimin γ from enterohemorrhagic *Escherichia coli* in *Citrobacter rodentium*. *Infection and immunity*, 68(8):4637-4646.

Harwood, V.J., Brownell, M., Perusek, W. and Whitlock, J.E., 2001. Vancomycin-resistant *Enterococcus* spp. isolated from wastewater and chicken feces in the United States. *Applied and Environmental Microbiology*, 67(10):4930-4933.

He, B., Wang, S., Guo, L. and Wu, X., 2019. Aridity change and its correlation with greening over drylands. *Agricultural and Forest Meteorology*, 278:107663.

Healy, R., 2007. Which diuretics are safe and effective for patients with a sulfa allergy.

Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicology letters*, 131(1-2):5-17.

Hedden, S. and Cilliers, J., 2014. Parched prospects-the emerging water crisis in South Africa. *Institute for Security Studies Papers*, 2014(11):16.

Herbig, F. J. W., 2019. Talking dirty - effluent and sewage irreverence in South Africa: A conservation crime perspective. *Cogent Social Sciences*, 5(1):1701359.

Heydari, F.E., Bonyadian, M., Moshtaghi, H. and Sami, M., 2020. Prevalence and antibiotic-resistance profile of Shiga toxin-producing *Escherichia coli* isolated from diarrheal samples. *Iranian Journal of Microbiology*, 12(4):289.

Hidayatullah, A.R., Effendi, M.H., Plumeriastuti, H., Wibisono, F.M., Hartadi, E.B. and Sofiana, E.D., 2020. A Review of the opportunistic pathogen *Citrobacter freundii* in piglets post weaning: Public Health Importance. *Sys. Rev. Pharm*, 11(9):767-773.

Hobbs P, De Meillon N., 2017. Hydrogeology of the Sterkfontein Cave System, Cradle of Humankind, South Africa. *South African Journal of Geology*, 120(3):403-20.

Hoko, Z. and Toto, T.N., 2020. Integration of water hyacinth plants into waste stabilization ponds: a case study of Donnybrook 4 Sewage Ponds in Mabvuku-Tafara, Harare, Zimbabwe. *Environmental Monitoring and Assessment*, 192(10):1-12.

Holman, I.P., Whelan, M.J., Howden, N.J., Bellamy, P.H., Willby, N.J., Rivas-Casado, M. and McConvey, P., 2008. Phosphorus in groundwater—an overlooked contributor to eutrophication?. *Hydrological Processes: An International Journal*, 22(26):5121-5127.

Holman, D.B., Klima, C.L., Gzyl, K.E., Zaheer, R., Service, C., Jones, T.H. and McAllister, T.A., 2021. Antimicrobial Resistance in *Enterococcus* spp. Isolated from a Beef Processing Plant and Retail Ground Beef. *Microbiology spectrum*, 9(3):e01980-21.

Hong, H., Qiu, J. and Liang, Y., 2010. Environmental factors influencing the distribution of total and faecal coliform bacteria in six water storage reservoirs in the Pearl River Delta Region, China. *Journal of Environmental Sciences*, 22(5):663-668.

Horn, S., Pieters, R. and Bezuidenhout, C., 2016. Pathogenic features of heterotrophic plate count bacteria from drinking-water boreholes. *Journal of water and health*, 14(6):890-900.

Horton, J.L., Kolb, T.E. and Hart, S.C., 2001. Physiological response to groundwater depth varies among species and with river flow regulation. *Ecological Applications*, 11(4):1046-1059.

Hosein, H.I., Azzam, R.A., Abo-Elwafa, M., Menshaw, A.M.S. and Rouby, S., 2019. Virulence profile of enteropathogenic escherichia coli (epec) isolated from the cases of neonatal calf diarrhea. *Adv. Anim. Vet. Sci*, 7(9):755-760.

Hossain, S., De Silva, B.C.J., Wimalasena, S.H.M.P., Pathirana, H.N.K.S. and Heo, G.J., 2017. Research Article High Prevalence of Quinolone Resistance Genes in *Citrobacter freundii* Isolated from Pet Turtles.

Hošťacká A, Čížnár I, Štefkovičová M. 2010. Temperature and pH affect the production of bacterial biofilm. *Folia microbiologica*, 55(1):75-8.

Hou, D., Huang, Z., Zeng, S., Liu, J., Wei, D., Deng, X., Weng, S., He, Z. and He, J., 2017. Environmental factors shape water microbial community structure and function in shrimp cultural enclosure ecosystems. *Frontiers in microbiology*, 8:2359.

Hua, Y., Bai, X., Zhang, J., Jernberg, C., Chromek, M., Hansson, S., Frykman, A., Yang, X., Xiong, Y., Wan, C. and Matussek, A., 2020. Molecular characteristics of eae-positive clinical Shiga toxin-producing Escherichia coli in Sweden. *Emerging microbes & infections*, 9(1):2562-2570.

Hubbard, A.T., Newire, E., Botelho, J., Reiné, J., Wright, E., Murphy, E.A., Hutton, W. and Roberts, A.P., 2020. Isolation of an antimicrobial-resistant, biofilm-forming, *Klebsiella grimontii* isolate from a reusable water bottle. *Microbiology Open*, 9(6):1128-1134.

Hudault, S., Gulnot, J., Selvin, A.L. 2001. *Escherichia coli* strains colonizing the gastrointestinal tract protect germ-free mice against *Salmonella typhimurium*. *Gut*, 49:47-55.

Hudzicki, J., 2009. Kirby-Bauer disk diffusion susceptibility test protocol.

Huff, R., Pereira, R.I., Pissetti, C., de Araújo, A.M., d'Azevedo, P.A., Frazzon, J. and GuedesFrazzon, A.P., 2020. Antimicrobial resistance and genetic relationships of enterococci from siblings and non-siblings *Heliconius erato* phyllis caterpillars. *PeerJ*, 8:e8647.

Huo, L., Pan, L., Chen, R., Shi, B., Wang, H. and He, S., 2021. Effects of disinfectants and particles on the occurrence of different microorganisms in drinking water distribution systems. *Environmental Science: Water Research & Technology*, 7(5):983-992.

Hussein, H.S. and Sakuma, T., 2005. Invited review: prevalence of Shiga toxin-producing *Escherichia coli* in dairy cattle and their products. *Journal of Dairy Science*, 88(2):450-465.

Hussain, A., Malik, M.Y., Khan, M. and Salahuddin, T., 2019. Application of generalized Fourier heat conduction law on MHD viscoelastic fluid flow over stretching surface. *International Journal of Numerical Methods for Heat & Fluid Flow*, 34(1):345:401.

Huys, G., Cnockaert, M., Janda, J.M. and Swings, J., 2003. *Escherichia albertii* sp. nov., a diarrhoeagenic species isolated from stool specimens of Bangladeshi children. *International journal of systematic and evolutionary microbiology*, 53(3):.807-810.

Hwang, S.B., Chelliah, R., Kang, J.E., Rubab, M., Banan-MwineDaliri, E., Elahi, F. and Oh, D.H., 2021. Role of Recent Therapeutic Applications and the Infection Strategies of Shiga Toxin-Producing *Escherichia coli*. *Frontiers in cellular and infection microbiology*, 11:450.

Iaria, C., Stassi, G., Costa, G.B., Di Leo, R., Toscano, A. and Cascio, A., 2005. Enterococcal meningitis caused by *Enterococcus casseliflavus*. First case report. *BMC Infectious Diseases*, 5(1):1-3.

Ilemobade, A.A., Gyang, E.O., Bida, S.A. and Addo, P.B., 1979. Cure of *Dermatophilus congolensis* infection in cattle by long-acting oxytetracycline. *Research in Veterinary Science*, 27(3):302-305.

Imran, M., Das, K.R. and Naik, M.M., 2019. Co-selection of multi-antibiotic-resistance in bacterial pathogens in metal and microplastic contaminated environments: An emerging health threat. *Chemosphere*, 215, pp.846-857.

Inagaki, K., Blackshear, C., Palmer, A. and Hobbs, C.V., 2018. Risk factors, geographic distribution, and healthcare burden of symptomatic congenital cytomegalovirus infection in the United States: analysis of a nationally representative database, 2000-2012. *The Journal of pediatrics*, 199:118-123.

Irvine, S.A. and Doughton, J.A., 2001. Salinity and sodicity, implications for farmers in Central Queensland. In *Proceedings of the 10th Australian agronomy conference*. (The Australian Society of Agronomy Inc.: Hobart) Available at <http://www.regional.org.au/au/asa/2001/3/b/irvine.htm> (verified 3 January 2006).

Isgren, C.M., 2021. Improving clinical outcomes via responsible antimicrobial use in horses. *Equine Veterinary Education*.

Islam, R., Faysal, S.M., Amin, R., Juliana, F.M., Islam, M.J., Alam, J., Hossain, M.N. and Asaduzzaman, M., 2017. Assessment of pH and total dissolved substances (TDS) in the commercially available bottled drinking water. *Journal of Nursing and Health Sciences*, 6(5):35-40.

Iversen, C., Waddington, M., On, S.L. and Forsythe, S., 2004. Identification and phylogeny of *Enterobacter sakazakii* relative to *Enterobacter* and *Citrobacter* species. *Journal of Clinical Microbiology*, 42(11):5368-5370.

Iweriebor, B.C., Gaqavu, S., Obi, L.C., Nwodo, U.U. and Okoh, A.I., 2015. Antibiotic susceptibilities of *Enterococcus* species isolated from hospital and domestic wastewater effluents in Alice, Eastern

Cape Province of South Africa. *International journal of environmental research and public health*, 12(4):4231-4246.

Jähne, B., Heinz, G. and Dietrich, W., 1987. Measurement of the diffusion coefficients of sparingly soluble gases in water. *Journal of Geophysical Research: Oceans*, 92(C10):10767-10776.

Jaiyeola, A.T. and Bwapwa, J.K., 2015. Dynamics of sedimentation and use of genetic algorithms for estimating sediment yields in a river: A critical review. *Natural Resource Modeling*, 28(3):207-218.

Jajere, S.M., 2019. A review of Salmonella enterica with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance. *Veterinary world*, 12(4):504.

Jang, H.J., Choi, Y.J., Ro, H.M. and Ka, J.O., 2012. Effects of phosphate addition on biofilm bacterial communities and water quality in annular reactors equipped with stainless steel and ductile cast iron pipes. *The Journal of Microbiology*, 50(1):17-28.

Jayalakshmi, K., Paramasivam, M., Sasikala, M., Tamilam, T.V. and Sumithra, A., 2017. Review on antibiotic residues in animal products and its impact on environments and human health. *Journal of Entomology and Zoology Study*, 5(3):1446-51.

Jensen, L.B., Frimodt-Møller, N. and Aarestrup, F.M., 1999. Presence of erm gene classes in gram-positive bacteria of animal and human origin in Denmark. *FEMS microbiology letters*, 170(1):151-158.

Jerse, A.E., Yu, J., Tall, B.D. and Kaper, J.B., 1990. A genetic locus of enteropathogenic Escherichia coli necessary for the production of attaching and effacing lesions on tissue culture cells. *Proceedings of the National Academy of Sciences*, 87(20):7839-7843.

Jett, B.D., Huycke, M.M. and Gilmore, M.S., 1994. Virulence of enterococci. *Clinical microbiology reviews*, 7(4):462-478.

Jiang, J., Hu, W., Xie, D., Yang, J., He, J., Gao, Y. and Wan, Q., 2019. 2D electric-double-layer phototransistor for photoelectronic and spatiotemporal hybrid neuromorphic integration. *Nanoscale*, 11(3):1360-1369.

John, D.E. and Rose, J.B., 2005. Review of factors affecting microbial survival in groundwater. *Environmental science & technology*, 39(19):7345-7356.

Jordaan, K. and Bezuidenhout, C.C., 2016. Bacterial community composition of an urban river in the North West Province, South Africa, in relation to physico-chemical water quality. *Environmental Science and Pollution Research*, 23(6):5868-5880.

Jubb, K.V.F., Kennedy, P.C. and Palmer, N., 2012. *Pathology of domestic animals*. Academic press.

Kafil HS, Mobarez AM, Moghadam MF. Adhesion and virulence factor properties of Enterococci isolated from clinical samples in Iran. *Indian Journal of Pathology and Microbiology*, 56(3):238.

Kalpoe, J.S., Hogenbirk, K., van Maarseveen, N.M., Gesink-Van der Veer, B.J., Kraakman, M.E.M., Maarleveld, J.J., van der Reyden, T.J.K., Dijkshoorn, L. and Bernards, A.T., 2008. Dissemination of *Bacillus cereus* in a paediatric intensive care unit traced to insufficient disinfection of reusable ventilator air-flow sensors. *Journal of Hospital Infection*, 68(4):341-347.

Kalule, J.B., Fortuin, S., Calder, B., Robberts, L., Keddy, K.H., Nel, A.J., Garnett, S., Nicol, M., Warner, D.F., Soares, N.C. and Blackburn, J.M., 2018. Proteomic comparison of three clinical diarrhoeagenic drug-resistant *Escherichia coli* isolates grown on CHROMagar™ STEC media. *Journal of proteomics*, 180:25-35.

Kalule-Sabiti, M. and Heath, R., 2008, May. Underground water—A key resource and the associated environmental issues in the North-West Province of South-Africa. In *Proceedings of the WISA Biennial Conference, Sun City, South Africa*, 18-22.

Kapoor, G., Saigal, S & Elongavan, A. 2017. Action and resistance mechanisms of antibiotics: A guide for clinicians. *Journal of Anaesthesiology and Clinical Pharmacology*, 33(3):300-305.

Karmali, M.A., 2018. Factors in the emergence of serious human infections associated with highly pathogenic strains of shiga toxin-producing *Escherichia coli*. *International Journal of Medical Microbiology*, 308(8):1067-1072.

Kaur, R., Bhullar, K.K., Deepali, S.M. and Handa, A., 2021. Comparison of Efficacy of 2.5% Vs 5.25% Concentrations of Sodium Hypochlorite Augmented by Different Irrigation Techniques in Eradication of Enterococcus Faecalis—An In-Vitro Study. *Annals of the Romanian Society for Cell Biology*, 45(7):6227-6236.

Kaushik, M., Kumar, S., Kapoor, R.K. and Gulati, P., 2019. Integrons and antibiotic-resistance genes in water-borne pathogens: threat detection and risk assessment. *Journal of medical microbiology*, 68(5):679-692.

Kelbe, B., Taylor, R. and Haldorsen, S., 2004. The groundwater dynamics and its importance to the ecological resilience of Lake St Lucia. *Groundwater*, 53:53.

Keynan, Y. and Rubinstein, E., 2007. The changing face of Klebsiella pneumoniae infections in the community. *International journal of antimicrobial agents*, 30(5):385-389.

Khachatourians, G.G., 1998. Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. *Cmaj*, 159(9):1129-1136.

Khan, F.Y., 2021. Enterococcal meningitis/ventriculitis in Qatar—Experience with eight patients. *Qatar Medical Journal*, 2020(3):46.

Khanum, I., Anwar, S. and Farooque, A., 2019. Enterococcal meningitis/ventriculitis: A tertiary care experience. *Asian journal of neurosurgery*, 14(1):102.

Kashid, S.S., Ghosh, S. and Maity, R., 2010. Streamflow prediction using multi-site rainfall obtained from hydroclimatic teleconnection. *Journal of Hydrology*, 395(1-2):23-38.

Kholdi, S., Motamedifar, M., Fani, F., Mohebi, S. and Bazargani, A., 2021. Virulence factors, serogroups, and antibiotic-resistance of Shiga-toxin producing *Escherichia coli* from raw beef, chicken meat, and vegetables in Southwest Iran. *Iranian Journal of Veterinary Research*, 22(3):180.

Kim M.-J., Han J.-K., Park J.-S., Lee J.-S., Lee S.-H., Cho J.-I., Kim K.-S. Various Enterotoxin and Other Virulence Factor Genes Widespread Among *Bacillus cereus* and *Bacillus thuringiensis* Strains. *J. Microbiol. Biotechnol.* Online journal

Kimera, Z.I., Mdegela, R.H., Mhaiki, C.J., Karimuribo, E.D., Mabiki, F., Nonga, H.E. and Mwesongo, J., 2015. Determination of oxytetracycline residues in cattle meat marketed in the Kilosa district, Tanzania: research communication. *Onderstepoort Journal of Veterinary Research*, 82(1):1-5.

King, D.T., Sobhanifar, S. and Strynadka, N.C., 2017. The mechanisms of resistance to b-lactam antibiotics. *Handbook of Antimicrobial Resistance*. New York: Springer.

Kivits, T., Broers, H.P., Beeltje, H., Van Vliet, M. and Griffioen, J., 2018. Presence and fate of veterinary antibiotics in age-dated groundwater in areas with intensive livestock farming. *Environmental pollution*, 241:988-998.

Klein, J.P. and Moeschberger, M.L., 2003. *Survival analysis: techniques for censored and truncated data*, New York: Springer.

Koda, E., Miskowska, A. and Sieczka, A., 2017. Levels of organic pollution indicators in groundwater at the old landfill and waste management site. *Applied Sciences*, 7(6):638.

Koike, S., Aminov, R.I., Yannarell, A.C., Gans, H.D., Krapac, I.G., Chee-Sanford, J.C. and Mackie, R.I., 2010. Molecular ecology of macrolide–lincosamide–streptogramin B methylases in waste lagoons and subsurface waters associated with swine production. *Microbial ecology*, 59(3):487-498.

Koo, H.J. and Woo, G.J., 2011. Distribution and transferability of tetracycline resistance determinants in *Escherichia coli* isolated from meat and meat products. *International journal of food microbiology*, 145(2):407-413.

Kritzinger, R.K., 2019. Antibiotic-resistant bacteria and-genes in raw water, and the implications for drinking water production (Doctoral dissertation, North-West University (South Africa). Potchefstroom Campus).

Kroll, P. and Von Appen, J., 2001. Post-spinel phases of silicon nitride. *Physica Status Solidi B (Basic Research)*, 226.

Kulakov, L.A., McAlister, M.B., Ogden, K.L., Larkin, M.J. and O'hlanon, J.F., 2002. Analysis of bacteria contaminating ultrapure water in industrial systems. *Applied and environmental microbiology*, 68(4):1548-1555.

Kumar, M. and Puri, A., 2012. A review of permissible limits of drinking water. *Indian journal of occupational and environmental medicine*, 16(1):40.

Kummu, M., De Moel, H., Salvucci, G., Viviroli, D., Ward, P.J. and Varis, O., 2016. Over the hills and further away from coast: global geospatial patterns of human and environment over the 20th–21st centuries. *Environmental Research Letters*, 11(3):034010.

Kwak, M.J., Kwon, S.K., Cho, S.H. and Kim, J.F., 2015. Genome sequences of the Shiga-like toxin-producing *Escherichia coli* NCCP15655 and NCCP15656. *Gut pathogens*, 7(1):1-8.

Labbate, M., Case, R.J. and Stokes, H.W., 2009. The integron/gene cassette system: an active player in bacterial adaptation. *Horizontal gene transfer*, 56:103-125.

Lammel, D.R., Barth, G., Ovaskainen, O., Cruz, L.M., Zanatta, J.A., Ryo, M., De Souza, E.M. and Pedrosa, F.O., 2018. Direct and indirect effects of a pH gradient bring insights into the mechanisms driving prokaryotic community structures. *Microbiome*, 6(1):1-13.

Landman, Willem A., Johan Malherbe, and Francois Engelbrecht. "South Africa's present-day climate." *Understanding the social and environmental implications of global change. Africa Sun Media, Stellenbosch* (2017): 7-12.

Lang, S., Demir, M., Duan, Y., Martin, A. and Schnabl, B., 2020. Cytolysin-positive *Enterococcus faecalis* is not increased in patients with non-alcoholic steatohepatitis. *Liver International*, 40(4):860-865.

Langdon, A., Crook, N. and Dantas, G., 2016. The effects of antibiotics on the microbiome throughout development and alternative approaches for therapeutic modulation. *Genome medicine*, 8(1):1-16.

Laverde Gomez, J.A., Hendrickx, A.P.A., Willems, R.J., Top, J. and Sava, I., 2011. Intra-and Interspecies Genomic Transfer of the *Enterococcus faecalis*.

Lautze, J. and Hanjra, M.A., 2014. Water scarcity. *In Key Concepts in Water Resource Management*, 8(1):27-44.

Lebreton, F., Willems, R.J. and Gilmore, M.S., 2014. *Enterococcus* diversity, origins in nature, and gut colonization. *Enterococci: from commensals to leading causes of drug resistant infection* . <https://www.ncbi.nlm.nih.gov/books/NBK190427/> accessed : 09/11/2021.

LeChevallier, M.W., 2003. Conditions favouring coliform and HPC bacterial growth in drinking water and on water contact surfaces. *Heterotrophic Plate Count Measurement in Drinking Water Safety Management. Geneva, World Health Organization*, 10(2):177-198.

LeChevallier, M.W., 2003. Heterotrophic plate counts and drinking-water safety. *World Health Organization (WHO), IWA Publishing, Houston*.

Leclercq, R., 2002. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. *Clinical infectious diseases*, 34(4):482-492.

Leidonald, R., Muhtadi, A., Lesmana, I., Harahap, Z.A. and Rahmadya, A., 2019, May. Profiles of temperature, salinity, dissolved oxygen, and pH in Tidal Lakes. In *IOP Conference Series: Earth and Environmental Science*, 26(1):12-75.

Li, J., Cheng, W., Xu, L., Strong, P.J. and Chen, H., 2015. Antibiotic-resistant genes and antibiotic-resistant bacteria in the effluent of urban residential areas, hospitals, and a municipal wastewater treatment plant system. *Environmental Science and Pollution Research*, 22(6):4587-4596.

Li, P., Wu, J., Qian, H., Lyu, X. & Liu, H. 2014. Origin and assessment of groundwater pollution and associated health risk: a case study in an industrial park, northwest China. *Environment of Geochemistry and Health*, 36:693–712.

Li, H., Yang, Q., Li, J., Gao, H., Li, P. and Zhou, H., 2015. The impact of temperature on microbial diversity and AOA activity in the Tengchong Geothermal Field, China. *Scientific reports*, 5(1):1-12.

Li, X., 2018. Gelatinase inhibitors: a patent review (2011-2017). *Expert opinion on therapeutic patents*, 28(1):31-46.

Li, Y.Q. and Gu, C.T., 2019. *Enterococcus pingfangensis* sp. nov., *Enterococcus dongliensis* sp. nov., *Enterococcus hulanensis* sp. nov., *Enterococcus nangangensis* sp. nov. and *Enterococcus songbeiensis* sp. nov., isolated from Chinese traditional pickle juice. *International journal of systematic and evolutionary microbiology*, 69(10):3191-3201.

Li, Y., Yang, X., Zhang, J., Yang, S., Zhang, S., Chen, M., Xue, L., Ding, Y., Zeng, H., Gu, Q. and Zhang, Y., 2021. Molecular characterisation of antimicrobial resistance determinants and class 1 integrons of *Salmonella enterica* subsp. *enterica* serotype Enteritidis strains from retail food in China. *Food Control*, 128:08191.

Liang, T., Werner, T.T., Heping, X., Jingsong, Y. and Zeming, S., 2021. A global-scale spatial assessment and geodatabase of mine areas. *Global and Planetary Change*, 204:103578.

Lin, B., Song, Z., Jia, Y., Zhang, Y., Wang, L., Fan, J. and Lin, Z., 2019. Biological characteristics and genome-wide sequence analysis of endophytic nitrogen-fixing bacteria *Klebsiella variicola* GN02. *Biotechnology & Biotechnological Equipment*, 33(1):108-117.

Lina, T.T., Rahman, S.R. and Gomes, D.J., 2007. Multiple-antibiotic-resistance mediated by plasmids and integrons in uropathogenic *Escherichia coli* and *Klebsiella pneumoniae*. *Bangladesh Journal of Microbiology*, 24(1):19-23.

Liu, J., Yang, L., Luan, M., Wang, Y., Zhang, C., Zhang, B., Shi, J., Zhao, F.G., Lan, W. and Luan, S., 2015. A vacuolar phosphate transporter essential for phosphate homeostasis in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, 112(47):E6571-E6578.

Liu, L., Feng, Y., Hu, Y., Kang, M., Xie, Y. and Zong, Z., 2018. *Klebsiella grimontii*, a new species acquired carbapenem resistance. *Frontiers in microbiology*, 9(1):170.

Liu, L., Lan, R., Liu, L., Wang, Y., Zhang, Y., Wang, Y. and Xu, J., 2017. Antimicrobial resistance and cytotoxicity of *Citrobacter* spp. in Maanshan Anhui Province, China. *Frontiers in microbiology*, 8:1357.

Liu, R., Miller, P.A. and Miller, M.J., 2021. Conjugation of Aztreonam, a Synthetic Monocyclic β -Lactam Antibiotic, to a Siderophore Mimetic Significantly Expands Activity Against Gram-Negative Bacteria. *ACS infectious diseases*.

Lowbury, E.J.L. and Hurst, L., 1959. The sensitivity of staphylococci and other wound bacteria to erythromycin, oleandomycin, and spiramycin. *Journal of clinical pathology*, 12(2):163.

Lu, S., Bai, X., Li, W. and Wang, N., 2019. Impacts of climate change on water resources and grain production. *Technological Forecasting and Social Change*, 143:76-84.

Luker, E. and Harris, L.M., 2019. Developing new urban water supplies: investigating motivations and barriers to groundwater use in Cape Town. *International Journal of Water Resources Development*, 35(6):917-937.

Luyt, C.D., Tandlich, R., Muller, W/J & Wilhemi, B.S. 2012. Microbial Monitoring of Surface Water in South Africa: An Overview. *International Journal of Environmental Research and Public Health*, 9(8):2669-93.

Maamar, B.S., Aquilina, L., Quaiser, A., Pauwels, H., Michon-Coudouel, S., Vergnaud-Ayraud, V., Labasque, T., Roques, C., Abbott, B.W. and Dufresne, A., 2015. Groundwater isolation governs chemistry and microbial community structure along hydrologic flowpaths. *Frontiers in microbiology*, 6:1457.

Mabeo, O.R., 2020. *Characterization of Clostridium, Aeromonas and heterotrophic bacteria in selected groundwater systems* (Doctoral dissertation, North-West University (South Africa)).

Macedo, A.S., Freitas, A.R., Abreu, C., Machado, E., Peixe, L., Sousa, J.C. and Novais, C., 2011. Characterization of antibiotic-resistant enterococci isolated from untreated waters for human consumption in Portugal. *International journal of food microbiology*, 145(1):L315-319.

Macler, B.A. and Merkle, J.C., 2000. Current knowledge on groundwater microbial pathogens and their control. *Hydrogeology Journal*, 8(1):29-40.

Macy, E., 2014. Penicillin and beta-lactam allergy: epidemiology and diagnosis. *Current allergy and asthma reports*, 14(11):.476.

Madu, C.E., 2019. *Occurrence of Vancomycin Resistant Enterococci (VRE) in two Durban wastewater treatment plants for effluent reuse* (Doctoral dissertation).

Mahabir, R., Crooks, A., Croitoru, A. and Agouris, P., 2016. The study of slums as social and physical constructs: Challenges and emerging research opportunities. *Regional Studies, Regional Science*, 3(1):399-419.

Mahapatra, N.R., O'Connor, D.T., Vaingankar, S.M., Hikim, A.P.S., Mahata, M., Ray, S., Staite, E., Wu, H., Gu, Y., Dalton, N. and Kennedy, B.P., 2005. Hypertension from targeted ablation of chromogranin A can be rescued by the human ortholog. *The Journal of clinical investigation*, 115(7):1942-1952.

Mahmood, A.E. and Atyah, A.A., 2021. Determination of some virulence factors of *Citrobacter freundii* isolated from Iraqi patients. *Iraqi Journal of Science*, 10:3358-3365.

Maje, M.D., Kaptchouang Tchatchouang, C.D., Manganyi, M.C., Fri, J. and Ateba, C.N., 2020. Characterisation of vibrio species from surface and drinking water sources and assessment of biocontrol potentials of their bacteriophages. *International journal of microbiology*, 2020.

Mandal, S.M., Sharma, S., Pinnaka, A.K., Kumari, A. and Korpole, S., 2013. Isolation and characterization of diverse antimicrobial lipopeptides produced by *Citrobacter* and *Enterobacter*. *BMC microbiology*, 13(1):1-9.

Marano, R.B. and Cytryn, E., 2017. The mobile resistome in wastewater treatment facilities and downstream environments. In *Antimicrobial Resistance in Wastewater Treatment Processes* (p. 129). Hoboken, NJ, USA: John Wiley & Sons, Inc..

Martínez-García, E., & de Lorenzo, V. 2011. Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of *Pseudomonas putida* KT2440. *Environmental microbiology*, 13(10):2702-2716.

Martin, M.S., Santos, I.C., Carlton Jr, D.D., Stigler-Granados, P., Hildenbrand, Z.L. and Schug, K.A., 2018. Characterization of bacterial diversity in contaminated groundwater using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Science of the Total Environment*, 622:1562-1571.

Martin, N.H., Trmčić, A., Hsieh, T.H., Boor, K.J. and Wiedmann, M., 2016. The evolving role of coliforms as indicators of unhygienic processing conditions in dairy foods. *Frontiers in microbiology*, 7:1549.

Martins, R.J., Pardo, R. and Boaventura, R.A., 2004. Cadmium (II) and zinc (II) adsorption by the aquatic moss *Fontinalis antipyretica*: effect of temperature, pH and water hardness. *Water Research*, 38(3):693-699.

Matchaya, G., Nhamo, L., Nhlengethwa, S. and Nhemachena, C., 2019. An overview of water markets in southern Africa: an option for water management in times of scarcity. *Water*, 11(5):1006.

Mateo-Sagasta, J., Zadeh, S.M., Turrall, H. and Burke, J., 2017. Water pollution from agriculture: a global review. Executive summary.

Mathebula, N.E., 2016. An Impossible Developmental State in the South African Context: A True Reflection of the Asian Tigers?. *Bangladesh e-journal of sociology*, 13(1):46-55.

Matloko, K., Fri, J., Ateba, T.P., Molale-Tom, L.G. and Ateba, C.N., 2021. Evidence of potentially unrelated AmpC beta-lactamase producing Enterobacteriaceae from cattle, cattle products and hospital environments commonly harboring the bla ACC resistance determinant. *Plos one*, 16(7):e0253647.

Matlou, D.P., Bissong, M.E.A., Tchatchouang, C.D.K., Adem, M.R., Foka, F.E.T., Kumar, A. and Ateba, C.N., 2019. Virulence profiles of vancomycin-resistant enterococci isolated from surface and ground water utilized by humans in the North West Province, South Africa: a public health perspective. *Environmental Science and Pollution Research*, 26(15):15105-15114.

Mbanga, J., Abia, A.L.K., Amoako, D.G. and Essack, S.Y., 2020. Quantitative microbial risk assessment for waterborne pathogens in a wastewater treatment plant and its receiving surface water body. *BMC microbiology*, 20(1):1-12.

Mbanga, J., Amoako, D.G., Abia, A.L., Allam, M., Ismail, A. and Essack, S.Y., 2021. Genomic Analysis of Enterococcus spp. Isolated From a Wastewater Treatment Plant and Its Associated Waters in Umgungundlovu District, South Africa. *Frontiers in Microbiology*, 12:1536.

McCafferty, J.R., Ellender, B.R., Weyl, O.L.F. and Britz, P.J., 2012. The use of water resources for inland fisheries in South Africa. *Water SA*, 38(2):327-344.

Mechie, S.C., Chapman, P.A. and Siddons, C.A., 1997. A fifteen month study of *Escherichia coli* O157: H7 in a dairy herd. *Epidemiology & Infection*, 118(1):17-25.

Mei, C., Chretien, R.L., Amaradasa, B.S., He, Y., Turner, A. and Lowman, S., 2021. Characterization of phosphate solubilizing bacterial endophytes and plant growth promotion in vitro and in greenhouse. *Microorganisms*, 9(9):1935.

Mekonnen, M.M. and Hoekstra, A.Y., 2016. Four billion people are facing severe water scarcity. *Science advances*, 2(2):pe1500323.

Melton-Celsa, A., Mohawk, K., Teel, L. and O'Brien, A., 2011. Pathogenesis of Shiga-toxin producing *Escherichia coli*. *Ricin and Shiga toxins*, 67-103.

Melton-Celsa, A.R., 2014. Shiga toxin (Stx) classification, structure, and function. *Microbiology spectrum*, 2(4):.2-4.

Memon, J.R., Memon, S.Q., Bhanger, M.I., Memon, G.Z., El-Turki, A. and Allen, G.C., 2008. Characterization of banana peel by scanning electron microscopy and FT-IR spectroscopy and its use for cadmium removal. *Colloids and Surfaces B: Biointerfaces*, 66(2):260-265.

Miescier, J.J. and Cabelli, V.J., 1982. Enterococci and other microbial indicators in municipal wastewater effluents. *Journal of Water Pollution Control Federation*, 1599-1606.

Miettinen, I.T., Vartiainen, T. and Martikainen, P.J., 1997. Phosphorus and bacterial growth in drinking water. *Applied and environmental microbiology*, 63(8):3242-3245.

Mihu-Pintilie, A., Romanescu, G. and Stoleriu, C., 2014. The seasonal changes of the temperature, pH and dissolved oxygen in the Cujele Lake, Romania. *Carpathian Journal of Earth and Environmental Sciences*, 9(2):113-123.

Mkwate, R.C., Chidya, R.C. and Wanda, E.M., 2017. Assessment of drinking water quality and rural household water treatment in Balaka District, Malawi. *Physics and Chemistry of the Earth, Parts A/B/C*, 100:353-362.

Mokhtar, S.M., Youssef, K.M. and Morsy, N.E., 2014. The effects of natural antioxidants on colour, lipid stability and sensory evaluation of fresh beef patties stored at 4 C. *Journal of Agroalimentary Processes and Technologies*, 20(3):282-292.

Molale, L.G., 2012. Surface water quality of the North West Province based on physico–chemical properties and faecal streptococci levels (Doctoral dissertation, North-West University).

Molale, L.G., 2016. *Characterization of enterococci isolates from water sources in the North West Province* (Doctoral dissertation, North-West University (South Africa)).

Molale-Tom, L.G. and Bezuidenhout, C.C., 2020. Prevalence, antibiotic-resistance and virulence of *Enterococcus* spp. from wastewater treatment plant effluent and receiving waters in South Africa. *Journal of Water and Health*, 18(5):753-765.

Molechan, C., Amoako, D.G., Abia, A.L.K., Somboro, A.M., Bester, L.A. and Essack, S.Y., 2019. Molecular epidemiology of antibiotic-resistant *Enterococcus* spp. from the farm-to-fork continuum in intensive poultry production in KwaZulu-Natal, South Africa. *Science of The Total Environment*, 692:868-878.

Momba, M.N. and Notshe, T.L., 2003. The microbiological quality of groundwater-derived drinking water after long storage in household containers in a rural community of South Africa. *Journal of Water Supply: Research and Technology—AQUA*, 52(1):67-77.

Monapathi, M.E., Bezuidenhout, C.C. and Rhode, O.H.J., 2017. Water quality and antifungal susceptibility of opportunistic yeast pathogens from rivers. *Water Science and Technology*, 75(6):1319-1331.

Monteiro, P.M.D., 2020. *Fighting the resistance: algae as key organisms against antibacterial resistance* (Doctoral dissertation, Universidade de Coimbra).

Monticelli, J., Knezevich, A., Luzzati, R. and Di Bella, S., 2018. Clinical management of non-faecium non-faecalis vancomycin-resistant enterococci infection. Focus on *Enterococcus gallinarum* and *Enterococcus casseliflavus/flavescens*. *Journal of Infection and Chemotherapy*, 24(4):237-246.

Montso, K.P., Dlamini, S.B., Kumar, A. and Ateba, C.N., 2019. Antimicrobial resistance factors of extended-spectrum beta-lactamases producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from Cattle Farms and Raw Beef in North-West Province, South Africa. *BioMed research international*, 2019.

Montwedi, M.G., Kumar, A. and Ateba, C.N., 2018. Persistence of vancomycin resistance genes and transposable elements in *Enterococcus faecalis* isolated from groundwater intended for human consumption: A public health implication. *Gene Reports*, 13:49-58.

Montwendi, M.G., 2013. *Phenotypic and Genotypic characterization vancomycin resistance determinants in Enterococcus faecalis isolated from groundwater in Mafikeng North West Province, South Africa* (Doctoral dissertation).

Morris, B.L., Lawrence, A.R., Chilton, P.J.C., Adams, B., Calow, R.C. and Klinck, B.A., 2003. Groundwater and its susceptibility to degradation: a global assessment of the problem and options for management.

Motina, E. and Rao, J.M., 2021. Molecular detection of *Enterococcus* with special reference to *Enterococcus faecalis* from water sources and its antimicrobial resistance.

Mpenyana-Monyatsi, L. and Momba, M.N.B., 2012. Assessment of groundwater quality in the rural areas of the North West Province, South Africa. *Scientific Research and Essays*, 7(8):903-914.

Mudau, M., Jacobson, R., Minenza, N., Kuonza, L., Morris, V., Engelbrecht, H., Nicol, M.P. and Bamford, C., 2013. Outbreak of multi-drug resistant *Pseudomonas aeruginosa* bloodstream infection in the haematology unit of a South African Academic Hospital. *PloS one*, 8(3):e55985.

Mudau, K.L., 2021. An assessment of water quality and occurrence of antibiotic-resistant bacteria in Naauwpoortspruit River, Mpumalanga province, South Africa (Doctoral dissertation).

Mulamattathil, S.G., Bezuidenhout, C., Mbewe, M. and Ateba, C.N., 2014. Isolation of environmental bacteria from surface and drinking water in Mafikeng, South Africa, and characterization using their antibiotic-resistance profiles. *Journal of pathogens*, 2014.

Muller, C., Massier, S., Le Breton, Y. and Rincé, A., 2018. The role of the CroR response regulator in resistance of *Enterococcus faecalis* to D-cycloserine is defined using an inducible receiver domain. *Molecular microbiology*, 107(3):416-427.

Mvandaba, V., Mwenge Kahinda, J-M., Hobbs, P., Nzuza, P., Le Roux, A. & Arnold, K. 2019. Green Book. The impact of climate change on South Africa's future groundwater availability. Pretoria: CSIR. Available at: <https://pta-gis-2-web1.csir.co.za/portal/apps/GBCascade/index.html?appid=73c7e05d7a1e4b0dbacc99b3709f7065>. Accessed on: 10/11/2021.

Naclerio, G.A. and Sintim, H.O., 2020. Multiple ways to kill bacteria via inhibiting novel cell wall or membrane targets. *Future Medicinal Chemistry*, 12(13):1253-1279.

Naidoo, S., Rodda, N., Schmidt, S., Dent, M., Buckley, C.A., Stenstrom, T.A., Bux, F., Hanke, N. and Fennemore, C., 2016. Water security in South Africa: perceptions on public expectations and municipal obligations, governance and water re-use. *Water SA*, 42(3):456-465.

Nappier, S.P., Liguori, K., Ichida, A.M., Stewart, J.R. and Jones, K.R., 2020. Antibiotic-resistance in Recreational Waters: State of the Science. *International Journal of Environmental Research and Public Health*, 17(21):8034.

NWRS: National Water resource strategy. 2013. Web: https://www.gov.za/sites/default/files/gcis_document/201409/finalwater.pdf

Nnadozie, R.C., 2011. Access to adequate water in post-apartheid South African provinces: an overview of numerical trends. *Water SA*:7(3).

Nthunya, L.N., Maifadi, S., Mamba, B.B., Verliefde, A.R. and Mhlanga, S.D., 2018. Spectroscopic determination of water salinity in brackish surface water in Nandoni Dam, at Vhembe District, Limpopo Province, South Africa. *Water*, 10(8):990.

Ntshangase, S., Mdanda, S., Naicker, T., Kruger, H.G., Baijnath, S. and Govender, T., 2019. Spatial distribution of elvitegravir and tenofovir in rat brain tissue: Application of matrix-assisted laser desorption/ionization mass spectrometry imaging and liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, 33(21):1643-1651.

Ntuli, V., Njage, P.M.K. and Buys, E.M., 2016. Characterization of *Escherichia coli* and *Enterobacteriaceae* in producer-distributor bulk milk. *Journal of dairy science*, 99(12):9534-9549.

Nqowana, T., Dube, C.S. and Tandlich, R., 2017. Monitoring of potable water quality in Makana local municipality, South Africa. *Aerul si Apa. Componente ale Mediului*, 8(2):345-350.

Nwankwo, C.O., Bamigboye, G.O., Davies, I.E. and Michaels, T.A., 2020. High volume Portland cement replacement: A review. *Construction and Building materials*, 260:120445.

NWDACE: North West Department of Agriculture, Conservation and Environment. 2008. Environmental outlook: A report of the state of the environment, Mmabatho. South Africa.

NWDC: North West Development Corporation. 2014. Investment North West.

NWPG: North West Province Government: North West Planning Commission. 2013, *Provincial development plan 2030*, North West Provincial Government, Mahikeng.

Obi, C.L., Potgieter, N., Bessong, P.O. and Matsaung, G., 2002. Assessment of the microbial quality of river water sources in rural Venda communities in South Africa. *Water SA*, 28(3):287-292.

Odonkor, S.T. and Ampofo, J.K., 2020. *Escherichia coli* as an indicator of bacteriological quality of water: an overview. *Microbiology research*, 4(1):5-11.

Odonkor, T.S. 2013. *E coli* as an indicator of bacteriological quality of water: An overview. *Microbiology research*, 4(1):1-5.

Olaniran, A.O., Naicker, K. and Pillay, B., 2009. Antibiotic-resistance profiles of *Escherichia coli* isolates from river sources in Durban, South Africa. *World Journal of Microbiology and Biotechnology*, 25(10):1743-1749.

Olaniran, A.O., Nzimande, S.B. and Mkize, N.G., 2015. Antimicrobial resistance and virulence signatures of *Listeria* and *Aeromonas* species recovered from treated wastewater effluent and receiving surface water in Durban, South Africa. *BMC microbiology*, 15(1):1-10.

Olaniran, E.I., Sogbanmu, T.O. and Saliu, J.K., 2019. Biomonitoring, physico-chemical, and biomarker evaluations of abattoir effluent discharges into the Ogun River from Kara Market, Ogun State, Nigeria, using *Clarias gariepinus*. *Environmental monitoring and assessment*, 191(1):1-17.

Oliver, J.D., 2005. The viable but nonculturable state in bacteria. *Journal of microbiology*, 43(spc1):93-100.

Olowe, B., Oluyeye, J., Famurewa, O., Ogunniran, A. and Adelegan, O., 2017. Molecular identification of *Escherichia coli* and new emerging enteropathogen, *Escherichia fergusonii*, from drinking water sources in Ado-Ekiti, Ekiti State, Nigeria. *Journal of Microbiology Resources*, 7:45-54.

Omar, K.B. and Barnard, T.G., 2010. The occurrence of pathogenic *Escherichia coli* in South African wastewater treatment plants as detected by multiplex PCR. *Water SA*, 36(2):172-176.

Omer, N.H., 2019. Water quality parameters. *Water quality-science, assessments and policy*, 18.

Osode, A.N. and Okoh, A.I., 2009. Impact of discharged wastewater final effluent on the physicochemical qualities of a receiving watershed in a suburban community of the Eastern Cape Province. *Clean–Soil, Air, Water*, 37(12):938-944.

Osterman, I.A., Dontsova, O.A. and Sergiev, P.V., 2020. rRNA Methylation and Antibiotic-resistance. *Biochemistry (Moscow)*, 85(11):1335-1349.

Oun, A., Kumar, A., Harrigan, T., Angelakis, A. and Xagorarakis, I., 2014. Effects of biosolids and manure application on microbial water quality in rural areas in the US. *Water*, 6(12):3701-3723.

Pal, M. and Ayele, Y., 2017. Public Health Significance of Verotoxin-Producing *Escherichia coli* O157: H7. *EC Microbiology*, 11(6):257-263.

Pal, P., 2014. Detection of coliforms in drinking water and its effect on human health-A review. *International Letters of Natural Sciences*, 12(2).

Palamuleni, L. and Akoth, M., 2015. Physico-chemical and microbial analysis of selected borehole water in Mahikeng, South Africa. *International journal of environmental research and public health*, 12(8):8619-8630.

Palmer, L.B. and Smaldone, G.C., 2014. Reduction of bacterial resistance with inhaled antibiotics in the intensive care unit. *American journal of respiratory and critical care medicine*, 189(10):1225-1233.

Pandey, P., Soupir, M.L., Wang, Y., Cao, W., Biswas, S., Vaddella, V., Atwill, R., Merwade, V. and Pasternack, G., 2018. Water and sediment microbial quality of mountain and agricultural streams. *Journal of environmental quality*, 47(5):985-996.

Pandey, N. and Cascella, M., 2020. Beta lactam antibiotics. *StatPearls*. Web:[Beta Lactam Antibiotics - StatPearls - NCBI Bookshelf \(nih.gov\)](https://www.ncbi.nlm.nih.gov/books/NBK537036/) (accessed: 25 May 2018).

Pandey, P.K., Kass, P.H., Soupir, M.L., Biswas, S. and Singh, V.P., 2014. Contamination of water resources by pathogenic bacteria. *Amb Express*, 4(1):1-16.

Papadaki, E., Botsaris, G., Athanasiadi, E. and Mantzouridou, F.T., 2020. Processing Wastewaters from Spanish-Style cv. Chalkidiki Green Olives: A Potential Source of *Enterococcus casseliflavus* and Hydroxytyrosol. *Microorganisms*, 8(9):1274.

Parin, U., Kirkan, S., Arslan, S.S. and Yuksel, H.T., 2018. Molecular identification and antimicrobial resistance of *Escherichia fergusonii* and *Escherichia coli* from dairy cattle with diarrhoea. *Veterinárni medicína*, 63(3):110-116.

Park, S.Y., Kim, K.M., Lee, J.H., Seo, S.J. and Lee, I.H., 2007. Extracellular gelatinase of *Enterococcus faecalis* destroys a defense system in insect hemolymph and human serum. *Infection and immunity*, 75(4):1861-1869.

Park, J.D. and Oh, S.Y., 2012. Methodology for the identification of impaired waters using LDC for the management of total maximum daily loads. *Journal of Korean Society on Water Environment*, 28(5):693-703.

Park, W., Park, J., Jang, J., Lee, H., Jeong, H., Cho, K., Hong, S. and Lee, T., 2013. Oxygen environmental and passivation effects on molybdenum disulfide field effect transistors. *Nanotechnology*, 24(9):095202.

Passet, V. and Brisse, S., 2018. Description of *Klebsiella grimontii* sp. nov. *International journal of systematic and evolutionary microbiology*, 68(1):377-381.

Patel, S., Mathivanan, N. and Goyal, A., 2017. Bacterial adhesins, the pathogenic weapons to trick host defense arsenal. *Biomedicine & Pharmacotherapy*, 93:763-771.

Patel, V. and Parikh, P., 2013. Assessment of seasonal variation in water quality of River Mini, at Sindhrot, Vadodara. *International journal of environmental sciences*, 3(5):1424-1436.

Pathak, S.P. and Gopal, K., 2008. Prevalence of bacterial contamination with antibiotic-resistant and enterotoxigenic fecal coliforms in treated drinking water. *Journal of Toxicology and Environmental Health, Part A*, 71(7):427-433.

Patil, P.N., Sawant, D.V. and Deshmukh, R.N., 2012. Physico-chemical parameters for testing of water—A review. *International journal of environmental sciences*, 3(3):1194-1207.

Patni, N.K., Masse, L. and Jui, P.Y., 1998. Groundwater quality under conventional and no tillage: I. Nitrate, electrical conductivity, and pH . *American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America*, 27(4):869-877.

Paton, J.C. and Paton, A.W., 1997. Instability of a Shiga toxin type 2 gene in *Enterobacter cloacae*. *Journal of Clinical Microbiology*, 35(7):1917-1917.

Pavlov, D., de Wet, C.M.E., Grabow, W.O.K. and Ehlers, M.M., 2002. Determination of cytotoxicity and invasiveness of heterotrophic plate count bacteria isolated from drinking water. *Water Science and Technology: Water Supply*, 2(3):115-122.

Pavlov, D., De Wet, C.M.E., Grabow, W.O.K. and Ehlers, M.M., 2004. Potentially pathogenic features of heterotrophic plate count bacteria isolated from treated and untreated drinking water. *International journal of food microbiology*, 92(3):275-287.

Penadés, J.R., Chen, J., Quiles-Puchalt, N., Carpena, N. and Novick, R.P., 2015. Bacteriophage-mediated spread of bacterial virulence genes. *Current opinion in microbiology*, 23:171-178.

Pepper, D.J., Jaswal, D., Sun, J., Welsh, J., Natanson, C. and Eichacker, P.Q., 2018. Evidence Underpinning the Centers for Medicare & Medicaid Services' Severe Sepsis and Septic Shock Management Bundle (SEP-1) A Systematic Review. *Annals of internal medicine*, 168(8):558-568.

Perumal-Pillay, V.A. and Suleman, F., 2017. Selection of essential medicines for South Africa-an analysis of in-depth interviews with national essential medicines list committee members. *BMC health services research*, 17(1):1-17.

Phung, D., Huang, C., Rutherford, S., Dwirahmadi, F., Chu, C., Wang, X., Nguyen, M., Nguyen, N.H., Do, C.M., Nguyen, T.H. and Dinh, T.A.D., 2015. Temporal and spatial assessment of river surface water quality using multivariate statistical techniques: a study in Can Tho City, a Mekong Delta area, Vietnam. *Environmental Monitoring and Assessment*, 187(5):1-13.

Pienaar, J.A., Singh, A. and Barnard, T.G., 2016. The viable but non-culturable state in pathogenic *Escherichia coli*: A general review. *African journal of laboratory medicine*, 5(1):1-9.

Pillay, D.G., Karas, J.A., Pillay, A. and Sturm, A.W., 1997. Nosocomial transmission of *Shigella dysenteriae* type 1. *Journal of Hospital Infection*, 37(3):199-205.

Plaatjie, M.T.A., 2019. Characteristics of antibiotic-resistant bacteria in raw and drinking water from water production facilities (Doctoral dissertation, North-West University (South Africa)).

Pokhrel, M.R., Cairns, S.C. and Andrew, N.R., 2020. Dung beetle species introductions: when an ecosystem service provider transforms into an invasive species. *Peer Journal*, 8:e9872.

Polianciuc, S.I., Gurzău, A.E., Kiss, B., Ştefan, M.G. and Loghin, F., 2020. Antibiotics in the environment: causes and consequences. *Medicine and Pharmacy Reports*, 93(3):231.

Pontes, J.G.D.M., Fernandes, L.S., dos Santos, R.V., Tasic, L. and Fill, T.P., 2020. Virulence factors in the phytopathogen–host interactions: an overview. *Journal of Agricultural and Food Chemistry*, 68(29):7555-7570.

Prinsloo, R.M.P., 2014. *Potential pathogenicity of heterotrophic plate count bacteria isolated from untreated drinking water* (Doctoral dissertation).

Qaiser, J., Seong, L.Y., Deok, J.H. and Young, K.K., 2018. Effect of plant growth-promoting bacteria *Bacillus amyloliquefaciens* Y1 on soil properties, pepper seedling growth, rhizosphere bacterial flora and soil enzymes. *Plant Protection Science*, 54(3):129-137.

Ramaraj, S., Kim, M.A., Rosa, V., Neelakantan, P., Shon, W.J. and Min, K.S., 2020. Combined Effect of Melittin and DNase on *Enterococcus faecalis* Biofilms and Its Susceptibility to Sodium Hypochlorite. *Materials*, 13(17):3740.

Ramos, M.C., Quinton, J.N. and Tyrrel, S.F., 2006. Effects of cattle manure on erosion rates and runoff water pollution by faecal coliforms. *Journal of Environmental Management*, 78(1):97-101.

Ramos, S., Silva, V., Dapkevicius, M.D.L.E., Igrejas, G. and Poeta, P., 2020. Enterococci, from harmless bacteria to a pathogen. *Microorganisms*, 8(8):1118.

Ravichandran, L., Sivaraman, U., Pramodhini, S., Srirangaraj, S. and Seetha, K.S., 2016. Prevalence of virulence factors among clinical isolates of *Enterococcus* spp. *Asian Journal of Pharmaceutical clinical Resources*, 9(9):72-5.

Rayne, N. and Aula, L., 2020. Livestock manure and the impacts on soil health: A review. *Soil Systems*, 4(4):64.

Raza, W., Ali, F., Raza, N., Luo, Y., Kim, K.H., Yang, J., Kumar, S., Mehmood, A. and Kwon, E.E., 2018. Recent advancements in supercapacitor technology. *Nano Energy*, 52:441-473.

Reid, K.C., Cockerill III, F.R. and Patel, R., 2001. Clinical and epidemiological features of *Enterococcus casseliflavus/flavescens* and *Enterococcus gallinarum* bacteremia: a report of 20 cases. *Clinical infectious diseases*, 32(11):1540-1546.

Rahmanian, N., Ali, S.H.B., Homayoonfard, M., Ali, N.J., Rehan, M., Sadeh, Y. and Nizami, A.S., 2015. Analysis of physiochemical parameters to evaluate the drinking water quality in the State of Perak, Malaysia. *Journal of Chemistry*, 20(15):34-56.

Rebelo, P., Costa-Rama, E., Seguro, I., Pacheco, J.G., Nouws, H.P., Cordeiro, M.N.D. and Delerue-Matos, C., 2021. Molecularly imprinted polymer-based electrochemical sensors for environmental analysis. *Biosensors and Bioelectronics*, 172:112719.

Rincé, A., Le Breton, Y., Verneuil, N., Giard, J.C., Hartke, A. and Auffray, Y., 2003. Physiological and molecular aspects of bile salt response in *Enterococcus faecalis*. *International journal of food microbiology*, 88(2-3):207-213.

Rivera, S.C., Hazen, T.C. and Toranzos, G.A., 1988. Isolation of faecal coliforms from pristine sites in a tropical rain forest. *Applied and Environmental Microbiology*, 54(2):513-517.

Roberts, M.C., Soge, O.O., Giardino, M.A., Mazengia, E., Ma, G. and Meschke, J.S., 2009. Vancomycin-resistant *Enterococcus* spp. in marine environments from the West Coast of the USA. *Journal of applied microbiology*, 107(1):300-307.

Rochegüe, T., Haenni, M., Mondot, S., Astruc, C., Cazeau, G., Ferry, T., Madec, J.Y. and Lupo, A., 2021. Impact of Antibiotic Therapies on Resistance Genes Dynamic and Composition of the Animal Gut Microbiota. *Animals*, 11(11):3280.

Rochelle-Newall, E., Nguyen, T.M.H., Le, T.P.Q., Sengtaheuanghoung, O. and Ribolzi, O., 2015. A short review of faecal indicator bacteria in tropical aquatic ecosystems: knowledge gaps and future directions. *Frontiers in microbiology*, 6:308.

Rohde, M.M., Froend, R. and Howard, J., 2017. A global synthesis of managing groundwater dependent ecosystems under sustainable groundwater policy. *Groundwater*, 55(3):293-301.

Rojas-Lopez, M., Monterio, R., Pizza, M., Desvaux, M. and Rosini, R., 2018. Intestinal pathogenic *Escherichia coli*: Insights for vaccine development. *Frontiers in microbiology*, 9:440.

Rowin, J., Xia, Y., Jung, B. and Sun, J., 2017. Gut inflammation and dysbiosis in human motor neuron disease. *Physiological reports*, 5(18):13443.

Roy, K., Islam, M.S., Paul, A., Levy, S., Talukder, M., Sobur, M.A., Ballah, F.M., Khan, M.S.R. and Rahman, M.T., 2021. Molecular detection and antibiotyping of multi-drug resistant *Enterococcus faecium* from healthy broiler chickens in Bangladesh. *Veterinary Medicine and Science*.

Rubin, M.A. and Samore, M.H., 2002. Antimicrobial use and resistance. *Current infectious disease reports*, 4(6):491.

Rusen, I.D., Fraser-Roberts, L., Slaney, L., Ombette, J., Lovgren, M., Datta, P., Ndinya-Achola, J., Talbot, J.A., Nagelkerke, N., Plummer, F.A. and Embree, J.E., 1997. Nasopharyngeal pneumococcal colonization among Kenyan children: antibiotic-resistance, strain types and

associations with human immunodeficiency virus type 1 infection. *The Pediatric infectious disease journal*, 16(7):656-662.

Ryan, M.P. and Pembroke, J.T., 2018. *Brevundimonas* spp: Emerging global opportunistic pathogens. *Virulence*, 9(1):480-493.

Saga, T. and Yamaguchi, K., 2009. History of antimicrobial agents and resistant bacteria. *Journal of Microbiology*, 52(2):103-108.

Sahulka, S.Q., Bhattarai, B., Bhattacharjee, A.S., Tanner, W., Mahar, R.B. and Goel, R., 2021. Differences in chlorine and peracetic acid disinfection kinetics of *Enterococcus faecalis* and *Escherichia fergusonii* and their susceptible strains based on gene expressions and genomics. *Water Research*, 203:117480.

Sakuma, T., Noda, T., Urata, S., Kawaoka, Y. and Yasuda, J., 2009. Inhibition of Lassa and Marburg virus production by tetherin. *Journal of virology*, 83(5):2382-2385.

Sandals, T. 2016. Microbiology laboratory techniques. *Pharmaceutical microbiology*, 11(6):63-80.

Sanders, C.C., 1987. Chromosomal cephalosporinases responsible for multiple resistance to newer β -lactam antibiotics. *Annual Reviews in Microbiology*, 41(1):573-594.

Santiago-Rodriguez, T.M., Rivera, J.I., Coradin, M. and Toranzos, G.A., 2013. Antibiotic-resistance and virulence genes in *Enterococcus* isolated from tropical recreational waters. *Journal of water and health*, 11(3):387-396.

Sarowska, J., Futoma-Koloch, B., Jama-Kmiecik, A., Frej-Madrzak, M., Ksiazczyk, M., Bugla-Ploskonska, G. and Choroszy-Krol, I., 2019. Virulence factors, prevalence and potential transmission of extraintestinal pathogenic *Escherichia coli* isolated from different sources: recent reports. *Gut pathogens*, 11(1):1-16.

Sasakova, N., Gregova, G., Takacova, D., Mojzisova, J., Papajova, I., Venglovsky, J., Szaboova, T. and Kovacova, S., 2018. Pollution of surface and ground water by sources related to agricultural activities. *Frontiers in Sustainable Food Systems*, 2:42.

Savin, M., Bierbaum, G., Hammerl, J.A., Heinemann, C., Parcina, M., Sib, E., Voigt, A. and Kreyenschmidt, J., 2020. Antibiotic-resistant bacteria and antimicrobial residues in wastewater and process water from German pig slaughterhouses and their receiving municipal wastewater treatment plants. *Science of the Total Environment*, 727:138788.

Savini, V., Catavittello, C., Talia, M., Manna, A., Pompetti, F., Favaro, M., Fontana, C., Febbo, F., Balbinot, A., Di Berardino, F. and Di Bonaventura, G., 2008. Multidrug-resistant *Escherichia fergusonii*: a case of acute cystitis. *Journal of clinical microbiology*, 46(4):1551-1552.

Scharping, R.J. and Garey, J.R., 2021. Relationship between aquifer biofilms and unattached microbial indicators of urban groundwater contamination. *Molecular Ecology*, 30(1):324-342.

Schmoll, O., Howard, G., Chilton, J. and Chorus, I. eds., 2006. Protecting groundwater for health: managing the quality of drinking-water sources. World Health Organization.

Schnappinger, D. and Hillen, W., 1996. Tetracyclines: antibiotic action, uptake, and resistance mechanisms. *Archives of microbiology*, 165(6):359-369.

Schneider, R., 1961. Correlation of groundwater levels and air temperature in the winter and spring in Minnesota.

Schwab, F., Gastmeier, P. and Meyer, E., 2014. The warmer the weather, the more gram-negative bacteria-impact of temperature on clinical isolates in intensive care units. *PloS one*, 9(3):91105.

Schreiner, W., Berczes, T. and Sztrik, J., 2014, January. Probabilistic model checking on HPC systems for the performance analysis of mobile networks. In *Annales Mathematicae et Informaticae*, 12:123-144.

Selleck, E.M., Van Tyne, D. and Gilmore, M.S., 2019. Pathogenicity of enterococci. *Microbiology spectrum*, 7(4).

Semedo-Lemsaddek, T., Pedroso, N.M., Freire, D., Nunes, T., Tavares, L., Verdade, L.M. and Oliveira, M., 2018. Otter fecal enterococci as general indicators of antimicrobial resistance dissemination in aquatic environments. *Ecological Indicators*, 85:1113-1120.

Seo, M., Lee, H. and Kim, Y., 2019. Relationship between coliform bacteria and water quality factors at weir stations in the Nakdong River, South Korea. *Water*, 11(6):1171.

Seong, Chi Nam, Joo Won Kang, Ji Hee Lee, So Yeon Seo, Jung Jae Woo, Chul Park, Kyung Sook Bae, and Mi Sun Kim., 2018. Taxonomic hierarchy of the phylum Firmicutes and novel Firmicutes species originated from various environments in Korea. *Journal of Microbiology*, 56(1):1-10.

Seppälä, H., Nissinen, A., Järvinen, H., Huovinen, S., Henriksson, T., Herva, E., Holm, S.E., Jahkola, M., Katila, M.L., Klaukka, T. and Kontiainen, S., 1992. Resistance to erythromycin in group A streptococci. *New England Journal of Medicine*, 326(5):292-297.

Serwecińska, L., 2020. Antimicrobials and antibiotic-resistant bacteria: a risk to the environment and to public health. *Water*, 12(12):3313.

Shanker, A.S., Vootla, P.K. and Pindi, P.K., 2020. Exploration of coliform diversity in drinking water resources by culture-independent approaches. *Journal of Water, Sanitation and Hygiene for Development*, 10(3):580-588.

Sharaf, O.Z. and Orhan, M.F., 2014. An overview of fuel cell technology: Fundamentals and applications. *Renewable and sustainable energy reviews*, 32:810-853.

Sharma, A. and Sharma, A., 2017. Screening of *Citrobacter* sp. for fibrinolytic protease production isolated from River Narmada. *Screening*, 2(6).

Sharma, A.K., Dhasmana, N., Dubey, N., Kumar, N., Gangwal, A., Gupta, M. and Singh, Y., 2017. Bacterial virulence factors: secreted for survival. *Indian journal of microbiology*, 57(1):1-10.

Sharma, K., Dhasmana, N., Dubey, N., Kumar, M., Gangwal, A., Gupta, M. & Singh, Y. 2016. Bacterial virulence factors: secreted for survival. *Indian Journal of Microbiology*, 57(1):1-10.

Sharpley, A.N., Weld, J.L., Beegle, D.B., Kleinman, P.J., Gburek, W.J., Moore, P.A. and Mullins, G., 2003. Development of phosphorus indices for nutrient management planning strategies in the United States. *Journal of Soil and Water Conservation*, 58(3):137-152.

Shaw, M.K., Marr, A.G. and Ingraham, J.L., 1971. Determination of the minimal temperature for growth of *Escherichia coli*. *Journal of Bacteriology*, 105(2):683-684.

Shibu, P., 2019. *Investigations of Carbapenem-resistant Klebsiella species and associated clinical considerations* (Doctoral dissertation, University of Westminster).

Shinde, K.G., Birajdar, U.M., Bhalekar, M.N. and Patil, B.T., 2012. Correlation and path analysis in eggplant (*Solanum melongena* L.). *Vegetable Science*, 39(1):108-110.

Shobo, C.O., Essack, S.Y. and Bester, L.A., 2021. Enterococcal contamination of hospital environments in KwaZulu-Natal, South Africa. *Journal of Applied Microbiology*.

Shroeder, M., Brooks, B. & Brooks, A.E. 2017. The Complex Relationship between Virulence and Antibiotic-resistance. *Genes*, 8(1):39-45.

Sidhu, J.P., Skelly, E., Hodgers, L., Ahmed, W., Li, Y. and Toze, S., 2014. Prevalence of Enterococcus species and their virulence genes in fresh water prior to and after storm events. *Environmental science & technology*, 48(5):2979-2988.

Sidhu, J.P.S., Gupta, V.V.S.R., Stange, C., Ho, J., Harris, N., Barry, K., Gonzalez, D., Van Nostrand, J.D., Zhou, J., Page, D. and Tiehm, A., 2020. Prevalence of antibiotic-resistance and

virulence genes in the biofilms from an aquifer recharged with stormwater. *Water Research*, 185:116269.

Siebert, S., Burke, J., Faures, J.M., Frenken, K., Hoogeveen, J., Döll, P. and Portmann, F.T., 2010. Groundwater use for irrigation—a global inventory. *Hydrology and earth system sciences*, 14(10):1863-1880.

Singal, A.G., Higgins, P.D. and Waljee, A.K., 2014. A primer on effectiveness and efficacy trials. *Clinical and translational gastroenterology*, 5(1):e45.

Singh, B., Singh, J.P., Kaur, A. and Singh, N., 2016. Bioactive compounds in banana and their associated health benefits—A review. *Food Chemistry*, 206;1-11.

Skočková, A., Kolářková, I., Bogdanovsogbanmuičová, K. and Karpíšková, R., 2015. Characteristic and antimicrobial resistance in *Escherichia coli* from retail meats purchased in the Czech Republic. *Food Control*, 47:401-406.

Sluiter, I.R.K., 2008. On the distribution, ecology and conservation status of three rare plant taxa *Zygophyllum compressum*, *Elachanthus glaber* and *Eremophila crassifolia* in southwestern New South Wales.

Snozzi, M., 2001. Nicholas J. Ashbolt, Willie OK Grabow and. *Water Quality Guidelines, Standards and Health: Assessment of risk and risk management for water-related infectious disease*, 289.

Soeung, V., Lu, S., Hu, L., Judge, A., Sankaran, B., Prasad, B.V. and Palzkill, T., 2020. A drug-resistant β -lactamase variant changes the conformation of its active-site proton shuttle to alter substrate specificity and inhibitor potency. *Journal of Biological Chemistry*, 295(52):18239-18255.

Sogbanmu, T.O., Aitsegame, S.O., Otubanjo, O.A. and Odiyo, J.O., 2019. Drinking water quality and human health risk evaluations in rural and urban areas of Ibeju-Lekki and Epe local government areas, Lagos, Nigeria. *Human and ecological risk assessment: An international journal*, 34(1):34-67.

Solaiman, S., Allard, S.M., Callahan, M.T., Jiang, C., Handy, E., East, C., Haymaker, J., Bui, A., Craddock, H., Murray, R. and Kulkarni, P., 2020. Longitudinal assessment of the dynamics of *Escherichia coli*, total coliforms, *Enterococcus* spp., and *Aeromonas* spp. in alternative irrigation water sources: a CONSERVE Study. *Applied and environmental microbiology*, 86(20):e00342-20.

Some, S., Mondal, R., Mitra, D., Jain, D., Verma, D. and Das, S., 2021. Microbial pollution of water with special reference to coliform bacteria and their nexus with environment. *Energy Nexus*, 1:100008.

Soon, L.K., Lihan, S., Dasthagir, F.F.G., Mikal, K.M., Collick, F. and Hua, N.K., 2014. Microbiological and physicochemical analysis of water from empurau fish (*Tor tam-broides*) farm in Kuching, Sarawak, Malaysian Borneo. *International Journal of Scientific & Technology Research*, 3(6):285-292.

Ssepunya, G., Wynants, E., Verreth, C., Crauwels, S., Lievens, B., Claes, J., Nakimbugwe, D. and Van Campenhout, L., 2019. Microbial characterisation of the edible grasshopper *Ruspolia differens* in raw condition after wild-harvesting in Uganda. *Food microbiology*, 77(2):106-117.

Stańczyk-Mazanek, E. and Stępnia, L., 2021. Analysis of migration of pathogenic drug-resistant bacteria to soils and groundwater after fertilization with sewage sludge. *bioRxiv*.

Stange, C., Sidhu, J.P.S., Tiehm, A. and Toze, S., 2016. Antibiotic-resistance and virulence genes in coliform water isolates. *International journal of hygiene and environmental health*, 219(8):823-831.

StatsSA: Statistics South Africa, 2016. Provincial profile: North West. Web: http://www.statssa.gov.za/?p=8501&qclid=Cj0KCQiAzMGNBhCyARIsANpUkzNDRVaM-DkgiiCqIQIMjUY1er14QZDRUB12iL5inxoFr-ThzcbkykaAsXyEALw_wcB (accessed: 24 May 2019).

StatsSA: Statistics South Africa, 2018. General Household survey. Web: <https://www.statssa.gov.za/publications/P0318/P03182018.pdf> (accessed 17 February 2022).

StatsSA: Statistics South Africa, 2019. Mid-year population estimates 2019. Statistical release P03022019. Web: <https://www.statssa.gov.za/publications/P0302/P03022019.pdf> (accessed: 20 June 2020).

StatsSA: Statistics South Africa, 2020. Measuring the progress of development in South Africa. Web: <http://www.statssa.gov.za/?p=13908> (accessed: 10 November 2021).

Stefanakis, A.I., Zouzias, D. and Marsellos, A.N.T.O.N.I.O.S., 2017. Groundwater pollution: human and natural sources and risks.

Stehr, F., Kretschmar, M., Kröger, C., Hube, B. and Schäfer, W., 2003. Microbial lipases as virulence factors. *Journal of molecular catalysis B: enzymatic*, 22(5-6):347-355.

Stevens, J.M., Galyov, E.E. and Stevens, M.P., 2006. Actin-dependent movement of bacterial pathogens. *Nature Reviews Microbiology*, 4(2):91-101.

Stewart, R.J., Goyal, S., Lee, S.H., Rammohan, A., Park, H.H., Min, K., Cho, E. and Heinz, H., 2019. Constructing surface models of silicate glasses using molecular dynamics to understand the effect of pH on the hydration properties. *The Journal of chemical physics*, 150(17):174-703.

Steyn, M., Genthe, B., Banoo, I., Thwala, M., Roos, T.H., Walters, C.R. and Ramakhwatho, F., 2020. A decision support tool for industrial water reuse in South Africa.

Stoll, C., Sidhu, J.P., Tiehm, A. and Toze, S., 2012. Prevalence of clinically relevant antibiotic-resistance genes in surface water samples collected from Germany and Australia. *Environmental science & technology*, 46(17):9716-9726.

Sukhwani, K.S., Bansal, N., Soni, M., Ramamurthy, A. and Gopalakrishnan, R., 2017. Enterococcal meningitis in association with Strongyloides hyperinfection syndrome. *Germs*, 7(1):28.

Sumby, P., Porcella, S.F., Madrigal, A.G., Barbian, K.D., Virtaneva, K., Ricklefs, S.M., Sturdevant, D.E., Graham, M.R., Vuopio-Varkila, J., Hoe, N.P. and Musser, J.M., 2005. Evolutionary origin and emergence of a highly successful clone of serotype M1 group A *Streptococcus* involved multiple horizontal gene transfer events. *The Journal of infectious diseases*, 192(5):771-782.

Sutcliffe, J., Grebe, T., Tait-Kamradt, A. and Wondrack, L., 1996. Detection of erythromycin-resistant determinants by PCR. *Antimicrobial agents and chemotherapy*, 40(11):2562-2566.

Sykes, R.B. and Bonner, D.P., 1985. Aztreonam: the first monobactam. *The American journal of medicine*, 78(2):2-10.

Szekeres, E., Chiriac, C.M., Baricz, A., Szőke-Nagy, T., Lung, I., Soran, M.L., Rudi, K., Dragos, N. and Coman, C., 2018. Investigating antibiotics, antibiotic-resistance genes, and microbial contaminants in groundwater in relation to the proximity of urban areas. *Environmental pollution*, 236:734-744.

Tague, C., Farrell, M., Grant, G., Lewis, S. and Rey, S., 2007. Hydrogeologic controls on summer stream temperatures in the McKenzie River basin, Oregon. *Hydrological Processes: An International Journal*, 21(24):3288-3300.

Takakura, T., Francis, R., Anani, H., Bilen, M., Raoult, D. and Khalil, J.B., 2019. *Enterococcus mediterraneensis* sp. nov., a new bacterium isolated from the stool of a 39-year-old Pygmy. *New microbes and new infections*, 32:00599.

Talabi, A.O. and Kayode, T.J., 2020. Groundwater pollution and remediation. *Journal of Water and Resource Protection*, 11: 1–19.

Tamma, P.D., Doi, Y., Bonomo, R.A., Johnson, J.K., Simner, P.J. and Antibacterial Resistance Leadership Group Tamma PD Doi Y Bonomo RA, 2019. A primer on AmpC β -lactamases:

necessary knowledge for an increasingly multidrug-resistant world. *Clinical Infectious Diseases*, 69(8):1446-1455.

Tan, Q., Qiu, J., Luo, X., Zhang, Y., Liu, Y., Chen, Y., Yuan, J. and Liao, W., 2018. Progress in one-pot bioconversion of cephalosporin C to 7-aminocephalosporanic acid. *Current pharmaceutical biotechnology*, 19(1):30-42.

Tao, Y., Deng, Y., Du, Y., Xu, Y., Leng, Z., Ma, T. and Wang, Y., 2020. Sources and enrichment of phosphorus in groundwater of the Central Yangtze River Basin. *Science of The Total Environment*, 737:139-837.

Taylor, R.G., Todd, M.C., Kongola, L., Maurice, L., Nahozya, E., Sanga, H. and MacDonald, A.M., 2013. Evidence of the dependence of groundwater resources on extreme rainfall in East Africa. *Nature Climate Change*, 3(4):374-378..

Tempelhoff, J.W. and Stopforth, G., 2014. The South African Water History Archival Repository (SAWHAR) Project at North-West University (Vaal) and a Historical Overview of the Waterlit Collection: Report to the Water Research Commission. Water Research Commission.

Thongkao, K. and Sudjaroen, Y., 2019. Beta-lactamase and integron-associated antibiotic-resistance genes of *Klebsiella pneumoniae* isolated from *Tilapia* fishes (*Oreochromis niloticus*). *Journal of Applied Pharmaceutical Science*, 9(1):125-30.

Thu, W.P., Sinwat, N., Bitrus, A.A., Angkittitrakul, S., Prathan, R. and Chuanchuen, R., 2019. Prevalence, antimicrobial resistance, virulence gene, and class 1 integrons of *Enterococcus faecium* and *Enterococcus faecalis* from pigs, pork and humans in Thai-Laos border provinces. *Journal of global antimicrobial resistance*, 18:130-138.

Titilawo, Y., Obi, L. and Okoh, A., 2015. Occurrence of virulence gene signatures associated with diarrhoeagenic and non-diarrhoeagenic pathovars of *Escherichia coli* isolates from some selected rivers in South-Western Nigeria. *BMC microbiology*, 15(1):1-14.

Trick, J.K., Stuart, M. and Reeder, S., 2008. Contaminated groundwater sampling and quality control of water analyses. In *Environmental geochemistry*, 15(1): 29-57.

Trofa, A.F., Ueno-Olsen, H., Oiwa, R. and Yoshikawa, M., 1999. Dr. Kiyoshi Shiga: discoverer of the dysentery bacillus. *Clinical infectious diseases*, 29(5):1303-1306.

Tsunoda, R., Usui, M., Tagaki, C., Fukuda, A., Boonla, C., Anomasiri, W., Sukpanyatham, N., Akapelwa, M.L., Nakajima, C., Tamura, Y. and Suzuki, Y., 2021. Genetic characterization of coliform bacterial isolates from environmental water in Thailand. *Journal of Infection and Chemotherapy*, 27(5): 722-728.

Tutmez, B., Hatipoglu, Z. and Kaymak, U., 2006. Modelling electrical conductivity of groundwater using an adaptive neuro-fuzzy inference system. *Computers & geosciences*, 32(4):421-433.

Ture, M. and Kutlu, I., 2018. Isolation of *Citrobacter freundii* from Rainbow Trout (*Oncorhynchus mykiss*) in Freshwater Cage. *Journal of Limnology and Freshwater Fisheries Research*, 4:85-89.

UK Essays. 2018. Drinking water analysis: South Africa. Web: <https://www.ukessays.com/essays/biology/analysis-water-quality-south-africas-2625.php> (accessed: 20 June 2020).

UN: World Water Assessment Programme, 2003. Water for People, Water for Life: The United Nations World Water Development Report: Executive Summary. Unesco Pub. Web: <https://www.unwater.org/publications/water-people-water-life/> (accessed: 08 December 2021).

Vadde, K.K., Wang, J., Cao, L., Yuan, T., McCarthy, A.J. and Sekar, R., 2018. Assessment of water quality and identification of pollution risk locations in Tiaoxi River (Taihu Watershed), China. *Water*, 10(2):183.

Van Weert, F., Van der Gun, J. and Reckman, J., 2009. Global overview of saline groundwater occurrence and genesis. International Groundwater Resources Assessment Centre.

Varol, M. and Balci, M., 2020. Characteristics of effluents from trout farms and their impact on water quality and benthic algal assemblages of the receiving stream. *Environmental Pollution*, 266:115101.

Vaz-Moreira, I., Nunes, O.C. and Manaia, C.M., 2017. Ubiquitous and persistent Proteobacteria and other Gram-negative bacteria in drinking water. *Science of the Total Environment*, 586:1141-1149.

Verhille, S., 2013. Understanding microbial indicators for drinking water assessment: interpretation of test results and public health significance. *National Collaborating Centre for Environmental Health*, 1-12.

Verlicchi, P. and Grillini, V., 2020. Surface water and groundwater quality in South Africa and mozambique—Analysis of the Most critical pollutants for drinking purposes and challenges in water treatment selection. *Water*, 12(1):305.

Vasilakopoulou, A., Vourli, S., Siafakas, N., Kavatha, D., Tziolos, N. and Pournaras, S., 2020. *Enterococcus casseliflavus* Bacteraemia in a Patient with Chronic Renal Disease. *Infectious Disease Reports*, 12(3):70-73.

Vineetha, V.P., Soumya, R.S. and Raghu, K.G., 2015. Phloretin ameliorates arsenic trioxide induced mitochondrial dysfunction in H9c2 cardiomyoblasts mediated via alterations in membrane permeability and ETC complexes. *European journal of pharmacology*, 754:162-172.

Waideman, M.A., Teixeira, V.P., Uemura, E.H., Stamford, T.M., Leal, D.A.G., Stangarlin-Fiori, L., Ferreira, S.M.R., Taconeli, C.A. and Beux, M.R., 2020. Enterococci used as complementary indicator of faecal contamination to assess water quality from public schools in the city of Curitiba, Paraná, Brazil. *Brazilian Journal of Food Technology*, 23.

Wandersman, C., 1989. Secretion, processing and activation of bacterial extracellular proteases. *Molecular microbiology*, 3(12):1825-1831.

Wang, J., Chu, L., Wojnárovits, L. and Takács, E., 2020. Occurrence and fate of antibiotics, antibiotic-resistant genes (ARGs) and antibiotic-resistant bacteria (ARB) in municipal wastewater treatment plant: an overview. *Science of The Total Environment*, 140997.

Water, S. and World Health Organization, 2006. Guidelines for drinking-water quality [electronic resource]: incorporating first addendum. Vol. 1, Recommendations.

Weisblum, B., 1995. Erythromycin resistance by ribosome modification. *Antimicrobial agents and chemotherapy*, 39(3);577-585.

Wen, X., Chen, F., Lin, Y., Zhu, H., Yuan, F., Kuang, D., Jia, Z. and Yuan, Z., 2020. Microbial Indicators and Their Use for Monitoring Drinking Water Quality—A Review. *Sustainability*, 12(6):2249.

Wertz, J.E., Goldstone, C., Gordon, D.M. and Riley, M.A., 2003. A molecular phylogeny of enteric bacteria and implications for a bacterial species concept. *Journal of evolutionary biology*, 16(6):1236-1248.

Whalen, J.G., Mully, T.W. and English, J.C., 2007. Spontaneous *Citrobacter freundii* infection in an immunocompetent patient. *Archives of dermatology*, 143(1):115-126.

White, J.F., Crawford, H., Torres, M.S., Mattera, R., Irizarry, I. and Bergen, M., 2012. A proposed mechanism for nitrogen acquisition by grass seedlings through oxidation of symbiotic bacteria. *Symbiosis*, 57(3):161-171.

Wiley, J.M., Sherwood, L.M., Woolverton, C.J. 2017. Prescott's Microbiology. 8th edition. New York: McGraw Hill.

Wilson, D.N., 2014. Ribosome-targeting antibiotics and mechanisms of bacterial resistance. *Nature Reviews Microbiology*, 12(1):35-48.

Winter, T.C., Harvey, J., Franke, O. and Alley, W., 1998. Natural processes of ground-water and surface-water interaction. *Ground Water and Surface Water: A Single Resource, US Geological Survey Circular*, 1139:2-50.

WHO: World Health Organization., 1996. Water quality parameters.

WHO: World Health Organization., 2007. pH in drinking water. Web: https://www.who.int/water_sanitation_health/dwg/chemicals/ph_revised_2007_clean_version.pdf (accessed: 14 April 2020).

WHO: World Health Organization., 2008. Guidelines: the current position. Web: https://www.who.int/water_sanitation_health/dwg/iwachap2.pdf?ua=1 (accessed: 12 January 2021).

World Health Organization, 2011. *Water safety in buildings*. World Health Organization.

WHO: World Health Organization and UNICEF., 2013. Progress on sanitation and drinking-water. World Health Organization.

WHO: World Health Organization., 2016. Nitrate and nitrites in drinking water. Web: https://www.who.int/water_sanitation_health/dwg/chemicals/nitrate-nitrite-background-jan17.pdf (accessed: 14 April 2020).

World Health Organization., 2016. Protecting surface water for health: Identifying, assessing and managing drinking-water quality risks in surface-water catchments.

WHO: World Health Organization., 2017. Progress on drinking water, sanitation and hygiene: 2017 update and SDG baselines.

World Health Organization, 2018. *Antimicrobial resistance and primary health care* (No. WHO/HIS/SDS/2018.56). World Health Organization.

Wu, D.L., Zhang, M., He, L.X., Zou, H.Y., Liu, Y.S., Li, B.B., Yang, Y.Y., Liu, C., He, L.Y. and Ying, G.G., 2020. Contamination profile of antibiotic-resistance genes in ground water in comparison with surface water. *Science of The Total Environment*, 715:136975.

Wu, G.Q., Bi, W.H., Lü, J.M. and Fu, G.W., 2011. Determination of chemical oxygen demand in water using near infrared transmission and UV absorbance method. *Spectroscopy and spectral analysis*, 31(6):1486-1489.

Xi, C., Zhang, Y., Marrs, C.F., Ye, W., Simon, C., Foxman, B. and Nriagu, J., 2009. Prevalence of antibiotic-resistance in drinking water treatment and distribution systems. *Applied and environmental microbiology*, 75(17):5714-5718.

Xiang, Y., Yang, Z., Zhang, Y., Xu, R., Zheng, Y., Hu, J., Li, X., Jia, M., Xiong, W. and Cao, J., 2019. Influence of nanoscale zero-valent iron and magnetite nanoparticles on anaerobic digestion performance and macrolide, aminoglycoside, β -lactam resistance genes reduction. *Bioresource technology*, 294:122139.

Yan, S.F., Yu, S.E., Wu, Y.B., Pan, D.F., She, D.L. and Ji, J., 2015. Seasonal variations in groundwater level and salinity in coastal plain of eastern China influenced by climate. *Journal of Chemistry*, 20(1):5.

Yang, X.J. and Seto, E.H.A.T., 2007. HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. *Oncogene*, 26(37):5310-5310.

Yao, T. and Yao, S., 2017. Magnetic ionic liquid aqueous two-phase system coupled with high performance liquid chromatography: A rapid approach for determination of chloramphenicol in water environment. *Journal of Chromatography A*, 1481:12-22.

Yang, F., Yang, L., Chang, Z., Chang, L. and Yang, B., 2018. Regulation of virulence and motility by acetate in enteropathogenic *Escherichia coli*. *International Journal of Medical Microbiology*, 308(7):840-847.

Yao, L., Li, Y., Li, Z., Shen, D., Feng, H., Zhou, H. and Wang, M., 2020. Prevalence of fluoroquinolone, macrolide and sulfonamide-related resistance genes in landfills from East China, mainly driven by MGEs. *Ecotoxicology and environmental safety*, 190:110131.

Yari, A.R., Mohammadi, M.J., Geravandi, S., Doosti, Z., Matboo, S.A., Jang, S.A. and Nazari, S., 2018. Assessment of microbial quality of household water output from desalination systems by the heterotrophic plate count method. *Journal of water and health*, 16(6):930-937.

Yeh, C.F., Liu, T.P., Cheng, C.W., Chang, S.C., Lee, M.H. and Lu, J.J., 2015. Molecular characteristics of disease-causing and commensal *Staphylococcus lugdunensis* isolates from 2003 to 2013 at a tertiary hospital in Taiwan. *PLoS One*, 10(8):e0134859.

Younger, P.L., 2009. *Groundwater in the environment: an introduction*. John Wiley & Sons.

Young, S., Rohr, J.R. and Harwood, V.J., 2019. Vancomycin resistance plasmids affect persistence of *Enterococcus faecium* in water. *Water research*, 166:115069.

Yousuf, J., Thajudeen, J., Rahiman, M., Krishnankutty, S., P. Alikunj, A. and A. Abdulla, M.H., 2017. Nitrogen fixing potential of various heterotrophic *Bacillus* strains from a tropical estuary and adjacent coastal regions. *Journal of basic microbiology*, 57(11):922-932.

Yu, C., Wei, X., Wang, Z., Liu, L., Liu, Z., Liu, J., Wu, L., Guo, H. and Jin, Z., 2020. Occurrence of two NDM-1-producing *Raoultella ornithinolytica* and *Enterobacter cloacae* in a single patient in China: probable a novel antimicrobial resistance plasmid transfer in vivo by conjugation. *Journal of Global Antimicrobial Resistance*, 22:835-841.

Yu, G., Wang, J., Liu, L., Li, Y., Zhang, Y. and Wang, S., 2020. The analysis of groundwater nitrate pollution and health risk assessment in rural areas of Yantai, China. *BMC Public Health*, 20(1):1-6.

Yu, Z., Wang, Y., Henderson, I.R. and Guo, J., 2021. Artificial sweeteners stimulate horizontal transfer of extracellular antibiotic-resistance genes through natural transformation. *The ISME Journal*, 1-12.

Zaffiri, L., Gardner, J. and Toledo-Pereyra, L.H., 2013. History of antibiotics: from fluoroquinolones to daptomycin (Part 2). *Journal of Investigative Surgery*, 26(4):167-179.

Zaheer, R., Cook, S.R., Barbieri, R., Goji, N., Cameron, A., Petkau, A., Polo, R.O., Tymensen, L., Stamm, C., Song, J. and Hannon, S., 2020. Surveillance of *Enterococcus* spp. reveals distinct species and antimicrobial resistance diversity across a One-Health continuum. *Scientific reports*, 10(1):1-16.

Zamxaka, M., Pironcheva, G. and Muyima, N., 2004. Microbiological and physico-chemical assessment of the quality of domestic water sources in selected rural communities of the Eastern Cape Province, South Africa. *Water Sa*, 30(3):333-340.

Zhang, J., Lü, F., Shao, L. and He, P., 2014. The use of biochar-amended composting to improve the humification and degradation of sewage sludge. *Bioresource technology*, 168:252-258.

Zhang, J., Buhe, C., Yu, D., Zhong, H. and Wei, Y., 2020. Ammonia stress reduces antibiotic efflux but enriches horizontal gene transfer of antibiotic-resistance genes in anaerobic digestion. *Bioresource technology*, 295:122191.

Zhang, K., Niu, Z.G., Lv, Z. and Zhang, Y., 2017. Occurrence and distribution of antibiotic-resistance genes in water supply reservoirs in Jingjinji area, China. *Ecotoxicology*, 26(9):1284-1292.

Zhang, Y., Hong, P.Y., LeChevallier, M.W. and Liu, W.T., 2015. Phenotypic and phylogenetic identification of coliform bacteria obtained using 12 coliform methods approved by the US Environmental Protection Agency. *Applied and environmental microbiology*, 81(17):6012-6023.

Zhang, Y.M. and Rock, C.O., 2008. Membrane lipid homeostasis in bacteria. *Nature Reviews Microbiology*, 6(3):222-233.

Zhao, F., Bi, X., Hao, Y. and Liao, X., 2013. Induction of viable but nonculturable *Escherichia coli* O157: H7 by high pressure CO₂ and its characteristics. *PLoS one*, 8(4):e62388.

Zhong, Z., Kwok, L.Y., Hou, Q., Sun, Y., Li, W., Zhang, H. and Sun, Z., 2019. Comparative genomic analysis revealed great plasticity and environmental adaptation of the genomes of *Enterococcus faecium*. *BMC genomics*, 20(1):1-13.

Zhong, Z., Zhang, W., Song, Y., Liu, W., Xu, H., Xi, X., Menghe, B., Zhang, H. and Sun, Z., 2017. Comparative genomic analysis of the genus *Enterococcus*. *Microbiological research*, 196:95-105.

Zhou, Z.C., Zheng, J., Wei, Y.Y., Chen, T., Dahlgren, R.A., Shang, X. and Chen, H., 2017. Antibiotic-resistance genes in an urban river as impacted by bacterial community and physicochemical parameters. *Environmental Science and Pollution Research*, 24(30):23753-23762.

Zin, P.P.K., Williams, G. and Fourches, D., 2020. SIME: synthetic insight-based macrolide enumerator to generate the V1B library of 1 billion macrolides. *Journal of cheminformatics*, 12(1):1-15.

APPENDIX

Appendix A

Table A1: GPS coordinates of the groundwater sample sites of interest

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	Hombre	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°5'57.38"E
PB22	H4	Botanical garden	26°40'53.68"S	27°5'42.46"E
PR6	A4	Laureus	26°41'44.34"S	27°5'20.25"E
Gerhard minnebron	Off-campus	Gerhard Minnebron	26.4767°S	27.1514°E
Site 1	Off-campus	Site 1	26°30'11.7"S	27°18'52.7"E
Site 2	Off-campus	Site 2	26°36'04.4"S	27°21'35.7"E
Site 3	Off-campus	Site 3	26°40'33.5"S	27°12'53.7"E
Site 4	Off-campus	Site 4	26°38'46.1"S	27°14'59.0"E
Site 5	Off-campus	Site 5	26°37'09.1"S	27°15'32.2"E
Site 6	Off-campus	Site 6	26°37'03.1"S	27°16'23.7"E
Site 7	Off-campus	Site 7	26°38'48.8"S	27°14'54.9"E

Table A2: p-value of correlations between microbial and physico-chemical parameters in autumn

Variable	Correlations (Autumn)											
	Coliform	Enterococci	HPCs	Temperature °C	EC	TDS	SALT	pH	COD	PO4	NO3	NH3N
Coliform	1.0000	-0.0184	-0.2549	-0.2422	-0.3202	0.7151	0.2449	0.3363	0.3637	0.1420	0.8368	0.4942
	p= ---	p= 0.948	p= 0.359	p= 0.384	p= 0.245	p= 0.003	p= 0.379	p= 0.220	p= 0.183	p= 0.614	p= 0.000	p= 0.061
Enterococci	-0.0184	1.0000	-0.1700	-0.2087	-0.1540	-0.2076	-0.0773	0.3194	-0.2018	-0.1167	0.0747	-0.0472
	p= 0.948	p= ---	p= 0.545	p= 0.455	p= 0.584	p= 0.458	p= 0.784	p= 0.246	p= 0.471	p= 0.679	p= 0.791	p= 0.867
HPCs	-0.2549	-0.1700	1.0000	-0.2455	0.0498	-0.1493	-0.1505	0.0845	0.2574	0.3431	-0.2068	-0.0054
	p= 0.359	p= 0.545	p= ---	p= 0.378	p= 0.860	p= 0.595	p= 0.592	p= 0.765	p= 0.354	p= 0.211	p= 0.460	p= 0.985
Temperature °C	-0.2422	-0.2087	-0.2455	1.0000	-0.0471	0.0110	0.0008	0.5649	0.2632	0.0077	-0.2663	0.0470
	p= 0.384	p= 0.455	p= 0.378	p= ---	p= 0.868	p= 0.969	p= 0.998	p= 0.028	p= 0.343	p= 0.978	p= 0.337	p= 0.868
EC	-0.3202	-0.1540	0.0498	-0.0471	1.0000	-0.2638	0.3069	0.1527	0.4517	-0.0240	-0.4498	-0.0114
	p= 0.245	p= 0.584	p= 0.860	p= 0.868	p= ---	p= 0.342	p= 0.266	p= 0.587	p= 0.091	p= 0.932	p= 0.093	p= 0.968
TDS	0.7151	-0.2076	-0.1493	0.0110	-0.2638	1.0000	0.1014	0.1237	0.0526	0.5971	0.5907	0.2295
	p= 0.003	p= 0.458	p= 0.595	p= 0.969	p= 0.342	p= ---	p= 0.719	p= 0.660	p= 0.852	p= 0.019	p= 0.020	p= 0.411
SALT	0.2449	-0.0773	-0.1505	0.0008	0.3069	0.1014	1.0000	0.1000	0.1181	0.2547	0.3536	-0.1162
	p= 0.379	p= 0.784	p= 0.592	p= 0.998	p= 0.266	p= 0.719	p= ---	p= 0.723	p= 0.675	p= 0.360	p= 0.196	p= 0.680
pH	0.3363	0.3194	0.0845	0.5649	0.1527	0.1237	0.1000	1.0000	0.1690	-0.0709	0.0884	0.3942
	p= 0.220	p= 0.246	p= 0.765	p= 0.028	p= 0.587	p= 0.660	p= 0.723	p= ---	p= 0.547	p= 0.802	p= 0.754	p= 0.146
COD	0.3637	-0.2018	0.2574	0.2632	0.4517	0.0526	0.1181	0.1690	1.0000	0.1614	-0.4180	-0.2898
	p= 0.183	p= 0.471	p= 0.354	p= 0.343	p= 0.091	p= 0.852	p= 0.675	p= 0.547	p= ---	p= 0.566	p= 0.121	p= 0.295
PO4	0.1420	-0.1167	0.3431	0.0077	-0.0240	0.5971	0.2547	-0.0709	0.1614	1.0000	0.2744	-0.0291
	p= 0.614	p= 0.679	p= 0.211	p= 0.978	p= 0.932	p= 0.019	p= 0.360	p= 0.802	p= 0.566	p= ---	p= 0.322	p= 0.918
NO3	0.8368	0.0747	-0.2068	-0.2663	-0.4498	0.5907	0.3536	0.0884	-0.4180	0.2744	1.0000	0.0873
	p= 0.000	p= 0.791	p= 0.460	p= 0.337	p= 0.093	p= 0.020	p= 0.196	p= 0.754	p= 0.121	p= 0.322	p= ---	p= 0.757
NH3N	0.4942	-0.0472	-0.0054	0.0470	-0.0114	0.2295	-0.1162	0.3942	-0.2898	-0.0291	0.0873	1.0000
	p= 0.061	p= 0.867	p= 0.985	p= 0.868	p= 0.968	p= 0.411	p= 0.680	p= 0.146	p= 0.295	p= 0.918	p= 0.757	p= ---

Table A3: p-value of correlations between microbial and physico-chemical parameters in winter

	Correlations (Winter) Marked correlations are significant at $p < .05000$ N=15 (Casewise deletion of missing data)											
Variable	Temperature °C	EC	TDS	SALT	pH	COD	PO4	NO3	NH3N	Coliform	Enterococci	HPCs
Temperature °C	1.0000	-.3606	.4194	.3482	-.0559	.3302	.0355	-.5107	.5967	-.0424	.2396	-.2291
	p= ---	p= .187	p= .120	p= .203	p= .843	p= .229	p= .900	p= .052	p= .019	p= .881	p= .390	p= .411
EC	-.3606	1.0000	.2428	.0595	.0637	-.1096	-.2072	-.1735	-.5178	.6016	-.1560	-.1969
	p= .187	p= ---	p= .383	p= .833	p= .822	p= .697	p= .459	p= .536	p= .048	p= .018	p= .579	p= .482
TDS	.4194	.2428	1.0000	.9208	-.1255	.0796	.0333	-.3096	.6590	.4855	-.0749	-.4801
	p= .120	p= .383	p= ---	p= .000	p= .656	p= .778	p= .906	p= .261	p= .008	p= .067	p= .791	p= .070
SALT	.3482	.0595	.9208	1.0000	-.0455	-.1350	.2462	-.1936	.7617	.3581	-.0696	-.4499
	p= .203	p= .833	p= .000	p= ---	p= .872	p= .632	p= .376	p= .489	p= .001	p= .190	p= .805	p= .092
pH	-.0559	.0637	-.1255	-.0455	1.0000	-.1375	.1388	.0841	-.1684	-.2665	-.3306	.3443
	p= .843	p= .822	p= .656	p= .872	p= ---	p= .625	p= .622	p= .766	p= .549	p= .337	p= .229	p= .209
COD	.3302	-.1096	.0796	-.1350	-.1375	1.0000	-.4675	-.0016	.0604	-.0826	-.2037	-.0529
	p= .229	p= .697	p= .778	p= .632	p= .625	p= ---	p= .079	p= .995	p= .831	p= .770	p= .467	p= .852
PO4	.0355	-.2072	.0333	.2462	.1388	-.4675	1.0000	.0983	.2460	-.0638	-.0222	-.3343
	p= .900	p= .459	p= .906	p= .376	p= .622	p= .079	p= ---	p= .728	p= .377	p= .821	p= .938	p= .223
NO3	-.5107	-.1735	-.3096	-.1936	.0841	-.0016	.0983	1.0000	-.0844	-.3321	-.0215	.1813
	p= .052	p= .536	p= .261	p= .489	p= .766	p= .995	p= .728	p= ---	p= .765	p= .226	p= .939	p= .518
NH3N	.5967	-.5178	.6590	.7617	-.1684	.0604	.2460	-.0844	1.0000	-.0139	.0075	-.3940
	p= .019	p= .048	p= .008	p= .001	p= .549	p= .831	p= .377	p= .765	p= ---	p= .961	p= .979	p= .146
Coliform	-.0424	.6016	.4855	.3581	-.2665	-.0826	-.0638	-.3321	-.0139	1.0000	-.0444	-.5332
	p= .881	p= .018	p= .067	p= .190	p= .337	p= .770	p= .821	p= .226	p= .961	p= ---	p= .875	p= .041
Enterococci	.2396	-.1560	-.0749	-.0696	-.3306	-.2037	-.0222	-.0215	.0075	-.0444	1.0000	.0589
	p= .390	p= .579	p= .791	p= .805	p= .229	p= .467	p= .938	p= .939	p= .979	p= .875	p= ---	p= .835
HPCs	-.2291	-.1969	-.4801	-.4499	.3443	-.0529	-.3343	.1813	-.3940	-.5332	.0589	1.0000
	p= .411	p= .482	p= .070	p= .092	p= .209	p= .852	p= .223	p= .518	p= .146	p= .041	p= .835	p= ---

Table A5: p-value of correlations between microbial and physico-chemical parameters in summer

	Correlations Summer Marked correlations are significant at p < .05000 N=14 (Casewise deletion of missing data)											
Variable	Temperature °C	EC	TDS	SALT	pH	COD	PO4	NO3	NH3N	Coliform	Enterococci	HPCs
Temperature °C	1.0000	-.1594	-.7175	-.1146	.1192	.3604	.5657	.4788	.6748	-.0840	.2093	.0199
	p= ---	p= .586	p= .004	p= .697	p= .685	p= .206	p= .035	p= .083	p= .008	p= .775	p= .473	p= .946
EC	-.1594	1.0000	.4662	.9926	-.2594	-.1227	-.3264	.1517	.1371	.3127	-.1529	-.4605
	p= .586	p= ---	p= .093	p= .000	p= .370	p= .676	p= .255	p= .605	p= .640	p= .276	p= .602	p= .097
TDS	-.7175	.4662	1.0000	.3808	-.1268	-.0047	-.2849	-.3933	-.7111	-.0742	-.1858	-.1250
	p= .004	p= .093	p= ---	p= .179	p= .666	p= .987	p= .324	p= .164	p= .004	p= .801	p= .525	p= .670
SALT	-.1146	.9926	.3808	1.0000	-.2394	-.1370	-.3328	.1853	.2290	.3228	-.1155	-.4733
	p= .697	p= .000	p= .179	p= ---	p= .410	p= .640	p= .245	p= .526	p= .431	p= .260	p= .694	p= .087
pH	.1192	-.2594	-.1268	-.2394	1.0000	.0762	-.1486	-.4348	-.0425	-.2403	-.0681	.0735
	p= .685	p= .370	p= .666	p= .410	p= ---	p= .796	p= .612	p= .120	p= .885	p= .408	p= .817	p= .803
COD	.3604	-.1227	-.0047	-.1370	.0762	1.0000	.4992	.0194	-.2226	-.0364	-.2116	-.3695
	p= .206	p= .676	p= .987	p= .640	p= .796	p= ---	p= .069	p= .947	p= .444	p= .902	p= .468	p= .194
PO4	.5657	-.3264	-.2849	-.3328	-.1486	.4992	1.0000	.4958	.1662	.0822	.3531	.0521
	p= .035	p= .255	p= .324	p= .245	p= .612	p= .069	p= ---	p= .071	p= .570	p= .780	p= .216	p= .860
NO3	.4788	.1517	-.3933	.1853	-.4348	.0194	.4958	1.0000	.6404	.4977	.5779	.0735
	p= .083	p= .605	p= .164	p= .526	p= .120	p= .947	p= .071	p= ---	p= .014	p= .070	p= .030	p= .803
NH3N	.6748	.1371	-.7111	.2290	-.0425	-.2226	.1662	.6404	1.0000	.1298	.4549	.1102
	p= .008	p= .640	p= .004	p= .431	p= .885	p= .444	p= .570	p= .014	p= ---	p= .658	p= .102	p= .708
Coliform	-.0840	.3127	-.0742	.3228	-.2403	-.0364	.0822	.4977	.1298	1.0000	.0654	-.2900
	p= .775	p= .276	p= .801	p= .260	p= .408	p= .902	p= .780	p= .070	p= .658	p= ---	p= .824	p= .315
Enterococci	.2093	-.1529	-.1858	-.1155	-.0681	-.2116	.3531	.5779	.4549	.0654	1.0000	.3788
	p= .473	p= .602	p= .525	p= .694	p= .817	p= .468	p= .216	p= .030	p= .102	p= .824	p= ---	p= .182
HPCs	.0199	-.4605	-.1250	-.4733	.0735	-.3695	.0521	.0735	.1102	-.2900	.3788	1.0000
	p= .946	p= .097	p= .670	p= .087	p= .803	p= .194	p= .860	p= .803	p= .708	p= .315	p= .182	p= ---

Table A4: p-value of correlations between microbial and physico-chemical parameters in summer

Variable	Correlations Spring Marked correlations are significant at $p < .05000$ N=13 (Casewise deletion of missing data)											
	Temperature °C	EC	TDS	SALT	pH	COD	PO4	NO3	NH3N	Coliform	Enterococci	HPCs
Temperature °C	1.0000	-.0850	-.1453	.1695	-.5095	-.3864	.0239	-.1130	.5315	-.4825	-.2987	-.3268
	p= ---	p= .782	p= .636	p= .580	p= .075	p= .192	p= .938	p= .713	p= .062	p= .095	p= .322	p= .276
EC	-.0850	1.0000	.6220	.1591	.3585	.7237	.2821	-.2538	.1806	.7637	.1142	.0323
	p= .782	p= ---	p= .023	p= .604	p= .229	p= .005	p= .350	p= .403	p= .555	p= .002	p= .710	p= .917
TDS	-.1453	.6220	1.0000	.1363	.1704	.9076	.4788	-.0071	-.2153	.6997	.2324	-.3677
	p= .636	p= .023	p= ---	p= .657	p= .578	p= .000	p= .098	p= .982	p= .480	p= .008	p= .445	p= .216
SALT	.1695	.1591	.1363	1.0000	.1989	.1372	.5417	-.4521	.0993	.0546	-.2722	-.3634
	p= .580	p= .604	p= .657	p= ---	p= .515	p= .655	p= .056	p= .121	p= .747	p= .859	p= .368	p= .222
pH	-.5095	.3585	.1704	.1989	1.0000	.5134	.1644	.0333	-.5352	.4088	.3481	.5225
	p= .075	p= .229	p= .578	p= .515	p= ---	p= .073	p= .591	p= .914	p= .059	p= .165	p= .244	p= .067
COD	-.3864	.7237	.9076	.1372	.5134	1.0000	.5195	-.0039	-.3327	.7653	.3622	-.1017
	p= .192	p= .005	p= .000	p= .655	p= .073	p= ---	p= .069	p= .990	p= .267	p= .002	p= .224	p= .741
PO4	.0239	.2821	.4788	.5417	.1644	.5195	1.0000	-.2961	-.0959	.1317	-.0232	-.5060
	p= .938	p= .350	p= .098	p= .056	p= .591	p= .069	p= ---	p= .326	p= .755	p= .668	p= .940	p= .078
NO3	-.1130	-.2538	-.0071	-.4521	.0333	-.0039	-.2961	1.0000	-.0460	-.2071	.7910	.2912
	p= .713	p= .403	p= .982	p= .121	p= .914	p= .990	p= .326	p= ---	p= .881	p= .497	p= .001	p= .334
NH3N	.5315	.1806	-.2153	.0993	-.5352	-.3327	-.0959	-.0460	1.0000	-.2999	-.0389	-.3331
	p= .062	p= .555	p= .480	p= .747	p= .059	p= .267	p= .755	p= .881	p= ---	p= .320	p= .900	p= .266
Coliform	-.4825	.7637	.6997	.0546	.4088	.7653	.1317	-.2071	-.2999	1.0000	.0252	.1418
	p= .095	p= .002	p= .008	p= .859	p= .165	p= .002	p= .668	p= .497	p= .320	p= ---	p= .935	p= .644
Enterococci	-.2987	.1142	.2324	-.2722	.3481	.3622	-.0232	.7910	-.0389	.0252	1.0000	.1691
	p= .322	p= .710	p= .445	p= .368	p= .244	p= .224	p= .940	p= .001	p= .900	p= .935	p= ---	p= .581
HPCs	-.3268	.0323	-.3677	-.3634	.5225	-.1017	-.5060	.2912	-.3331	.1418	.1691	1.0000
	p= .276	p= .917	p= .216	p= .222	p= .067	p= .741	p= .078	p= .334	p= .266	p= .644	p= .581	p= ---

Annexure B

TableB: Accession numbers

Species	Accession number
<i>Escherichia fergusonii</i>	OK030997.1
<i>Escherichia fergusonii</i>	OK030988.1
<i>Escherichia fergusonii</i>	OK030994.1
<i>Escherichia fergusonii</i>	OK030991.1
<i>Escherichia fergusonii</i>	OK030989.1
<i>Citrobacter freundii</i>	OK030996.1
<i>Enterobacter cloacae</i>	OK030995.1
<i>Klebsiella grimontii</i>	OK030998.1
<i>Enterococcus faecalis</i>	MZ675458.1
<i>Enterococcus faecalis</i>	MZ675449.1
<i>Enterococcus faecalis</i>	MZ675451.1
<i>Enterococcus faecalis</i>	MZ675445.1
<i>Enterococcus faecalis</i>	MZ675454.1
<i>Enterococcus casseliflavus</i>	MZ675446.1
<i>Enterococcus casseliflavus</i>	MZ675451.1
<i>Enterococcus casseliflavus</i>	MZ675457.1
<i>Enterococcus gallinarum</i>	MZ675452.1
<i>Enterococcus gallinarum</i>	MZ675456.1
<i>Enterococcus faecalis</i>	MZ680541.1
<i>Enterococcus gallinarum</i>	MZ680537.1
<i>Enterococcus casseliflavus</i>	MZ680534.1
<i>Enterococcus faecalis</i>	MZ680532.1

<i>Enterococcus casseliflavus</i>	MZ680533.1
<i>Enterococcus casseliflavus</i>	MZ680554.1
<i>Enterococcus casseliflavus</i>	MZ680551.1
<i>Enterococcus casseliflavus</i>	MZ680543.1
<i>Bacillus simplex</i>	OK065837.1
<i>Stenotrophomonas maltophilia</i>	OK065835.1
<i>Stenotrophomonas maltophilia</i>	OK065836.1
<i>Stenotrophomonas maltophilia</i>	OK065842.1
<i>Stenotrophomonas maltophilia</i>	OK065842.1
<i>Fictibacillus phosphorivans</i>	OK065844.1
<i>Sphingopyxis soli</i>	OK065834.1
<i>Bacillus cereus</i>	OK087670.1
<i>Bacillus cereus</i>	OK087655.1
<i>Stenotrophomonas maltophilia</i>	OK065843.1
<i>Stenotrophomonas maltophilia</i>	OK065835.1
<i>Stenotrophomonas maltophilia</i>	OK065830.1
<i>Brevundimonas albigilva</i>	OK065838.1
<i>Brevundimonas aurantiaca</i>	OK065652.1
<i>Pseudimonas geniculata</i>	OK065648.1
<i>Stenotrophomonas maltophilia</i>	OK065650.1
<i>Pseudomonas geniculata</i>	OK065850
<i>Pseudomonas hibiscicola</i>	OK065634
<i>Pseudomonas geniculata</i>	OK065651
<i>Xanthomonas floridensis</i>	OK087639
<i>Lysobacter hankyongensis</i>	OK087638
<i>Tahibacter aquaticus</i>	OK065644
<i>Nevskia aquatilis</i>	OK065640
<i>Hydrogenophaga caeni</i>	OK065646
<i>Vogesella perlucida</i>	OK065637
<i>Vogesella facilis</i>	OK065650
<i>Vogesella facilis</i>	OK065829
<i>Acinetobacter kookii</i>	OK065826
<i>Acinetobacter lwoffii</i>	OK087648
<i>Acinetobacter lwoffii</i>	OK065647
<i>Aeromonas molluscorum</i>	OK065636
<i>Pseudomonas songnenensis</i>	OK087661
<i>Pseudomonas aeruginosa</i>	OK065648
<i>Pseudomonas songnenensis</i>	OK087638
<i>Pseudomonas monteillii</i>	OK087643
<i>Pseudomonas fulva</i>	OK087635
<i>Pseudomonas monteillii</i>	OK065652
<i>Elstera litoris</i>	OK065625
<i>Sphingobium yanoikuyae</i>	OK065649
<i>Sphingobium yanoikuyae</i>	OK087645
<i>Sphingomonas panacis</i>	OK065631
<i>Sphingomonas trueperi</i>	OK065828
<i>Sphingopyxis bauzanensis</i>	OK065628
<i>Sphingopyxis soli</i>	OK065834

<i>Ensifer adhaerens</i>	OK065629
<i>Rhizobium zeae</i>	OK087629
<i>Caulobacter segnis</i>	OK065643
<i>Brevundimonas denitrificans</i>	OK065626
<i>Brevundimonas albigilva</i>	OK065839
<i>Brevundimonas nasdae</i>	OK087554
<i>Brevundimonas aurantiaca</i>	OK087641
<i>Brevundimonas aurantianca</i>	OK065840
<i>Microbacterium chocolatum</i>	OK065641
<i>Microbacterium</i>	OK065832
<i>Microbacteriu aurum</i>	OK065645
<i>Microbacterium zeae</i>	OK065642
<i>Paenibacillus glucanolyticus</i>	OK087649
<i>Paenibacillus lautus</i>	OK065842
<i>Paenibacillus yonginensis</i>	OK065622
<i>Brevibacillus ginsengsoli</i>	OK065848
<i>Exiguobacterium mexicanum</i>	OK087627
<i>Lysinibacillus sinduriensis</i>	OK065639
<i>Fictibacillus phosphorivorans</i>	OK065846
<i>Fictibacillus phosphorivans</i>	OK065627
<i>Bacillus niacini</i>	OK087666
<i>Bacillus simplex</i>	OK065838
<i>Brevibacterium frigoritolerans</i>	OK087650
<i>Bacillus cereus</i>	OK087669
<i>Bacillus cereus</i>	OK087670
<i>Bacillus tropicus</i>	OK065633
<i>Bacillus halotolerans</i>	OK087658
<i>Bacillus halotolerans</i>	OK087662
<i>Bacillus piscis</i>	OK087631
<i>Bacillus pumilus</i>	OK087656
<i>Bacillus safensis</i>	OK087638
<i>Bacillus tropicus</i>	OK065635
<i>Bacillus pumilus</i>	OK087663
<i>Bacillus safensis</i>	OK087664
<i>Flavobacterium aquidureense</i>	OK087642
<i>Chryseobacterium cucumeris</i>	OK065654
<i>Chryseobacterium phosphatilyticum</i>	OK065833

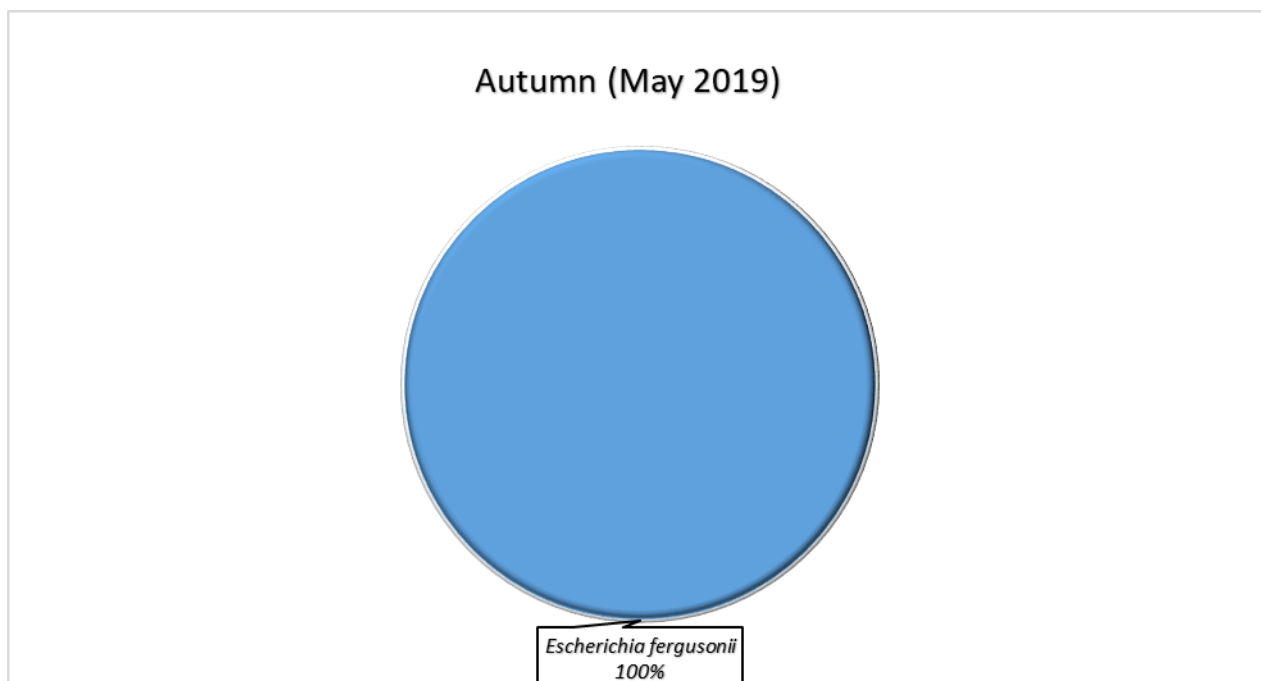


Figure 4.6a- Diversity of coliforms in autumn

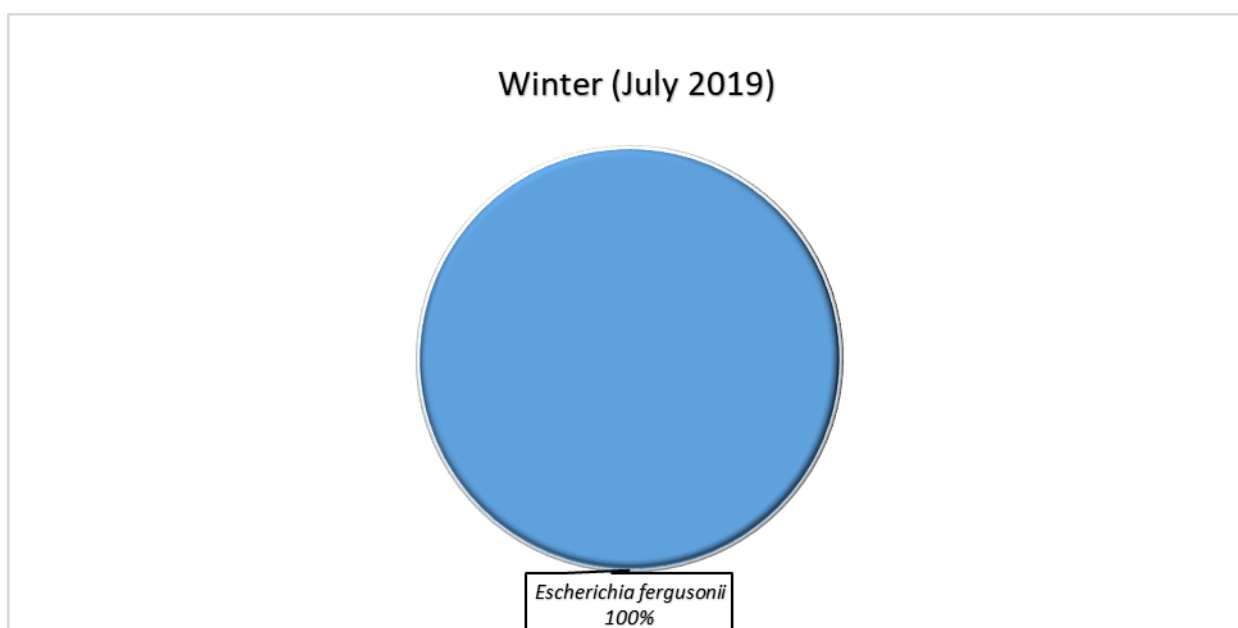


Figure 4.6b- Diversity of coliforms in winter

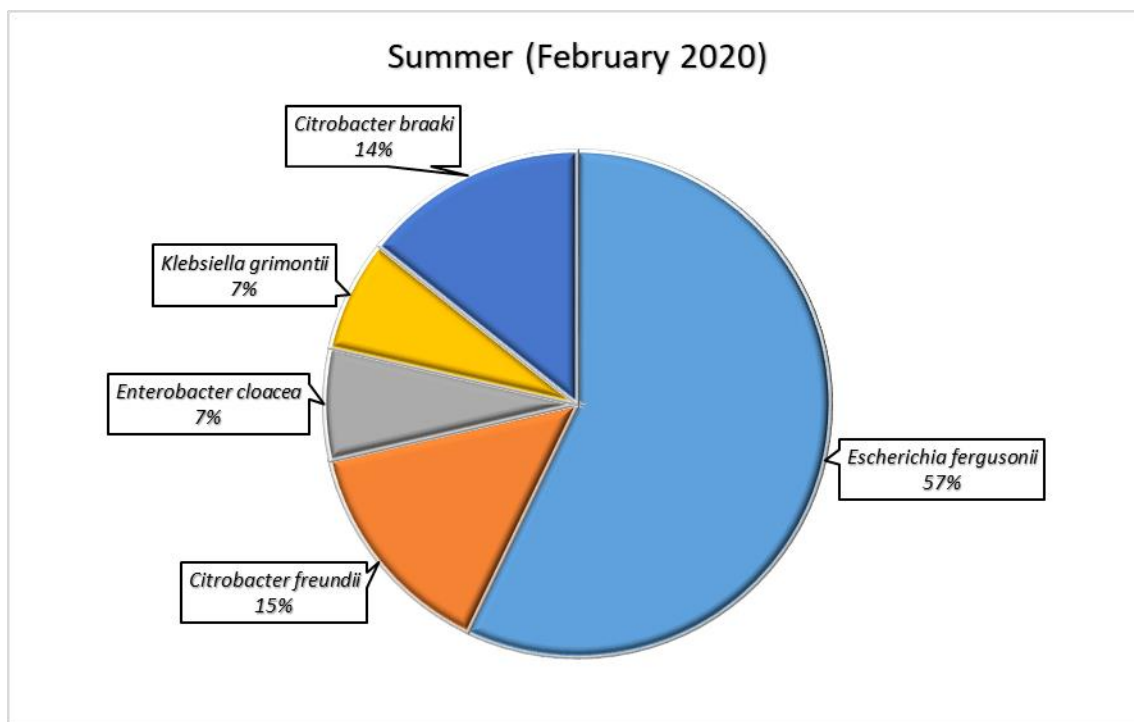


Figure 4.6c- Diversity of coliforms in summer

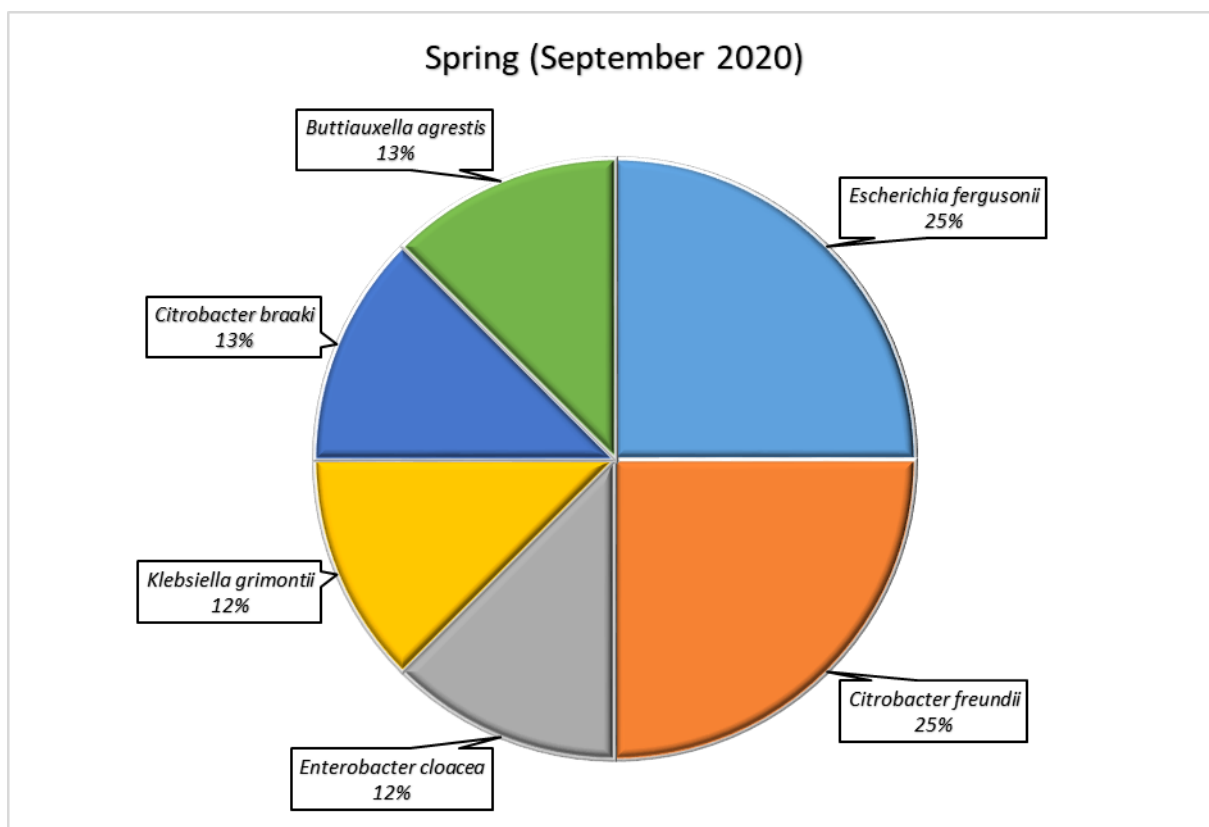


Figure 4.6d- Diversity of coliforms in spring

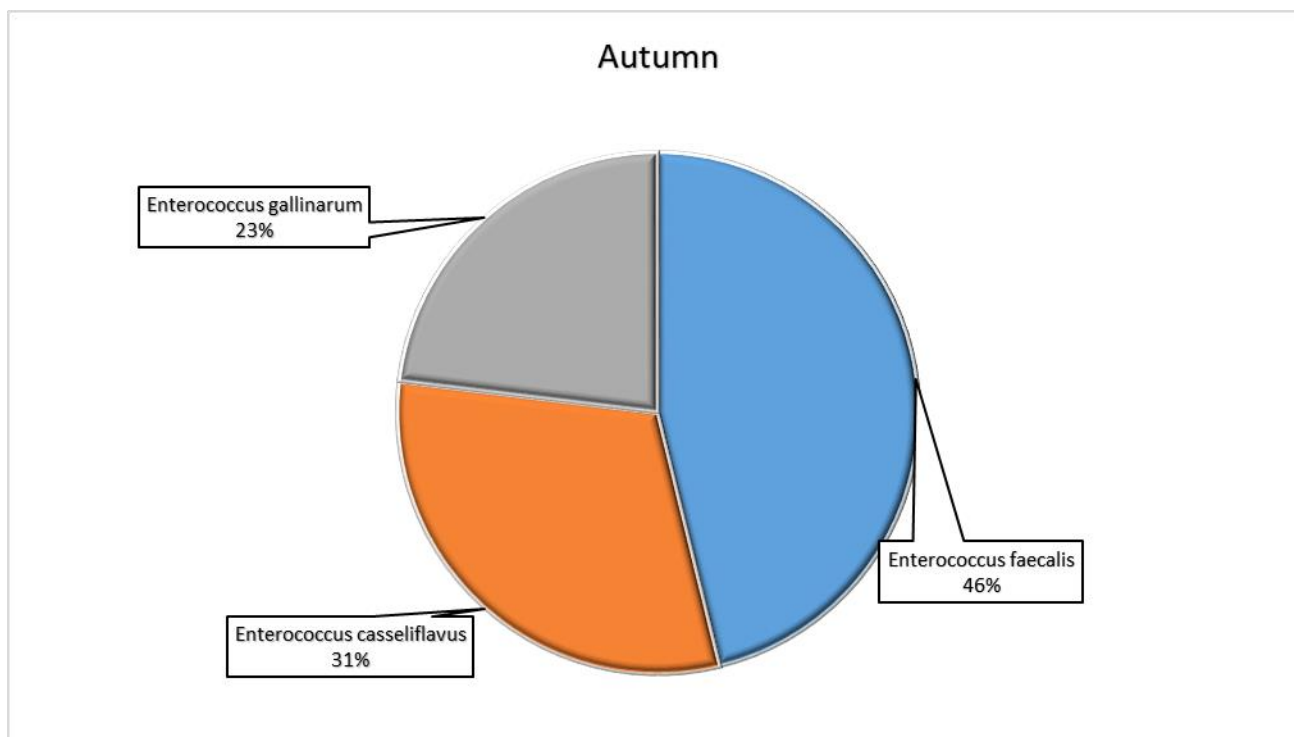


Figure 4.7a- Diversity of *Enterococcus* spp. in autumn

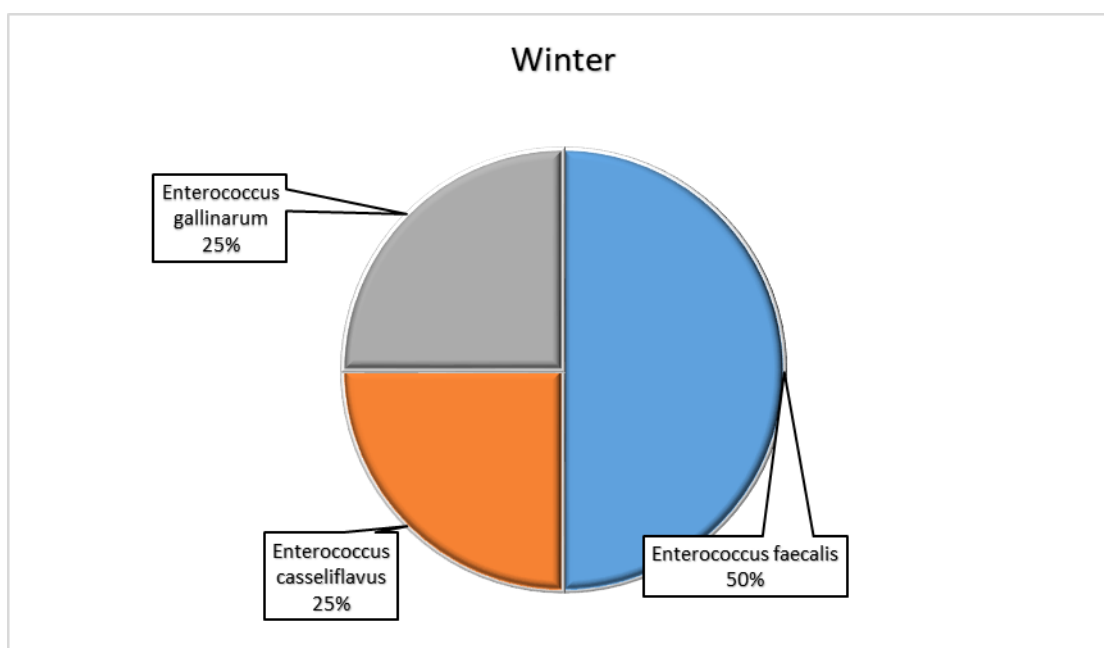


Figure 4.7b- Diversity of *Enterococcus* spp. in winter

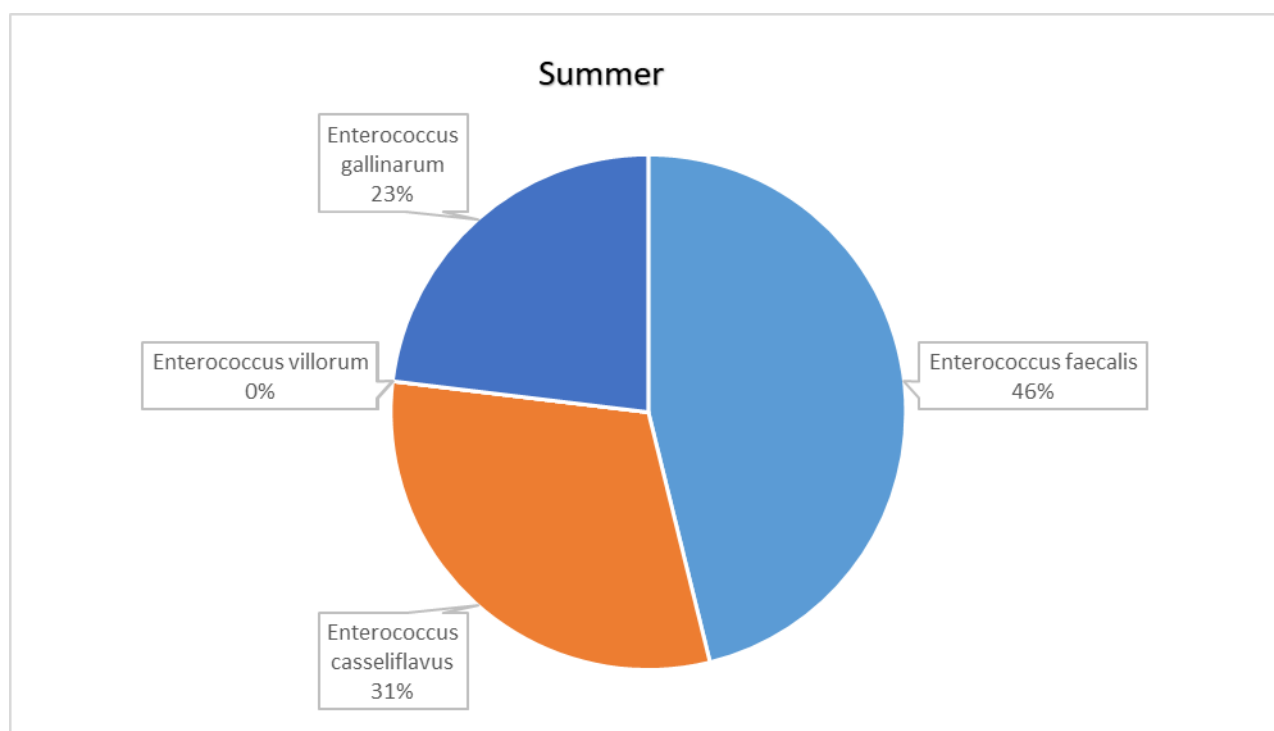


Figure 4.7c- Diversity of *Enterococcus* spp. in summer

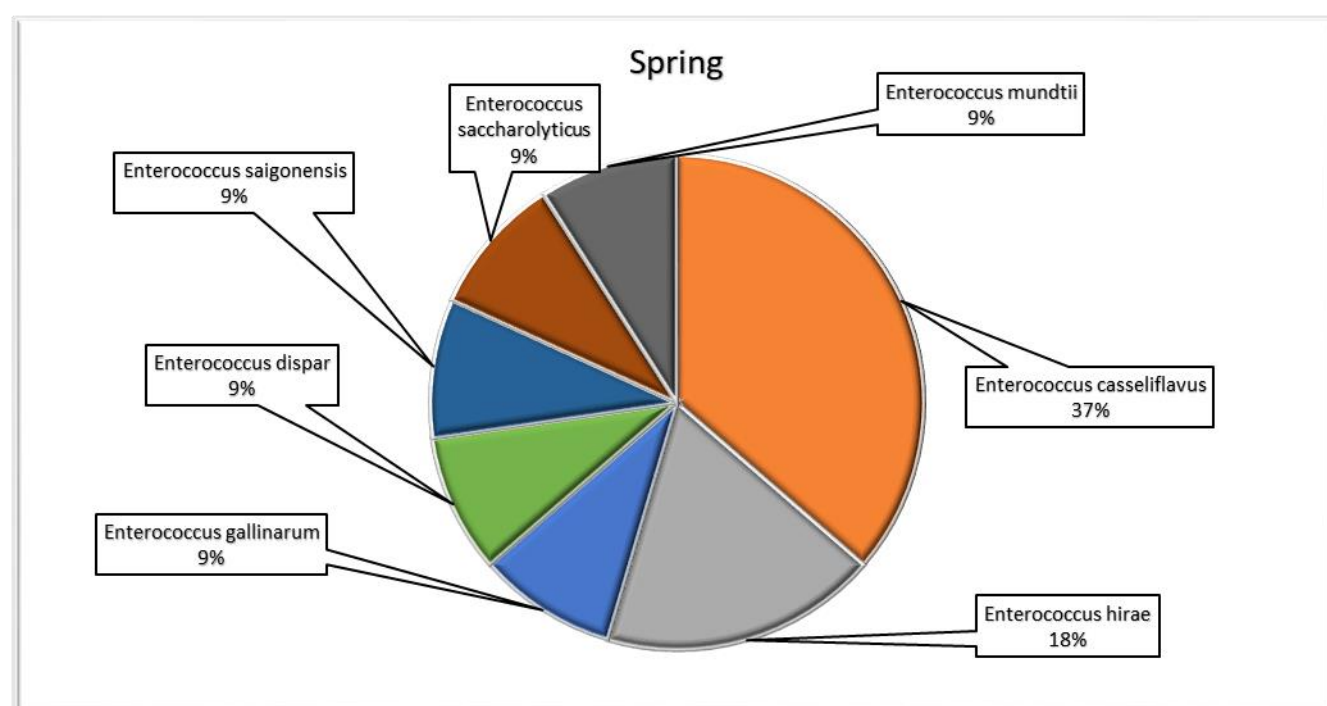


Figure 4.7d- Diversity of *Enterococcus* spp. in spring

Appendix C

Table A6: The number of alpha and beta isolates in groundwater across the North West Province

Season	Site name	Number of α/β haemolytic coliforms	Number of α/β haemolytic <i>Enterococcus</i> spp. isolates	Number of α/β haemolytic HPC isolates	Total isolates per site
Autumn	S1	N/A	4	2	6
	S2	N/A	N/A	4	4
	S3	N/A	N/A	1	1
	S4	N/A	2	2	4
	S5	N/A	N/A	2	2
	S6	2	2	8	12
	S7	N/A	2	1	3
	BG	N/A	1	1	2
	DK	N/A	N/A	1	1
	SG	N/A	N/A	N/A	0
	FD	N/A	1	1	2
	H	N/A	1	N/A	1
	LS	N/A	N/A	N/A	0
	LV	N/A	N/A	N/A	0
	GM	N/A	N/A	2	2

	Total isolates	2	13	27	42
Winter	S1	N/A	2	N/A	2
	S2	N/A	N/A	1	1
	S3	1	N/A	N/A	1
	S4	N/A	1	N/A	1
	S5	N/A	N/A	1	1
	S6	1	N/A	3	4
	S7	N/A	1	N/A	1
	BG	N/A	N/A	3	3
	DK	N/A	N/A	2	2
	SG	N/A	N/A	2	2
	FD	N/A	N/A	1	1
	H	N/A	N/A	4	4
	LS	N/A	N/A	2	2
	LV	N/A	N/A	1	1
	GM	N/A	N/A	5	5
	Total isolates	2	4	24	30
Summer	S1	2	1	N/A	3
	S2	N/A	N/A	1	1

	S3	N/A	N/A	N/A	0
	S4	N/A	N/A	2	2
	S5	1	N/A	N/A	1
	S6	N/A	N/A	4	4
	S7	N/A	1	N/A	1
	BG	2	N/A	N/A	2
	DK	N/A	N/A	2	2
	SG	N/A	N/A	2	2
	FD	N/A	2	N/A	2
	H	N/A	4	N/A	4
	LS	1	N/A	1	2
	LV	N/A	N/A	1	1
	GM	4	3	N/A	7
	Total isolates	10	11	13	34
Spring	S1	1	N/A	3	4
	S2	N/A	N/A	2	2
	S3	N/A	N/A	N/A	0
	S4	N/A	N/A	2	2
	S5	N/A	5	3	8

	S6	1	N/A	4	5
	S7	N/A	2	N/A	2
	BG	1	N/A	N/A	1
	DK	N/A	1	N/A	1
	SG	N/A	N/A	2	2
	FD	1	3	2	6
	H	N/A	N/A	2	2
	LS	N/A	N/A	2	2
	LV	2	N/A	1	3
	GM	N/A	N/A	N/A	N/A
	Total isolates	6	11	23	40
GRAND TOTAL		20	39	87	146

N/A – Not applicable (No isolates were beta or alpha haemolytic). BG- Botanical gardens, SG- Sportsground, LV- LaVaria, LS-Lareus, GM- METSI, DK-Draak, FD- Fanie; H- Hombre.

Extracellular enzymes

Table A7 – Percentage of extracellular enzyme producing coliform species

Species name	Overall percentage (%)	Proteinase %	Lipase %	DNase %	Gelatinase %
<i>Citrobacter braakii</i>	17	0	0	20	0
<i>Citrobacter freundii</i>	17	20	0	0	0
<i>Escherichia fergusonii</i>	66	80	0	80	100
Total	100 (6)	100 (5)	0	100 (5)	100 (1)

Table A8 – Percentage of extracellular enzyme producing *Enterococcus* species

Species name	Overall percentage (%)	Proteinase	Lipase	DNase	Gelatinase
<i>Enterococcus casseliflavus</i>	42	42	33.3	25	20
<i>Enterococcus dispar</i>	3	3	0	0	0
<i>Enterococcus faecalis</i>	17	17	0	25	20
<i>Enterococcus gallinarum</i>	17	17	33.3	25	20
<i>Enterococcus hirae</i>	9	9	33.3	0	20
<i>Enterococcus mundtii</i>	3	3	0	0	0
<i>Enterococcus saccharolyticus</i>	3	3	0	0	0
<i>Enterococcus saigonensis</i>	3	3	0	25	20
<i>Enterococcus villorum</i>	3	3	0	0	0
Total number	100 (35)	100 (35)	100 (3)	100 (4)	100 (5)

Table A9: Percentage of extracellular enzyme producing HPC isolates

Species name	Grand total	Proteinase	Lipase	DNase	Gelatinase
<i>Acinetobacter johnsonii</i>	2%	3%	7%	0%	0%
<i>Acinetobacter kookii</i>	2%	3%	7%	0%	0%
<i>Acinetobacter lwoffii</i>	2%	3%	0%	3%	0%
<i>Aeromonas molluscorum</i>	2%	3%	0%	0%	0%
<i>Bacillus cereus</i>	4%	5%	14%	6%	0%
<i>Bacillus halotolerans</i>	2%	3%	0%	0%	0%
<i>Bacillus pumilus</i>	4%	0%	0%	6%	0%
<i>Bacillus safensis</i>	2%	3%	7%	0%	0%
<i>Bacillus simplex</i>	2%	3%	0%	3%	0%
<i>Bacillus tropicus</i>	2%	3%	0%	0%	0%
<i>Brevundimonas aurantiaca</i>	2%	0%	0%	3%	33%
<i>Chryseobacterium phosphatilyticum</i>	2%	3%	0%	3%	33%
<i>Ensifer adhaerens</i>	2%	3%	0%	3%	0%
<i>Fictibacillus aquaticus</i>	2%	3%	0%	3%	0%
<i>Fictibacillus phosphorivorans</i>	12%	8%	7%	14%	0%
<i>Lysobacter hankyongensis</i>	2%	3%	7%	0%	0%

<i>Microbacterium saccharophilum</i>	2%	3%	0%	3%	0%
<i>Microbacterium zeae</i>	2%	3%	0%	0%	0%
<i>Paenibacillus lautus</i>	2%	3%	7%	0%	0%
<i>Pseudomonas aeruginosa</i>	2%	3%	0%	0%	0%
<i>Pseudomonas fulva</i>	2%	3%	0%	3%	0%
<i>Pseudomonas geniculate</i>	2%	0%	7%	0%	0%
<i>Pseudomonas songnenensis</i>	4%	5%	0%	3%	0%
<i>Pseudomonas taiwanensis</i>	2%	0%	0%	3%	0%
<i>Sphingobium yanoikuyae</i>	2%	3%	0%	3%	0%
<i>Sphingomonas ginsenosidimutans</i>	2%	3%	0%	3%	0%
<i>Sphingomonas trueperi</i>	2%	3%	0%	0%	0%
<i>Sphingopyxis soli</i>	2%	0%	0%	3%	0%
<i>Stenotrophomonas maltophilia</i>	23%	16%	36%	31%	0%
<i>Tahibacter aquaticus</i>	2%	3%	0%	0%	0%
<i>Vogesella facilis</i>	2%	3%	0%	3%	33%
<i>Vogesella perlucida</i>	2%	3%	0%	0%	0%
<i>Xanthomonas floridensis</i>	2%	3%	0%	3%	0%

Appendix D

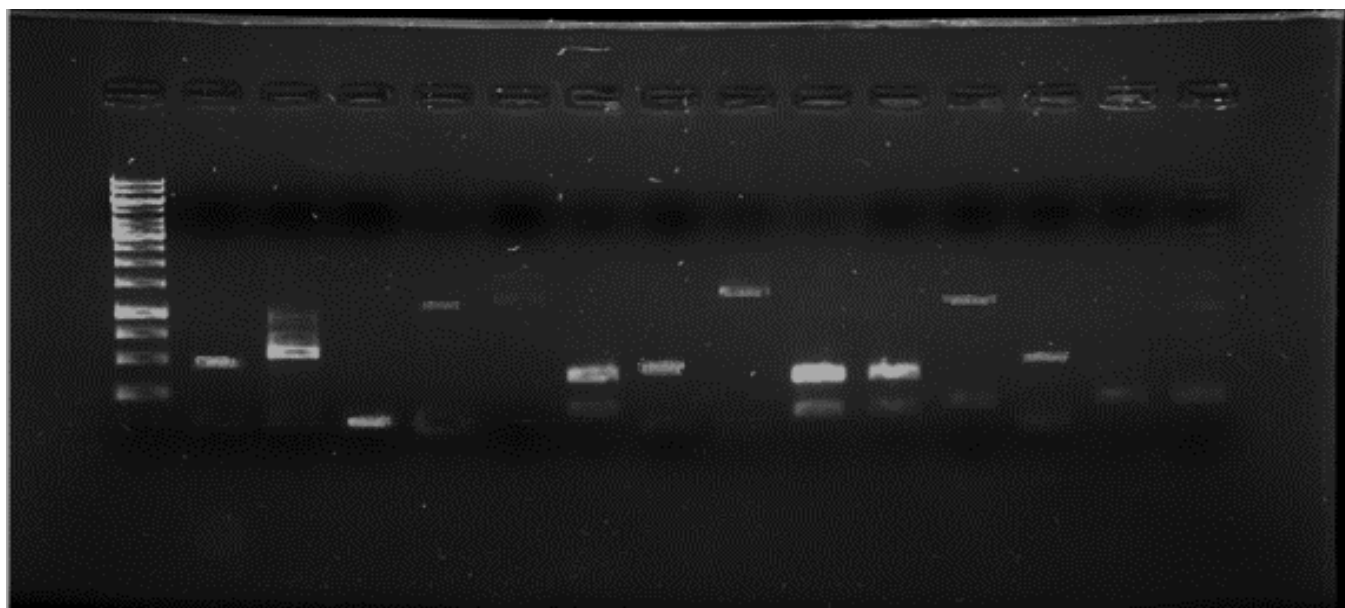


Figure C: An image of some of the amplified genes.