Characteristics of antibiotic resistant bacteria in raw and drinking water from water production facilities

MTA Plaatjie
orcid.org 0000-0002-8321-6469

Dissertation submitted in fulfilment of the requirements for the degree Masters of Science in Microbiology at the North-West University

Supervisor: Prof CC Bezuidenhout
Co-supervisor: Dr CMS Mienie

Graduation May 2019
24355062
Abstract

Water is a limited resource, and the quality of raw water has an impact on drinking water production. Regular testing is done to ensure that the quality is compliant with regulations. Heterotrophic bacteria are widely used as indicators of drinking water quality. According to SANS 241 (2015), the amount of heterotrophic plate count (HPC) bacteria in drinking water should not exceed more than 1000 colony forming units (CFU)/ml. There is currently a growing concern about the presence of HPC bacteria in drinking water. Many of these might be potentially pathogenic microorganisms, and they are associated with secondary infections in immuno-compromised individuals. The South African population has a higher potential infection rate of HPC bacteria compared to other countries due to the escalating percentage of patients living with HIV. These potential pathogens have been reported in drinking water systems. Therefore, this current study aimed to determine antibiotic resistant bacteria and potentially pathogenic HPC bacteria in the drinking water. Samples were collected from two water production facilities, one in the North West (NW-D) and the other in Gauteng (GH-T) Province. Physico-chemical parameters measured were free chlorine, TDS, pH, nitrate, nitrite, COD, phosphates and temperature. HPC bacteria were isolated and purified with a culture-based method, identified by Gram stain and 16S rRNA sequencing, and tested for α- or β-haemolysis on blood agar. Further tests were done for the production of extracellular enzymes such as DNase, hyaluronidase, lipase, proteinase, chondroitinase and lecithinase. Total coliforms and faecal coliforms were enumerated on MLG Agar, using standard procedures. The levels of these were only used for water quality purposes. Antibiotic susceptibility of the HPC bacteria and whether antibiotic resistant genes (ARGs) could be associated with antibiotic resistance phenotypes were determined. Environmental DNA (eDNA) was also isolated to determine if ARGs could be detected directly in selected samples. Turbidity and nitrite levels were alarmingly high, and in some cases, both exceeded the SANS 241 (2015) of drinking water. The other physico-chemical parameters were mainly within the recommended levels. All microbiological parameters were detected at levels below the standard in drinking water, however the higher levels of heterotrophic bacteria after treatment should not be ignored. Fifty one percent (51%) of the isolated HPC bacteria displayed α- or β-haemolysis. These haemolytic isolates revealed the production of various enzymes: proteinase (74% from GH-T), DNase (58% from NW-D) for the 2017 sampling run and chondroitinase (58% from NW-D) for 2016. Both DNase and proteinase enzymes were produced by most of the haemolysin producing isolates. Hyaluronidase and lecithinase were the least detected enzymes. Among all the isolates, resistance to individual antibiotics were observed in the following order of decrease: ampicillin (89%), trimethoprim (77%), cephalothin (62%) and penicillin G (53%) in water after treatment and drinking water. There
was high a percentage susceptibility, and low intermediate resistance percentage of HPC isolates to neomycin, erythromycin, and streptomycin. Penicillin and ciprofloxacin were detected at low levels by liquid chromatography-mass spectrometry analysis. Isolates were resistant to more than two antibiotics classes. The most prevalent MAR phenotype was AMP-TM-KF-PEN-G-VAN-OT. Genes encoding for macrolide-lincosamide-streptogramin (MLS) (ermF and ermB), were detected. However, ermB was more prevalent in the MAR isolates (50%), while ermF gene was found in only one isolate (3%). The tetM and IntI1 genes could not be detected in any of the isolates. β-lactam (blaTEM and ampC) genes were also detected in samples from the water treatment system. Moreover, ermB and intI1 was found in raw water when eDNA was tested. The identified species included Bacillus cereus, Bacillus thuringiensis, Bacillus pumilus, Bacillus licheniformis, Micrococcus luteus, Shinella sp., Chryseobacterium sp., Paenibacillus chitinolyticus, Bacillus anthracis, Bacillus wiedmannii, Bacillus Toyonensis and Bacillus pumilus. The predominant genus was Bacillus, for all the sampling sites in both production facilities. These species are linked to various infections, including wound infections and skin and soft-tissue infections. This study confirmed the presence of potentially pathogenic HPC bacteria in the raw and treated water from two water production facilities. The obtained results showed that ARGs were not completely removed during the drinking water production process, and they can also thus be present in the distribution system. This is a cause for concern, particularly for the immune-compromised individuals. The results show a need for education and awareness programmes in the communities about contaminants entering the water source, and ways to treat water at house level. This will help prevent water-borne diseases but particularly the spread of antibiotic resistance through water systems.

Keywords: Antibiotic-resistant bacteria (ARB); Antibiotic-resistant genes (ARGs); Distribution system; HPC bacteria; Potential pathogens; Water quality.
Preface

The research discussed in this dissertation for the M.Sc. degree in Microbiology was conducted in the Unit for Environmental Sciences and Management, North-West University, Potchefstroom Campus, South Africa. This work was conducted over a two-year period, under the supervision of Prof. Carlos Bezuidenhout and Dr. Charlotte Mienie.

The research done and presented in this dissertation represents original work undertaken by the author and has not been previously submitted for degree purposes to any other university. The use of work of other researchers is duly acknowledged in the text. References were done according to the specifications provided by the NWU Harvard Referencing Guide.
Acknowledgements

To my Heavenly Father, thank you for giving me this opportunity to study. Your love surpasses all knowledge.

I would like to express my sincere appreciation and gratitude to the following people for their support towards the completion of this study:

- Prof. C.C. Bezuidenhout, for guidance, time, support and for constructive advice in making all of this possible. It has been a great experience to further my research under your supervision.
- Dr. C. Mienie for your patience, guidance and understanding. Your input in my project is highly appreciated. Thank you.
- Dr. Jaco Bezuidenhout for assistance with the statistical analysis of this study and for giving helpful advice regarding this study.
- Mr Abraham, for being my mentor: you were very supportive throughout the study, thank you for your support.
- My family: Thank you mama for your prayers and for allowing me to follow my dreams. To my brother, you’ve always believed in me, I appreciate you. Thank you: aunty Racheal for constantly reminding me who God created me to be.
- Tumelo, for being so understanding, for helping me with lab work, and all the time you have invested in this project is highly appreciated. My niece and nephew, Tshepang and Ompolokile, thank you for your words of encouragement.
- Kabelo: You have been a good friend, thank you for our long conversations and for allowing me to disturb you.
- Karabo and Rinaldo: I will always treasure everything that you both did. This project was fun because you were part of it.
- Refilwe, Mzimkhulu, Lee, Dr Marlin: I appreciate you all.
- All postgraduate students and Microbiology Department (for helping me with all my several inquiries).
- To my close friends (Morwa, Lebo, Andrew, Manana, Mpho, Celiwe, Nthabi, and Tsholo): Thank you for your love and for taking part in this great journey.

The Water Research Commission of South Africa (K5/2585//3) is acknowledged for financial support.

“You saw me before I was born. Every day of my life was recorded in your book. Every moment was laid out before a single day had passed”.

Psalm 139:16
Table of Contents

Abstract............................................................................................................................. 1
Preface ................................................................................................................................. iii
Acknowledgements............................................................................................................. iv
List of abbreviations ........................................................................................................ x
List of figures .................................................................................................................... xii
List of tables ..................................................................................................................... xiv
Chapter 1............................................................................................................................ 1
Introduction ......................................................................................................................... 1
  1.1 General introduction ................................................................................................. 1
  1.1.1 Problem statement .............................................................................................. 2
  1.2 Aim and objectives ................................................................................................. 3
  1.2.1 Aim ..................................................................................................................... 3
  1.2.2 Objectives .......................................................................................................... 3
Chapter 2............................................................................................................................ 4
Literature review ............................................................................................................... 4
  2.1 Drinking water production ..................................................................................... 4
    (a) Coagulation and flocculation .............................................................................. 4
    (b) Sedimentation ...................................................................................................... 5
    (c) Filtration ............................................................................................................... 5
    (d) Disinfection ......................................................................................................... 5
    (e) Advanced treatment processes .......................................................................... 5
  2.2 Drinking water quality ............................................................................................ 6
  2.3 Drinking water quality management ....................................................................... 7
  2.3.1 Laws and regulations that govern safe drinking water ..................................... 7
  2.3.2 South African National Standard (SANS) 241 for drinking water ................. 8
  2.3.3 Drinking Water Quality Framework ................................................................ 8
  2.3.4 Blue Drop Certification Programme ................................................................ 9
  2.3.5 Water Safety Plans .......................................................................................... 10
  2.4 Physico-chemical parameters .............................................................................. 10
    2.4.1 Free chlorine .................................................................................................. 10
    2.4.2. Total dissolved solids (TDS) ...................................................................... 11
    2.4.3. pH ............................................................................................................... 11
    2.4.4 Nitrate and nitrite ......................................................................................... 11
    2.4.5 Chemical Oxygen Demand (COD) .............................................................. 12
    2.4.6 Phosphates ................................................................................................... 13
  2.5 Temperature ........................................................................................................... 13
3.4.2 Indicator organisms (E. coli and total coliforms) .................................................. 34
3.5 Gram staining ............................................................................................................. 34
3.6 Production of haemolysin on blood agar (pathogenic screening) ............................ 34
3.7 Extracellular enzyme production .......................................................................... 34
3.7.1 DNase .................................................................................................................. 34
3.7.2 Protease .............................................................................................................. 35
3.7.3 Lipase .................................................................................................................. 35
3.7.4 Hyaluronidase .................................................................................................... 35
3.7.5 Chondroitinase .................................................................................................. 35
3.7.5 Lecithinase ....................................................................................................... 36
3.7.6 Gelatinase ......................................................................................................... 36
3.8 Antibiotic susceptibility profiles of HPC bacteria ....................................................... 36
3.9 DNA extraction ....................................................................................................... 37
3.10 Agarose gel electrophoresis of DNA. .................................................................... 37
3.11 PCR amplification .................................................................................................. 37
3.11.1 16S rRNA gene amplification .......................................................................... 37
3.11.2 ermB and ermF .................................................................................................. 38
3.11.3 blaTEM ............................................................................................................. 38
3.11.4 tetM .................................................................................................................. 38
3.11.5 ampC and intI 1 ............................................................................................... 38
3.12 Environmental DNA (eDNA) extraction. ............................................................... 38
3.13 Analysis of antimicrobial compounds/pharmaceuticals ......................................... 39
3.14 Sequencing of the 16S rRNA genes and identification of the isolates .................. 41
3.15 Statistics applied in this study .............................................................................. 41
Chapter 4 ..................................................................................................................... 43
Results ......................................................................................................................... 43
4.1 Water samples collected in 2016 and 2017 .............................................................. 43
4.1.1 Physico-chemical analysis ................................................................................. 43
4.2 Microbiological analysis ......................................................................................... 48
4.2.1 Microbiological results for 2016 ...................................................................... 48
4.2.2 Microbiological results for 2017 ...................................................................... 49
4.3 Relationship between physico-chemical and enumerated HPC bacteria ............... 50
4.3.1 PCA of 2016 for Plant NW-D ........................................................................... 51
4.3.2 RDA of 2017 Plant for NW-D and GH-T ......................................................... 52
4.4 Potential pathogenic testing results ........................................................................ 54
4.4.1 Haemolysin results for 2016 and 2017 .............................................................. 54
4.4.2 Extracellular enzyme production ..................................................................... 55
6.1.3 Determination of antibiotic resistance patterns and their associated ARGs ............ 85
6.1.4 Potential pathogenic features associated with HPC that are MAR......................... 86
6.1.5 Identification of HPC bacteria (16S rRNA gene sequencing).................................. 86
6.2 Recommendations ..................................................................................................... 86
References ...................................................................................................................... 88
Appendix ........................................................................................................................ 128
List of abbreviations

The following abbreviations have been used throughout this dissertation.

AIDS  Acquired immunodeficiency syndrome
AMP   Ampicillin
ARB   Antibiotic resistant bacteria
ARGs  Antibiotic resistant genes
BLAST Basic Logic Alignment Search Tool
CHL   Chloramphenicol
CIP   Ciprofloxacin
COD   Chemical oxygen demand
DWAF  Department of Water Affairs and Forestry
E.coli Escherichia coli
ERY   Erythromycin
HIV   Human immunodeficiency virus
HPC   Heterotrophic plate count
KAN   Kanamycin
KF    Cephalothin
MAR   Multiple antibiotic resistance
MEGA  Molecular Evolutionary Genetics Analysis
NCCLS National Committee for Clinical Laboratory Standards
NEO   Neomycin
O-T   Oxy-tetracycline
PCA   Principal Component Analysis
PCR:  Polymerase Chain Reaction
PEN-G Penicilllin G
RDA   Redundancy analysis
STRE  Streptomycin
TDS   Total dissolved solids
TM    Trimethoprim
VAN   Vancomycin
WHO   World Health Organisation
WPF   Water Production Facility
WSA   Water Services Authority
WSP   Water Service Provider
α     Alpha
List of figures

**Figure 1.1:** Schematics of conventional water treatment process (Katayon *et al.*, 2006). ..................4

**Figure 3.1:** Schematic representation of Plant NW-D. .................................................................31

**Figure 3.2:** Schematic representation of Plant GH-T. .................................................................32

**Figure 3.3:** Illustration of different colony morphology (Pelczar, 1957). .................................33

**Figure 4.1:** PCA biplot indicating the relationship of physico-chemical and microbiological data of the drinking water at NW-D measured over a one year period for 2016. HPC: heterotrophic plate count; temp: temperature; turb: turbidity; PO4: phosphates; free Cl: free chlorine; COD: chemical oxygen demand; NO2: nitrites; NO3: nitrates; TDS: total dissolved solids. 1: March; 2: May; 3: August. D: Distributed; AT: After treatment. ...................................................51

**Figure 4.2:** RDA biplot illustrating the correlation between the physico-chemical parameters (pH, temp: Temperature; TDS: total dissolved solids; COD: chemical oxygen demand; Free Cl: free chlorine; NO2: nitrites; NO3: nitrates and PO4: phosphates) and the microbiological indicators (HPC: heterotrophic plate count bacteria) and total coliforms species levels, during the following sampling period (NWD-1: May; 2: October; GHT- 1: June and 2: October) of 2017. The red arrows represent the physico-chemical parameters, whereas the blue arrows represent the microbiological parameters. .............................................................................52

**Figure 4.3:** Redundancy analysis (RDA) biplot illustrating the correlation between the physico-chemical parameters (pH, temp: Temperature; TDS: total dissolved solids; COD: chemical oxygen demand; Free Cl: free chlorine; NO2: nitrite; NO3: nitrate and PO4: phosphates) and the microbiological indicators (HPC: heterotrophic plate count bacteria, total coliforms and faecal coliforms species levels, during the following sampling period seasons (NWD1- May; 2: October; GHT- 1: June and 2: October) of 2016 and 2017 for drinking water in 2 water production facilities surface. The red arrows represent the physico-chemical parameters, whereas the blue arrows indicate the microbiological parameters. .......................................................53

**Figure 4.4:** Total percentages of isolates testing positive for each enzyme from NW-D for 2016. ........................................................................................................6

**Figure 4.5:** Total percentages of enzymes produced by haemolytic HPC isolates from NW-D for 2017. ....................................................................................................................................56

**Figure 4.6:** Total percentages of enzymes produced by haemolytic HPC isolates from GH-T for 2017. ....................................................................................................................................57

**Figure 4.7:** 1.5 % agarose gels with successful amplified ampC. Lane C represents the no template DNA control and MW represents the 1kb molecular size marker (O’ GeneRulerTM 1kb DNA ladder, Fermentas Life Science, US). Electrophoresed at 80 V for 45 minutes. For ampC (amplicon size: 550bp); successful amplification is shown in lane:1-3 and 7-8. While lane: Lane: 4-6 shows no amplification. .................................................................................63

**Figure 4.8:** 1.5 % agarose gels with successful amplified blaTEM. MW represents the 1kb molecular size marker (O’ GeneRulerTM 1kb DNA ladder, Fermentas Life Science, US). Electrophoresed at 80 V for 45 minutes. For blaTEM (amplicon size: 1080bp); Lane 1-3 and 6-7 shows successful amplification. While lane: 4-5 indicates no amplification. .................................................................................63
Figure 4.9: Agarose gels with successful amplified ermB. Lane C represents the no template DNA control and MW represents the 1kb molecular size marker (O’ GeneRuler™ 1kb DNA ladder, Fermentas Life Science, US). Electrophoresed at 80 V for 45 minutes. For ermB (amplicon size: 639). .................................................................63

Figure 4.10: 1.5% agarose gel of 16S rRNA amplicons with the expected size of 1 465 bp. Lane 1-11: isolates from Plant NW-D and GH-T. Lane C: no template DNA control; MW: 1kb molecular size marker (O’ GeneRuler™ 1kb DNA ladder, Fermentas Life Science, US). Electrophoresed at 80V for 45 minutes. ................................................................................64

Figure 4.11: Pie chart displaying bacterial species identified with the 16s rRNA gene from all the sampling runs for NWD and GH-T. ................................................................................65

Figure 4.12: Neighbour-Joining phylogenetic tree representing relationship of 16s gene sequences from GenBank database and the sequences of HPC bacteria isolated from water production facilities (NW-D and GH-T) in 2016 and 2017. E.coli was used as an outgroup. ........................................................................................................67
List of tables

**Table 2.1**: Antibiotic resistance genes (ARGs), antibiotics associated with them and their water source. ..................................................................................................................................................20

**Table 3.1**: Summary of the sampling period (2016-2017), sampling sites and sampling months for both WPFs.

**Table 3.2**: Oligonucleotide primers for PCR amplification of 16S rRNA,ermB, ermF, blaTEM, tetM, ampC and int 1. F- Forward primer and R- Reverse primer. ..................................................40

**Table 4.1**: Physical parameters results from 2016 and 2017 for Plant NW-D & GH-T. ..................................................................................................................................................46

**Table 4.2**: Chemical parameters results from 2016 and 2017 for Plant NW-D & GH-T. ...............................................................................................................................................47

**Table 4.3**: Results of heterotrophic plate counts of water before treatment, after treatment and in the distribution for 2016 during March, May and August. .....................................................................48

**Table 4.4**: Microbiological results for each sampling point 2017. .................................................................................................50

**Table 4.5**: Results obtained for haemolysin production for each individual site from raw water in plant NW-D & GH-T (2016 to 2017). ..................................................................................................................54

**Table 4.6**: Results obtained for haemolysin production for each individual site from after treatment and drinking water in plant NW-D & GH-T (2016 to 2017). .....................................................................................56

**Table 4.7**: Antimicrobials detected in source, final and the distribution system of NW-D. ..........................................................................58

**Table 4.8**: Percentage of selected HPC isolates resistant (R), intermediate resistant (IR) and susceptible (S) to antibiotics during the 2016 to 2017 sampling period. ...............................................................59

**Table 4.9**: Highest antibiotic resistance percentage (%) of selected HPC isolates resistant to antibiotics during the 2016 to 2017 sampling period. ................................................................................................59

**Table 4.10**: Antibiotic resistance genes (ampC, ermB, blaTEM and ermF) in varied identified species isolated from raw, after treatment and distribution water in Plant NW-D and GH-T. ...................62

**Table 4.11**: Summary of characteristics of the potential pathogenic HPC bacteria. .................................................................68
Chapter 1
Introduction

1.1 General introduction
Access to safe and clean drinking water is a vital human need, and thus a basic right. This right is recorded in the Bill of Rights of South Africa (Constitution of the Republic of South Africa Act, No.108 of 1996). People use water for a variety of purposes in the homes, industries, agriculture and for recreation purposes (Ilyas et al., 2017). Improving access to safe drinking water can result in significant benefits to health. Therefore, every effort should be made in achieving drinking water that is safe for human consumption by water suppliers (Arnone and Walling, 2007), particularly in South Africa, as it is a water-stressed country (Mulamattathil et al., 2015). There are many communities with inadequate water supply who are left with no other means of getting safe drinking water. Thus, such communities resort to collecting water from available sources such as springs, wells, ponds, rivers, lakes and rainwater to meet their domestic water needs. Water from these sources is mostly contaminated and consumed without any treatment process (Edokpayi et al., 2018).

The quality of drinking water is categorised on the basis of water parameters (chemical, physical, and microbiological). The standards of water quality differ for domestic, agricultural and industrial uses. In South Africa, the South African National Standards for drinking water (SANS 241, 2015), specifies the acceptable levels of these parameters at the point of delivery. Physical water quality is usually not of direct public health concern but affect the aesthetic properties of water. The quality of drinking water may be affected by chemical contaminants that may pose health risks to consumers (Abrams, 2001). Microbiological quality of water is measured by the use of indicators such as faecal coliforms (E. coli), total coliforms and heterotrophic plate count (HPC). This is done to safeguard the consumer from drinking water that is contaminated by pathogens such as bacteria, protozoa and viruses (Figueras and Borrego, 2010). According to SANS 241 (2015), HPC bacteria found in drinking water should not be above 1000 colony forming units (CFUs)/ml. In the past, many studies showed an association between human disease and HPC bacteria (Payment et al., 2003; Hellard et al., 2001). However, recent studies have associated HPC bacteria with gastrointestinal illness, while showing some potential pathogenic features in some genera of these HPC bacteria (Rusin et al., 1997; Pavlov et al., 2004; Horn et al., 2016). These potential pathogenic bacteria are associated with infections (primary and secondary) in immunocompromised individuals (Pavlov et al., 2004).

The phenomenon of antibiotic resistance of bacteria has become a serious problem all over
the world (Bergeron et al., 2015). The widespread of these antibiotic resistant bacteria and their possible genes in the environment can be a public health concern (Pruden et al., 2006; Barancheshme and Munir, 2018). Furthermore, their presence in drinking water, surface water and wastewater is well documented (Luczkiewicz et al., 2010; Mulamathathil et al., 2014). Various studies have indicated that the disinfection process, especially chlorination can select for antibiotic-resistant genes (Armstrong et al., 1982; Shi et al., 2013) and their spread in the distribution systems (Xi et al., 2009).

In South Africa, many studies have shown the presence of multiple antibiotic-resistant pathogens in source and drinking water (Mulamathathil et al., 2014; Kinge et al., 2010). This threatens the ability to treat common infectious diseases with widely used antibiotics (Lupan et al., 2017). A Global Action Plan on Antimicrobial Resistance was developed by the World Health Organisation, to address this challenge (Vikesland et al., 2017).

1.1.1 Problem statement

In the North-West and Gauteng provinces, activities in catchment areas of local rivers, both down and upstream of water treatment plants (purification plants) include active and suspended mining, industrial and agricultural processes as well as urbanization (Van Eeden, et al., 2009; Masondo and Evans, 2011). Some of these are potential sources of hazardous contamination that affect the quality of water available for drinking water production. It is therefore imperative that these activities are effectively regulated, as this might have negative effects on the water quality in the future (McCarthy and Humphries, 2013). Thus, regular physico-chemical analysis of water at source and distribution must be done to determine or to assess the effectiveness of treatment processes.

South Africa has the highest HIV infections in the world. In 2016, there was an estimation of 7.1 million people in South Africa living with HIV (Kufa-Chakezha, 2018). North West (NW) Province is the fourth highest amongst other provinces with an HIV testing coverage of 35.2%; followed by Gauteng Province (ranked fifth) at 12.4% (SANAC, 2016). In 2016, children living with HIV in South Africa was estimated at 320,000 (AVERT, 2018). Thus, safe drinking water is of utmost importance to these immuno-compromised individuals to minimise infections. It is thus important that studies are conducted to investigate impacts of deteriorating water quality on drinking water production processes with a focus on risk factors, such as antibiotic-resistant bacteria and their associated resistant genes.
1.2 Aim and objectives

1.2.1 Aim
The aim of this study is to determine the prevalence of antibiotic resistant bacteria in raw and drinking water and potentially pathogenic bacteria in the drinking water supplied by selected water production facilities.

1.2.2 Objectives
Specific objectives include:
- To assess the physico-chemical and microbiological quality of raw and drinking water
- To isolate and purify HPC bacteria as well as determine antibiotic resistance patterns and associated ARGs.
- To test for potential pathogenic features (extracellular enzyme production) that are associated with HPC that are resistant to multiple antibiotics.
- To identify HPC bacteria isolates that are antibiotic resistant and also potentially pathogenic using 16S rRNA gene sequencing.
Chapter 2
Literature review

2.1 Drinking water production
Water utilities produce drinking water through appropriate treatment technologies (Charrois and Jeffrey, 2010). The water is sourced from free-flowing rivers, dams and subsurface resources. Most water treatment plants use similar basic water treatment process to ensure safe drinking water (Hunter Water, 2006). However, this might differ in some cases, in relation to the location and the technology of the plant. It is imperative to recognise that some treatment processes are only suitable for use in some treatment plants and not applicable in another place. This mainly depends on the accessibility of resources such as electricity and materials, as well as the quality of operator skills (WHO, 1997).

There are various activities in the catchment areas that may impact the quality of the resource water. Therefore, the raw water must undergo various appropriate steps during the purification process. This is to safeguard the final water and to ensure that it meets the requirements/standards set for drinking water (USEPA, 2004). There are five commonly accepted steps in the treatment process, and these include coagulation, flocculation, sedimentation, filtration and disinfection (Figure 1.1) (Momba et al., 2009).

![Schematics of conventional water treatment process](Katayon et al., 2006).

**Figure 2.1**: Schematics of conventional water treatment process (Katayon et al., 2006).

(a) Coagulation and flocculation
Coagulation is a process of destabilising colloid particles (Rand Water, 2016). During this process, coagulants such as aluminium sulphate are added to the water to attract suspended particles. During the stirring of water, large particles are formed, and they are then removed by sedimentation or filtration (DWAF, 2002). During the flocculation step, individual destabilised particles collide with one another forming larger floc particles which also collide with the precipitate formed by the coagulant (Apostol et al., 2011).
(b) Sedimentation
Sedimentation is the oldest and the most commonly used treatment process (Goula et al., 2008). It is known to improve the filtration process by removing particulate material (Gregory and Edzwald, 2010). During this process, the water together with the floc flows slowly into a large sedimentation tank (also intended for the removal of sludge) where the floc settles to the bottom of the tank and is now called sludge. This is then sucked out by desludging bridges followed by its deposition to the sludge deposit site (Saminu et al., 2013; Rand Water, 2016).

(c) Filtration
The filtration process allows for water to flow through a filter media, designed to remove particles that are still present in water after sedimentation. This happens by means of chemical adsorption, where the passage of the contaminants is blocked. Such a process is very important as it enhances the effectiveness of the disinfection step. The several stages of filtration allow drinking water to be pure and free from contamination (USEPA, 2004).

(d) Disinfection
The goal of disinfection of public water supplies is to eliminate, deactivate or kill pathogenic microorganisms (Achour and Chabbi, 2014). The treatment of drinking water often involves both primary and secondary disinfection. Chlorine is used as a primary disinfectant in water treatment but also added to provide a disinfectant residual to preserve the water in distribution (Dore et al., 2013; USEPA, 2004). The effectiveness of chlorine is dependent on its concentration, contact time, turbidity, temperature and pH (LeChevallier and Kwok-Keung, 2004). Secondary disinfection refers to the disinfectant added just before the treated water is distributed, to maintain the water quality within the distribution system (EPA, 2011). In addition, it also acts as a final barrier to help maintain the microbial safety of the water by controlling bacterial contamination and regrowth within the distribution system (Stanfield et al., 2003).

(e) Advanced treatment processes
More clean and safe drinking water will be needed due to increased population growth and water pollution, as well as people’s increasing demands for better life quality (Schutte, 2006). To deal with this problem, various types of drinking water treatment methods have been established by governments in different countries. The conventional treatment process often produce water that is not suitable for human consumption, hence advanced treatment processes are used (DWAF, 2002). The commonly used methods for advanced drinking water treatment include ozonation, desalination, distillation and reverse osmosis (Schutte, 2006; Maurel, 2006). Ozonation has excellent disinfectant properties, thus used in the treatment process. Furthermore, it can inactivate microorganisms such as protozoa which are very resistant to conventional disinfectants (Van der Walt et al., 2009). Desalination refers to the
removal of dissolved salts from the water by making use of distillation, membrane processes and ion exchange. Distillation is one of the oldest water treatment processes used to remove most of the dissolved materials. Membrane processes such as reverse osmosis (RO) and nanofiltration (NF) are alternatives for drinking water treatment where a high product quality is desired. RO and NF membranes can successfully remove organic and inorganic compounds and microorganisms from water (Koyuncu, 2002; Drewes et al., 2003). Studies have showed that finished water produced by RO membrane treatment produced higher inorganic and natural organic water quality than conventional treatment systems (Liu et al., 2007). Thus, the RO process is an important solution for generating safe potable water. Although advanced methods overcome most of the problems mentioned above, they are less used due to technological limitations such as potentially high start-up costs, frequent back-flushing and/or replacement of filters and membranes and high energy consumption (Wimalawansa, 2013; Bremere et al., 2001).

2.2 Drinking water quality

Drinking water quality is defined as water with acceptable physical, chemical, and microbiological properties. Many of these properties are controlled or influenced by substances which are either dissolved or suspended in the water (DWAF, 1996). The quality of drinking water should comply with microbiological, physical, aesthetic and chemical determinant numeric limits as specified in SANS 241 (2015). According to Momba et al. (2003; 2006), in South Africa, the drinking water quality in non-metropolitan areas is still questionable. The majority of drinking water treatment works in these areas fail to produce water according to required or acceptable world standards.

The physical quality of drinking water refers to water quality that may be determined by physical methods, namely, conductivity, pH and turbidity measurement. These properties mainly affect the aesthetic quality (taste, odour and appearance) of water (WRC, 1998). The physical properties do not have a direct public health risk. However, consumers can easily detect them, so they can have significant effects on perceptions of water quality and acceptability (Dietrich, 2006). Furthermore, turbidity is used to indicate the efficiency of the water treatment process and can be used to determine risks and problems in the infrastructure of the treatment process (Obi et al., 2008; Ramavandi, 2014). It may affect its acceptability to consumers and also affect markedly its benefit in certain industries (EPA, 2001).

The chemical quality of drinking water is categorised by dissolved substances such as organic chemicals, salts, and metals (WRC, 1998). Some of the chemical substances in water are essentially part of the daily required intake, but at maximum levels, they may pose health risks.
to public health. The SANS 241 (2015) specifies acceptable daily intake levels of a range of chemicals that have been listed in three categories as macro, micro and organic determinants. The effects of these chemical determinants may be either aesthetic, operational and/or health (SANS 241, 2015). Bartram and Howard (2003) emphasises the need to control chemical safety in drinking water through the development of numerical limits. Some hazardous chemicals that may occur in drinking water can be of concern because of effects that may arise from consequences of exposures over a short period. Examples of such chemicals include nitrate, arsenic and fluoride (Feldman et al., 2007; SANS 241, 2015). According to the World Health Organization (WHO, 2008a) and Calderon (2000), they may cause diseases such as cancer, cardiovascular disease, methemoglobinemia, neurological disease, and miscarriage respectively if they exceed SANS 241 (2015) standards.

Microbial quality is one of the primary indicators for the safety of a drinking water supply (Salgado et al., 2003). It is influenced by the presence of disease-causing organisms (pathogens) in drinking water. These pathogens are mainly of faecal origin, including bacteria, viruses and protozoa. Normally, microbial quality is expressed in terms of the presence of indicator bacteria. The most commonly used indicator bacteria are the faecal coliforms (Cabral, 2010), due to their specificity to faecal sources of contamination (WHO, 2011a). The dominant risk to health is from ingestion of water contaminated with faeces containing pathogens that may cause infectious diseases such as cholera and other diarrheal diseases, dysenteries, and enteric fevers (Bain et al., 2014). According to Department of Health (2001), improper treatment of water was one source of a cholera outbreak in KwaZulu-Natal in 2001. Previous studies (Momba and Kaleni, 2002; Momba et al., 2003) isolated heterotrophic bacteria from on-site reservoir and distribution systems, which included Pseudomonas aeruginosa and Enterobacter cloacae. Pseudomonas aeruginosa mostly infects the urinary tract, pulmonary tract, burn-, and other wounds (Baghal et al., 2013), while Enterobacter cloacae is responsible for lower respiratory tract, urinary tract, and bone joint infections. Immuno-compromised patients are more susceptible to contract such infections (Lin et al., 2006; Baghal et al., 2013). Therefore, it is important to monitor drinking water quality on a regular basis.

2.3 Drinking water quality management

2.3.1 Laws and regulations that govern safe drinking water

According to the Department of Water Affairs and Forestry (DWAF, 2005a), successful drinking water quality management involves a clear understanding of the entire drinking water supply system. This includes the hazards and events that can compromise drinking water
quality, the counteractive and preventative measures, along with the operational controls necessary to ensure a safe and dependable drinking water supply.

The South African Bill of Rights states that everyone has the right to have access to an environment that is not harmful to their health or their well-being (Constitution of the Republic of South Africa, 1996). This includes constant provision of clean and safe water (Momba et al., 2010). According to the South African Constitution (Act No. 107 of 1996) and the Water Services Act (Act No. 108 of 1997) water service delivery is the primary responsibility of the Local Government, i.e. Water Services Authorities (WSAs), therefore, carrying out this responsibility perfectly and lawfully should be the goal (DWAF, 2005a; Haigh et al., 2010). Furthermore, WSAs also have a responsibility to regulate the quality of water supplied by Water Service Providers (WSPs) (DWAF, 2005a). When capacity complications are identified that may prevent a WSA from being compliant, different possibilities of support will be explored until such time that the WSA is capable of being compliant. Meanwhile, the Department of Water and Sanitation (DWS) role of Sector Regulator is employed to provide support in a progressive manner. The emphasis is thus on incentive-based regulation (DWAF, 2005b; Hodgson and Manus, 2006).

2.3.2 South African National Standard (SANS) 241 for drinking water
In South Africa, safe drinking water complies with the South African National Standard (SANS) 241 (2015) for Drinking Water Specification. This is because of the different sensitivities that may occur in various life stages (e.g. of infants and the elderly) and also the immune compromised such as individuals living with HIV/AIDS (Hodgson and Manus, 2006; Momba et al., 2010).

The SANS 241 (2015) specifies acceptable numerical limits for drinking water quality determinants at the point of delivery in South Africa in terms of physical, microbiological, aesthetic and chemical quality. Water that does not comply with the specified parameters in SANS 241 is suggested to present acceptable health risk for lifetime consumption (SANS 241, 2015).

2.3.3 Drinking Water Quality Framework
South Africa has set up a Drinking Water Quality Framework to enable effective management of drinking water quality. This framework is based on a protective approach by implementing risk management (Hodgson and Manus, 2006) which helps with the understanding of the entire water supply system. This also includes the events that can compromise drinking water quality and the operational control essential for improving drinking water quality to protect public health (DWAF, 2005b). A study by Momba et al. (2009), indicates a key to produce
water of such desired quality is to implement multiple barriers, which help in controlling microbiological pathogens, and chemical contaminants that may enter the water supply system. This consists of adopting sound management practices and continually revisiting both the state of the water treatment and the distribution infrastructure, as well as the quality of water produced.

Although monitoring of drinking water is imperative, more attention should also be on reducing the probability of contaminants entering raw water supplies in the first place (DWAF, 2005b). The WHO (2011a) suggested that the prevention of contaminants entering the raw sources should be a crucial step in terms of ranking risks, as a result, this will help the Water Services Institutions (WSI) to decrease the amount of treatment chemicals that will be mandatory for water treatment. When source or raw water is less polluted, less chemicals may be used for water treatment. Hence, the need for WSI to understand the identified risks from the catchment to the point of use (DWA, 2013).

2.3.4 Blue Drop Certification Programme

Drinking water often was considered to be of poor quality in many non-metropolitan areas in South Africa (DWAF, 2005a). As a remedial method, the Department of Water Affairs introduced the incentive-based programme in 2008 with the aim of maintaining and improving drinking water quality management in the Republic of South Africa (DWA, 2010). This was called the Blue Drop Certification (BDC) Programme. This programme is used to encourage performance of the drinking water quality management in the country and to provide correct statistical information to the public on drinking water quality performance (DWAF, 2009b; DWA, 2010). The BDC programme consists of annual assessment of WSAs’ management and service rendering of the potable water supply in their particular geographical areas of responsibility (Nealer and Mtsweni, 2013). This award is only granted when 95% compliance with the Blue Drop Certification (BDC) programme criteria is met (DWAF, 2009b). Blue Drop status is awarded as an indication of recognising excellence in the approach that the WSI are managing drinking water (DWA, 2013).

The first Blue Drop assessments were conducted in 2009 whereby, nationally a total of 107 municipalities and 402 water supply systems in 2009 were assessed. The 2009 Blue Drop score was 51.4% while the 2010 (153 municipalities and 787 systems) improved status was 67.2% (DWA, 2011). This improved status did not remain for long, as the 2014 Blue Water Service Audit (BWSA) Report indicates a sudden failure in drinking water service provision with a decrease in the National Blue Drop Score to 79.64% in 2014, from the 2012 value of 87.6%; a decrease of 8% (DWA, 2014).
The best performing province in 2014 was Gauteng Province with a BWSA score of 92.1%. In the North West, there was a decrease of 15.3% reported, taking the Province’s BWSA score to 63% (DWA, 2014).

2.3.5 Water Safety Plans

In 2004, the WHO Guidelines for Drinking Water Quality recommended that water suppliers should develop and implement Water Safety Plans to effectively maintain the safety of drinking water supplies. This was done through the use of a broad risk assessment and risk management approach that includes all systematic steps in water supply from catchment to consumer (WHO, 2004). WSPs regulates step-by-step preventions for risk management concerning water contamination (WHO, 2010). The introduction of the Department of Water and Sanitation’s (DWS) Blue Drop Certification programme in 2008 has done a lot to ensure that municipalities in South Africa put Water Safety Plans in place (DWAF, 2009a). Water Safety Plans has therefore been adopted as a tool to help proactively identify potential risks to supplies and implement preventive barriers that improve safety (Bartram et al., 2009).

2.4 Physico-chemical parameters

Throughout the world, the quantity and quality of water is affected by increased anthropogenic activities or any pollution (physical or chemical) causing changes to the quality of the receiving water body. Water contains different types of dissolved, suspended, microbiological and bacteriological impurities that could possibly threaten human health (Sagar et al., 2015). Therefore, it is essential to test different physico-chemical parameters of water before using it for drinking, domestic, agricultural or industrial purposes (Dixit et al., 2015). Physico-chemical parameters used to determine the quality of drinking water are explained below.

2.4.1 Free chlorine

Free chlorine refers to the free chlorine concentration that remains in the water after disinfection (WRC, 1998). The presence of free residual chlorine in drinking water is linked to the absence of pathogenic organisms and thus is regarded as an indirect measure of the potability of water. If residual chlorine is present in acceptable concentrations, it can prevent secondary contamination of a treated water source, thus providing safe water. The WHO (2011a) set the free chlorine residual in drinking water to be in the order of 0.2 - 0.5 mg/l for 30 min contact time (WHO, 2004). High chlorine concentrations at the point of entry may lead to taste and odour problems or disinfection by-products that may be harmful to human health (Milot et al., 2002; Blokker et al., 2014). According to Momba and Brouckaert (2005) the WSP
needs to enlighten consumers of the reason for the change in taste and reassure them that it makes the water safer to drink.

2.4.2. Total dissolved solids (TDS)
Total dissolved solids (TDS) refer to inorganic salts and small amounts of organic matter present in water (WHO, 2008a; Rahmanian et al., 2015). Dissolved minerals, gases and organic constituents may produce aesthetically displeasing colour, taste and odour in water (Durmishi et al., 2015). There are no dependable data on possible health effects associated with the ingestion of TDS in drinking water (WHO, 2008a). Elevated concentrations of salts are reported to give an unpleasant taste to water. In addition, some physiological effects may be directly related to high concentrations of dissolved salts including; laxative effects, and some effects on kidney function (DWAF, 1996; Durmishi et al., 2015).

2.4.3. pH
pH is defined as the hydrogen ion activity and it relates to the measure of acidity and alkalinity of the water. In drinking water, the pH is classed as one of the most important water quality parameters (Rahmanian et al., 2015). This is because the concentration of hydrogen ions (H+) affects almost all of the chemical and biological processes in water (Trick et al., 2008). pH value is a good indicator of whether water is hard or soft. The pH ranges from 0-14, with 7 being neutral. pH of less than 7 is considered acidic, pH above 7 is alkaline (Boyd et al., 2011). Although an ideal pH level of drinking water should be between 6-8.5, living organisms are able to maintain constant pH equilibrium and will not be affected by water consumption (Dirisu et al., 2016). High acidity in water can lead to corrosion of metal pipes and plumbing systems and cause aesthetic problems (Rahmanian et al., 2015). For effective drinking water disinfection by chlorination, the pH should preferably be lower than 8 (WHO, 2011a). Failure to control pH might result in contamination of drinking water and in adverse effects on its taste, appearance and odour (WHO, 2007).

2.4.4 Nitrate and nitrite
Nitrate and nitrite are naturally occurring ions, made up of both oxygen and nitrogen and also forming part of the nitrogen cycle. Nitrite is usually not present in notable concentrations except in a reducing environment, because nitrate is the more stable oxidation state. It is known to form chemically by Nitrosomonas bacteria during stagnation of nitrate-containing drinking water in galvanized steel pipes. Nitrate and nitrite are increased by an excess of free ammonia entering the distribution system, leading to nitrification (WHO, 2011a).
The most common sources of both nitrate and nitrite in water include agricultural activities (inorganic fertilizers and manure), wastewater treatment, nitrogenous waste products from humans and discharges from industrial processes (Parvizishad et al., 2017). Nitrate is removed during drinking water production processes and is regulated in drinking water mainly because excess levels (above 40 mg/l) can cause methemoglobinemia, or “blue baby” disease (Durmishi et al., 2015), which is more commonly to occur in bottle-fed infants. To cause methemoglobinemia, nitrate must be converted to nitrite. There is strong evidence showing that gastrointestinal infections greatly increase nitrate excretion, which increases the risk of methaemoglobinemia because of the increase in reduction of nitrate to nitrite (WHO, 2011a).

Although nitrate levels that affect infants do not pose a direct threat to older children and adults, they do indicate the possible presence of other more serious residential or agricultural contaminants, such as pesticides or harmful bacteria (Kumar and Puri, 2012). Nitrate in drinking water is a health concern because it can be readily converted in the gastrointestinal tract to nitrite as a result of bacterial reduction (DWAF, 1996). This conversion results in the formation of nitrosamines, which are known to be carcinogenic (Farren et al., 2015).

In the absence of nitrites, the presence of nitrates indicates an old contamination. Elevated levels of nitrite generally indicate that the activity producing the nitrite is very recent and/or closeby (Kumar and Puri, 2012). Elevated levels of nitrate together with increased phosphate concentrations indicate contamination due to fertilisers (Scheierling, 2007).

2.4.5 Chemical Oxygen Demand (COD)
Chemical oxygen demand (COD) is a measurement of the oxygen equivalent of the organic matter in a water sample (Kumar et al., 2010). It is one of the important water quality parameters. A greater amount of oxidizable organic matter in the sample will lead to increased levels of COD and will reduce dissolved oxygen. The higher the COD, the higher the amount of pollution in the water sample (Yin et al., 2011). The oxidizing agents have been found to split organic compounds of high molecular weight to simple organic acids, which increases the potential for growth of heterotrophic microbes in the drinking water systems (Daniel et al., 1993). This microbially unstable portion of organic carbon, i.e., assimilable organic carbon, is suggested by Lehtola et al. (2001) to be the main nutrient for microbial regrowth in distribution systems. There is no SANS 241 (2015) for COD for drinking water. However, the South African Water Quality Guidelines recommends COD levels of 0 to 30 mg/l for category 3 industrial processes, which also include domestic use (DWAF, 1996).
2.4.6 Phosphates

Phosphates are chemical compounds containing the element phosphorus and is necessary for growth of plants and animals. The extreme use of fertilizer is the main source of phosphate which comes from agricultural or residential cultivated land into surface waters with storm runoff (Gupta et al., 2017). Phosphates do not have any health effect on humans unless they are present in very high concentrations (Kumar and Puri, 2012). High phosphate levels cause muscle damage, problem with breathing and kidney failure (Nyamangara et al., 2013). It has been observed that phosphorus availability in drinking water can regulate microbial growth. This observation creates new possibilities to regulate microbial growth in water distribution systems by developing technologies to remove phosphorus efficiently from drinking water (Miettinen et al., 1997). There is no SANS 241 (2015) standard for phosphates in drinking water available. However, WHO (2008a) has a recommendation of a maximum 5 mg/l for phosphate in drinking water.

2.5.7 Temperature

Temperature of the water is important because it affects the physico-chemical properties of water and biological reactions of organisms (Dixit et al., 2015). It also affects the efficiency of treatment units (Jayalakshmi et al., 2011). High temperature leads to faster regrowth of microorganisms, and low temperatures can slow down microbial growth. Furthermore, an increase in temperature causes the pH of water to reach neutrality and thus favours microorganism growth (Tokajian et al., 2000; Zamxaka et al., 2004). It may also increase odour, taste, colour, and corrosion problems (WHO, 2004).

2.5.8 Turbidity

Turbidity is one of the key parameters in drinking water analysis; it is the measure of how clear the water is. It relates to the content of disease-causing organisms in water, which may come from soil runoff (Rahmanian et al., 2015). Turbidity does not always represent a direct threat to public health (De Roos et al., 2017); however, it can interfere with disinfection treatment or provide a medium for microbial growth (Obi et al., 2008). Moreover, it is commonly used for operational monitoring of control measures included in water safety plans, the recommended approach to managing drinking water quality in the WHO Guidelines for Drinking-water Quality (WHO, 2017). The point of detection is significant in considering potential impacts. High levels of turbidity in source water can indicate pollution events in the catchment (e.g. heavy rain, spills or contamination of groundwater). This can challenge the efficiency of coagulation, disinfection, clarification and filtration (WHO, 2008a; Preston et al., 2010). Turbidity of drinking water should ideally be kept below 1 Nephelometric Turbidity Unit (NTU) because of the
recorded effects on disinfection (WHO, 2017). In drinking water, the higher the turbidity level, the higher the risk that people may develop diseases (Mann et al., 2007).

2.5 Microbiological parameters
The analysis of microbiological quality of water is one of the key points directly related to personal and public health. Its purpose is to ensure the consumer is protected from pathogenic bacteria, viruses and protozoa (Figueras and Borrego, 2010). The most common indicators used for assessing microbiological safety and quality of water are mainly total coliforms, faecal coliforms and heterotrophic bacteria (Whitlock et al., 2002; Pavlov et al., 2004). Furthermore, these indicators are also assessed to obtain the most reliable indication of potential risks of infection by pathogenic microorganisms. Bacteria can be used as indicators of faecal pollution (faecal indicator bacteria) or to indicate the effectiveness of a water treatment system (HPC levels; Wingender and Flemming, 2011).

2.5.1 Heterotrophic plate counts (HPC)
Heterotrophs are microorganisms that need organic carbon for growth, including bacteria, yeasts and moulds (Allen et al., 2004). These organisms are naturally present in the environment and can be found in soil, sediment, food, water and in human and animal faeces (Olson et al., 1991; Lillis and Bissonnette, 2001). Although they are generally considered harmless, some heterotrophic microorganisms are at times opportunistic pathogens, which have virulence factors that could affect the health of consumers with suppressed immune systems (Bartram et al., 2003).

The term “heterotrophic plate count” refers to a range of simple culture-based tests that are intended to recover a wide range of microorganisms from water (World Health Organisation Sustainable Development and Healthy Environments, 2002). The commonly used practices for HPC determination is based on the pour-plate method, membrane filtration and the spread plate method with varying results across the culture methods. Variability further arises from differences in the resources and temperature of cultivation (Sartory et al., 2008).

HPC is mainly used to assess the general microbial water quality (Sartory et al., 2008). The test is simple and inexpensive, and yields results in a relatively short period of time. It has been proved as one of the most reliable and sensitive indicators of treatment or disinfection failure (Burgess and Pletschke, 2010). In addition, HPC can also be used to measure the regrowth of organisms that may or may not pose a health risk (WHO, 2002). SANS 241 standard for HPCs is <1000 CFU/ml (SANS 241: 2015). Venter (2010) isolated HPC from a water distribution system biofilm from the North West Province and identified pathogenic
microorganisms such as *B. cereus, B. megaterium, B. subtilis, Kocuria rosea, B. pumilus,* and *B. licheniformis*.

### 2.5.2 Total coliforms
Total coliforms are a group of bacteria usually found in the aquatic environment in soil and vegetation and in the intestines of mammals, including humans (Bej *et al.*, 1990). Total coliform bacteria consist of faecal and non-faecal origin. Their presence in water is mostly an indication of the hygienic quality of water, as well as possible failures in distribution systems (Zamxaka *et al.*, 2004; NHMRC, 2003). In addition, they can also indicate a lack of system integrity (Besner *et al.*, 2002). The SANS 241 (2015) standard for total coliforms in drinking water is ≤10 CFU/100 ml.

In a study by Momba and co-workers (2004) conducted in the Alice Water Treatment Plant, total coliforms were above 5 CFU/100ml, which is the recommended limit for no risk. The distribution system had high levels of total coliforms. These results indicated poor water treatment and suggested that the overall sanitary qualities of the drinking water, in terms of total coliform counts, were unacceptable (Momba and Binda, 2002).

### 2.5.3 Faecal coliforms (*E. coli*)
Faecal coliforms are a subgroup of the coliform genera. Their presence in water samples indicates recent faecal pollution or post-treatment faecal contamination. According to some older published works, faecal coliforms such as *Escherichia coli* are not very persistent in environmental conditions (Gabutti *et al.*, 2000; Payment and Robertson, 2004). Hence, it is documented as the best indicator of faecal pollution in water sources (WHO, 2004; Paruch and Maehlum, 2012). However, recent studies have shown that *E. coli* can survive for long periods of time in the environment, and potentially replicate in water (Berthe *et al.*, 2013; Dublan *et al.*, 2014).

In comparison to all the contaminants present in drinking water, those originating from human and animal faeces are known to pose an extreme danger to public health. Therefore, this supports the need to detect faecal contamination in drinking water to guarantee public safety (Tyagi *et al.*, 2006). The SANS 241 (2015) standard for *E. coli* in drinking water is 0 CFU/100 ml.

### 2.6 Potentially pathogenic HPC bacteria
Control of HPC bacteria in a water distribution system is a significant tool to minimize human exposure to pathogenic microorganisms (Chowdhury, 2012). According to the WHO (2002)
and Bartram et al. (2004), it was concluded that heterotrophic bacteria in drinking water are not a health concern to the general public. However, some studies have indicated that the immuno-compromised individuals might be vulnerable to some HPC bacteria (Kunimoto et al. 2003; Payment and Robertson, 2004). These individuals include the very young (0 to 5 years) and very old (+65 years), as well as patients with immune-compromising infections such as AIDS, patients under medical treatment for various forms of cancer and organ transplant patients (Pavlov et al., 2004). The following genera have been associated with opportunistic infections: Acinetobacter, Aeromonas, Bacillus, Flavobacterium, Klebsiella, Legionella, Moraxella, Mycobacterium, Staphylococcus, Serratia, Pseudomonas, and Xanthomonas (Kudinha et al., 2000; Van der Kooij, 2005; Pavlov et al., 2004). South Africans may be at a higher risk of infection by HPC bacteria. This is due to the increasing number of immuno-compromised individuals such as HIV positive patients (Bor et al., 2013). In 2016, there were 7.1 million people estimated to be living with HIV (Kufa-Chakezha, 2018). Obi and co-workers (2006) have demonstrated the vulnerability of HIV-positive individuals to water-borne pathogens, thus safe drinking water is more critical to these individuals. Results from a study by Lule et al. (2005) showed that there was a 25% reduction of diarrhoea episodes after the implementation of a safe water system among HIV positive individuals.

2.7 Water-borne disease outbreaks associated with HPC bacteria

Water can be extremely dangerous when it becomes the vehicle of transmission of diseases (WHO-SEARO, 2010). According to the WHO, water-borne diseases are estimated to cause more than 3.4 million deaths each year, commonly in developing countries and have been the major cause of mortality and morbidity (Berman, 2009). Water-borne diseases are caused by enteric pathogens such as viruses, bacteria and parasites which are transmitted via the faecal-oral route (Theron and Cloete, 2002; Ashbolt, 2004). These include cholera, shigellosis and typhoid fever among others, which are transmitted in contaminated fresh water, food, washing, crops and direct or indirect contact such as domestic recreational or occupational activities (Schwarzenbach et al., 2006; Prüss-Ustün, 2014). The incidence of these diseases can be reduced by improved water supply and proper sanitation. However, outbreaks of water-borne diseases still frequently occur, even in developed countries. Thus, it is important to monitor the levels of contamination and to prevent disease outbreaks from both an economic and public health perspective (WHO-SEARO, 2010; Shakya et al., 2012). Water-borne diseases resulting from infection depend on the causal agent. Furthermore, this will also affect the severity of the infection. Studies by Rusin et al. (1997) and Pavlov et al. (2004) gave an indication of the relative significance of opportunistic HPC bacteria in causing human infection.
Studies by Momba et al. (2002; 2003) have shown that consumers are at risk of water-borne diseases because the majority of small water works in South Africa struggle to provide adequate treatment and disinfection. In 2000, outbreaks of cholera and typhoid infection in the South African provinces of KwaZulu-Natal, Mpumalanga and Eastern Cape were reported. The cholera outbreak spread to eight provinces. Moreover, by the end of 2001 there were 239 deaths and 106 866 cholera reported cases, with KwaZulu-Natal being the most affected (99%) (Department of Health, 2001). In the year 2005, a diarrhoea and typhoid outbreak was reported in Mpumalanga Province (Delmas Town). This led to five deaths, with a total of 561 with typhoid infection and 3 000 people with diarrhoea (Groenewald and Dibetle, 2005; Masinga, 2005). In December 2008, there were an additional 1 279 cases, and 12 deaths reported. WHO (2008b), reported that the majority of the cases (1 194) occurred in the Limpopo Province.

There is currently over 500 water-borne potential pathogens of concern in drinking waters, identified by the US Environmental Protection Agency (EPA) including bacteria (Legionella longbeacheae, Escherichia coli O157:H7, Pseudomonas aeruginosa) viruses (Hepatitis A and E, Enteroviruses, Norwalk viruses, Adenoviruses, Rotaviruses Astroviruses) (Adetunde and Glover, 2010) and protozoa (Balamuthia mandrillaris, Naegleria, Cyclospora, Septata spp.; Soller et al., 2010; Straub and Chandler, 2003). Water-borne disease surveillance data collection is beneficial and guarantees that risks could be identified, and processes be put in place to ensure the safety of drinking and recreational water (Macler and Merkle, 2000).

2.8 Antibiotic resistant bacteria in drinking water

Antibiotics are one of the most important drugs used to treat infectious diseases (Rodriguez-Mozaz and Weinberg, 2010). However, considerable quantities of these compounds are released into municipal wastewater due to common use of antibiotics in human therapy and agricultural practices (Wright, 2010). Furthermore, the wide use and abuse of antibiotics has led to the emergence of antibiotic resistant bacteria (ARB), compromising the efficiency of antimicrobial therapy because the infectious organisms are becoming more resistant to most antibiotics (Pruneau et al., 2011). Aquatic ecosystems are recognised as reservoirs for ARB (Biyela et al., 2004; Martinez, 2008; Zhang et al., 2009). Previous studies have reported that ARB are common in drinking water systems from source to finished water (Ramteke et al., 1990; Shrivastava et al., 2004; Pathak and Gopal, 2008).

Resistance is a result of inappropriate use, such as not completing a prescription or over-use of the drugs (Xi et al., 2009). Other reasons include the selective pressure of antibiotic use,
as well as change in genome that improves the transmission of resistant organisms. Naturally occurring ARB and ARGs in the aquatic environment are selected for, and enriched for by antibiotics found in sewage and agricultural runoff, which result from the widespread and increased use of antibiotics (Baquero et al., 2008). Studies by Rutala et al. (1997) and Fraise (2002) demonstrated that the susceptibility of ARB and antibiotic-susceptible bacteria to a disinfectant is similar. This indicates that disinfection does not select ARB but instead encourages the development of antibiotic resistance. The most commonly used disinfection process, chlorination, had been directly linked to select for ARBs (Shi et al., 2013; Chiao et al., 2014, Shrivastava et al., 2004; Khan et al., 2016).

Multiple antibiotic resistance (MAR) indicates resistance to two or more antibiotics as reported in previous studies (Guan et al., 2002; Mulamattathil et al., 2004). The MAR index is a useful tool for bacterial source tracking. This tool is useful for health risk assessment which identify if isolates are from a region of high or low antibiotic use (Davis and Brown, 2016). It is also used to assist in differentiating between human and non-human faecal sources (Scott et al., 2002).

2.9 Antibiotic resistance genes (ARGs)
According to WHO (2014), dissemination of antibiotic resistance genes in the environment is highlighted as an emerging problem. Hence an increasing amount of attention is placed on investigating antibiotic resistance in the natural environment (Martinez, 2008; Biyela et al., 2004; Pruneau et al., 2011; Bergeron et al., 2015). ARGs can be found in almost all environmental compartments, including drinking water sources and tap water, eventually threatening human health (Pruden et al., 2006; Becerra-Castro et al., 2015). Thus, their abundance in drinking water sources and removal from drinking water systems need to be understood (Su et al., 2018). Table 2.1 summarises all the antibiotic resistant genes, antibiotics associated with them and their probable water source.

2.9.1 β-lactamase resistance genes (ampC and blaTEM)
Beta-lactamase is a family of enzymes that could hydrolyze β-lactam rings, consequently deactivating β-lactam antibiotics (Walsh et al., 2005). They are a major defense mechanism of Gram-negative bacteria against β-lactam antibiotics (Jacoby, 2009; Zeng and Lin, 2013). Resistance to β-lactam compounds is mostly due to the production of β-lactamases that hydrolyze and thus inactivate β-lactam antibiotics (Jacoby and Munoz-Price, 2005).

AmpC β-lactamases are a class C or group I cephalosporinases that confer resistance to a wide variety of β-lactam antibiotics including penicillins, cephamycins, cephalosporins,
monobactams and oxyiminocephalosporins (Parveen et al., 2010; El-Hady and Adel, 2015). Genes for ampC β-lactamases are commonly found on the chromosomes of various Gram negative bacilli, such as Pseudomonas aeruginosa and Enterobacter spp., Acinetobacter spp., Aeromonas spp., C. freundii, E. coli, S. marcescens, etc. (Barlow and Hall, 2002; Singhal et al., 2005).

The TEM β-lactamase, conferring resistance to the penicillin family antibiotics such as ampicillin, is encoded by the blaTEM gene (Bailey et al., 2011). Previous studies by Adefisoye and Okoh (2016), and Singh et al. (2018) have reported high prevalence of the blaTEM gene in aquatic environments. The blaTEM gene is mainly associated with Enterobacteriaceae. It is used as an indicator for anthropogenic antibiotic resistance contamination when found in the environment (Lachmayr et al., 2009).
Table 2.1: Antibiotic resistance genes (ARGs), antibiotics associated with them and their water source.

<table>
<thead>
<tr>
<th>ARGs</th>
<th>Antibiotics resistant to</th>
<th>Associated bacterial isolates</th>
<th>Water source*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ampC</strong></td>
<td>Cephalothin, ceafazolin, cefoxitin and most penicillins</td>
<td><em>Citrobacter, Enterobacter, E. coli,</em> viable but non-cultivable bacteria</td>
<td>RW, DW</td>
<td>(Schwartz et al., 2003; Volkmann et al., 2004; Zhang et al., 2009; Xu et al., 2016)</td>
</tr>
<tr>
<td><strong>blaTEM</strong></td>
<td>Cephalothin and penicillins</td>
<td><em>E. coli,</em> Heterotrophic plate count bacteria</td>
<td>RW, TW, DW</td>
<td>Xi et al., 2009; Zhang et al., 2009; Xu et al., 2016</td>
</tr>
<tr>
<td><strong>ermB</strong>, <strong>ermF</strong></td>
<td>Macrolides, lincosamide and streptogramin</td>
<td><em>Bacillus, Enterococcus</em></td>
<td>RW, TW, DW</td>
<td>(Zhang et al., 2009; Xu et al., 2016)</td>
</tr>
<tr>
<td><strong>tetM</strong></td>
<td>Tetracycline</td>
<td><em>Aeromonas, Bacillus, Escherichia, Lactococcus, Pseudoalteromonas, Vibrio</em></td>
<td>RW</td>
<td>Zhang et al. (2009)</td>
</tr>
<tr>
<td><strong>IntI 1</strong></td>
<td>Depends on genes present in cassettes</td>
<td><em>E. coli, Vibrio</em></td>
<td>RW, TW, DW</td>
<td>(Ozgumus et al., 2007; Taviani et al., 2008; Xu et al., 2016)</td>
</tr>
</tbody>
</table>

* RW = Raw Water; TW = Tap Water; DW = Drinking Water
2.9.2 Tetracycline resistance efflux pumps genes (tetM)
Tetracyclines is one of the most widely used family of broad-spectrum antibiotics in clinics and agriculture. This is due to their excellent therapeutical index, few side effects, oral administration and low cost (Chopra and Roberts, 2001; Thaker et al., 2010). Tetracycline inhibits protein synthesis in Gram-positive and Gram-negative bacteria by impairing stable binding of aminoacyl-tRNA molecules to the 30S ribosomal subunit (Chopra and Roberts, 2001).

To date, more than 40 different tetracycline resistance genes have been identified, which are involved mainly through three mechanisms: active efflux, ribosomal protection proteins, and enzymatic inactivation of the tetracycline (Brown et al., 2008; Roberts, 2005). Among the tet genes, tetM has the widest host range of any tetracycline genes (Roberts, 2005), since it is often associated with mobile genetic elements which improve its transferability from one bacteria to another. Several studies have reported a variety of genotypes of the tetM gene from natural environments or resistance isolates (Rizzotti et al., 2009).

2.9.3 Macrolide-lincosamide-streptogramin (MLS) resistance genes (ermF and ermB)
Macrolide (erythromycin) antibiotic, inhibit protein synthesis via binding to the bacterial 50S ribosomal subunit and are extremely effective (Roberts, 2008). However, recent studies show that macrolides have an overlapping binding site with lincosamides and streptogramins on the bacterial ribosome. This has led to an increase in the number of genes generating resistance to these antimicrobials, i.e., MLS resistance genes (Saribas et al., 2006; Liu et al., 2014).

The ermB and ermF genes have been proposed as indicators for assessing the antibiotic resistance status of a specific environment (Berendonk et al., 2015). Together, ermB and ermF genes can assist on possible differences in genes that are closely related and operate via similar resistance mechanisms (Echeverria-Palencia et al., 2017). Recent studies indicate a high prevalence of ermB in surface water (Stoll et al., 2012; Rieke et al., 2018). Wang et al. (2016) suggest a potential role for ermB in the proliferation of resistance genes and support the use of it as a potential indicator gene in assessing water quality.

2.9.4 Integrons genetic markers (intI1)
Integrons are widespread mobile genetic elements that are able to capture, recognise and mobilize antibiotic resistance gene cassettes, and are frequently found inserted into mobile elements (Fluit and Schmitz, 2004; Cambray et al., 2010). Integrons contain the genetic information of a site-specific recombination system, consisting of an int gene encoding a site-specific recombinase and adjacent recombination site (Collis et al., 2002). Integrons can
assemble genes that confer advantageous phenotypes. Consequently, they are regarded as one of the major drivers contributing to the evolution and acquisition of bacterial resistance (Gillings et al., 2008; Rosewarne, 2010).

Studies by Koczura et al. (2014) and Mokracka et al. (2012) show integrons detected in the genomes of cultivable heterotrophs isolated from surface waters with different anthropogenic pressure, ground water and wastewater. On the basis of the integrase genes (intI), integrons have been grouped into three classes; Class 1, 2, and 3 (Deng et al., 2015). Class 1 resistance integrons (whose integrase is called intI1) are often located on mobile elements such as transposons and plasmids (Dominguez et al., 2015) and thus are involved in spread of antibiotic resistance genes in bacteria by lateral gene transfer (Stokes and Gillings, 2011).

2.10. Analysis of some pharmaceuticals in drinking water
Pharmaceuticals and personal care products (PPCPs) are a structurally diverse class of emerging contaminants that have been detected in aquatic environments, including drinking water (Kumar et al., 2010). These compounds include prescription pharmaceuticals, over-the-counter medications and naturally occurring compounds that trigger a physiological effect (WHO, 2011b). They commonly enter the environment through treated and untreated wastewater. Some of these compounds may also enter the environment via other routes, such as urban or agricultural runoff (Chander et al., 2014). Once in the environment, pharmaceuticals are attenuated by processes such as dilution, microbial biodegradation, adsorption to solids, or other abiotic transformation (WHO, 2011b).

Recent studies have reported that current wastewater treatment methods do not sufficiently remove pharmaceuticals and/or their metabolites, therefore they can reach surface, ground, and drinking waters (Benotti and Brownawell, 2007; Joss et al., 2008). Although the concentrations of individual pharmaceuticals and/or PPCPs are reported in investigated water bodies worldwide, they are detected at low levels and may not lead to harmful health effects. It has been reported that chronic exposure to a mixture of such compounds may disturb the balance in the human body and enhance resistance to antibiotics (Shraim et al., 2017). Monitoring antibiotics in environmental water is important as it will provide insights into trends and whether there is an increase over time.
2.11 Approaches and media used to enumerate and identify HPC and indicator bacteria

2.11.1 Membrane filtration and media

The membrane filtration technique is an effective, accepted technique used by municipal water treatment plants for monitoring drinking and surface water for presence of coliform bacteria, including faecal coliforms. It involves minimal preparation compared to other traditional methods and is one of few methods that will allow the isolation and enumeration of microorganisms. Using the membrane filtration technique, the water sample is passed through the membrane (generally 0.45 μm) using a vacuum system (Rompré et al., 2002). Bacteria in the sample are retained on the surface of the membrane, which is placed on a suitable selective medium and incubated at an appropriate temperature. If coliforms and/or faecal coliforms are present in the water sample, discrete colonies form (Wang and Wanda, 2007). Subsequently, it can be directly counted, and the results are expressed as colony forming units per 100 ml (CFU/100ml) (Edge and Hill, 2007).

R2A agar is a low nutrient medium (Uhl and Schaule, 2000), and it favours the growth of water-based oligotrophic bacteria in combination with ambient temperature incubation (20 to 28° C) as well as longer incubation time (5 to 7 days) (Allen et al., 2004). It is used for enumeration of HPC in water, mainly for potable water (Bartram et al., 2003; Reasoner, 2004).

MLG agar is a medium used for differentiation and enumeration of total coliforms and E. coli (Fricker et al., 2008). The medium contains laurel sulphate to inhibit growth of Gram positive bacteria. Identification of coliforms and E. coli in MLG agar is facilitated through the following principle: lactose fermentation is indicated by the dye phenol red which gives colonies a yellow colour due to acid production (Fricker et al., 2008; Carstens et al., 2014). Coliforms are lactose positive, so colonies will appear yellow on the agar, while E. coli is both lactose positive and has glucuronidase enzyme and as a result, colonies will appear green (Fricker et al., 2008; Oxoid Limited, 2017). Hallas et al. (2008) and Fricker et al. (2008) used MLGA to detect coliforms and E. coli and total coliforms in potable and non-potable water.

2.11.2 Gram staining technique

The Gram stain is important since it is used for the initial classification of unknown isolates (Beveridge, 2009). This procedure is used to differentiate organisms of domain Bacteria according to their cell wall structure and then to classify bacteria according to their sizes, forms and cellular morphologies (Lamanna and Mallette, 1950). Gram staining allows the separation of all bacteria into two large groups: “Gram positive” bacteria retain the initial violet stain (has a purple or blue colour), and their cells have a thick peptidoglycan layer, whereas those that
are “Gram negative” retain safranin (has a red to pink colour) and consist of a thin peptidoglycan layer (Smith and Hussey, 2005).

2.11.3 16S gene sequencing
Since its first use three decades ago, 16S rRNA gene sequencing has represented an important step in bacteria identification and provides vital information for their classification (Woo et al., 2008). This gene (16S rRNA gene) is the most used housekeeping genetic marker to study bacterial phylogeny and taxonomy, and it is used for a number of reasons: (1) it is found in almost all bacteria, frequently exists as a multigene family or operons; (2) there is no change in the function of 16 rRNA gene over time, and this suggests that there is more accurate measure of time (evolution) by random sequence changes; and (3) the 16S rRNA gene can be used for informatics purposes because of its size (1,500 bp) (Janda and Abbott, 2007). However, 16S rRNA gene sequencing provides genus identification in most cases (>90%) but less with regard to species (65 to 83%; Michael, 2007). Inomata et al. (2009) used 16S rRNA gene sequencing to identify haemolytic HPC bacteria from drinking water. The identification included: *Bacillus*, *Mycobacterium*, *Sphyngomonas*, *Staphylococcus* and *Mikkrococcus*.

2.12 Methods to determine potentially pathogenic HPC bacteria
Pathogenicity is the organism’s ability to cause disease. Virulence is referred to as the degree of pathogenicity (Willey et al., 2008). Virulence is determined by any features such as the structure, genetic or biochemical, allowing the pathogen to cause disease within the host (Todar, 2009). Pathogenic potential of the bacteria is determined by methods such as the haemolysin assay and other extracellular enzyme production analysis (Pavlov et al., 2004). Ahmad et al. (2010) and Willey et al. (2011) have shown that haemolysis is a common virulence factor found in many potentially pathogenic bacteria, thus it is used as the first toxins tested for when screening for pathogenic potential. Microorganisms have shown to also produce various extracellular enzymes that help in their pathogenicity (De Assis et al., 2003).

Pavlov and co-workers (2004) showed that HPC bacteria that are α- or β-haemolytic and produce two or more extracellular enzymes are potentially pathogenic. There are several enzymes involved with bacterial pathogenesis, which include DNase, protease, lipase, hyaluronidase, chondroitinase, gelatinase and lecithinase.

2.12.1 Haemolysin assay
Haemolysin is responsible for the lysis of red blood cells (Willey et al., 2008) and causes haemoglobin to be released. Haemolysin is one of the first toxins tested for when screening
for pathogenic potential. Blood agar is used because it is high in nutrient content and hence it is able to support the growth of a varied range of bacteria (Payment et al., 1994; BookRags Staff, 2005). There are three types of haemolysins: alpha (α), beta (β) or gamma (γ) (Payment et al., 1994; Mulamattathil et al., 2014). α-haemolysin is defined as a partial breakdown of blood cells, forming a green discoloration on the medium. β-haemolysin is responsible for full lysis of blood cells, causing a clear zone around the organism. γ-haemolysin is a sign for no haemolysis. It is indicated by a brownish growth on blood agar (BookRags Staff, 2005; Mulamattathil et al., 2014). Previous studies made use of enzymes involved in pathogenicity to determine the pathogenic potential of HPC bacteria present in treated and untreated water. These included gelatinase DNase, protease, lipase, hyaluronidase, chondroitinase, lecithinase (Pavlov et al., 2004; Horn et al., 2016). The results showed that enzymes were produced by many of the haemolytic positive isolates.

2.12.2 DNase
DNase is a class of extracellular endonucleases capable of hydrolysing polymerised DNA into smaller oligonucleotides. DNases are capable of inactivating the genetic machinery of phagocytes once they have engulfed the DNase-producing bacteria. DNase induces the degradation of nucleic acids and is DNA-specific (Pavlov et al., 2004; Haas et al., 2014).

2.12.3 Proteinase
Proteinase, also known as protease, are enzymes that can attack peptide bonds within peptides and proteins (Barrett et al., 2004). Proteinase production is an inherent capacity of all microorganisms. Bacteria are the main group of alkaline proteinase producers, with the genus Bacillus being the most common source (Singh et al., 2015). Todar (2009) suggested that proteinase may play a role in bacterial metabolism or nutrition and may have a direct or indirect role in invasion of host cells.

2.12.4 Lipase
Lipases hydrolyze the ester bonds in triacylglycerols, resulting in the release of fatty acids and glycerol (Subramoni et al., 2010). The production of lipases can affect microbial fitness and virulence (Stehr et al., 2003). Schaller et al. (2005) and Stehr et al. (2000) suggested that roles of microbial extracellular lipases include: digestion of lipids for nutrient acquisition, initiation of inflammatory processes by affecting immune cells and adhesion to host cells and host tissues.

2.12.5 Hyaluronidase and Chondroitinase
Hyaluronidase is an enzyme known to degrade hyaluronic acid of connective tissue, however, some of these enzymes are also able to cleave chondroitin sulphate. It is known as the
“spreading factor” because of its functionality of spreading bacteria and their toxins in host tissues (Hynes and Walton, 2000). Chondroitinase enzymes are known to degrade chondroitin sulfate (Penttinen et al., 2016). Hyaluronidase and chondroitinase are categorised as virulence factors because they allow for infecting microbes to penetrate tissue (De Assis et al., 2003).

2.12.6 Lecithinase
Lecithinase is also known as phospholipase C or phosphatidylcholine phosphohydrolase and is a type of phospholipase that hydrolyses lecithin, which is a lipid component of eukaryotic membranes (Sharaf et al., 2014). This enzyme is known to destroy the integrity of cytoplasmic membranes of many cells (Ghannoum, 2000). Furthermore, it can act as hemolysin, causing lysis of red blood cells in addition to destroying cells of other tissues (Beecher and Wong, 2000). Iron released from the lysed blood cells allows the pathogen to grow in an environment that has sufficient concentration of this essential growth nutrient (Atlas, 1997).

2.12.7 Gelatinase
Gelatinase enzyme allows microorganisms to hydrolyze gelatin into its sub-compounds (polypeptides, peptides and amino acids) that can cross the cell membrane and used by the organism. Forms of gelatinases are expressed in several bacteria including Pseudomonas aeruginosa, Staphylococcus aureus, Clostridium perfringens and Serratia marcescens (Balan et al., 2012). Gelatinase can contribute to biofilm formation (Hancock and Perego, 2004) and is also thought to contribute to virulence by degradation of a wide range of host substrates, including collagen, fibrinogen, fibrin, etc. (Thurlow et al., 2010).

2.13. Method and media used to determine antibiotic resistance profiles
Muller-Hinton agar is a growth medium that is mostly used for antibiotic susceptibility testing (National Committee for Clinical Laboratory Standards, 2000). The test involves the growing of a bacterial lawn on the medium on which paper disks are placed which are impregnated with an antibiotic. The antibiotic disks are standardised to contain a specific amount of antibiotic (Jorgensen and Ferraro, 2009). Clinical and Laboratory Standards Institute approved standards recommend/select Muller-Hinton agar for the diffusion of antimicrobial agents impregnated on discs through an agar gel for several reasons: (1) Good batch-to-batch reproducibility is demonstrated for susceptibility testing; (2) most non-fastidious bacterial pathogens are supported hence they grow in the media; and (3) there has been a lot of experience and data regarding its performance. The medium is rich in nutrients thus it is used with complete confidence, and it can also grow fastidious organisms (NCCLS, 1979). Previous studies by Carstens et al. (2014) and Pavlov et al. (2004) have used the disk method due to
its simplicity and the fact that it does not require any special equipment. Furthermore, the provision of categorical results can easily be interpreted, and it is the least costly of all susceptibility methods (Jorgensen and Ferraro, 2009).

2.13 Molecular-based methods to determine the presence of antibiotic resistance genes

In the last few decades, we have seen remarkable development of novel molecular methods to detect antibiotic resistance genes from environmental settings. Most of these methods are faster, and they can improve the main shortcoming related to culture-based methods (Anjum et al., 2017; Wang et al., 2018). These methods are either used independently or in combination to investigate levels of the relevant ARGs. Molecular methods mostly used to detect ARGs in the environment include PCR, qPCR, whole genome sequencing, next-generation sequencing, multiplex PCR and other metagenomic approaches (Xi et al., 2009; Luby et al., 2016; Fernando et al., 2016; Moran et al., 2017).

Polymerase chain reaction (PCR) is an in vitro technique used to amplify a specific DNA fragment from a complex pool of DNA (Garibyan and Avashia, 2013). It is the most used technique in molecular biology because it is easy, quick and inexpensive (Hongbao, 2005). DNA is separated into its complementary strands and DNA polymerase is used to synthesize new DNA molecules. (Hongbao, 2005). There are two methods mainly used for visualizing the PCR products: (1) using a chemical dye (ethidium bromide) to stain the amplified DNA product; and (2) using fluorescent dyes for labelling the PCR primers or nucleotides prior to PCR amplification (Garibyan and Avashia, 2014).

PCR-based molecular methods are commonly used to detect ARGs in bacteria. Recently, many studies have demonstrated the high capability of molecular methods such as PCR and gel electrophoresis; these methods are increasingly used for their specific, rapid, reliable, and accurate detection of bacteria and genes of interest (El-Seedy et al., 2017). Therefore, PCR primers can be designed to target the conserved regions of particular genes of interest, thereby amplifying the variable sequences of the gene. PCR-based techniques are also useful when working with environmental DNA (eDNA). This technique can be used to amplify the target gene if no inhibiting products are present in the DNA sample. Previous studies detected ARGs by PCR directed to the following genes; *ampC*, *IntI*, *ermB*, *ermC*, *ermF*, *tetO*, *tetW* (Schwartz et al., 2003; Beukers et al., 2018; Selvaraj et al., 2018) in aquatic environments.

2.14 Chapter summary

In the preceding literature review, it was demonstrated that various anthropogenic activities or chemical/physical pollution in the catchment areas may impact the quality of the source water.
This could possibly threaten human health. Thus, it is crucial for WSAs to analyse both sets of parameters by SANS 241 of drinking water.

More advanced treatment processes are used due to failure of conventional treatment processes to produce water fit for domestic use. This problem is mainly reported in non-metropolitan areas. There are laws and programmes in place that govern safe drinking water. This is to encourage performance of the drinking water quality management and to implement preventive barriers that will improve drinking water quality performance of the country.

In the past, heterotrophic bacteria in drinking water were not regarded as a health concern to the general public. However, few studies have indicated that the immuno-compromised individuals might be vulnerable to some HPC bacteria, as they are considered potential pathogens. This is alarming especially for South Africa since it has a higher risk of infection by HPC bacteria, due to an ever-increasing number of individuals with HIV/AIDS.

The wide use and abuse of antibiotics have led to the emergence of ARB and ARGs, compromising the efficiency of antimicrobial therapy. Previous studies have reported that ARB and ARGs are common in drinking water systems from source to drinking water. The most commonly used disinfection process, chlorination, had been shown to select for ARB and ARGs. The literature review also discussed various physico-chemical as well as microbiological methods available to study water quality. It also compared some methods that could be used to characterize HPC bacteria isolated from distribution water systems as well as ARGs developing in such systems.
3.1. Study sites

3.1.1 Water production facility of Plant NW-D
The drinking water production facility NW-D is in the North West Province and it provides more than 28 000 households with piped water. Potable water in this facility is produced from raw water obtained from surface and groundwater. There are two dams that store raw water, and from one dam it is transported to the water purification plant of the city in a 12-km long uncovered cement canal (Annandale and Nealer, 2011). Hereafter, it undergoes several water production processes (illustrated in Figure 3.1) to ensure that the water meets SANS 241 (2015) standards before it is distributed to consumers.

The NW-D water purification plant uses flocculation-coagulation, sedimentation, sand filtration, activated carbon treatment and disinfection (chlorination) to treat source water (Figure 3.1). It was decided to add an activated carbon filtration step at the start of the treatment plant. This is to eliminate the bad taste during the dry periods when the dam levels are low and algae growth is high. Water Service Authority and Provider of NW-D area received a Blue Drop Score of 95.11% in 2009 by the Department of Water Affairs. It retained its Blue Drop Status for consecutive years. In 2014, this municipality was the only one that received the highest score (97%) in the North West Province (DWA, 2014). There are potential sources of hazards that could affect NW-D’s Local Municipality. This include, natural and anthropogenic activities from both point and non-point sources of pollutants in the catchment area (Heath et al., 2009). The main economic sectors in this region are mining, finance, business services, manufacturing, trade, construction, government services and agriculture (LGH, 2012).

3.1.2 Water production facility of Plant GH-T
Drinking water production facility GH-T is situated in Gauteng Province and supplies on average 3 200 million litres of water to more than 12 million people on a daily basis. It achieved Blue Drop Certification status with compliance of 95.48% to 97.22% from 2011 to 2014 (DWA, 2014). The facility receives its raw water from a dam through a canal and a gravity pipeline. The water goes through purification processes vital to guarantee that the water meets the standards set for potable water (Nel and Haarhof, 2011).
The GH-T water purification plant uses flocculation-coagulation, sedimentation, sand filtration, granular activated carbon treatment, UV irradiation and disinfection (chlorination) to treat source water (Figure 3.2). A multi-barrier approach is used to achieve the water quality target in accordance with SANS 241 (2015). The UV plant was installed to ensure efficient removal of protozoan pathogens (WHO, 2004). Furthermore, chlorine and ammonia are added to form monochloramine, which protects the water against bacterial growth. Physical attributes and chemical composition of water are continually monitored so that corrective action can be taken to prevent the water quality from differing from the prescribed limits (Nel and Haarhof, 2011). Once the water is purified, it is then pumped to several reservoirs (55) located in the area of supply.

3.2 Sample collection
In each of the two systems (NW-D and GH-T), water samples were collected from three different sampling points (inflow- raw water, outflow- immediately after treatment and in the drinking water distribution - point of use). There were three different sampling sites in the distribution system. Figure 3.1 and 3.2 shows the various drinking water purification steps for each WPF. The experimental period was from 2016 to 2017. The water was sampled in 1 litre Schott bottles (sterile) and kept on ice in a cooler box. Water samples were analysed in the laboratory within six hours of collection. In both figures below, sampling sites are indicated with triangles (blue - raw water; red - water after treatment; yellow - distribution water) for different months. May to August was considered to be a cooler, dry season and September to March the rainy, warm season.

Table 3.1: Summary of the sampling period (2016-2017), sampling sites and sampling months for both WPFs.

<table>
<thead>
<tr>
<th>WPF (Year)</th>
<th>Sampling sites</th>
<th>Number of samples</th>
<th>Sampling Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>NW-D (2016)</td>
<td>RW</td>
<td>1</td>
<td>March, May and August</td>
</tr>
<tr>
<td></td>
<td>AT</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distribution</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>NW-D (2017)</td>
<td>RW</td>
<td>1</td>
<td>May and October</td>
</tr>
<tr>
<td></td>
<td>AT</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distribution</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>GH-T (2017)</td>
<td>RW</td>
<td>1</td>
<td>June and October</td>
</tr>
<tr>
<td></td>
<td>AT</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distribution</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

RW: Raw water; AT: After treatment.
Figure 3.1: Schematic representation of Plant NW-D.
Figure 3.2: Schematic representation of Plant GH-T.
3.3 Physico-chemical analysis
Water quality parameters (pH, temperature and total dissolved solids) were measured in situ using a multi-350 probe analyser (Merck, Germany). The HACH DR 2800 spectrophotometer (HACH, USA) was used to measure nitrate (method 8039), nitrite (method 8153), phosphates (method 8178), free chlorine (method 8021) and COD (method 8000). Measurements were recorded as milligrams per litre (mg/l). Turbidity was measured using HACH 21000P TurbidiMeter (HACH, USA). The beaker was cleaned with distilled water and also rinsed with the water sample before taking a new measurement. The probe was rinsed with distilled water before and after taking measurements.

3.4 Enumeration of bacteria

3.4.1 Heterotrophic plate count (HPC)
Heterotrophic plate count bacteria were enumerated by plating a 10-fold dilution series up to 10⁻⁵. Hundred (100) μL of each diluent was spread inoculated onto the surface of R2A agar plates (Becton, Dickinson and Company, France) and incubated for 5-7 days at room temperature. Colonies were counted, and the total number of HPC bacteria were expressed as CFU/ml (Venieri et al., 2010). Subsequently, the bacteria were differentiated into various morphologies (according to colour, shape, description etc.) (Figure 3.3) and purified by subculturing on R2A agar.

![Bacterial Colony Morphology](image)

**Figure 3.3:** Illustration of different colony morphology (Pelczar, 1957)
3.4.2 Indicator organisms (E.coli and total coliforms)

Hundred milliliter (100 ml) aliquots of water were filtered through 0.45 μm-pore size membrane filters (PALL Corporation, USA) using the membrane filtration technique. Individually filtered membranes were placed onto MLG (MERCK, Germany) agar and incubated at 35°C for 24 hours. The analyses were conducted in triplicate. Green colonies indicated faecal coliforms, or possibly E. coli and yellow colonies indicated total coliforms. These were counted and levels recorded, but were not used for further studies.

3.5 Gram staining

Single selected HPC colonies were streaked onto R2A agar and purified. Gram staining was performed to differentiate between Gram positive and negative organisms. The Gram staining procedure was done as described (Burke and Barnes, 1929; Coico, 2005). Only fresh cultures were Gram stained. The Gram reaction was recorded and later compared to the identification by 16S rRNA gene sequencing.

3.6 Production of haemolysin on blood agar (pathogenic screening)

Pure isolates were aseptically spot inoculated onto 5% sheep blood agar plates, as the first measure to establish potential pathogenic features of HPC bacteria. Blood agar plates were incubated for 24 hours at 37°C and haemolytic activities were classified as follows: β-haemolysin production was indicated by a colourless zone surrounding the bacterial colonies; the presence of a distinct greenish-darkening around the inoculums indicates α-haemolytic activity and γ-haemolysin left the agar under and around colonies unchanged (there was only bacterial growth), indicating that haemolysin was not produced (Atlas, 1997; Pavlov et al., 2004).

3.7 Extracellular enzyme production

Assessment of extracellular enzymes involved using a media that contain substrates specific for particular enzymes (Israil et al., 2011). HPC bacterial isolates that produced α- and β-haemolysin were selected to determine if they also produced the various extracellular enzymes. The bacterial production of the following enzymes was determined: DNase, protease, lipase, hyaluronidase, chondroitinase and lecithinase. Once again, fresh cultures were spot inoculated on the respective media.

3.7.1 DNase

DNase agar (Oxoid, UK) was prepared according to manufacturer’s instructions and supplemented with (0.01%) toluidine blue O (Sigma Aldrich, US) which acted as an indicator by binding to hydrolysed DNA (Sen et al., 2010). Plates were incubated for 48 hr at 37°C. After incubation, the petridishes were flooded with a 1 M HCl (Merck, Germany) solution. A positive
result was indicated by a clear zone or a pink halo around the bacterial growth, which indicated DNase activity (Pavlov et al., 2004).

3.7.2 Protease
A medium was prepared by first preparing 50 ml of 3% (w/v) skim milk (Oxoid, England) solution, 50 ml Brain Heart Infusion Broth (Merck, Germany) and 100 ml agar (Merck, Germany) (Pavlov et al., 2004). The ingredients were prepared and autoclaved separately after which they were mixed and dispensed (Saran et al., 2007). Isolates were spot inoculated onto the petridishes and incubated at 37°C for 48h. A positive result was seen as a clear zone around the colonies, which indicated proteolytic activity (Boominadhan et al., 2009).

3.7.3 Lipase
Tryptone soy agar (Merck, Germany) was prepared and supplemented with 1% Tween 80 (polyoxyethylene sorbitan monooleate) (Sigma Aldrich, Germany) and served as the substrate for lipase production (Kumar et al., 2012). A turbid halo around the bacterial colony (after 24 to 48 hours) indicated a positive result (Edberg et al., 1996; Pavlov et al., 2004).

3.7.4 Hyaluronidase
One gram (1 g) of Noble agar (Conda, Spain) was added to 100 ml of Brain Heart Infusion Broth (Merck, US). The medium was autoclaved and allowed to cool down (Edberg et al., 1996). An aqueous solution of 2 mg/ml hyaluronic acid (Merck, Germany) and 5% bovine albumin fraction V (final concentration) (Roche, Germany) were prepared. The solution was filtered with a 0.22-µm filter (Corning, USA) before the two substrates were mixed together and poured into petridishes (De Assis et al., 2003). Pure 24 h bacterial cultures were inoculated, and plates incubated at 37°C for 72 h (Edberg et al., 1996). A positive result was indicated by a turbid halo around the inoculum spot.

3.7.5 Chondroitinase
Brain Heart Infusion Broth (Merck, US) was prepared to which 1 g of Noble agar (Conda, Spain) was added for each 100 ml (Pavlov et al., 2004). This was supplemented 4 mg/ml chondroitin sulphate A from bovine trachea (Roth, Germany) and 5% bovine albumin fraction V (final concentration) (Hyclone labs, USA). Both of these solutions were poured through a 0.22 µm filter to sterilize (De Assis et al., 2003). After inoculation, incubation followed for 48 h at 37°C. A clear zone around the bacterial colonies was indicative of a positive result.
3.7.5 Lecithinase

Bacto McClung-Toabe agar (Merck, Germany) was prepared according to manufacturer’s instructions and sterilized. After cooling it was supplemented with 10 ml of a 50% egg yolk enrichment emulsion added to 90 ml of sterilized Bacto McClung-Toabe agar (Merck, Germany) to serve as the growth media in determining the production of lecithinase. Petri dishes were examined for evidence of egg yolk degradation after 72 h of incubation at 37°C (Edberg et al., 1996). A white precipitate around or beneath the inoculum spot on the egg yolk agar indicated the production of lecithinase (Jula et al., 2011).

3.7.6 Gelatinase

Gelatinase production was detected by inoculating isolates onto agar plates containing 40 g/L tryptone soy agar, (Merck, Germany) and 3% (w/v) gelatine powder (Merck, Germany). Isolates were spot inoculated onto the plates and incubated at 37°C for 24-hours. After incubation, petridishes were cooled and the appearance of a turbid halo or clear zone around colonies was considered to be a positive indication of gelatinase production (Vergis et al., 2003).

3.8 Antibiotic susceptibility profiles of HPC bacteria

Antibiotic susceptibility tests were performed on HPC isolates using the Kirby-Bauer disk diffusion method (Horn et al., 2016). These bacteria were inoculated in R2A broth for 6 days and spread onto Mueller Hinton agar (Merck, Germany). Antibiotic discs were placed on the spread plates. Twelve antibiotics were chosen for the study, because they are widely used in veterinary and human medicine (Gomba et al., 2016). Furthermore, recent studies in South Africa have report resistance to these antibiotics in source and drinking water (Mulamattathil et al., 2014; Horn et al., 2016). They included: Erythromycin (15 μg), Cephalothin (30 μg), Ciprofloxacin (5 μg), Chloramphenicol (30 μg), Trimethoprim (5 μg), Oxytetracycline (30 μg), Streptomycin (25 μg), Neomycin (30 μg), Kanamycin (30 μg), Ampicillin (10 μg), Penicillin G (10 μg) and Vancomycin (30μg). All antibiotics were obtained from Mast Diagnostics (UK). Isolates were incubated at 37°C for 24 hours. Inhibition zones were measured in millimetres (mm) and divided into three groups classifying the isolates as resistant (R), susceptible (S) or intermediate resistant (I). This was compared to the Performance Standards for Antimicrobial Disk Susceptibility Tests (NCCLS, 1999).

The data obtained was used to determine multiple antibiotic resistant (MAR) phenotypes, and indices of each sample area were determined. Only antibiotics that had more than 20% of isolates resistant to it was included in the most prevalent MAR phenotype. MAR indices were calculated using the following formula (Guan et al., 2002).
3.9 DNA extraction

DNA was isolated from the HPC isolates grown in R2A broth and incubated at room temperature for 6 days. The Chemagic DNA Bacteria Kit (PerkinElmer, Germany) was used for bacterial DNA extraction following the manufacturer’s protocol. After isolation, the NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, US) was used to determine DNA quality and quantity.

3.10 Agarose gel electrophoresis of DNA

Gel electrophoresis was used to determine the integrity of DNA from HPC bacteria. Two microliters (2 µl) of the sample DNA was mixed with 2 µl 6x Orange Loading Dye (Thermo Fischer Scientific, US) containing GelRed (10x, Biotium, US) and was loaded onto a 1.5% (w/v) agarose gel. A 1 kb molecular weight marker (Fermentas, US) was used to confirm the integrity of the individual bands. The electrophoresis conditions were set at 80 V for 45 minutes in an electrophoresis system (Bio-Rad, US). For the electrophoresis buffer 1 X TAE (20 mM Acetic acid, 100 mM EDTA, 40 mM Tris at pH 8.0) was used. Gels were viewed using a ChemiDoc MP (Bio-Rad, US) imager.

3.11 PCR amplification

The preparation for all PCR reactions was done in a laminar flow (Bioflow I) cabinet. An iCycler Thermocycler (Bio-Rad, US) was used for PCR amplification. All PCR reactions had a final volume of 25 µl, containing 2 X PCR Master Mix (0.4 mM dNTPs, 4 mM MgCl2 and 0.05 U/µl Taq DNA polymerase (Fermentas Life Science, US)), nuclease free water (Fermentas Life Sciences, US) and 1 µl of gDNA (20-80 ng/µl). For the 16S rRNA, *ermB*, *ermF*, *blaTEM*, *tetM*, *ampC* and *intI* 1 genes, 0.4 µM of specific primer sets were added to the reaction mixture. All primers were synthesised by Inqaba Biotech, South Africa and the sequences are presented in Table 3.1. Agarose electrophoresis (Section 3.9) was used to determine whether the PCRs were successful.

3.11.1 16S rRNA gene amplification

The 27F and 1492R primers were used to amplify the 1 465 bp 16S rRNA gene (Weisburg *et al.*, 1991). PCR conditions were as follows: initial denaturation at 95°C for 300 seconds, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 30
seconds and extension at 72°C for 60 seconds. The final extension step was at 72°C for 600 seconds.

3.11.2 *ermB* and *ermF*
For the amplification of the *ermB* and *ermF*, the PCR conditions used were as follows: denaturing at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 2 minutes. Each cycle was repeated 35 times. For the amplification of the *ermB* gene, the same conditions were used, with the exception of annealing temperature at 48°C for 1 minute.

3.11.3 *blaTEM*
The *blaTEM* gene was amplified under the following PCR conditions: initial denaturing step was set at 95°C for 5 minutes. The 35-cycle step consisted of denaturation step at 94°C for 60 seconds, annealing at 63°C for 30 seconds and extension at 72°C for 60 seconds. The reaction was finished by an extension step of 72°C for 10 minutes.

3.11.4 *tetM*
For the amplification of *tetM* gene, the reactions underwent 35 cycles which began with denaturing at 95°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 30 seconds. The reaction was completed by an additional extension at 72°C for 5 minutes.

3.11.5 *ampC* and *intI*
The successful amplification of *ampC* and *intI* has previously been described by Coertze and Bezuidenhout (2018). PCR conditions were as follows: initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes. For the *ampC* gene the initial denaturation step was 10 minutes, the annealing temperature 49°C and the final extension time 7 minutes.

3.12 Environmental DNA (eDNA) extraction
In order to extract eDNA, a large quantity of water (specifically treated- and drinking water) was filtered to increase the concentration of eDNA for extraction. The methodology used by Ma et al. (2017) in order to achieve such high concentrations were followed and adapted accordingly. Therefore, adaptations and modifications were made to a LifeStraw filter (Vestergaard Frandsen, Switzerland) so that it can be screwed onto a tap. The hollow fibre filter has an optimum filter capacity of 1000 L. Approximately a 1000 L were filtered through these filters. After filtration, the hollow fibre filter was cut into a Schott bottle with 300 ml of autoclaved distilled water. This was then sonicated for 10 min to release the bacteria and
eDNA. The sonicated water was then filtered onto a 0.45 µm, 47 mm grid PALL Corporation sterilised filter membrane [(PALL Life Sciences, Mexico) (CAT No: GN-6 Metricel Membrane 66191)].

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

3.13 Analysis of antimicrobial compounds/pharmaceuticals
The list of analytes that were tested for included; ampicillin, chloramphenicol, cephalothin, ciprofloxacin, kanamycin, benzylpenicillin (Penicillin G), erythromycin, neomycin, oxytetracycline, streptomycin, trimethoprim, colistin, penicillin, vancomycin and sulfamethoxazole.

Quantification of the selected antibiotics, antiseptics and other antimicrobials were carried out for samples collected following the analytical methodology of an analytical facility at the North-West University. Glass containers (1 L) were used for sampling. Contaminants from previously used glassware were removed using the procedure following the USEPA, 2007 method. Pharmaceuticals were extracted from the water using the methods of Ferrer et al. (2010). This was done by spiking the water and further using a SPE-DEX system (Horizon Technology, Salem, NH, USA) and 47 mm Oasis HLB disks (Horizon Technology). The pharmaceuticals were eluted and the eluent concentrated to near dryness using nitrogen gas and heat (Dry-Vap system, Horizon Technology, Salem, New Hampshire). Eluents filtered through 0.22 µm PTFE filters were subjected to UHLC-QTOF for analysis.
<table>
<thead>
<tr>
<th>primer</th>
<th>Sequence (5'-3')</th>
<th>Amplicon size (bp)</th>
<th>Annealing Temp (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rDNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27F</td>
<td>5'-AGA GTT TGA TCM TGG CTC AG- 3'</td>
<td>1465</td>
<td>54</td>
<td>Jiang et al., 2006</td>
</tr>
<tr>
<td>1492R</td>
<td>5'-GG TTA CCT TGT TAC GAC TT- 3'</td>
<td>638</td>
<td>48</td>
<td>Tran et al., 2013</td>
</tr>
<tr>
<td>ermB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ermB-F</td>
<td>5' -GAA AAG GTA CTC AAC CAA ATA- 3'</td>
<td>466</td>
<td>50</td>
<td>Chung et al., 1999</td>
</tr>
<tr>
<td>ermB-R</td>
<td>5' -AGT AAC GGT ACT TAA ATT GTT TAC- 3'</td>
<td>466</td>
<td>50</td>
<td>Chung et al., 1999</td>
</tr>
<tr>
<td>ermF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ermF1</td>
<td>5' -GAA AAG GTA CTC AAC CAA ATA- 3'</td>
<td>1150</td>
<td>63</td>
<td>Costa et al., 2007</td>
</tr>
<tr>
<td>ermF2</td>
<td>5' -AGT AAC GGT ACT TAA ATT GTT TAC- 3'</td>
<td>473</td>
<td>60</td>
<td>Labbate et al., 2008</td>
</tr>
<tr>
<td>tetM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blaTEM</td>
<td>5' -ATT CTT GAA GAC GAA AGG GC- 3'</td>
<td>550</td>
<td>49</td>
<td>Schwartz et al., 2003</td>
</tr>
<tr>
<td>TEM-F</td>
<td>5' -ACG CTC AGT GGA ACG AAA AC- 3'</td>
<td>550</td>
<td>49</td>
<td>Schwartz et al., 2003</td>
</tr>
<tr>
<td>TEM-R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ampC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ampC-F</td>
<td>5' -TTC TAT CAA MAC TGG CAR CC- 3'</td>
<td>473</td>
<td>60</td>
<td>Labbate et al., 2008</td>
</tr>
<tr>
<td>ampC-R</td>
<td>5' -CCY TTT TAT GTA CCC AYG A- 3'</td>
<td>473</td>
<td>60</td>
<td>Labbate et al., 2008</td>
</tr>
<tr>
<td>int1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HS463A</td>
<td>5' -CTG GAT TTC GAT CAC GGC ACG- 3'</td>
<td>473</td>
<td>60</td>
<td>Labbate et al., 2008</td>
</tr>
<tr>
<td>HS464</td>
<td>5' -ACA TGC GTG TAA ATC ATC GTC G- 3'</td>
<td>473</td>
<td>60</td>
<td>Labbate et al., 2008</td>
</tr>
</tbody>
</table>
3.14 Sequencing of the 16S rRNA genes and identification of the isolates

A silica resin procedure, described by Li et al. (2010), was used to purify all PCR products that were successfully amplified. This was done to remove all residual primer dimers. Briefly, 150 µl of 6 M NaI was added to the 25 µl PCR product and was mixed well (by inverting the tube) and 10 µl silica matrix (0.2 g Silicon dioxide (Sigma-Aldrich; US) in 2 ml Milli-Q® water) was added and incubated at room temperature (28°C) for 5 min. The mixture was then centrifuged for 10 seconds at 13 400 rpm (maximum speed) and the supernatant was removed by pipetting. The pellet was then washed with 500 µl Washing Buffer (50 mM NaCl; 10 mM Tris pH 7.5, 2.5 mM EDTA, 50% v/v Ethanol and Milli-Q® water for a final volume of 50 ml) followed by centrifugation at maximum speed for 10 seconds.

The wash steps were repeated, centrifuged again for 1 min, after which all traces of liquid were removed by pipetting. The pellet was then left open to air dry for 5 min. The pellet was resuspended in 20 µl Milli-Q® water and centrifuged for 2 min at 13 400 rpm. The DNA eluate was then transferred to a new sterile PCR tube. DNA quantity and quality was determined by a NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, US).

A Cycle Sequencing BigDye Terminator v3.1 Kit (Applied Biosystems, UK) was used to perform the sequencing PCR. The reaction mix consisted of: 4 µl Ready Reaction mix (0.25x), 2 µl BigDye Sequencing Buffer (5x), 3.2 pmol 27F primer (Inqaba Biotech, SA), 1 µl Template DNA (5-20 ng) and 9.8 µl of water (Fermentas Life Sciences, US) up to a final volume of 20 µl. Thermal cycling conditions were set at 96°C for 60 seconds initial denaturation that was followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 240 seconds. A Zymo Research DNA-Sequencing Clean-up kit (Zymo Research, US), was used to purify the sequencing PCR products according to the manufacturer's protocol.

Amplicons were sequenced in-house, using an ABI 3130 Genetic Analyser (Applied Biosystems, UK). Geospiza Finch TV (version 1.4) software was used to view chromatograms and BLAST (Basic Local Alignment Search Tool) searches were used to determine the identity of the amplified sequences (http://www.ncbi.nlm.nih.gov/BLAST).

3.15 Statistics applied in this study

The statistical analysis of all physico-chemical and microbiological parameters such as averages and standard deviation were conducted in Statistica version 10 (StatSoft, US) and Microsoft Office Excel 2013. Canonical ordination redundancy analyses (RDA) and principal component analysis (PCA) was also used to determine the correlation between environmental (temperature, pH, TDS, COD, nitrate, nitrite, turbidity and phosphate) and microbiological
variables, as well as sampling sites. Canoco software version 4.5 (developed by Ter Braak, 1990) was used to perform the multivariate analysis, and the results were visualised by mean triplots. The smaller the angle is between the factors, the greater the correlation between these factors are. MEGA 7 software programme was used for phylogenetic analyses (Tamura et al., 2013). A phylogenetic tree (neighbour-joining) was constructed, with a bootstrap of 1000 replicates. The Jukes-Cantor model was used to calculate evolutionary distance.
Chapter 4
Results

This chapter includes the results from the 2016 and 2017 sampling periods. Physico-chemical and microbiological results of the three sites (inflow, outflow, and distribution) from 2 water production facilities (NW-D and GH-T) sampled are given in Section 4.1 and 4.2. The values are represented in averages and standard deviations. Furthermore, these measured values are compared to the South African National Standards (SANS 241:2015) for drinking water. In Section 4.3, microbiological and physico-chemical results are compared using PCA and RDA biplots. Section 4.4, contains two parts (haemolysin and extracellular enzymes) for confirmation of potential pathogenic isolates. Section 4.5, contains three parts (results for antibiotic profiles of HPC isolates and the levels of pharmaceuticals, MAR phenotypes and the presence of ARGs), followed by the identification and confirmation of the isolates (Section 4.6). These results were then used to construct a phylogenetic tree (Section 4.7) to observe a possible close association between identified HPC bacteria species in the present study and those obtained from the NCBI database.

4.1 Water samples collected in 2016 and 2017

This section includes the physico-chemical and microbiological quality of two water production facilities sampled in 2016 and 2017.

4.1.1 Physico-chemical analysis

Table 4.1 and 4.2, briefly summarises the following physico-chemical parameters of plant NW-D and GH-T measured at 3 sampling sites: turbidity; pH; temperature; total dissolved solids (TDS); phosphorus; nitrite; free chlorine; chemical oxygen demand (COD); and nitrate. These parameters were compared to the South African National Standards (SANS 241:2015) for drinking water.

The values recorded for free chlorine levels in all of the sampling sites comply with the SANS 241 (2015) standard which is ≤5 mg/l (Table 4.2). The levels were very low during May (0.07 mg/l) and October 2017 for site NW-D (0.05 mg/l). Similar results were recorded during June at site GH-T (0.08 mg/l) for the same year (2017). These low levels may cause regrowth of microorganisms in the distribution system. GH-T had the highest levels of free chlorine (1.66 mg/l) during November for 2017 sampling period. Such high chlorine levels may have aesthetic effects.
TDS values obtained complied with the SANS 241 (2015) standard of drinking water of ≤1200 ppm. Values ranged from 425 ppm (site NW-D) in 2017 to 552 ppm (site NW-D) in 2016. There was a decline in the TDS levels after the water had undergone treatment at site NW-D during May 2017 (515 mg/l) and November 2017 (538 mg/l) for site GH-T (Table 4.1).

The pH levels measured at all sampling points during 2016 and 2017 complied with the SANS 241 (2015) standard of ≥5 to ≤9.7 pH units for drinking water (Table 4.1). It therefore, does not pose any health threats. A slight decrease in the pH levels from July to October 2017 was observed in the raw and drinking water. NW-D had the highest average pH of 8.86 (raw water), and GH-T had the lowest pH with a value of 7.24 (drinking water).

The nitrate levels in the raw water ranged from 0 to 6.09 mg/l. Values were low during March 2016 and May 2017 (Table 4.2). GH-T had the highest level of nitrate in the raw water (6.09 mg/l) and drinking water (5.03 mg/l) during November 2017. The nitrate levels were reduced after treatment but was still elevated during this period.

During August 2016 and October 2017, NW-D also had high nitrate levels for raw water (2.10 mg/l) for both months. It was observed that the water after it was treated, and in distribution, had higher levels of nitrates than in the raw water (Table 4.2). This indicated that nitrates were not removed during the water treatment process. No nitrate was detected in the raw water of NW-D during March 2016, but was detected in drinking and after treatment water. The nitrate levels of all sampling sites complied with the SANS 241 (2015) standard of ≤11 mg/l for drinking water, but the elevated levels of GH-T during 2017 was noted.

Nitrite levels, however, were alarmingly high in drinking water (Table 4.2) for NW-D during March (1.70 mg/l), May (1.53 mg/l) and August (2.75 mg/l) of 2016. This is a concern as high levels may influence the quality of the drinking water. However, levels recorded for May and October 2017 for the same sampling sites complied with the standard (SANS 241:2015). The average nitrite levels of site GH-T were within the SANS 241(2015) for drinking water (≤0.9) sampled in 2017, except for one side during June in the drinking water (1.00 mg/l). The lowest levels were recorded during November 2017 at site GH-T (0.03 mg/l).

There is no specified value for COD concerning drinking water in the SANS 241 (2015). Values were higher in May compared to August 2016. NW-D had the highest COD levels in water after treatment (7 mg/l) and drinking water (6 mg/l) (Table 4.2). For GH-T, COD levels from June and November 2017 decrease from raw to drinking water (4.24 to 1.45 mg/l).
SANS 241 (2015) does not specify a standard value for phosphates in drinking water. NW-D had the highest phosphate level in raw water during October 2017 (5.04 mg/l) and drinking water (4.95 mg/l) (Table 4.2). Phosphate levels were higher in water after treatment for all production facilities (March to May 2016), including 2017 for May to November. These chemical results demonstrate that nutrients (PO₄; NO₃; COD) enter the source and are present in the distribution system to sustain bacterial regrowth/growth and would allow for the formation of biofilms.

The temperature of water measured at all respective sites ranged from 11.9 to 23.6°C for 2016 (Table 4.1). These temperature ranges reflected the seasonal variation of the sampling periods. NW-D had the lowest overall average water temperature (11.9°C). All the NW-D and GH-T samples taken during the warm season had high temperature levels. However, the highest temperatures were observed at NW-D (23.6°C) for 2016. Higher temperatures may promote the growth of HPC bacteria. There is no SANS (2015) standard for temperature.

Turbidity levels were high in the raw water of plant NW-D (3.87 and 3.65 NTU respectively) for March and May 2016, and for GH-T (3.33 NTU) during June 2017 (Table 4.1). NW-D had the highest turbidity level in water after treatment during May (2.24 NTU) and August 2016 (2.11 NTU). These high levels do not comply with the SANS 241 (2015) standard of ≤1 for drinking water. However, the levels of turbidity in May and October 2017 was lower (0.42 to 0.60 NTU) in water after treatment. The levels of turbidity for GH-T for all the sampling periods were always within the SANS 241 (2015) values for drinking water.
Table 4.1: Physical parameters results from 2016 and 2017 for Plant NW-D & GH-T.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Turbidity (NTU)</th>
<th>pH at 25°C (pH units)</th>
<th>Temperature (°C)</th>
<th>TDS (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤1</td>
<td>≥5 to ≤9.7</td>
<td></td>
<td>≤1200</td>
</tr>
<tr>
<td>SANS 241: 2015</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NW-D (March 2016)</td>
<td>AV 3.87, 1.63, 0.52</td>
<td>R 7.90, AT 7.40, D 7.62</td>
<td>R 22.5, AT 23.5, D 23.6</td>
<td>R 495, AT 499, D 494</td>
</tr>
<tr>
<td></td>
<td>SD 0, 0.04, 0.01</td>
<td>0.07, 0.14, 0.38</td>
<td>0.07, 0.71, 1.31</td>
<td>4.24, 4.95, 3.61</td>
</tr>
<tr>
<td>Plant NW-D (May 2016)</td>
<td>AV 3.65, 2.24, 0.47</td>
<td>R 8.86, AT 8.24, D 7.24</td>
<td>R 17.5, AT 19.0, D 19.2</td>
<td>R 518, AT 533, D 515</td>
</tr>
<tr>
<td></td>
<td>SD 0.06, 0.01, 0.03</td>
<td>0.03, 0.04, 0.02</td>
<td>0.14, 0.14, 1.20</td>
<td>4.24, 6.36, 11.0</td>
</tr>
<tr>
<td>NW-D (August 2016)</td>
<td>AV 2.39, 2.11, 0.44</td>
<td>R 8.22, AT 8.52, D 8.70</td>
<td>R 11.9, AT 12.6, D 14.2</td>
<td>R 543, AT 552, D 542</td>
</tr>
<tr>
<td></td>
<td>SD 0.08, 0.02, 0.15</td>
<td>0.28, 0.11, 0.31</td>
<td>0.06, 0.12, 1.11</td>
<td>0.58, 1.00, 3.04</td>
</tr>
<tr>
<td>NW-D (May 2017)</td>
<td>AV 2.33, 0.42, 0.23</td>
<td>R 8.46, AT 8.12, D 8.01</td>
<td>R 16.5, AT 15.5, D 18.6</td>
<td>R 520, AT 515, D 514</td>
</tr>
<tr>
<td></td>
<td>SD 0.69, 0.03, 0.08</td>
<td>0.02, 0.02, 0.04</td>
<td>0.60, 0.40, 1.17</td>
<td>0, 0.02, 0.04</td>
</tr>
<tr>
<td>NW-D (October 2017)</td>
<td>AV 0.67, 0.60, 1.47</td>
<td>R 8.63, AT 8.30, D 8.36</td>
<td>R 18.6, AT 17.4, D 16.7</td>
<td>R 436, AT 444, D 425</td>
</tr>
<tr>
<td></td>
<td>SD 0.03, 0.03, 0.24</td>
<td>0.28, 0.02, 0.05</td>
<td>0.15, N/A, 5.52</td>
<td>1.53, 0.58, 14.18</td>
</tr>
<tr>
<td>GH-T (June 2017)</td>
<td>AV 3.33, 0.67, 0.63</td>
<td>R 8.46, AT 8.19, D 8.02</td>
<td>R 17.5, AT 20.5, D 19.9</td>
<td>R 492, AT 489, D 491</td>
</tr>
<tr>
<td></td>
<td>SD 0.03, 0.02, 0.02</td>
<td>0, 0.10, 0.65</td>
<td>0.12, SD 0.03</td>
<td>1.53, 1.73, 1.67</td>
</tr>
<tr>
<td>GH-T (November 2017)</td>
<td>AV 9.99, 0.25, 0.67</td>
<td>R 8.16, AT 8.14, D 8.08</td>
<td>R 20.3, AT 21.2, D 21.0</td>
<td>R 551, AT 538, D 530</td>
</tr>
<tr>
<td></td>
<td>SD 0, 0.04, 0.29</td>
<td>0.08, 0.02, 0.03</td>
<td>0.20, 0.20, 0.85</td>
<td>1.53, 0.58, 6.11</td>
</tr>
</tbody>
</table>

R: Raw water; AT: After treatment; D: Distribution; AV: Average; SD: Standard Deviation; N/A: Not applicable.
Table 4.2: Chemical parameters results from 2016 and 2017 for Plant NW-D & GH-T.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Phosphate (mg/l)</th>
<th>Nitrite (mg/l)</th>
<th>Free chlorine (mg/l)</th>
<th>Chemical Oxygen Demand (mg/l)</th>
<th>Nitrate (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SANS 241: 2015</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NW-D (March 2016)</td>
<td>R</td>
<td>AT</td>
<td>D</td>
<td>R</td>
<td>AT</td>
</tr>
<tr>
<td>AV</td>
<td>0.68</td>
<td>0.70</td>
<td>1.23</td>
<td>1.51</td>
<td>1.00</td>
</tr>
<tr>
<td>SD</td>
<td>0.42</td>
<td>0.07</td>
<td>N/A</td>
<td>4.95</td>
<td>2.42</td>
</tr>
<tr>
<td>NW-D (May 2016)</td>
<td>AV</td>
<td>1.11</td>
<td>1.40</td>
<td>1.8</td>
<td>1.26</td>
</tr>
<tr>
<td>SD</td>
<td>N/A</td>
<td>0.42</td>
<td>0.39</td>
<td>0.14</td>
<td>0.07</td>
</tr>
<tr>
<td>NW-D (August 2016)</td>
<td>AV</td>
<td>1.01</td>
<td>0.02</td>
<td>1.80</td>
<td>1.00</td>
</tr>
<tr>
<td>SD</td>
<td>0.07</td>
<td>0.03</td>
<td>0.54</td>
<td>0.71</td>
<td>0.01</td>
</tr>
<tr>
<td>NW-D (May 2017)</td>
<td>AV</td>
<td>0.38</td>
<td>0.71</td>
<td>1.10</td>
<td>1.72</td>
</tr>
<tr>
<td>SD</td>
<td>0.06</td>
<td>0.54</td>
<td>0.71</td>
<td>0.58</td>
<td>0.56</td>
</tr>
<tr>
<td>NW-D (October 2017)</td>
<td>AV</td>
<td>5.04</td>
<td>4.95</td>
<td>2.33</td>
<td>0.03</td>
</tr>
<tr>
<td>SD</td>
<td>0.41</td>
<td>0.18</td>
<td>0.80</td>
<td>0.05</td>
<td>N/A</td>
</tr>
<tr>
<td>GH-T (June 2017)</td>
<td>AV</td>
<td>0.38</td>
<td>0.71</td>
<td>1.01</td>
<td>1.73</td>
</tr>
<tr>
<td>SD</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>GH-T (November 2017)</td>
<td>AV</td>
<td>2.00</td>
<td>1.50</td>
<td>1.30</td>
<td>0.17</td>
</tr>
<tr>
<td>SD</td>
<td>0.12</td>
<td>0.04</td>
<td>0.05</td>
<td>0.02</td>
<td>0.02</td>
</tr>
</tbody>
</table>

R: Raw water; AT: After treatment; D: Distribution; AV: Average; SD: Standard Deviation; N/A: Not applicable. T
4.2 Microbiological analysis

HPC results obtained in 2016 during March to November are listed in Table 4.3. In 2017, total and faecal coliforms were added to the microbiological parameters (Table 4.4). Total coliforms and faecal coliforms were only used for primary screening of water quality. They were added as a confirmation step, because they are normally found in the raw water but not in drinking water. This trend is confirmed by the results (Table 4.4), although a decreased levels of total coliforms was measured in treated water (in acceptable limits).

4.2.1 Microbiological results for 2016

HPC bacteria were detected in all the sites that were studied (Table 4.3). It is evident from the table that levels of detection varied between the different sites. The amount of HPC bacteria detected in the source water ranged from 1 370 to 5 900 CFU/ml for site NW-D. HPC bacteria levels from water after treatment and drinking water ranged from 70.3 to 740 CFU/ml, where the lowest levels were detected during May. Overall, all the values recorded complied with the SANS 241 (2015) for drinking water of ≤1 000 CFU/ml. This indicates an effective removal of HPC bacteria from drinking water.

Table 4.3: Results of heterotrophic plate counts of water before treatment, after treatment and in the distribution for 2016 during March, May and August.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>HPC bacteria (CFU/ml)</th>
<th>R</th>
<th>AT</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SANS 241: 2015</td>
<td>≤1000 CFU/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant NW-D (March 2016)</td>
<td>AV 5 900</td>
<td>280</td>
<td>463</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD 122.0</td>
<td>68.9</td>
<td>4.40</td>
<td></td>
</tr>
<tr>
<td>Plant NW-D (May 2016)</td>
<td>AV 1 370</td>
<td>160</td>
<td>70.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD 36.0</td>
<td>20.0</td>
<td>47.2</td>
<td></td>
</tr>
<tr>
<td>Plant NW-D (August 2016)</td>
<td>AV 2 360</td>
<td>740</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD 24.0</td>
<td>13.1</td>
<td>13.6</td>
<td></td>
</tr>
</tbody>
</table>

R: Raw water; AT: After treatment; D: Distribution; AV: Average; SD: Standard Deviation.
4.2.2 Microbiological results for 2017

In Table 4.4, total coliform levels were very high in the raw water of plant NW-D during May (398 CFU/100ml) and October (684 CFU/100ml). Total coliform levels were lower after treatment and at the distribution site during May and October. This is an indication that the treatment process removed total coliforms from the raw water. No total coliforms were detected in water after treatment and in the drinking water of GH-T, and also in NW-D during May. Except, for low levels that were detected in NW-D for the October sampling period. These results indicate that all the measured levels of total coliforms measured were within the SANS 241 (2015) standard of drinking water.

Faecal coliform levels (Table 4.4) were very high in the raw water during May and October (241 to 417 CFU/100 ml) for NW-D, except for the GH-T levels, which were low during June and November (12 to 10 CFU/ 100 ml). No faecal coliforms were detected in the drinking water of all production facility systems and thus complied with the SANS 241 (2015) standard of 0 CFU/100 ml. This indicated that the treatment process effectively removed all the faecal coliforms from the raw water.

The HPC bacteria levels measured at all sampling points complied with the SANS 241 (2015) standard of ≤1 000 CFU/ml for drinking water (Table 4.4). HPC bacteria levels were relatively high in the raw water, especially plant NW-D during October 2017 (296 CFU/ml). The levels decreased in the water after treatment in all the sampling sites, except for GH-T during November.
Table 4.4: Microbiological results for each sampling point 2017

<table>
<thead>
<tr>
<th>SANS 241: 2015</th>
<th>Total coliforms (CFU/100ml)</th>
<th>Faecal coliforms (CFU/100ml)</th>
<th>HPC bacteria (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤10 CFU/100ml</td>
<td>0 CFU/100ml</td>
<td>≤1 000 CFU/ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>R</th>
<th>AT</th>
<th>D</th>
<th>R</th>
<th>AT</th>
<th>D</th>
<th>R</th>
<th>AT</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant NW-D</td>
<td>AV</td>
<td>398</td>
<td>0</td>
<td>0</td>
<td>241</td>
<td>0</td>
<td>0</td>
<td>567</td>
<td>230</td>
</tr>
<tr>
<td>(May 2017)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>78.4</td>
<td>N/A</td>
<td>N/A</td>
<td>24.6</td>
<td>N/A</td>
<td>N/A</td>
<td>10.1</td>
<td>17.3</td>
</tr>
<tr>
<td>Plant NW-D</td>
<td>AV</td>
<td>684</td>
<td>4.00</td>
<td>3.00</td>
<td>417</td>
<td>0</td>
<td>0</td>
<td>296</td>
<td>106</td>
</tr>
<tr>
<td>(October 2017)</td>
<td>SD</td>
<td>54.4</td>
<td>1.53</td>
<td>2.59</td>
<td>14.8</td>
<td>N/A</td>
<td>N/A</td>
<td>38.9</td>
<td>93.2</td>
</tr>
<tr>
<td>Plant GH-T</td>
<td>AV</td>
<td>35.3</td>
<td>0</td>
<td>0</td>
<td>12.0</td>
<td>0</td>
<td>0</td>
<td>167</td>
<td>120</td>
</tr>
<tr>
<td>(June 2017)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>18.2</td>
<td>N/A</td>
<td>N/A</td>
<td>7.39</td>
<td>N/A</td>
<td>N/A</td>
<td>44.5</td>
<td>10</td>
</tr>
<tr>
<td>Plant GH-T</td>
<td>AV</td>
<td>15.0</td>
<td>0</td>
<td>0</td>
<td>10.0</td>
<td>0</td>
<td>0</td>
<td>110</td>
<td>300</td>
</tr>
<tr>
<td>(November 2017)</td>
<td>SD</td>
<td>3.53</td>
<td>N/A</td>
<td>N/A</td>
<td>2.12</td>
<td>N/A</td>
<td>N/A</td>
<td>7.07</td>
<td>28.3</td>
</tr>
</tbody>
</table>

R: Raw water; AT: After treatment; D: Distribution; AV: Average; SD: Standard, Deviation; N/A: Not applicable.

4.3 Relationship between physico-chemical and enumerated HPC bacteria

In Figure 4.1, a principal component analysis (PCA) was used to indicate possible correlations between the physico-chemical parameters and the HPC bacteria (CFU/ml) in drinking water, for 2016 sampling period. This analysis procedure was selected since only one set of microbiological data was available and RDA could not be used.

A RDA correlation biplot was used to determine the correlation between the physico-chemical and microbiological variables (HPC, total coliforms and faecal coliform) during 2017 sampling run, for water production facilities. The RDA results (Figure 4.2 and 4.3) are presented in two segments, the one being for drinking water and the other for raw water. The plots span over a duration of two years, 2016 to 2017, and were constructed by using the averages of the physico-chemical and microbiological parameters of each water production facility for each month.
4.3.1 PCA of 2016 for Plant NW-D

In Figure 4.1, the relationship between the measurements was demonstrated. A weak positive correlation can be observed between the HPC bacteria and two physico-chemical parameters (temperature and turbidity). These parameters had a great effect on the levels of HPC bacteria. When comparing the levels of HPC bacteria to COD, PO₄, NO₂, TDS and pH, a negative correlation is noticed. This analysis demonstrates that some of the physico-chemical parameters had impacts on the bacterial levels observed in the water production facilities.
4.3.2 RDA of 2017 Plant for NW-D and GH-T

The RDA plots, shown in Figure 4.2 and 4.3, illustrates the correlation between the physico-chemical parameters (pH; Temperature; Total dissolved solids; Chemical oxygen demand; Free chlorine; nitrites; nitrates and phosphates) and the microbiological indicators (HPC bacteria) and total coliform species levels, for 2016-2017 sampling period.

![Figure 4.2: RDA biplot illustrating the correlation between the physico-chemical parameters (pH, temp: Temperature; TDS: total dissolved solids; COD: chemical oxygen demand; Free Cl: free chlorine; NO₂: nitrites; NO₃: nitrates and PO₄: phosphates) and the microbiological indicators (HPC: heterotrophic plate count bacteria) and total coliforms species levels, during the following sampling period (NWD-N1: May; N2: October; GHT- G1: June and G2: October) of 2017. The red arrows represent the physico-chemical parameters, whereas the blue arrows represent the microbiological parameters.](image)

In Figure 4.2, a strong positive correlation can be observed among the HPC bacteria levels and the free chlorine and nitrate. The GH-T after treatment and distribution for 2017 (November; G2AT_17 and G2D_17) were the greatest impacted by these two parameters. Temperature and TDS showed a positive association and impacted treated drinking water in the NW-D during the first 2017 sampling. Total coliform levels associated with pH, COD, phosphates and turbidity. These parameters also impacted the drinking water of the NW-D during the second 2017 sampling period (Figure 4.2).
Figure 4.3: Redundancy analysis (RDA) biplot illustrating the correlation between the physico-chemical parameters (pH, temp: Temperature; TDS: total dissolved solids; COD: chemical oxygen demand; Free Cl: free chlorine; NO₂: nitrite; NO₃: nitrate and PO₄: phosphates) and the microbiological indicators (HPC: heterotrophic plate count bacteria, total coliforms and faecal coliforms species levels, during the following sampling period seasons (NWD-N1: May; N2: October; GHT- G1: June and G2: October) of 2016 and 2017 for drinking water in 2 water production facilities surface. The red arrows represent the physico-chemical parameters, whereas the blue arrows indicate the microbiological parameters.

In the raw water, the following trends were observed (Figure 4.3). The HPC bacteria levels correlated strongly with nitrite, and temperature. The raw water of plant NW-D collected in May 2016 was strongly impacted by these parameters. On the other hand, turbidity, TDS and nitrates correlated. Raw water for the GHT plant (both sampling) was impacted by these parameters. The raw water sampled during the second and third sampling sessions were also impacted by the mentioned parameters. Phosphates, faecal and total coliforms correlated and these impacted the raw water of second sampling of 2017.
4.4 Potential pathogenic testing results
Haemolysin results obtained for 2016 and 2017 sampling period are listed in Table 4.5. Bacteria that produced haemolysin were regarded as virulent. Purified HPC bacterial isolates were grown on blood agar plates to determine whether they are haemolytic.

4.4.1 Haemolysin results for 2016 and 2017
Thirty seven pure colonies from raw water sampling sites were tested for the production of haemolysin from both water production facilities (Table 4.5). Sixteen of the HPC isolates displayed α- or β-haemolysis. The number of isolates that tested positive for α- or β-haemolysis ranged from 5 to 6.

Fifty two pure colonies from drinking water (after treatment and distribution) were tested for the production of haemolysin from plant NW-D (2016 and 2017). Thirty of these HPC isolates displayed α- or β-haemolysis. The numbers ranged from 2 to 15. Haemolysin was mostly produced by isolates from drinking water, as compared to source and after treatment water. Among the isolates from Plant GH-T, 50% produced haemolysin in drinking water (Table 4.6).

Table 4.5: Results obtained for haemolysin production for each individual site from raw water in plant NW-D & GH-T (2016 to 2017)

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of isolates streaked</th>
<th>Isolates indicating α- or β-hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>NW-D 2016</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>NW-D 2017</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>GH-T 2017</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>16</td>
</tr>
</tbody>
</table>

R: Raw water; AT: After treatment; D: Distribution; AV: Average; SD: Standard Deviation
Table 4.6: Results obtained for haemolysin production for each individual site from after treatment and drinking water in plant NW-D & GH-T (2016 to 2017).

<table>
<thead>
<tr>
<th>Year</th>
<th>Sampling site</th>
<th>Number of isolates streaked</th>
<th>Isolates indicating α- or β-hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>NW-D</td>
<td>AT</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>2016</td>
<td>D</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>31</td>
<td>12</td>
</tr>
<tr>
<td>NW-D</td>
<td>AT</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>2017</td>
<td>D</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>GH-T</td>
<td>AT</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2017</td>
<td>D</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>16</td>
<td>8</td>
</tr>
</tbody>
</table>

AT: After treatment; D: Distribution

4.4.2 Extracellular enzyme production
The ability of the 54 haemolytic HPC isolates (Table 4.5 and Table 4.6) to produce 7 different extracellular enzymes was tested. These enzymes included DNase, lipase, proteinase, hyaluronidase, chondroitinase, gelatinase and lecithinase (methods are described in Section 3.6). The results are depicted in Figures 4.4, 4.5 and 4.6.

Figure 4.4: Total percentages of isolates testing positive for each enzyme from NW-D for 2016.
a) Plant NW-D for 2016
The percentages of HPC isolates that produced several enzymes varied (Figures 4.4, 4.5, 4.6). When considering Figure 4.4 (NW-D 2016), it is evident that DNase, lipase, proteinase, hyaluronidase and gelatinase were produced by between 20% and 40% of isolates from raw water. On the other hand, DNase, proteinase, gelatinase and lecinthinase was produced by between 10% and 89% of isolates from drinking water. Lipase and hyaluronidase were also produced by some of the treated water samples. None of the isolates produced chondrontinase.

Figure 4.5: Total percentages of enzymes produced by haemolytic HPC isolates from NW-D for 2017.

b) Plant NW-D for 2017
The overall percentages of HPC isolates that produced the different enzymes varied. Of the 18 haemolytic positive isolates in drinking and after treatment water, chondroitinase was produced by 84% of the isolates; proteinase by 74%, DNase by 59%, hyaluronidase and gelatinase by 50%, lipase by 42% and lecinthinase by 25%. All isolates in after treatment water produced hyaluronidase (100%), while gelatinase was produced by all isolates from the drinking water (100%). There were only two enzymes (DNase and proteinase) produced by isolates from raw water. Isolates from after treatment water produced 6 different enzymes. Figure 4.5, shows that isolates from drinking and after treatment water produced more enzymes as compared to raw water.
c) Plant GH-T for 2017

From plant GH-T (Table 4.6), of the 8 haemolytic positive isolates from drinking and water after treatment, chondroitinase was produced by 84%, DNase and proteinase by 57%, lipase by 41%, hyaluronidase and lipase by 17%, gelatinase by 10% and lecithinase by 6.5%. Isolates from water after treatment produced 7 different enzymes, drinking water 3 and raw water 2.

4.5 Antibiotic profiles of HPC isolates and levels of pharmaceuticals

Twelve common antibiotics that are used in human therapeutics were used to determine the susceptibility, intermediate resistance and resistance of all HPC isolates. Table 4.8 shows the 2016 and 2017 results for overall percentage of isolates that were resistant, intermediate resistant and susceptible to the antibiotics. A total of 183 HPC isolates for the two sampling periods (2016 to 2017) for both production facilities were tested. This table also gives the average MAR indices for each sample site (see Table A1 to A7 in Appendix A for the antibiotic profiles for each isolate tested), as well as the most prevalent resistance phenotype at each site. Only antibiotics that more than 20% of isolates were resistant to was included in the latter phenotype. The levels of antimicrobials compounds in raw, after treatment, and drinking water were analysed, but only for plant NW-D.

4.5.1 Plant NW-D 2016

The following patterns are demonstrated in Table 4.8. Of the total 91 HPC isolates tested, most were resistant to ampicillin (62%) and vancomycin (56%), followed by trimethoprim (52%), and cephalothin (39%). Twenty eight percent (28%) were intermediate resistant to
erythromycin. More than 75% of the isolates were susceptible to streptomycin and ciprofloxacin.

### 4.5.2 Plant NW-D 2017

The isolates from 2017 had a slightly different resistance pattern. Ninety six percent (96%) of isolates were resistant to trimethoprim, followed by the two β-lactam antibiotics, ampicillin and cephalothin (92% and 82% respectively). A large proportion of the isolates were susceptible to chloramphenicol (88%) and neomycin (80%). Forty three percent (43%) of the isolates had intermediate and susceptible resistance to oxy-tetracycline.

### 4.5.3 Plant GH-T 2017

Among the 41 HPC isolates, all were resistant to trimethoprim. Percentage resistance to ampicillin, cephalothin, oxy-tetracycline and penicillin G, was also high (95%, 93%, 78%, and 68% respectively). Ninety eight percent (98%) of the isolates were susceptible to neomycin. Only 20% of the total HPC isolates tested were intermediate resistant to chloramphenicol and ciprofloxacin. Most isolates were susceptible to aminoglycosides (neomycin, erythromycin, and streptomyacin).

In Table 4.9, it is observed that isolates from all sampling periods (both water production facilities) were mostly resistant to ampicillin (87%). All the Gram positive isolates tested from drinking water (GH-T: 2017) were resistant to pen-G (100), while there is a reduced susceptibility of isolates from the source water. Similar observations were made with respect to susceptibility patterns to trimethoprim and cephalothin.

### 4.5.4 Detection of antimicrobial compounds

Of all the antimicrobials tested (15) for, only three were detected in all the sampling sites. These included ciprofloxacin and penicillin detected in water after treatment and drinking water. Ciprofloxacin and streptomycin were detected in source water (Table 4.7). The values are qualitative thus no units are given. The results could only be interpreted as present/absent.

#### Table 4.7: Antimicrobials detected in source, final and the distribution system of NW-D.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Classification</th>
<th>Raw</th>
<th>After treatment</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>Antibiotic</td>
<td>121 030</td>
<td>167 425</td>
<td>1 832 294</td>
</tr>
<tr>
<td>Penicillin</td>
<td>Antibiotic</td>
<td>N/D</td>
<td>8 400</td>
<td>5 957</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Antibiotic</td>
<td>14 211</td>
<td>N/D</td>
<td>N/D</td>
</tr>
</tbody>
</table>

N/D: Not detected.
Table 4.8: Percentage of selected HPC isolates resistant (R), intermediate resistant (IR) and susceptible (S) to antibiotics during the 2016 to 2017 sampling period.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>AMP</th>
<th>KF</th>
<th>CHL</th>
<th>CIP</th>
<th>ERY</th>
<th>KAN</th>
<th>NEO</th>
<th>O-T</th>
<th>PEN-G</th>
<th>STREP</th>
<th>TM</th>
<th>VAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>NW-D-2016 (March+May+August)</td>
<td>%R</td>
<td>62</td>
<td>39</td>
<td>16</td>
<td>12</td>
<td>13</td>
<td>34</td>
<td>19</td>
<td>22</td>
<td>44</td>
<td>13</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>%IR</td>
<td>13</td>
<td>6</td>
<td>18</td>
<td>13</td>
<td>28</td>
<td>13</td>
<td>18</td>
<td>15</td>
<td>13</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>%S</td>
<td>25</td>
<td>56</td>
<td>67</td>
<td>75</td>
<td>60</td>
<td>53</td>
<td>63</td>
<td>64</td>
<td>44</td>
<td>13</td>
<td>56</td>
</tr>
<tr>
<td>n=91</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAR:0.275</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NW-D-2017 (May+October)</td>
<td>%R</td>
<td>92</td>
<td>82</td>
<td>8</td>
<td>18</td>
<td>14</td>
<td>27</td>
<td>8</td>
<td>14</td>
<td>68</td>
<td>14</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>%IR</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>10</td>
<td>8</td>
<td>14</td>
<td>12</td>
<td>43</td>
<td>11</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>%S</td>
<td>8</td>
<td>14</td>
<td>88</td>
<td>73</td>
<td>78</td>
<td>59</td>
<td>80</td>
<td>43</td>
<td>21</td>
<td>61</td>
<td>4</td>
</tr>
<tr>
<td>n=51</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAR:0.355</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH-T-2017 (June+November)</td>
<td>%R</td>
<td>95</td>
<td>93</td>
<td>7</td>
<td>5</td>
<td>12</td>
<td>61</td>
<td>0</td>
<td>78</td>
<td>68</td>
<td>32</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>%IR</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>20</td>
<td>7</td>
<td>15</td>
<td>2</td>
<td>7</td>
<td>20</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>%S</td>
<td>5</td>
<td>7</td>
<td>73</td>
<td>76</td>
<td>80</td>
<td>24</td>
<td>98</td>
<td>15</td>
<td>12</td>
<td>66</td>
<td>0</td>
</tr>
<tr>
<td>n=41</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAR:0.461</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AMP- Ampicillin; KF- Cephalothin; ERY- Erythromycin; CHL- Chloramphenicol; CIP- Ciprofloxacin; KAN- Kanamycin; NEO- Neomycin; STREP- Streptomycin; VAN- Vancomycin; PEN-G- Penicillin G; O-T- Oxy-tetracycline; TM- Trimethoprim.
Table 4.9: Summary of the antibiotics to which a large proportion of HPC isolates were resistant to during the 2016 to 2017 sampling period.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Source</th>
<th>AMP%</th>
<th>KF%</th>
<th>NEO%</th>
<th>PEN-G%</th>
<th>STREP%</th>
<th>TM%</th>
</tr>
</thead>
<tbody>
<tr>
<td>NW-D-2016 (March+May+August)</td>
<td>Raw</td>
<td>67</td>
<td>54</td>
<td>14</td>
<td>21</td>
<td>14</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>AT</td>
<td>83</td>
<td>38</td>
<td>4</td>
<td>39</td>
<td>8</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>75</td>
<td>66</td>
<td>16</td>
<td>38</td>
<td>11</td>
<td>75</td>
</tr>
<tr>
<td>NW-D-2017 (May+October)</td>
<td>Raw</td>
<td>100</td>
<td>87</td>
<td>25</td>
<td>100</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>AT</td>
<td>100</td>
<td>50</td>
<td>17</td>
<td>67</td>
<td>17</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>86</td>
<td>79</td>
<td>3</td>
<td>36</td>
<td>17</td>
<td>97</td>
</tr>
<tr>
<td>GH-T-2017 (June+November)</td>
<td>Raw</td>
<td>90</td>
<td>6</td>
<td>81</td>
<td>25</td>
<td>100</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>AT</td>
<td>100</td>
<td>92</td>
<td>0</td>
<td>40</td>
<td>34</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>88</td>
<td>88</td>
<td>0</td>
<td>100</td>
<td>40</td>
<td>100</td>
</tr>
</tbody>
</table>

AT: After treatment; D: Distribution AMP- Ampicillin; KF- Cephalothin; NEO- Neomycin; STRE- Streptomycin; PEN-G- Penicillin G; TM- Trimethoprim.

4.5.5 Multiple antibiotic resistant phenotypes of all sampling sites (2016 and 2017)
A summary of the MAR phenotypes and some isolates that displayed the specific phenotype per site is summarized in Table 4.8. The MAR phenotype in which isolates were resistant to eight of the antibiotics AMP-KF-ER-KA-OT-PE-TM-VA, was observed in the distribution sites for both years.

Site NW-D (2016) had the lowest MAR index value (0.275), as well as the MAR phenotype with the least antibiotic representatives (AMP-VAN-TM-PEN-G-KF-KAN-OT) to which more than 20% of isolates were resistant to. Site NW-D (2017) had a MAR index value of 0.355. More than 35% of the HPC isolates from the site were resistant to six of the twelve antibiotics tested (AMP-TM-KF-PEN-G-VAN-KAN). The highest MAR index value of 0.461 was observed among isolates from site GH-T (2017). The most prevalent MAR phenotype for this site was TM-AMP-KF-O-T-PEN-G-KAN-VAN-STREP. The overall MAR phenotype for all isolates was (AMP-TM-KF-PEN-G-VAN-OT), but not in the same order (Table 4.8).

4.5.6 Presence of antibiotic resistance genes in HPC isolates
HPC isolates were screened for the presence of six antibiotic resistance genes using PCR amplification. This was performed for selected MAR isolates that were resistant to the antibiotic for which the specific gene encoded resistance to. Table 4.10 shows four of the genes that were found among antibiotic-resistant isolates obtained from raw and treated
drinking water (after treatment and distribution water). Figures 4.7 to 4.9 show representative amplicons on agarose gels of the four ARGs, \textit{ampC}, \textit{ermF}, \textit{bla}_{TEM} and \textit{ermF}, that were successfully amplified through PCR. The presence of ARGs using eDNA as the potential source was studied for plant NW-D only.

Of all the HPC isolates resistant to β-lactamase antibiotics (ampicillin and cephalothin), 5 different isolates harbour the \textit{ampC} and \textit{bla}_{TEM} genes. All genes (Figure 4.7 and 4.8 respectively) were found in isolates obtained from all sampling sites (mostly from site GH-T). Moreover, these β-lactamase encoding genes were found in most of the \textit{Bacillus} species such as \textit{Bacillus cereus}, \textit{Bacillus thuringiensis} and \textit{Bacillus licheniformis}. The expected band sizes were approximately 550 base pairs, although a variation in gene size was observed.

Of the two macrolide-lincosamide-streptogramin (MLS) resistance genes (\textit{ermB} and \textit{ermF}), the \textit{ermB} gene (Figure 4.9) was present in 15 of the isolates, and only 1 was positive for the \textit{ermF} gene. Furthermore, these \textit{erm} genes were found in most of the \textit{Bacillus} species (13 isolates), followed by \textit{Shinella} with one isolate. Most of these isolates were obtained from the GH-T plant. None of the isolates tested positive for the \textit{intI} 1 and the \textit{tetM} gene. An eDNA approach was used to detect the same resistance genes, and only two of the genes (\textit{ermB} and \textit{intI} 1) were detected in the source water.
Table 4.10: Antibiotic resistance genes (ampC, ermB, blaTEM and ermF) in varied identified species isolated from raw, after treatment and distribution water in Plant NW-D and GH-T

<table>
<thead>
<tr>
<th>Antibiotic resistant gene</th>
<th>Sampling site</th>
<th>Isolate name</th>
<th>Water production plant</th>
<th>Identified species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ampC (n= 5)</strong></td>
<td>After treatment</td>
<td>Z</td>
<td>NW-D</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td></td>
<td>Distribution</td>
<td>T</td>
<td>NW-D</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td></td>
<td>Raw</td>
<td>C4</td>
<td>NW-D</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td></td>
<td>Raw</td>
<td>F13</td>
<td>GH-T</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td></td>
<td>Distribution</td>
<td>F29</td>
<td>GH-T</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td><strong>ermB (n= 15)</strong></td>
<td>Distribution E1</td>
<td>NW-D</td>
<td><em>Bacillus cereus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distribution E7</td>
<td>NW-D</td>
<td><em>Bacillus thuringiensis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Raw</td>
<td>C4</td>
<td>NW-D</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td></td>
<td>Distribution C15</td>
<td>NW-D</td>
<td><em>Shinella curvata</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distribution C20</td>
<td>NW-D</td>
<td><em>Bacillus cereus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distribution C25</td>
<td>NW-D</td>
<td><em>Bacillus pumilus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distribution C26</td>
<td>NW-D</td>
<td><em>Bacillus cereus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distribution E2</td>
<td>NW-D</td>
<td><em>Bacillus thuringiensis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Raw</td>
<td>D</td>
<td>NW-D</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td></td>
<td>After treatment</td>
<td>D13</td>
<td>GH-T</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td></td>
<td>Distribution P</td>
<td>NW-D</td>
<td><em>Bacillus anthracis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distribution Y</td>
<td>NW-D</td>
<td><em>Bacillus cereus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distribution D22</td>
<td>GH-T</td>
<td><em>Bacillus cereus</em></td>
<td></td>
</tr>
<tr>
<td><strong>blaTEM (n= 5)</strong></td>
<td>Distribution E1</td>
<td>NW-D</td>
<td><em>Bacillus thuringiensis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distribution E2</td>
<td>NW-D</td>
<td><em>Bacillus thuringiensis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distribution E18</td>
<td>NW-D</td>
<td><em>Chryseobacterium sp.</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Raw</td>
<td>E25</td>
<td>NW-D</td>
<td><em>Bacillus licheniformis</em></td>
</tr>
<tr>
<td></td>
<td>Raw</td>
<td>F16</td>
<td>GH-T</td>
<td><em>Paenibacillus chitinolyticus</em></td>
</tr>
<tr>
<td><strong>ermF (n=1)</strong></td>
<td>Distribution E7</td>
<td>NW-D</td>
<td><em>Bacillus thuringiensis</em></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.7: 1.5 % agarose gels with successful amplified *ampC*. Lane C represents the no template DNA control and MW represents the 1 kb molecular size marker (O’ GeneRulerTM 1kb DNA ladder, Fermentas Life Science, US). Electrophoresed at 80 V for 45 minutes. For *ampC* (amplicon size: 550bp); successful amplification is shown in lane:1-3 and 7-8. While lane: Lane: 4-6 shows no amplification.

Figure 4.8: 1.5 % agarose gels with successful amplified blaTEM. MW represents the 1kb molecular size marker (O’ GeneRulerTM 1kb DNA ladder, Fermentas Life Science, US). Electrophoresed at 80 V for 45 minutes. For *blaTEM* (amplicon size: 1080bp); Lane 1-3 and 6-7 shows successful amplification. While lane: 4-5 indicates no amplification.

Figure 4.9: Agarose gels with successful amplified *ermB*. Lane C represents the no template DNA control and MW represents the 1kb molecular size marker (O’ GeneRulerTM 1kb DNA ladder, Fermentas Life Science, US). Electrophoresed at 80 V for 45 minutes. For *ermB* (amplicon size: 639)
4.7 Identification and confirmation of HPC isolates

4.7.1 Identification by 16S rRNA

To confirm their identity, genomic DNA of isolated HPC species were amplified by using polymerase chain reaction (PCR) and sequencing under conditions described in (Section 3.9). Figure 4.7 below shows a 1.5% (w/v) agarose gel of 11 isolates that were successful in the 16S rRNA amplification, with a no template DNA control (C). All amplicons were at an expected size of approximately 1 465 bp, with no non-specific bands and primer dimers present. No contamination was found as supported by the absence of a band in the no template DNA control. Amplified fragments were sequenced in-house using ABI 3130 Analyser (Applied Biosystems, UK), as described in Section 3.10.

![Image]

**Figure 4.10**: 1.5% agarose gel of 16S rRNA amplicons with the expected size of 1 465 bp. Lane 1-11: isolates from Plant NW-D and GH-T. Lane C: no template DNA control; MW: 1kb molecular size marker (O’GeneRulerTM 1kb DNA ladder, Fermentas Life Science, US). Electrophoresed at 80V for 45 minutes.

The amplified sequences were identified by BlastN searches against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). The identification of HPC bacteria from various sites are summarised in Table B1, in Appendix B. These tables also include additional information such as the isolate name, accession number from GenBank, and the percentage (%) similarity.

A total of five different genera were identified in plant NW-D and GH-T for 2016 and 2017, collectively. Figure 4.11 depicts the composition of all HPC isolates identified through 16S rRNA sequencing. The most abundant genera identified were Bacillus with 44 isolates (92%). The least abundant genera identified were Micrococcus, Chryseobacterium, Shinella and Paenibacillus with only one isolate each. Bacillus species identified included; B. Cereus

Plant NW-D (2017) had a greater diversity of species with the following genera; *Bacillus*, *Chryseobacterium* and *Shinella*. Both plant GH-T (2017) and NW-D (2016) had diverse genera including *Micrococcus*, *Bacillus* and *Paenibacillus*, *Bacillus* respectively.

![Pie chart displaying bacterial species identified with the 16S rRNA gene from all the sampling runs for NWD and GH-T.](image)

**Figure 4.11:** Pie chart displaying bacterial species identified with the 16S rRNA gene from all the sampling runs for NWD and GH-T.

### 4.8. Phylogenetic analysis

Sequences were compared to 16S rRNA gene sequences within the GenBank database through BLASTn searches. Only those with high sequence similarities (≥ 97 %) were used for phylogenetic analysis. The sequencing results used to construct the phylogenetic tree is found in Table B1 (in Appendix B). The sequences used were from both production facilities (2016 to 2017).

Figure 4.12 illustrates the phylogenetic relationship of the HPC bacteria. The tree is divided into seven (A, B, C, D, E, F and G) clades. Species from the same genus were grouped together (Figure 4.12). This is supported by strong Bootstrap values, with the majority of species groupings supported by values between 90% and 100%. The number in brackets represents the number of isolates that were identified as the same species in the current study.
The scale in the tree is 0.050, indicating that there is less than 1% genetic change. This shows that there is a common evolutionary ancestor and that they are descendant groups.

In Figure 4.12, some of the relationships observed are briefly discussed: The F29, R, E14, GG, D sequences clustered with *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. wiedmanni*, *B. toyonensis* complex. This indicates a strong relationship between the species, as they form part of the genus *Bacillus*. There is a strong Bootstrap support of 90% for these species. Two separate clades are observed within the *Bacillus* genus. *Bacillus licheniforms* (E24) clustered with *B. licheniforms* (KT719714.1 and KT719487.1) with bootstrap support of 82% and 100%, respectively. *Bacillus pumilus* (C25) clustered with *B. safensis* (KR780976.1) with a strong Bootstrap support of 97%. *Micrococcus luteus* (B37) clustered very well with *M. yunnanensis* (MH2984951 and KU925163.1) and *M. luteus* with a strong Bootstrap support (100%), indicating that they are members of the *Micrococcus* genus. The C15 complex clustered with *S. curvata* (KY996858.1) with 100% bootstrap support and also clustered with a reduced bootstrap support (75%). This observation indicates that these species are members of the same genus, namely *Shinella*.

Table 4.11 gives a summary of all identified potential pathogens for this study, as well as health effects associated with these pathogens. In addition, the most prevalent phenotype of these bacteria are also shown. It can be observed that *Bacillus* sp. had the highest potential for being pathogenic as compared to the other species. There is more resistance (100%) to ampicillin in all the potential pathogenic bacteria, followed by cephalothin.
Figure 4.12: Neighbour-Joining phylogenetic tree representing relationship of 16S gene sequences from GenBank database and the sequences of HPC bacteria isolated from water production facilities (NW-D and GH-T) in 2016 and 2017. *Escherichia coli* was used as an outgroup.
<table>
<thead>
<tr>
<th>Production facility</th>
<th>Sampling site</th>
<th>Identified isolates</th>
<th>Extracellular enzymes</th>
<th>Prevalence phenotype</th>
<th>Health effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT and D</td>
<td>Bacillus cereus (9)</td>
<td>G, D, P, Le, Li, H</td>
<td>AMP-KF-CHL-ER-KAN-NE-ST-TM</td>
<td>Gastrointestinal illness including, diarrhea and emesis (Chaabouni et al. 2015)</td>
<td>(Molva et al., 2009; Pavlov et al., 2004; Kumari and Sarkar, 2014)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bacillus pumilus(1)</td>
<td>P, Li</td>
<td>AMP-KF-PEN-TM</td>
<td>Cutaneous infection (Tena et al., 2007)</td>
<td>Sangeetha et al. 2007</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bacillus licheniformis(1)</td>
<td>D, P, Li</td>
<td>AMP-KF-TM</td>
<td>Bacteremia, peritonitis, food poisoning and eye infections (Haydushka et al., 2012)</td>
<td>Sangeetha et al. 2007</td>
<td></td>
</tr>
<tr>
<td>Production facility</td>
<td>Sampling site</td>
<td>Identified isolates</td>
<td>Extracellular enzymes</td>
<td>Prevalence</td>
<td>Health effects</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------</td>
<td>----------------</td>
<td>---------------------</td>
<td>-----------------------</td>
<td>------------</td>
<td>----------------</td>
<td>-----------</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Bacillus thuringiensis</em> (2)</td>
<td>D, Le, P, Li</td>
<td>AMP-KF-PEN-TRI</td>
<td>Diarrhoea and emesis, as well as some non-gastrointestinal infections (Kotiranta et al., 2000)</td>
<td>(Molva et al., 2009); Horn et al., 2016)</td>
</tr>
<tr>
<td>GH-T 2017</td>
<td>Raw</td>
<td><em>Bacillus cereus</em> (1)</td>
<td>D, P</td>
<td>AMP-KF-KA-PE-TM-VA</td>
<td>Food poisoning including, diarrhea and emesis (Chaabouni et al., 2015)</td>
<td>(Molva et al., 2009; Pavlov et al., 2004; Kumari and Sarkar, 2014)</td>
</tr>
<tr>
<td>GH-T 2017</td>
<td>AT and D</td>
<td><em>Bacillus thuringiensis</em> (2)</td>
<td>P, D,</td>
<td>AMP-KF-CHL-KA-OT-PE-ST-TM</td>
<td>Diarrhoea and emesis, as well as some non-gastrointestinal infections (Kotiranta et al., 2000)</td>
<td>Molva et al. (2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Bacillus cereus</em> (2)</td>
<td>D, C, P,</td>
<td>AMP-KF-KA-PE-TM-VA</td>
<td>Food poisoning including, diarrhea and emesis (Chaabouni et al., 2015).</td>
<td>(Molva et al., 2009; Pavlov et al., 2004; Sarkar, 2014)</td>
</tr>
</tbody>
</table>

AMP- Ampicillin; KF- Cephalothin; ER- Erythromycin; CHL- Chloramphenicol ; CIP- Ciprofloxacin; KA- Kanamycin; NEO- Neomycin; ST- Streptomycin; VA- Vancomycin; PE- Penicillin G; OT- Oxy-tetracycline; TM- Trimethoprim. AT: After treatment; D: Distribution. AT: After treatment; D: Distribution. G: Gelatinase; D: DNase; P: Proteinase; Le: Lecithinase; Li: Lipase; H: Hyaluronidase. The number in brackets represents the number of isolates that were identified for that organisms.
5.1 Introduction
The purpose of water treatment systems is to provide consumers with drinking water that is sufficiently free of microbial pathogens to prevent water-borne diseases. Furthermore, water treatment for domestic use must produce an aesthetically acceptable (in terms of taste, appearance, and odour) and chemically stable water (i.e. it must not cause corrosion or form deposit in pipes or fixtures) (Momba et al., 2009). Recent studies (Momba and Brouckaert, 2005; Momba et al., 2006; Obi et al., 2007) conducted in South Africa have confirmed that water treatment plants are not producing the desired water quantity or quality. The occurrence and spread of ARB and ARGs have become a pressing public health problem worldwide. Aquatic ecosystems are a recognized reservoir for these contaminants (Xi et al., 2009). Thus, recent studies have placed more focus on investigating antibiotic resistance in the aquatic environment (Biyela et al., 2004; Bergeron et al., 2015). This present study was undertaken to investigate the drinking water quality and the prevalence of ARB in two water production systems (NW-D and GH-T). This was done by isolating the HPC bacteria from raw and drinking water. Isolates were also tested for pathogenic potential by determining their ability to produce certain virulence-associated enzymes. Purified HPC isolates were subjected to PCR amplification and sequencing to identify potential pathogenic bacteria associated with these isolates. Followed by potential resistance genes.

5.2 Physico-chemical analysis
The physico-chemical results obtained during 2016 and 2017 are discussed in this section. SANS 241:2015 was used as a guide to assess the physico-chemical quality of the drinking water.

5.2.1 Free chlorine
Free chlorine is regulated by the SANS 241 (2015) and should not exceed 5 mg/l. The results in Table 4.2, shows that free chlorine levels comply with the SANS 241 (2015) for drinking water, where the levels ranged from 0.05 to 1.66 mg/l. Two sampling periods, (March: 2016) for NW-D and (November: 2017) for GH-T levels were above 1 mg/l. This is a problem, as high chlorine levels have been associated with the formation of toxic disinfection by-products, odour and taste complaints (Bowden et al., 2006; WHO, 2011a).

The WHO suggests that free residual chlorine for water that is treated at the point of delivery should fall within 0.2 to 0.5 mg/l (WHO, 2011a). For NW-D (2017), free chlorine levels during
May and October were very low, which is a concern, because these low levels may cause regrowth of microorganisms in the distribution system (DiGiano et al., 2000). These results are in line with the study conducted by Karikari and Ampofo (2013) in which they investigated the drinking water sample from a water treatment plant of Accra-Tema Metropolis, Ghana. The mean free chlorine value for this study varied from the lowest value of 0.13 to 1.4 mg/l.

This low amount of residual chlorine in the drinking water may have affected the microbial content of water as total coliform counts were detected in water after treatment and drinking water during October for NW-D: 2017). This was also observed by Sara and Qaiser (2014) who investigated drinking water from different sectors of Islamabad, Pakistan. The low amount of residual chlorine in drinking water affected the microbial content of water as total coliform counts were detected at few sampling sites. The mean free chlorine ranged from 0.04 to 0.83 mg/l. Karikari and Ampofo (2013) suggested that the lack of a detectable level of free residual chlorine will cause pathogens to survive in drinking water distribution pipelines and these could potentially be delivered to consumers at the tap.

The correlations between the levels of the respective microbial indicators in final water and water at point of use, suggest that the microorganisms detected at point of use may have come from the treated water and have thrived due to low residual chlorine levels (Obi et al., 2007). Considering the levels of free chlorine in the two drinking water production plants in the present study, isolating HPCs from the drinking water (immediately after treatment and in the distribution system) comes as no surprise.

5.2.2 Total dissolved solids (TDS)
SANS 241 (2015) regulates TDS for drinking water and should not exceed 1 200 mg/l. In Table 4.1, TDS ranged from 425 to 551 mg/l in both treatment systems. The TDS in all the water samples were far below the SANS 241 (2015) maximum allowable limit, making these water sources suitable for drinking. Water with very low concentrations of TDS may be unacceptable due to its flat and tasteless flavour. However, there are no dependable data on possible health effects associated with the ingestion of TDS in drinking water (WHO, 2008a). The concentration of TDS increased after the water was treated for all the months (the concentration ranged from 444 to 552 mg/l), except for August in NW-D (2016 to 2017). High TDS levels (>500 mg/l) could result in excessive scaling in water pipes, boilers, water heaters and household appliances. In addition, the water may taste salty, bitter and might have unpleasant odours (Mezgebe et al., 2015).
5.2.3 pH
SANS 241 (2015) regulates pH for drinking water. The pH values should be in the range of 5 to 9.7. The results in Table 4.1, shows that pH was within the SANS 241 (2015) of drinking water. The pH value for after treatment and drinking water ranged from 7.40 to 8.70 in both treatment plants. The values for all the sampling periods were more or less the same. The normal drinking water pH range mentioned in WHO guidelines is between 6.5 and 8.5. The values (8.52 and 8.70 respectively) recorded for NW-D during August (2016) exceeded this standard. Failure to control this might lead to contamination of drinking water. This will have adverse effects on its taste, appearance and odour (WHO, 2007).

5.2.4 Nitrate
Nitrate is regulated by the SANS 241 (2015) and should not exceed 11 mg/l. In Table 4.2, the nitrate concentrations ranged between 0.05 to 1.72 mg/l in drinking water and after treatment (in both treatment systems). This is acceptable for drinking water. The nitrate levels observed in this study did not pose a health risk to humans. This is positive for both plants in the two provinces (North West and Gauteng), as they are known for their agricultural practices, which might have contributed to the high concentrations of this parameter (Kalule-Sabiti and Heath, 2008). The results also show nitrate concentrations varied seasonally and spatially. Site GH-T (2017) during November, were again noted to have the highest nitrate concentrations (4.12 to 5.03 mg/l, for drinking water) in the warmer months, incidentally also the rainy months. In contrast, NW-D (2016) also had high concentration of nitrate (1.25 to 1.72 mg/l for drinking water) during May. No nitrate was detected in the raw water of NW-D during March 2016, but was detected in drinking water and after treatment. Similar results were obtained in other studies (Eliku and Sulaiman, 2015; Edokpayi et al., 2018).

5.2.5 Nitrite
Nitrite is regulated by the SANS 241 (2015) and should not exceed 0.9 mg/l. The results, seen in Table 4.2 ranged between 0.03 to 2.75 mg/l in drinking water and after treatment. This means that the concentrations of nitrite exceeded the SANS 241 (2015) standards for drinking water in some instances. The highest nitrite concentration was recorded at NW-D (2016) for the month of August in the drinking water. This might have been due to surface runoff from farms in the catchment area, into the water sources during early rain as well as from sewage treatment plants (Razak et al., 2009).

Only one site in GH-T did not comply with the SANS 241 (2015) for drinking water. Nitrite is known to react directly with haemoglobin in human blood to produce methemoglobin. Methemoglobin destroys the ability of red blood cells to transport oxygen. This condition is especially serious in babies under three months of age (Kumar and Puri, 2012). Therefore,
high levels of nitrite in the drinking water of plant NW-D is a concern. Nitrite can also react with secondary amines in the human stomach to generate N-nitrosamines that are carcinogenic (Pegg and Shahidi, 2000). However, data from epidemiological studies does not support an association between high nitrite exposure from drinking water and increased cancer rates in humans (WHO, 2007).

5.2.6 Chemical Oxygen Demand (COD)
COD is not regulated by the SANS 241 (2015) for drinking water. The highest level of COD was recorded in water after treatment during August (NW-D: 2016). The COD levels ranged from 1 to 7 mg/l for all the sampling periods (Table 4.2) and were thus very low. High levels of COD may indicate a serious water pollution (Yin et al., 2011). It was observed that after the water was treated, no COD was detected during March (2016) at NW-D. COD levels for GH-T were high in the raw water, but gradually decreased after treatment for the June (2017) sampling period. This indicates that the disinfection process effectively removed organic matter. But overall, the COD in all the water samples was far below the maximum allowable limit. These levels can be used for monitoring and controlling the amount of organic pollutants found in surface water after the correlation with biological oxygen demand (BOD) has been established (Swartz et al., 2004). It has been suggested that organic waste from wastewater treatment plants are a food source for water-borne bacteria. Bacteria remove these organic carbon molecules by consuming them, using and thus reducing the dissolved oxygen (Davies, 2005).

In a study by Mamba and co-workers (2009) conducted in the Sedibeng and Midvaal Treatment plants, values for COD ranged between 19.04 and 24.06 mg/l and between 13.28 and 110.73 mg/l for the Midvaal and Sedibeng water treatment plants, respectively. A decrease in the COD levels was observed after the water treatment step and is in line with the results for this study except for one sampling site during October (NW-D: 2017), which showed an increase in COD after the water was treated. They also observed a sharp decrease after sand filtration at Sedibeng suggesting that the sand-filtration process removed most of the oxidisable organic matter.

5.2.7 Phosphates
Phosphate is also not regulated by the SANS 241 (2015) for drinking water. WHO recommended a maximum phosphate of 5 mg/l. The highest phosphate levels were recorded at NW-D (2017: October) from water after treatment with a concentration of 4.95 mg/l, while the lowest concentration was 0.38 mg/l recorded at GH-T and NW-D (2017) in raw water. Only once (October 2017) did the NW-D not meet WHO recommended maximum of 5 mg/l. This may pose health effects such as kidney damage and osteoporosis (Kipngetich et al., 2013).
Increased concentration of phosphates could be attributed to the agricultural activities being undertaken where there is heavy use of phosphate fertilizers in the catchment area (de Villiers and Thiart, 2007). Increases in phosphates can also favour biofilm formation in the pipelines (Chu et al., 2005). There were slightly low levels recorded in the water after treatment, which might be a problem in the future as these levels could influence the coagulation process.

5.2.8 Temperature
The temperature of drinking water is not regulated by the SANS 241 (2015). Based on the WHO recommended value (<15°C), this is the threshold temperature that makes drinking water palatable (WHO, 2003). There was a significant temperature variation in all sampling sites. As expected the highest average temperature (Table 4.1) was recorded in March by 23.6 °C from NW-D (2016). The lowest was recorded in August (11.9°C) from NW-D in the same year. This is in line with the observation from Henne et al. (2013), as this shows a strong seasonal variation between sampling periods. The temperature range of drinking and treatment water of both treatment systems were 12.6°C to 23.6°C. The NW-D (November: 2017) sampling run maintained a low temperature range throughout, from the source to the drinking water (18.6 to 16.7°C). High temperatures (19.9 to 20.5) in GH-T (2017) in June might have caused a reduction in residual chlorine (0.06 to 0.08 mg/l) since chlorine volatilizes as chlorine gas more readily at higher temperatures. Therefore, this might increase microorganism growth since the high temperature increases the metabolism of the organisms and the low residual chlorine levels are not enough to destroy microbes (Momba and Notshe, 2003; Lautenschlager et al., 2013; Henne et al., 2013). This may lead to corrosion problems or increase odour, taste and colour (UNICEF, 2008).

5.2.9 Turbidity
Turbidity is regulated by the SANS 241 (2015) and should not exceed 1 mg/l. The results, seen in Table 4.1 ranged between 0.23 to 9.99 mg/l in all the sampling sites. Raw water had the higher turbidity (0.67 to 3.87 NTU), and this was generally reduced after treatment to values lower than the SANS 241 (2015) levels. However, high turbidity levels (1.63 to 2.24 NTU) were recorded in NW-D (2016) in water after treatment. Momba et al. (2009) reported that in the North West Province 28% of the plants did not comply with the maximum turbidity limits at the point of treatment as well as the point of use. Highly turbid water interferes with the disinfection process because excessive turbidity can protect pathogenic microorganisms from the effects of disinfectants, and also stimulate the growth of bacteria during storage (USEPA, 2007; Momba et al., 2009). This is also observed by Amin et al. (2012). These pathogenic microorganisms can cause nausea, cramps, diarrhoea and headaches (USEPA, 2007). Turbidity levels for all the sampling sites in plant GH-T complied with the SANS 241 (2015).
5.3 Microbiological parameters

The microbiological parameters that were measured during 2016 and 2017 are discussed in this section. These include HPC bacteria and total and faecal coliforms.

5.3.1 Heterotrophic plate count (HPC) bacteria

HPC bacteria are an indicator of the general microbial water quality and should not exceed 1 000 CFU/ml for drinking water (SANS 241, 2015). Lehtola et al. (2004), reported that there is a challenge regarding microbiological quality control for safety management of water from water treatment plants used for drinking. Hence it is essential to frequently monitor drinking water quality from source to the distribution system (taps), to ensure that the water is compliant with quality standards and to protect consumers from any health risk. In the present study, the average HPC bacteria numbers detected at the 5 sites are presented in Table 4.3.

HPC bacteria levels were very high in the raw water during 2016 and 2017, but lower in the water after treatment in all of the sampling periods (Table 4.3). In NW-D (2016), the counts of HPC bacteria enumerated at room temperature ranged from (160 to 740 CFU/ml, and 70.3 to 500 CFU/ml, respectively) in water after treatment and drinking water. Higher counts were detected in August.

All the counts of HPC bacteria were within the SANS 241 (2015) for drinking water. In 2017, it was observed that lower levels of HPC bacteria were enumerated in both production facilities as compared to the 2016 results. There is a concern in GH-T during the November sampling, where HPC bacteria counts in water after treatment were higher than those in the raw water. This might be subjective and such a finding need to be repeated during similar sampling periods. Higher levels in HPC counts could be symptoms of treatment failure, installation, repair, or influence of microbial growth in the distribution system and presence of biofilm (Amanidaz et al., 2015).

The source water within the NW-D catchment area is exposed to pollution from mining, agricultural and industrial activities (Venter, 2001), which results in pollution in an aquatic ecosystem. Thus there are high levels of HPC in the raw water.

According to Farhadkhani et al. (2014), there is another factor that might have influenced an increase of contamination in distribution systems, and that is higher temperature (during the month October, the temperature was 21°C), that might have sped up the bacterial growth.
5.3.2 Total coliforms and faecal coliforms

The SANS241 (2015) regulates total and faecal coliform numbers. Total coliform may not exceed 10 count/100 ml, and faecal coliform may not exceed 0 count/100 ml. Table 4.5 shows that there was no faecal coliform detected in water after treatment and also in drinking water. NW-D had high levels of total and faecal coliforms in the water before treatment. The same trend is also observed in the study of Momba et al. (2009) where 0 to 4 count/100 ml of total coliform were detected. Total coliforms should be absent in the drinking water after treatment, but in the current study low levels were detected. Their presence potentially points toward treatment inefficiency if found after treatment (WHO, 2005). According to the South African Water Quality Guidelines (DWAF, 1996) for drinking water and WHO (2005), total coliforms should not exceed 5 CFU/100 ml. The results from the present study indicate that with respect to the SANS241 (2015) the water was acceptable.

Total coliform bacteria do not cause serious health effects since they are considered harmless. However, this does not mean that the problem of the occurrence of total coliform group bacteria should be neglected (Moosa et al., 2015), since total coliforms can be used as an indicator of treatment efficiency and integrity of the distribution system (Sapkota et al., 2012).

From the results above, in some cases of the water distribution system with 0.2 to 0.5 mg/l of residual chlorine, more than 500 heterotrophic bacteria CFU/ml were isolated. This indicates that some bacteria may survive in high concentrations of free residual chlorine. Amanidaz et al. (2015) further added that the presence of more than 500 CFU/ml heterotrophic bacteria in the distribution water suppress coliform of 1 to 10 CFU/ml. Temperature and HPC also showed a medium to strong relationship in the current study.

5.4. Haemolysin assay

The haemolysin assay was the first step to screen for potentially pathogenic properties of HPC bacteria. Fifty-four of hundred and five isolates displayed α- and β-haemolysis. Table 4.5 shows the number of HPC isolates purified from a specific sampling site. Isolates that proved positive for α- and β-haemolysis ranged from 3 to 15 isolates per water sampling site. HPC from all sampling sites displayed α- and β-haemolysis. There were more isolates (24 in total) that displayed haemolysis in drinking water as compared to the other sampling sites.

Previous studies (Payment et al., 1994; Pavlov et al., 2004; Inomata et al., 2009) have found haemolytic isolates among HPC bacteria isolated from water after treatment. A study by Horn et al. (2016) focused on untreated water, and isolated HPC bacteria also displayed α- and β-haemolysis. The ratio of haemolytic isolates among all isolated HPC bacteria was 51%
(54/105) for the current study, which agrees with a previous reported ratio (55%) (Pavlov et al., 2004). Therefore, the haemolytic assay is a useful tool to determine virulence of HPC bacteria.

5.5 Extracellular enzyme production
In combination with the haemolysin assay, extracellular enzyme production was determined. Haemolytic isolates that produced more than one enzyme were classified as potentially pathogenic (Edberg et al., 1996). The results are depicted in Figure 4.4 to 4.6.

Previous studies have investigated the production of extracellular enzymes because of their role in pathogenesis (Pavlov et al., 2004; Horn et al. 2016). Proteinase (62%) and DNase (52%) were produced by most of the isolates in NW-D from 2016 to 2017. The trend is different for GH-T (2017), as chondroitinase was produced by 56% of the isolates, followed by proteinase and DNase (49%). The overall trend in percentage of isolates that produced extracellular enzymes: proteinase > DNase > lipase > chondroitinase > gelatinase > hyaluronidase > lecithinase. Most of these extracellular enzymes are associated with virulence and screening for their presence may, therefore, contribute to determine the pathogenic potential of an organism (Georgescu et al., 2016).

Of the fifty-four (54) isolates that displayed haemolysis, twenty-two (40%) produced more than one enzyme and are thus regarded as potentially pathogenic. As seen in Table 5.1, these potential pathogens were identified by sequencing (as described in Section 3.11). The most abundant genus identified with more than one enzyme was Bacillus (20 isolates). In a study by Bal et al. (2009), large numbers of isolates from Bacillus spp. produced protease and lecithinase. Molva et al. (2009) also isolated large numbers of isolates from Bacillus spp. (specifically Bacillus cereus and Bacillus thuringiensis) that produced protease, DNase and lecithinase. This is in line with the findings of the current study.

The Bacillus spp. found in this study produced all the tested extracellular enzymes. However, this ability was differentially distributed across isolates and water production facilities. Bacillus pumilus was responsible for the production of lipase and proteinase. This is supported by findings of Sangeetha et al. (2007). Bacillus licheniformis also produced lipase, proteinase (Sangeetha et al., 2007) as well as DNase.

In the current study, Bacillus wiedmannii produced three enzymes; hyaluronidase, DNase and proteinase. A recent study of Sherpa et al. (2018) found that Bacillus wiedmannii species could produce amylase, but not proteinase.
*Bacillus cereus* produced all enzymes tested for, except for chondroitinase (Table 5.1). This is supported by Kumari and Sarkar (2014) who investigated the prevalence and characterization of *Bacillus cereus*. Ninety-three percent of the isolates exhibited β-haemolysis. Ninety-six and ninety-seven percent of the haemolytic isolates were capable of producing proteinase and lipase respectively.

*Micrococcus luteus* produced the highest number of extracellular enzymes (5). This is in contrast with a previous study conducted by Pavlov *et al.* (2004), in which *Micrococcus luteus* did not produce any of the extracellular enzymes. However, results from a study by Akbar *et al.* (2014) showed that *M. luteus* could produce proteinase and lipase.

### 5.6 Antibiotic resistance

The resistance, intermediate resistance and susceptibility of selected HPC isolates from water after treatment and raw water were tested in 2016 and 2017 against 12 commonly used antibiotics. In 2016 and 2017, 91 and 51 isolates respectively were tested against the selected antibiotics from NW-D. The antibiotic profiles for each isolate tested can be seen in Appendix A (Tables A1 to A7). It provides the resistance, intermediate resistance and susceptibility of HPC isolates in a specific area.

Table 4.9 shows that the quantities of most isolates resistant to the antibiotics which were tested for is greater in water after treatment and drinking water than source water. The size of the general population of resistant bacteria was as follows: water after treatment > distribution water > source water. This might be a potential risk to human health (Pruden *et al.*, 2006; Becerra-Castro *et al.*, 2015).

There are increased levels of specialized groups of ARB in distribution water when compared to water after treatment and source water. It may indicate that water treatment processes result in antibiotic resistant bacteria surviving the processes (Xi *et al.*, 2009). This could be the results of disinfection processes as previously suggested (Chiao *et al.*, 2014; Shrivastava *et al.*, 2004; Khan *et al.*, 2016), or regrowth within drinking water distribution systems. This suggests that some ARBs present in the source water may survive the drinking water treatment process and manifest in the distribution system (Xi *et al.*, 2009). Furthermore, cross- or co-resistance of disinfectants and ARGs might be the underlying mechanisms responsible for the antibiotic resistance promotion (Shi *et al.*, 2013).
Few of the isolates (13%) for all sampling periods (2016 to 2017) were resistant to erythromycin. This is not in line with previous studies (Ehlers et al., 2003; Biyela et al., 2004) where most of the isolates were resistant to erythromycin. In addition, Islam et al. (2010) also found that most isolates were highly resistant to erythromycin (90%). They also found the highest number were susceptible towards chloramphenicol (80%) and ciprofloxacin (60%). The same trend is also observed in the present study, as many of the isolates were susceptible to chloramphenicol (76%). Many of the isolates from GH-T (2017), were resistant to most antibiotics.

A large portion of the isolates (from all sampling periods) had reduced susceptibility (intermediate resistance) to streptomycin, ciprofloxacin and neomycin which agrees with the results observed by Mulamattathil et al. (2014) and Biyela et al. (2004). They also found the highest resistance towards trimethoprim followed by ß-lactams and tetracycline. This is also observed in the current study, especially in site GH-T (2017), whereby all isolates were resistant to trimethoprim (100%), followed by ß-lactams (ampicillin and cephalothin by 95% and 93% respectively) and oxytetracycline by 78%. Moreover, isolates were also resistant to kanamycin (68%). From these results, it is evident that chloramphenicol, ciprofloxacin and streptomycin were the most effective, because all or a large proportion of the isolates were susceptible to them.

The average MAR index for the isolates of NW-D for the 2016 sampling period was 0.275, with the most prevalent MAR phenotype being AMP-VAN-TM-PEN-G-KF-KAN-OT. In 2017, for the same site (NW-D), results were different and the MAR indices were higher (0.355). MAR indices for both sampling periods were greater than 0.2, indicating a high risk of contamination (Osundiya et al., 2013).

The highest MAR index value of 0.461 was observed among isolates from site GH-T (2017), where the value indicates that the 41 isolates from this sampling period were resistant, on average, to more than four of the antibiotics tested against. The overall MAR phenotype for all isolates was AMP-TM-KF-PEN-G-VAN-OT. This observation raises a concern. The problem with multiple antibiotic resistance is the challenges that individuals (especially those whose immune system is compromised) might encounter if they are infected by these opportunistic pathogens (Pavlov et al., 2004). Moreover, the results of this study on bacterial resistance profiles are consistent with previous studies in other surface and drinking water systems (Pavlov et al., 2004; Biyela et al., 2004; Mulamattathil et al., 2014; Carstens et al., 2014; Piotrowska et al., 2017).
With regards to the presence of antimicrobials and PPCPs in source and drinking water, results obtained in this study showed that these substances enter drinking water production facilities as part of the source water. Only ciprofloxacin and penicillin were detected. Ciprofloxacin was detected in all sampling sites (raw, after treatment and drinking water), while penicillin was only detected in water after treatment and drinking water in all the sampling sites. Ebele et al. (2017) mentioned that the detection of these compounds does not necessarily mean that it is of concern or may cause harm. However, major concerns arise from the detection of chemicals could mean that they present selective pressures for selecting and maintaining resistant bacterial populations.

5.7 Antibiotic resistant genes (ARGs) detected
The presence of ARGs in water is a major public health concern (Pruden et al., 2006). Various studies have reported ARGs in drinking water systems from source to finished water (Shrivastava et al., 2004; Pathak and Gopal, 2008; Xi et al., 2009; Bergeron et al., 2015; Bai et al., 2015).

tetM genes were not identified in any of the MAR isolates for this study even though they are considered the most widely distributed tetracycline resistance determinants (Zhang et al., 2009). However, the findings of the present study were similar to results reported by Adesoji et al. (2015), where none of the isolates from raw and drinking water had the tetM gene present. Santamaria et al. (2011) suggested that tetM genes might be present at low frequency in water, making it difficult to detect by PCR. Agerso et al. (2002), mentioned that the tet(M) gene has not previously been found in the genus Bacillus, but can be introduced into this species in the laboratory. Bacillus spp. were the most isolated HPC bacteria that were surveyed for the presence of tetM genes.

Among the ARGs present, MLS resistance genes were the most prevalent. The ermB gene is the most widespread of macrolide resistance genes, and they have been found in a variety of environments (Berglund, 2015). Fifty percent (50%; 15/30) of the MAR isolates in the present study was positive for this gene. These findings are similar to those of Shi et al. (2013), who also reported the presence of ermB from filtered, disinfection and tap water. Stoll et al. (2012) also found high numbers of isolates from surface water positive for ermB genes. In the present study, these ermB genes were mainly detected in Bacillus species (14 isolates), followed by Shinella with only one isolate. The ermF gene was only found in one isolate from drinking water. The presence of ermF gene has previously been found in higher percentages in bacteria from aquatic environments (Adesoji et al., 2015). Furthermore, bacteria can easily transfer macrolide resistance (encoded by erm genes) as these genes are associated with
plasmids and transposons and can be horizontally transferred (Liu et al., 2007; Roberts, 2004). This might possibly explain the high presence of ermB in the various HPC resistant isolates.

Integrons are known to act as facilitators in the prevalence of ARGs in environment. Based on the results, intI1, although a major mechanism for the acquisition of antibiotic resistance, was not detected in the resistant isolates. This opposes the extensively reported circulation of resistance associated with intI1 in aquatic environmental samples (Ozgumus et al., 2007; Taviani et al., 2008; Xu et al., 2016).

As shown in Table 5.1, 17% of the resistant isolates tested positive for blaTEM. A previous study by Adesoji and Ogunjobi (2016) conducted in Nigeria, showed that among 164 multiple antibiotic resistant (MAR) isolates from raw, treated, and tap water, 61 were carrying bla genes. The most predominant gene family was blaTEM, present in 50 out of 61 PCR-positive isolates. Similar results were reported by Xi et al. (2009) who also detected blaTEM in tap water. Lachmayr et al. (2009) mentioned that blaTEM can be used as an indicator for anthropogenic antibiotic resistance contamination when found in the environment.

In the present study, five (17%) of the MAR isolates tested positive for the detection of the β-lactam ampC gene. Shi et al. (2013) reported the presence of ampC in disinfected and tap water samples. In the current study, the ampC gene occurred only in Bacillus cereus species.

Xi et al. (2009) stated that water distribution systems might serve as reservoirs for the spread of antibiotic resistance. The results highlighted the prevalence of ARGs in chlorinated drinking water, indicating the promotion phenomenon of water treatment and distribution processes on these ARGs. This phenomenon was seen for all the tested resistant genes, especially ermB. This increase of ARGs in drinking water distribution systems was confirmed in various studies (Ramteke et al., 1990; Pathak and Gopal, 2008; Adesoji and Ogunjobi, 2016). This is a concern for water treatment plants.

From the results presented, it was observed that eDNA gave less information with regard to the detection of ARGs in the various samples, as compared with the single isolate approach. However, the ermB and intI1 genes were detected in eDNA. This is interesting as none of the intI1 gene was detected in the MAR isolates. As previously mentioned, there are many anthropogenic activities in the catchment site of NW-D. Integron class 1 genes are known to be present in such environments, and their role in acquiring and disseminating antibiotic
resistance determinants has been reported (Heath et al., 2009; Gillings et al., 2008; Gillings et al., 2015). This factor might possibly explain the presence of this gene in the environment when using eDNA. The presence of \textit{ermB} gene by this approach further emphasises its prevalence in the environment as previously mentioned (Berglund, 2015).

5.8 Identification of HPC bacteria

The initial selection of HPC isolates was based on morphological characteristics of the different colonies on the original plate. Identification of all HPC bacteria from different sampling sites are summarised in Table B1 (in Appendix B). A neighbour-joining phylogenetic tree was constructed using MEGA 7. The phylogenetic tree in Figure 4.12 illustrates the relationship of HPC bacteria species from water production facilities (NW-D and GH-T) with reference sequences from Genbank.

The presence of both Gram-negative and Gram-positive bacteria was confirmed in all the sampling sites. Finally, a total of five different genera were identified (\textit{Bacillus}, \textit{Micrococcus}, \textit{Chryseobacterium}, \textit{Shinella} and \textit{Paenibacillus}). The predominant genera identified were \textit{Bacillus} with 43 isolates. This is supported by a study of Goja (2013) and Khan et al. (2016), where the following bacterial species were identified \textit{Bacillus}, \textit{Micrococcus}, \textit{Paenibacillus}. Farhadkhani et al. (2014) also had \textit{Bacillus} species as a dominant genus in drinking water. These results are similar to what the present study is showing.

Bacteria from genus \textit{Micrococcus} was only resistant to one antibiotic (ampicillin), but showed potential pathogenic properties (production of extracellular enzymes and haemolysis). \textit{Micrococcus} sp. is generally regarded as harmless saprophytes that inhabit or can contaminate the skin, mucosa, and to some extend also the oropharynx. However, they can also be regarded as opportunistic pathogens for the immunocompromised. They have been linked to various infections, including continuous ambulatory peritoneal dialysis peritonitis and bacteremia (Kocur et al., 2006). Cases of infective endocarditis due to \textit{Micrococcus luteus} have been reported in the literature (Miltiadous and Elisaf, 2011).

\textit{Bacillus} sp. are Gram positive, rod-shaped bacteria that were isolated from water distribution system in the present and previous studies. Some of the identified species of \textit{Bacillus} (\textit{B. cereus}, \textit{B. thuringiensis}, \textit{B. toyonensis} and \textit{B. safensis}) in this study are mostly associated with foodborne illness (Miller et al., 2018). \textit{Bacillus cereus} (dominant species in this study) are capable of producing spores known to be resistant to disinfection and environmental stressors (Farhadkhani et al., 2014). These characteristics are potentially why they could survive drinking water purification processes.
*Chryseobacterium* sp. are ubiquitous in nature. They are found in soil, fresh and marine water, and also in chlorine-treated municipal water supplies. *Chryseobacteria* have been reported to cause clinical conditions such as endocarditis, wound infections and skin and soft-tissue infections (Alfouzan *et al*., 2014).

*Shinella* sp. have been isolated from various environmental samples, such as soils, water and active sludge. Members of the genus are Gram-negative and non endospore-forming (Subhash and Lee, 2016). *Shinella* sp. have been reported negative in tests for amylase, protease, lipase, cellulose, xylanase, DNase, gelatinase and urease. *Shinella curvata* have not been associated with any negative health effects (An *et al*., 2006).

*Paenibacillus* sp. are Gram-positive, aerobic or facultative anaerobic rod-shaped, endospore-forming bacteria. They have been isolated from a variety of environments, with many of the species being relevant to humans, animals, plants, and the environment. Furthermore, they are occasionally present as opportunistic infections in humans (Lorentz *et al*., 2006; Grady *et al*., 2016).

**5.9 Phylogenetic analysis**

Sequences with high sequence similarities (97%) were used to construct the neighbour-joining phylogenetic trees (Figures 4.9). The neighbour-joining method is frequently used because of its demonstrated accuracy for smaller data sets and its computational speed (Tamura *et al*., 2004). Neighbour-joining trees have been used in various studies that analysed microbial communities in water treatment plants (Liao *et al*., 2014; Kampfer *et al*., 2016; Santos *et al*., 2017).

In Figure 4.12, the evolutionary relationship of the 16S rRNA gene sequences from treatment plants can be observed. The species from the same genus clustered together based on sequence similarities. Many single clusters formed between the selected bacterial groups. These groupings were supported with strong bootstrap values (above 82%).

A monophyletic clade of the genus *Bacillus* is observed, of which *B. cereus* (F) clustered with *B. anthracis* (E and R), *B. toyonensis* (E14), *B. wiedmannii* (D), *B. thuringiensis* (I) with bootstrap support of 90% and 100%. Similar results were observed by Wang and Ash (2015) that showed a monophyletic clade between *B. anthracis, B. thuringiensis* and *B. cereus*. 
According to Priest et al. (2004), these species (clade A) have revealed extensive genomic similarities and few consistent differences amongst them, thus they form one clade.

These results are in agreement with the results from DNA-DNA hybridization analysis and Multi Locus Enzyme Electrophoresis, which showed high identity amongst B. anthracis, B. cereus, and B. thuringiensis strains (Priest et al., 1994). These species are thus grouped under one clade which is Bacillus cereus sensu lato (Priest et al., 2004).

The Bacillus genus is further divided into two clades; the Bacillus licheniformis (E24) clustered with B. licheniformis (KT719714.1 and KT719487.1) with bootstrap support of 82% and 100%, respectively. Bacillus pumilus (C25) clustered with B. pumilus (MG719573.1) and B. safensis (KR780976.1) with a high Bootstrap support of 99% and 97%, respectively. There are some Bacillus isolates that are closely related to B. pumilus, making it difficult to distinguish them from each other by 16S rRNA gene sequence alone. The B. pumilus group contains 5 species, namely, B. pumilus, B. altitudinis, B. stratosphericus, B. safensis and B. aerophilus. These species are closely identical in 16S rRNA gene sequence, and they share similarity over 99.5% (Liu et al., 2013). B. pumilus and B. safensis form one clade (C) (Figure 4.12). By observation, B. licheniformis, B. safensis and B. pumilus have proved to be a paraphyletic group to species on clade A.

The relationship between B37 (M. Luteus) complex and M. yunnanensis (MH298495.1 and KU925163.1) had 100 % bootstrap support with M. luteus, which indicated that they are members of the Bacillus genus. There was also a high bootstrap support (99%) between F16 complex and P. chitinolyticus and P. agaridevorans. These results are similar with Zhao et al. (2009) who mentioned that the levels of DNA–DNA relatedness supported that of strain M. yunnanensis, thus it is used to represents a novel species of the genus Micrococcus.

A close relationship was observed between C15 (S. curvata) complex and S. Zoogloeoides (KY996858.1) with S. Curvata (MK039092.1 and NR_152074.1) with 100% and 75% bootstrap support. This grouping indicated a positive morphological identification of the species. The relationship between Chryseobacterium sp. (DQ530125.1 and GQ915087.1) and the E18 complex had 100% and 91% bootstrap support respectively, indicating a close relationship.
Chapter 6
Conclusions and Recommendations

6.1 Conclusion
The aim of the study was to determine the prevalence of antibiotic resistant bacteria (ARB) in raw and drinking water and potentially pathogenic bacteria in the drinking water supplied by selected water production facilities (NW-D and GH-T). Samples were collected from 2016-2017 sampling period (for different months). In achieving the aim, the following objectives were completed.

6.1.1 Physico-chemical and microbiological quality in drinking water
The physico-chemical parameters for both production facilities were determined. The majority of the selected physico-chemical parameters were in ranges set by SANS 241 (2015) for drinking water except for turbidity and nitrite in drinking water. Levels of heterotrophic bacteria, total and faecal coliform bacteria in the drinking water were determined. Of particular concern were the considerably high heterotrophic plate count bacteria detected in water after treatment, and the prospects that they may have masked counts of total coliforms and faecal bacteria (Amanidaz et al., 2015). However, the levels of all microbiological parameters were within the SANS 241 (2015) of drinking water.

6.1.2 Isolation and purification of HPC bacteria
R2A agar was successfully employed for the isolation of HPC bacteria from three sampling sites in both production facilities. Hundred and five isolates could be purified for further analysis. The overall results obtained in the present study indicated that the organisms in treated water may have survived the treatment process (Shi et al., 2013). This was observed for all the sampling periods. However, all the results showed that the drinking water is potable and of a very high standard. This is not a surprise as both of the production facilities have constantly achieved the Blue Drop Certification status with compliance of more than 95% (Blue Drop Report, 2014).

6.1.3 Determination of antibiotic resistance patterns and their associated ARGs
The results revealed that a large number of HPC isolates were resistant to some of the common antibiotics in human medicine such as β-lactams (ampicillin, cephalothin and Penicillin G) and trimetroprim. Isolates of Bacillus spp. were more resistant to β-lactams; this genus is known to be capable of producing spores known to be resistant to disinfection and
environmental stressors. Some of the source water isolates showed high susceptibility to ciprofloxacin, streptomycin, neomycin and kanamycin. The increased levels of specialized groups of ARB and ARGs in distribution water when compared to water after treatment and source water, may indicate that water treatment (chlorination) could increase the antibiotic resistance of surviving bacteria (Chiao et al., 2014; Shrivastava et al., 2004; Khan et al., 2016). The situation is more pronounced in GH-T, compared to NW-D. This might indicate that the distribution system is not designed to eliminate these ARB (Bai et al., 2015). Several of the HPC isolates were resistant to multiple antibiotics, with the highest prevalence of resistance to β-lactam antibiotics. The overall MAR phenotype for all isolates was AMP-TM-KF-PEN-G-VAN-OT. ARGs (ermB blatem, ampC and ermF), especially ermB gene was distributed in the water treatment system. This is a concern, considering the number of immuno-compromised in South Africa and particularly in Gauteng and North West Province.

6.1.4 Potential pathogenic features associated with HPC that are MAR
Twenty-two of the fifty-four haemolytic isolates produced more than one enzyme. This indicates that these isolates from the distribution systems were potential pathogens. Two or more extracellular enzymes were produced by Bacillus spp. and Micrococcus. Micrococcus luteus isolate were the most pathogenic as it produced five enzymes from drinking water. Proteinase and DNase were mostly produced by these haemolytic isolates.

6.1.5 Identification of HPC bacteria (16S rRNA gene sequencing)
Five different genera were identified, and Bacillus spp. was the most abundant. Identified bacteria also included several species that had previously been reported as opportunistic pathogens. Studies showed that some strains of bacterial species which form part of the HPC bacteria and can be a source of infections in a person with a compromised immune system. Results such as those presented in this study offer evidence that could be helpful in the identification of future studies and initiatives aimed at improvement of water quality in water production facilities.

6.2 Recommendations

- New methods should be put in place to reduce the accumulation of ARB in the drinking water.

- Antibiotic resistance surveillance can be used as a tool to control the problem of antibiotic resistance and to educate the public on the consequences of misusing antibiotics and also to regulate the usage of drugs in both human and veterinary medicine.
Guidelines should be formulated as they might be helpful for optimal use of antibiotics.

A need for education and awareness programmes for the communities, that teaches about water pollution and/or parameters that might lead to contaminated drinking water. This way, communities can learn to treat water in their own home, by either boiling or safe storage practices.

Whole genome sequencing can be used to get an overview of ARGs associated with Bacillus genus, because a wide range of their species dominated in all sampling sites.

It is also very important that findings from studies such as this one should be circulated to the relevant stakeholders and the affected communities especially the results of ARB.

The collected data for the current study has demonstrated that potentially pathogenic HPC bacteria are present in raw water and end up in the drinking water distribution system. Thus, it can be concluded that the overall aim and the specific objectives of this study were successfully achieved.
References


Molva, C., Sudagidan, M. and Okuklu, B. 2009. Extracellular enzyme production and enterotoxigenic gene profiles of *Bacillus cereus* and *Bacillus thuringiensis* strains isolated from cheese in Turkey. *Food control*, 20:829-834.


Paruch, A.M. and Maehlum, T. 2012. Specific features of Escherichia coli that distinguish it from coliform and thermotolerant coliform bacteria and define it as the most accurate indicator of faecal contamination in the environment. Ecological Indicators, 23:140-142.


### Appendix A

Table A1: Antibiotic susceptibility patterns of the HPC isolates for Plant NW-D (March) from 2016

<table>
<thead>
<tr>
<th>Site</th>
<th>Isolate</th>
<th>Gram reaction</th>
<th>AMP</th>
<th>KF</th>
<th>Chl</th>
<th>CIP</th>
<th>Ery</th>
<th>Kan</th>
<th>Neo</th>
<th>O-T</th>
<th>Pen-G</th>
<th>Strep</th>
<th>TM</th>
<th>Van</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>A1</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>A4</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>A5</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>A6</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>A7</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>A9</td>
<td>+</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>A10</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>R</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>A11</td>
<td>-</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>A12</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A13</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>N/G</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>AT</td>
<td>B17</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>D</td>
<td>F</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>-</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>I</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td></td>
</tr>
</tbody>
</table>

AMP - Ampicillin; KF - Cephalothin; Ery - Erythromycin; Chl - Chloramphenicol; CIP - Ciprofloxacin; Kan - Kanamycin; Neo - Neomycin; Strep - Streptomycin; Van - Vancomycin; Pen-G - Penicillin G; O-T - Oxy-tetracycline; TM - Trimethoprim. (S = Susceptible, R = resistance, I = Intermediate resistant, NG = no growth). D – Distribution. AT- after treated water.
Table A2: Antibiotic susceptibility patterns of the HPC isolates for Plant NW-D (May) from 2016

<table>
<thead>
<tr>
<th>Site</th>
<th>Isolate</th>
<th>Gram reaction</th>
<th>AMP</th>
<th>KF</th>
<th>Chl</th>
<th>CIP</th>
<th>Ery</th>
<th>Kan</th>
<th>Neo</th>
<th>O-T</th>
<th>Pen-G</th>
<th>Strep</th>
<th>TM</th>
<th>Van</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>G1</td>
<td>-</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td>G2</td>
<td>-</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td>G3</td>
<td>-</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
</tr>
<tr>
<td>G5</td>
<td>-</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
</tr>
<tr>
<td>G6</td>
<td>-</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
</tr>
<tr>
<td>G7</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
</tr>
<tr>
<td>G8</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>R</td>
<td>R</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>G9</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>NG</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>I</td>
<td>R</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>G10</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td>G11</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>G12</td>
<td>-</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
</tr>
<tr>
<td>G13</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
</tr>
<tr>
<td>G14</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
</tr>
<tr>
<td>G15</td>
<td>-</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>NG</td>
<td>S</td>
<td>NG</td>
<td>NG</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>G16</td>
<td>-</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>NG</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>G17</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>NG</td>
<td>NG</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
</tr>
<tr>
<td>G18</td>
<td>-</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>G19</td>
<td>-</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>NG</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

AMP- Amoxicillin; KF – Cephalothin; Ery- Erythromycin; Chl - Chloramphenicol ; CIP- Ciprofloxacin; Kan – Kanamycin; Neo- Neomycin ; Strep- Streptomycin; Van- Vancomycin; Pen-G - Penicillin G; O-T – Oxy-tetracycline; TM – Trimethoprim. (S = Susceptible, R = resistance, I = Intermediate resistant, NG – no growth). D – Distribution. AT- after treated water.
<table>
<thead>
<tr>
<th>Site</th>
<th>Isolate</th>
<th>Gram reaction</th>
<th>AMP</th>
<th>KF</th>
<th>Chl</th>
<th>CIP</th>
<th>Ery</th>
<th>Kan</th>
<th>Neo</th>
<th>O-T</th>
<th>Pen-G</th>
<th>Stre</th>
<th>TM</th>
<th>Van</th>
</tr>
</thead>
<tbody>
<tr>
<td>G20</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>NG</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>NG</td>
<td>S</td>
<td>N/A</td>
</tr>
<tr>
<td>G21</td>
<td>+</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>G22</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G23</td>
<td>-</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>I</td>
<td>S</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>G24</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>G26</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>G27</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>G28</td>
<td>-</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>G29</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>AT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>B4</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>B5</td>
<td>-</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>R</td>
<td>S</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>B7</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>B15</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>B33</td>
<td>-</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>B37</td>
<td>+</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>H1</td>
<td>+</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>H2</td>
<td>+</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>H3</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>H4</td>
<td>-</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

AMP- Ampicillin; KF- Cephalothin; Ery- Erythromycin; Chl- Chloramphenicol; CIP- Ciprofloxacin; Kan- Kanamycin; Neo- Neomycin ; Strep- Streptomycin; Van- Vancomycin; Pen-G- Penicillin G; O-T- Oxy-tetracycline; TM- Trimethoprim. (S = Susceptible, R = resistance, I = Intermediate resistant, NG- no growth, NA- not applicable). D- Distribution. AT- after treated water.
<table>
<thead>
<tr>
<th>Site</th>
<th>Isolate</th>
<th>Gram reaction</th>
<th>AMP</th>
<th>KF</th>
<th>Chl</th>
<th>CIP</th>
<th>Ery</th>
<th>Kan</th>
<th>Neo</th>
<th>O-T</th>
<th>Pen-G</th>
<th>Strep</th>
<th>TM</th>
<th>Van</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>H5</td>
<td>+</td>
<td></td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>H6</td>
<td>+</td>
<td></td>
<td>S</td>
<td>N/G</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>N/G</td>
<td>R</td>
<td>N/G</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
</tr>
<tr>
<td>H7</td>
<td>-</td>
<td></td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td>H8</td>
<td>+</td>
<td></td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H9</td>
<td>+</td>
<td></td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>N/G</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>H10</td>
<td>+</td>
<td></td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>H11</td>
<td>+</td>
<td></td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>H12</td>
<td>-</td>
<td></td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
</tr>
<tr>
<td>H13</td>
<td>+</td>
<td></td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>H14</td>
<td>+</td>
<td></td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>H15</td>
<td>+</td>
<td></td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
</tbody>
</table>

AMP- Ampicillin; KF- Cephalothin; Ery- Erythromycin; Chl- Chloramphenicol; CIP- Ciprofloxacin; Kan- Kanamycin; Neo- Neomycin ; Strep- Streptomycin; Van- Vancomycin; Pen-G- Penicillin G; O-T- Oxy-tetracycline; TM- Trimethoprim. (S = Susceptible, R = resistance, I = Intermediate resistant, NG= no growth, NA- not applicable). D- Distribution. AT- after treated water.
Table A3: Antibiotic susceptibility patterns of the HPC isolates for Plant NW-D (August) from 2016

<table>
<thead>
<tr>
<th>Site</th>
<th>Isolate</th>
<th>Gram reaction</th>
<th>AMP</th>
<th>KF</th>
<th>Chl</th>
<th>CIP</th>
<th>Ery</th>
<th>Kan</th>
<th>Neo</th>
<th>O-T</th>
<th>Pen-G</th>
<th>Strep</th>
<th>TM</th>
<th>Van</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>B</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>J</td>
<td>-</td>
<td>N/G</td>
<td>N/G</td>
<td>N/G</td>
<td>N/G</td>
<td>N/G</td>
<td>N/G</td>
<td>N/G</td>
<td>N/G</td>
<td>N/G</td>
<td>N/G</td>
<td>N/G</td>
<td>N/G</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td>AT</td>
<td>L</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>I</td>
<td>N/A</td>
<td>R</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>Z</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>HH</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>N/A</td>
<td>R</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td>D</td>
<td>E</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>D</td>
<td>S</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>EE</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

AMP- Ampicillin; KF- Cephalothin; Ery- Erythromycin; Chl- Chloramphenicol ; CIP- Ciprofloxacin; Kan- Kanamycin; Neo- Neomycin ; Streptomycin; Van- Vancomycin; Pen-G- Penicillin G; O-T- Oxy-tetracycline; TM- Trimethoprim. (S = Susceptible, R = resistance, I = Intermediate resistant, NG- no growth, NA- not applicable). D- Distribution. AT- after treated water.
<table>
<thead>
<tr>
<th>Site</th>
<th>Isolate</th>
<th>Gram reaction</th>
<th>AMP</th>
<th>KF</th>
<th>Chl</th>
<th>CIP</th>
<th>Ery</th>
<th>Kan</th>
<th>Neo</th>
<th>O-T Pen</th>
<th>Strep</th>
<th>TM</th>
<th>Van</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>C1</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C3</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C4</td>
<td>+</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C5</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>N/A</td>
<td>I</td>
<td>N/A</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>C6</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C7</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>N/A</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C8</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C9</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C10</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C11</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C12</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>C27</td>
<td>+</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>C13</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C14</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C15</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C16</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C17</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C18</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C19</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C20</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>C21</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

**Notes:**
- AMP, KF, Chl, CIP, Ery, Kan, Neo, O-T Pen, Strep, TM, and Van indicate the sensitivity of the isolate to respective antibiotics.
- '+' indicates sensitive, '-' indicates resistant, and 'N/A' indicates not applicable or no data available.
<table>
<thead>
<tr>
<th>Site</th>
<th>Isolate</th>
<th>Gram reaction</th>
<th>AMP</th>
<th>KF</th>
<th>Chl</th>
<th>CIP</th>
<th>Ery</th>
<th>Kan</th>
<th>Neo</th>
<th>O-T</th>
<th>Pen-G</th>
<th>Strep</th>
<th>TM</th>
<th>Van</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>E17</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>E18</td>
<td>-</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>N/A S</td>
</tr>
<tr>
<td></td>
<td>E19</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>E20</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>E21</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>N/A</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>E22</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>AT</td>
<td>E23</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>E24</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>E25</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>E26</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
</tr>
</tbody>
</table>

AMP- Ampicillin; KF- Cephalothin; Ery- Erythromycin; Chl- Chloramphenicol; CIP- Ciprofloxacin; Kan- Kanamycin; Neo- Neomycin ; Strep- Streptomycin; Van- Vancomycin; Pen-G- Penicillin G; O-T- Oxy-tetracycline; TM- Trimethoprim. (S = Susceptible, R = resistance, I = Intermediate resistant, NA= not applicable). D- Distribution. AT- after treated water.

Table A5: Antibiotic susceptibility patterns of the HPC isolates for Plant NW-D (October) from 2017
<table>
<thead>
<tr>
<th>Site</th>
<th>Isolate</th>
<th>Gram reaction</th>
<th>AMP</th>
<th>KF</th>
<th>Chl</th>
<th>CIP</th>
<th>Ery</th>
<th>Kan</th>
<th>Neo</th>
<th>O-T</th>
<th>Pen-G</th>
<th>Stre p</th>
<th>TM</th>
<th>Van</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>E1</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>E3</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>E4</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>E5</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>E6</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>E7</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>E8</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>E9</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>E10</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>E11</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>E12</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>I</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>E13</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>N/A</td>
<td>I</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>E14</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>R</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>E15</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>E16</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
</tbody>
</table>

AMP- Ampicillin; KF- Cephalothin; Ery- Erythromycin; Chl- Chloramphenicol ; CIP- Ciprofloxacin; Kan- Kanamycin; Neo- Neomycin ; Strep- Streptomycin; Van- Vancomycin; Pen-G- Penicillin G; O-T- Oxy-tetracycline; TM- Trimethoprim. (S = Susceptible, R = resistance, I = Intermediate resistant, NG- no growth, NA- not applicable). D- Distribution. AT- after treated water.
Table A6: Antibiotic susceptibility patterns of the HPC isolates for Plant GT-H (June) from 2017

<table>
<thead>
<tr>
<th>Site</th>
<th>Isolate</th>
<th>Gram reaction</th>
<th>AMP</th>
<th>KF</th>
<th>Chl</th>
<th>CIP</th>
<th>Ery</th>
<th>Kan</th>
<th>Neo</th>
<th>O-T</th>
<th>Pen-G</th>
<th>Strep</th>
<th>TM</th>
<th>Van</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>D2</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>R</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>D17</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>D3</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>D4</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>D6</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>AT</td>
<td>D7</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>D8</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>D9</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>D10</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>N/A</td>
<td>R</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>D12</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>D13</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>D</td>
<td>D21</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>D22</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>D24</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>D5</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

AMP- Ampicillin; KF- Cephalothin; Ery- Erythromycin; Chl- Chloramphenicol; CIP- Ciprofloxacin; Kan- Kanamycin; Neo- Neomycin; Strep- Streptomycin; Van- Vancomycin; Pen-G- Penicillin G; O-T- Oxy-tetracycline; TM- Trimethoprim. (S = Susceptible, R = resistance, I = Intermediate resistant, NA- not applicable). D- Distribution. AT- after treated water.
Table A7: Antibiotic susceptibility patterns of the HPC isolates for Plant GT-H (November) from 2017

<table>
<thead>
<tr>
<th>Site</th>
<th>Isolate</th>
<th>Gram reaction</th>
<th>AMP</th>
<th>KF</th>
<th>Chl</th>
<th>CIP</th>
<th>Ery</th>
<th>Kan</th>
<th>Neo</th>
<th>O-T Pen</th>
<th>Strep</th>
<th>TM</th>
<th>Van</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>F1</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F5</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>F6</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F7</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F8</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F9</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F11</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F12</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F13</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F15</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F16</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>N/A</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F21</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>AT</td>
<td>F27</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F28</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F29</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F31</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F32</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F34</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F37</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F40</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F44</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
</tr>
</tbody>
</table>

137 | Page
<table>
<thead>
<tr>
<th>Site</th>
<th>Isolate</th>
<th>Gram reaction</th>
<th>AMP</th>
<th>KF</th>
<th>Chl</th>
<th>CIP</th>
<th>Ery</th>
<th>Kan</th>
<th>Neo</th>
<th>O-T</th>
<th>Pen-G</th>
<th>Strep</th>
<th>TM</th>
<th>Van</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>F47</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>F48</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>F49</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>F53</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
<td>S</td>
<td>R</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>F55</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

AMP- Ampicillin; KF- Cephalothin; Ery- Erythromycin; Chl- Chloramphenicol; CIP- Ciprofloxacin; Kan- Kanamycin; Neo- Neomycin; Strep- Streptomycin; Van- Vancomycin; Pen-G- Penicillin G; O-T- Oxy-tetracycline; TM- Trimethoprim. (S = Susceptible, R = resistance, I = Intermediate resistant, NA = not applicable). D- Distribution. AT- after treated water.
### Appendix B

**Table B1**: GenBank identification of the amplified HPC isolates from NW-D (2016-2017)

<table>
<thead>
<tr>
<th>Year (Plant)</th>
<th>Sampling site</th>
<th>Isolate name</th>
<th>GenBank ID</th>
<th>% Identity number</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2016 NW-D</strong></td>
<td>Raw</td>
<td>A9</td>
<td><em>Bacillus cereus</em></td>
<td>87%</td>
<td>KR780449.1</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td><em>Bacillus thuringiensis</em></td>
<td>95%</td>
<td>KF818643.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td><em>Bacillus wiedmannii</em></td>
<td>99%</td>
<td>MG890254.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td><em>Bacillus cereus</em></td>
<td>94%</td>
<td>KX641888.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td><em>Bacillus cereus</em></td>
<td>95%</td>
<td>EU982473.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>After treatment</td>
<td>B7</td>
<td><em>Bacillus cereus</em></td>
<td>100%</td>
<td>CP026678.1</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td><em>Bacillus cereus</em></td>
<td>98%</td>
<td>KP813644.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z</td>
<td><em>Bacillus cereus</em></td>
<td>97%</td>
<td>KR780449.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td><em>Bacillus cereus</em></td>
<td>97%</td>
<td>KM596528.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distribution</td>
<td>I</td>
<td><em>Bacillus thuringiensis</em></td>
<td>97%</td>
<td>HF545006.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td><em>Bacillus thuringiensis</em></td>
<td>99%</td>
<td>KT714039.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G</td>
<td><em>Bacillus cereus</em></td>
<td>97%</td>
<td>HM179550.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y</td>
<td><em>Bacillus cereus</em></td>
<td>95%</td>
<td>KJ534420.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td><em>Bacillus cereus</em></td>
<td>98%</td>
<td>LC215052.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GG</td>
<td><em>Bacillus toyonensis</em></td>
<td>98%</td>
<td>KX881447.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td><em>Bacillus cereus</em></td>
<td>96%</td>
<td>CPO15589.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td><em>Bacillus anthracis</em></td>
<td>91%</td>
<td>KF875584.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td><em>Bacillus cereus</em></td>
<td>98%</td>
<td>HQ238566.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EE</td>
<td><em>Bacillus cereus</em></td>
<td>96%</td>
<td>CP020937.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td><em>Bacillus thuringiensis</em></td>
<td>90%</td>
<td>CPO15150.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B37</td>
<td><em>Micrococcus luteus</em></td>
<td>99%</td>
<td>MG597316.1</td>
</tr>
<tr>
<td><strong>2017 NW-D</strong></td>
<td>Raw</td>
<td>C3</td>
<td><em>Bacillus thuringiensis</em></td>
<td>98%</td>
<td>KF971833.1</td>
</tr>
<tr>
<td></td>
<td>C4</td>
<td><em>Bacillus cereus</em></td>
<td>99%</td>
<td>KC519400.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E18</td>
<td><em>Chryseobacterium sp.</em></td>
<td>98%</td>
<td>JF899297.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>After treatment</td>
<td>E23</td>
<td><em>Bacillus cereus</em></td>
<td>100%</td>
<td>KJ812448.1</td>
</tr>
<tr>
<td>Year (Plant)</td>
<td>Sampling site</td>
<td>Isolate name</td>
<td>GenBank ID</td>
<td>% Identity number</td>
<td>Accession number</td>
</tr>
<tr>
<td>-------------</td>
<td>---------------</td>
<td>--------------</td>
<td>------------</td>
<td>-------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>After treatment</td>
<td>E24</td>
<td><em>Bacillus licheniformis</em></td>
<td>MF321846.1</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>Distribution</td>
<td>C15</td>
<td><em>Shinella curvata</em></td>
<td>LT545981.1</td>
<td>98%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C17</td>
<td><em>Bacillus cereus</em></td>
<td>KP992166.1</td>
<td>98%</td>
<td></td>
</tr>
<tr>
<td>2017 (NW-D)</td>
<td>Distribution</td>
<td>C20</td>
<td><em>Bacillus cereus</em></td>
<td>KU877653.1</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td>C22</td>
<td><em>Bacillus safensis</em></td>
<td>KR780976.1</td>
<td>98%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C25</td>
<td><em>Bacillus pumilus</em></td>
<td>JX680128.1</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D13</td>
<td><em>Bacillus cereus</em></td>
<td>KF295678.1</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E1</td>
<td><em>Bacillus cereus</em></td>
<td>MF953999.1</td>
<td>98%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td><em>Bacillus thuringiensis</em></td>
<td>KP997272.1</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E3</td>
<td><em>Bacillus cereus</em></td>
<td>KF731616.1</td>
<td>95%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E7</td>
<td><em>Bacillus thuringiensis</em></td>
<td>CP013274.1</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E14</td>
<td><em>Bacillus toyonensis</em></td>
<td>MG737481.1</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>2017 (GH-T)</td>
<td>Raw</td>
<td>D3</td>
<td><em>Bacillus cereus</em></td>
<td>KX495491.1</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td>F16</td>
<td><em>Paenibacillus chitinolyticus</em></td>
<td>NR_113797.1</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F13</td>
<td><em>Bacillus cereus</em></td>
<td>EF535591.1</td>
<td>98%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td><em>Bacillus thuringiensis</em></td>
<td>CP016589.1</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F48</td>
<td><em>Bacillus cereus</em></td>
<td>CP017060.1</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>After treatment</td>
<td>D7</td>
<td><em>Bacillus thuringiensis</em></td>
<td>CP016588.1</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D8</td>
<td><em>Bacillus cereus</em></td>
<td>KU551240.1</td>
<td>*99%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D13</td>
<td><em>Bacillus thuringiensis</em></td>
<td>CP016588.1</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D21</td>
<td><em>Bacillus thuringiensis</em></td>
<td>KC414686.1</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D22</td>
<td><em>Bacillus cereus</em></td>
<td>MG407612.1</td>
<td>96%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F29</td>
<td><em>Bacillus thuringiensis</em></td>
<td>KT714050.1</td>
<td>99%</td>
<td></td>
</tr>
</tbody>
</table>