

Antibiotic resistant bacteria and -genes in raw water, and the implications for drinking water production

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ABSTRACT

The introduction of antibiotic treatment in the 1940s was a revolutionary breakthrough in medicine. However, the wide and inappropriate use of antibiotics ever since have created the perfect conditions for increasing the selective pressures on bacteria to develop resistance against antibiotics. Antibiotic resistance truly threatens the success of modern medicine and finding such antibiotic resistant bacteria in drinking water distribution systems (DWDS) in South Africa has recently been demonstrated. The aim of this study was thus to determine the general drinking water quality and antibiotic resistance profiles of isolated heterotrophic bacteria in two DWDS in South Africa. Samples were collected in 2016 and 2017 and included raw water, treated water and sampling points in the distribution system. Selected physico-chemical properties that must adhere to the South African National Standards (SANS 241) for drinking were measured for each sample. Heterotrophic plate count (HPC) bacteria were enumerated on R2A Agar using standard methods. These were isolated by a successive streak plate method to purify the isolates. The isolates were identified by sequencing the 16S rRNA gene. The Kirby Bauer disc diffusion method was used to determine antibiotic susceptibility of the isolates. Conventional PCRs were run in order to detect antibiotic resistance genes in the isolates such as *ampC*, *TEM1*, *ermB*, *ermF*, *tetM* and *int1*. The presence of antibiotics within water samples were determined. The potential pathogenicity of isolates was determined by testing for the production of haemolysins, proteinase, lecithinase, lipase and DNase. Environmental DNA (eDNA) was isolated from raw water, treated water and distribution water then conventional PCR was used to detect presence of the same six antibiotic resistance genes. Six *Bacillus* isolates isolated from the raw water, treated water and drinking water were used for whole genome sequencing. The physico-chemical properties mostly complied to the SANS 241 (2015) except for the turbidity levels that in some cases reached 3.30 mg/L and 3.85 mg/L at the both drinking water treatment plants (DWTP) respectively. Also the nitrites showed high levels that reached levels of 5.00 mg/L and 10.50 mg/L for both treatment plants respectively. Identification of HPC isolates showed that *Bacillus spp.* represented 35% and 31% of HPC bacteria in the raw water at both DWTP respectively. In the drinking water *Bacillus spp.* represented 73% and 39% of HPC bacteria at both DWTP respectively. High resistance patterns were observed for

ampicillin, cephalothin, penicillin and trimethoprim. The average number of isolates showing resistance against these four antibiotics in the raw water, treated water and distribution water ranged from 47% - 100%. A clear trend was observed that the average number of isolates resistant to antibiotics increased from the raw water into the drinking water (treated water and distribution water). The average multiple antibiotic resistance (MAR) index showed values higher than 0.2 at both DWTP in all the samples indicating antibiotic pollution. Only one isolate amplified the *ampC* gene whereas the rest of the antibiotic resistant genes were not detected. For eDNA, only the *int1* gene was detected in the raw water and distribution water at one of the DWTP. For both DWTP more than 76% of isolates tested positive for haemolysins, proteinase and lecithinase whereas between 19% and 50% of the isolates tested positive for lipase and DNase. Nine isolates produced all the enzymes. The high levels of turbidity and nitrites might be an indication of biofilm formation in the distribution system or ineffective filtering of water. A clear trend can be observed where selective pressure takes place from the raw water to the drinking water regarding heterotrophic bacteria and antibiotic resistance. Together with that we prove that heterotrophic bacteria can produce enzymes that can make them potentially pathogenic. Antibiotic resistance is a phenomenon that presents itself even within drinking water of which we are dependent on a daily basis. Together with its pathogenicity potential it can pose health risks to the immunocompromised, elderly as well as young children.

Keywords: Antibiotic resistance, heterotrophic bacteria, general drinking water quality, potential pathogenicity, antibiotic resistance genes, 16S rRNA gene, MAR index, conventional PCR, *Bacillus spp.*

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Water is the most fundamental resource we have on our planet. It is a molecule that has sustained complex life for approximately 3.8 billion years. The structure of a water molecule is very unique which permits it to be the only substance that occurs in the liquid, gas and solid phase. This allows it to be a dynamic entity that can circulate between the soil, air and oceans which enables for biological, physical and chemical interaction on a very large scale (Van As *et al.*, 2012). Therefore, water related research requires the recognition, appreciation as well as the understanding of processes at environmental interfaces and improved connections between biological, physical, and chemical processes. Such an integrated perspective and understanding will allow for the advancement in our ability to forecast, plan and prevent social issues such as human health, economic prosperity, environmental quality, global warming, over population and sustainable development (Lin, 2010; Tempelhoff, 2011).

Micro-organisms maintain the biogeochemical and elemental cycles on this planet, they produce important components of the atmosphere, soil and water, and they represent a large portion of life's genetic diversity. This planet indeed belongs to the micro-organisms and has been so for billions of years. They are everywhere, including in our water sources and drinking water (Whitman *et al.*, 1998; Kowalchuk *et al.*, 2008). According to Whitman *et al.* (1998), there are approximately a million bacterial cells in one millilitre of fresh water which underlines the importance of water management and the production of safe drinking water.

The fact that we depend on these micro-organisms, does not mean that they are not dangerous - in fact, we are in serious trouble. The introduction of antibiotic treatment in the 1940s was a revolutionary breakthrough in medicine (Madigan, 2012). However, only 80 years later, bacteria have developed resistance to most of these antibiotic treatments (DoH, 2014). The wide and inappropriate use of antibiotics have increased the selective pressure for resistant bacteria and when these resistant bacteria are pathogenic, they become untreatable (Madigan, 2012).

By assessing the antibiotic resistant bacteria (ARBs) and antibiotic resistant genes (ARGs) found in our water sources and drinking water, we can start to gather information and try to understand the dynamics, function and structure of this global crisis. Physico-chemical analysis is also essential because of its effects on human health when safety limits are conceded and the direct influence they have on the bacterial communities found in water (Obi *et al.*, 2004).

Increasingly, governments around the world are beginning to pay attention to antibiotic resistance that is so serious it threatens the achievements of modern medicine (DoH, 2014). This is because we have reached a post-antibiotic era in which multidrug resistant bacterial infections are becoming increasingly common and difficult to treat (Mendelson, 2015; WHO, 2014). The management and surveillance of antimicrobial resistance is of critical importance. However, South Africa has not yet effectively implemented such systems into policies although there are a few surveillance activities that participate in the understanding of infections and antimicrobial resistance (DoH, 2014; Suleman and Meyer, 2012). With water being a fundamental resource to all living organisms for billions of years, anthropogenic activities are destroying its value and sustainability in a very short time. This means that water can become our enemy and greatest threat.

1.1. Research aim and objectives

The aim of this study was to determine the antibiotic resistance profiles of bacteria and to correlate the findings with antibiotic resistant genes found in the drinking water of two drinking water production facilities.

The objectives of this study were to:

- i. analyse the selected physico-chemical properties of drinking water
- ii. identify the HPC bacteria
- iii. determine the potential pathogenicity of isolated bacteria
- iv. determine the antibiotic resistance profiles of HPC bacteria and detect the presence of antibiotic resistance genes (ARGs)
- v. do whole genome sequencing on *Bacillus* species

1.2. A perspective on sustainability

The concept of sustainability can be a difficult term to define due to the complex world we live in. There are approximately three hundred definitions and interpretations of “sustainability” and “sustainable development”, however the emphasis can be laid on the fact that we need to meet fundamental human needs while we preserve the life-support systems of planet Earth (Chang *et al.*, 2017). The United Nations in 1997 gave the following definition regarding sustainability: “Development is a multidimensional undertaking to achieve a higher quality of life for all people. Economic development, social development and environmental protection are interdependent and mutually reinforcing components of sustainable development”.

1.3. A perspective on water and sustainability

The Sustainable Development Goals (SDGs) are a set of 17 objectives set by the Nations General Assembly in 2015 which aimed to eradicate poverty and hunger, improve health and education, transform cities to be more sustainable, combat climate change, and protect the oceans and forests. Goal 6 of the SDGs concerns the matter for water and sanitation where access to water and sanitation facilities matters to every part of human dignity. Water contributes to the improvement of social well-being and inclusive growth that will affect the lives of billions of people (UNDP, 2016). Our understanding of water related challenges lie with water studies as well as the integration of interdisciplinary research where politics, economics, engineering, philosophy and of course science are accommodated (Tempelhoff, 2011; Funke *et al.*, 2014; Kohler, 2016).

1.4. Water in South Africa

South Africa is a water-scarce country (Van As *et al.*, 2012). The country receives approximately an average of 450 mm annual rainfall compared to the world average of 860 mm (DEA, 2012). According to Sershen *et al.* (2016), the country has built many dam systems but the water resources within these systems are threatened by the presence of alien plant species, mining activities, effluent discharge of industries, high evaporative rates and plenty other factors. The river ecosystems vary from sub-tropical

in the north-east to semi-arid and arid in the interior, and cool temperate rivers of the Fynbos biome. The diversity of rivers in South Africa can be seen by the 223 river ecosystem types that have been identified based on the geology, soil, vegetation, climate, flow, and the slope of the river channel (DEA, 2012).

The country is also susceptible to periodic and sometimes long lasting droughts as can be seen in the Eastern- and Western Cape currently (DWS, 2018). The surface water quality in most parts of the country is threatened by industries that produce chemical waste; mines that introduce metals to these sources; wastewater treatment plants that discharge untreated effluents into surface water bodies that increases the levels of nutrients, phosphates, coliforms; and agricultural activities that use excessive amounts of pesticides, herbicides and fertilizers that also flow into the surface waters through runoff (DEA, 2012). It is therefore vital that the water we do have to our usage, we utilize and manage effectively so that there can be a balance between resource sustainability and sustainable economic growth (Kohler, 2016).

1.5. Brief historic perspective on drinking water production

Our technical understanding of water safety is more sophisticated than ever before. From the consumer's point of view, the water should look attractive which means that it should be clear, free from obvious solid matter and usually of low colour (Lewis, 1980; WHO, 2017).

Our conception of safety evolves over time and across cultures, informed by a society's understanding of disease, technological capabilities and aversion to risk (Salzman, 2012). Water have already been subjected to some forms of filtration approximately 2000 - 3000 years BC as can be seen from Sanskrit writings in Egypt (Lewis, 1980; Schutte, 2006; Salzman, 2012). Although boiling water has been an ancient but only partly effective way of treating water, the most common technology to clarify water was slow sand filtration which gained popularity when the first municipal plant was built in Paisley, Scotland in 1832. However, with the work of Robert Koch and Dr John Snow in the 19th century on cholera epidemics, they showed empirically that the disease was transmitted through contaminated water that was not filtered and treated sufficiently. Robert Koch realised that filtration applied to remove suspended

matter from water and render it a more attractive appearance, also had a beneficial effect in reducing the number bacteria in the water (Lewis, 1980; Schutte, 2006).

But the most significant development in drinking water treatment occurred at the turn of the twentieth century, with the realization that the addition of chlorine to water would kill most of the microorganisms (Salzman, 2005, 2012). Chlorinating drinking water supplies now seems an obvious decision. At the time however, it was highly controversial. Adding a chemical to water to make it safer had never been done before on a large scale. It was in 1902, Middelkerke, Belgium, where the first chlorine disinfectant system was installed. In 1908, Jersey City in the United States, provided chlorinated drinking water to an entire city. Chlorination was easy to apply, inexpensive, and persistent in the drinking water. From here water departments and commissions in the United States established the first drinking water standards in 1913. By 1941, 85% of the United States' more than five thousand water treatment systems chlorinated their drinking water. The ability to chlorinate drinking water changed the approach and perspective of consumers. Now municipal drinking water became "modern". Not only did chlorination add a commodity value to drinking water, more importantly it improved public health as waterborne diseases had been neutered. In the early parts of the 20th century, our knowledge on coagulation-flocculation, sedimentation and filtration as basic water treatment processes further improved and enhanced the production of potable drinking water (Schutte, 2006; Salzman, 2012).

1.6. Conventional water treatment

Conventional treatment normally refers to the treatment of surface water and ground water that undergo a series of processes that aims to remove suspended and colloidal matter from the water, disinfects the water and then stabilizing the water chemically before distributing it to customers. Methods for removing suspended and colloidal matter from the water include the coagulation-flocculation process where small colloidal particles are destabilized and form aggregates that settle out of suspension through the addition of chemicals (Figure 1). In order to optimize the formation and growth of aggregates and flocs, the flow-rate and velocity of the water needs to be controlled. This will increase the probability for the colloidal particles and aggregates to collide more often (Schutte, 2006).

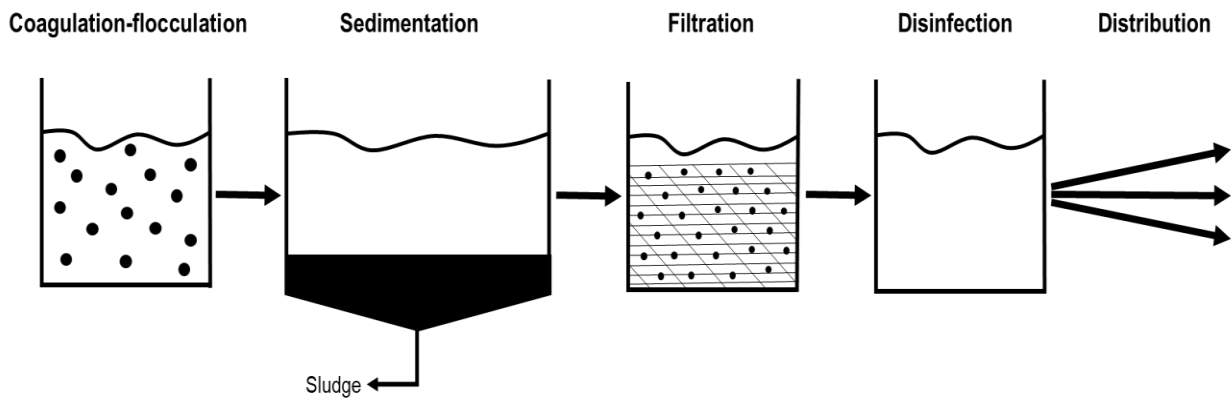


Figure 1: Demonstration of a conventional treatment process.

Sedimentation is the next process in which the aggregates that have been formed settle out of suspension to the bottom of the sedimentation tank through gravitation. The flocs collect as sludge which needs to be removed regularly. The clean water leaves the sedimentation tank at the top. Sedimentation is suitable for flocs that form from clay and silt as it settles readily to the bottom of the tank (Schutte, 2006).

Sand filtration is a simple process where water is allowed to filter through a layer of sand and the remaining flocculated particles are removed by the sand grains. However, there are two types of sand filtration processes: rapid gravity sand filtration and slow sand filtration. With rapid gravity sand filtration, the filters can be open and exposed to the atmosphere. Water will filter downward through the sand by means of gravity. Some sand filters are not open to the atmosphere and would operate under pressure. These filters are usually used in package treatment plants. Slow sand filtration has a slow rate of filtration and can be employed as stand-alone treatment process (Schutte, 2006).

Although large amounts of bacteria and microorganisms are removed from the water through the filtration process, disinfection is still necessary to remove remaining microorganisms and prevent water-borne diseases from spreading. Disinfection entails the addition of a chemical agent to the water with sufficient contact time between the water and the disinfectant. The most commonly used disinfectant is chlorine gas. But other disinfectants can include ozone, chlorine dioxide, calcium hypochlorite (HTH), sodium hypochlorite (bleach) and monochloramine (Schutte,

2006). However, other than chemical disinfection, physical disinfection can include either irradiation with UV-light or the boiling of the water.

Finally, the water that leaves the treatment plant should be chemically stable. The chemical stability of the water can affect the tendency of the water to become corrosive or to form chemical scale in pipes and fixtures. The stabilization of the water therefore involves the addition of chemical agents that will make the water supersaturated with calcium carbonate. A very thin layer of calcium carbonate will precipitate onto the surface of the pipes and therefore protect it against corrosion (Schutte, 2006).

1.7. Drinking water in South Africa and the North-West Province

Access to potable, reliable and safe drinking water is a basic human right and essential to a consumers' health. The South African National Standard (SANS) 241 Drinking Water Specification is a guideline from 2015 to which the drinking water quality must comply to ensure that the water does not pose any health risk over a lifetime of consumption or between life stages (babies and infants, the immunocompromised and elderly) (DWAF, 2005; Hodgson and Manus, 2006). According to Hodgson and Manus (2006), incidences of poor drinking quality and a lack in compliance to SANS 241 (2015) can be attributed to the lack of understanding by the water service authority (WSA) regarding the requirements for effective drinking water quality management. Furthermore, inadequate monitoring of drinking water services, WSA institution capacity (funding, staffing, education, experience, expertise), and a lack of intervention to address poor drinking water quality outcomes (DWAF, 2005; Hodgson and Manus, 2006). The Blue Drop Certification Program is a legislative requirement that was initiated in 2008 by the Department of Water and Environmental Affairs (DWEA). The certification process judged municipalities' water supply systems on a criterion such as the skills levels of process controllers, operation and management, operational and compliance monitoring, and prove that this information and data are used to improve maintenance and processing. This program also helps to provide public awareness on drinking water quality and the standard of drinking water that they receive (van Vuuren, 2009; Foulds, 2014; Sershen *et al.*, 2016).

The responsibility of drinking water distribution lies directly or indirectly with municipality owned enterprises or water service authorities (WSA); or with private companies. South Africa has 169 WSAs that include 15 government owned water boards and need to provide drinking water to 231 municipalities. Government owned water boards operate dams, bulk water supply infrastructure, retail infrastructure and wastewater systems. Municipalities or WSAs would buy produced drinking water from these government-owned water boards or private companies and sell it to consumers. The three largest water boards include Rand Water (Gauteng), Umgeni Water (Kwazulu Natal) and Overberg Water (Western Cape) (DWS, 2014).

1.8. Physico-chemical properties of drinking water

The physico-chemical properties of drinking water can affect the appearance, colour, odour and safety of the water. Based on this criteria, the consumers will evaluate the quality and acceptability of the water (WHO, 2011). Although the methodology of physico-chemical assessments have limitations (Dallas and Day, 2004), it certainly gives us an overview of the condition of the water throughout a period of time.

1.8.1. Physical properties

1.8.1.1. Temperature

Water temperature can have an influence on the treatment and evaluation of water supplies, which is important for the health of all consumers (De Zuane, 1997). Also, water temperature influences the physical, chemical and biological processes in water. A rise in temperature generally increases the chemical reactions, metabolic rates of microorganisms, growth rates, turbidity and depletion of oxygen (Chapman and Kimstach, 1996). This can support the formation and re-growth of bacteria and particularly opportunistic pathogens and form biofilms in the distribution system (Wingender and Flemming, 2011). Drinking water quality is also dependant on aesthetic properties. Therefore, increased temperatures can also impact the odour, taste and colour of water (UNICEF, 2008). But according to De Zuane (1997), the health risks associated with temperature are insignificant.

1.8.1.2. pH

"The pH is a measure of the acid balance of a solution and dependant on the hydrogen ion concentration" (Chapman and Kimstach, 1996). Therefore, at a given temperature, the pH designate that a solution is acidic or basic and is controlled by the chemical and biological processes in that solution. It is important that the pH of water entering the distribution systems, be managed to reduce the corrosion effects on water mains and pipes in household water supply systems (WHO, 2011). Although the pH doesn't have a direct impact on consumers and doesn't pose any health risks, it plays an important role in the disinfection of the water during treatment. In order for successful disinfection by the use of chlorine, the pH must be less than 8. The optimum pH for household use will vary according to the type and construction of the distribution system and the composition of the water itself. Therefore, a pH-range of ≥ 5 to ≤ 9.7 is recommended by SANS 241 (2015).

1.8.1.3. Total dissolved solids (TDS)

TDS refers to the total amount of dissolved material which can be organic and inorganic, or ionized and unionized in a water sample (Dallas and Day, 2004). TDS levels in drinking water less than 600 mg/L is generally considered to be good and palatable. TDS levels higher than 1000 mg/L makes drinking water unpalatable (UNICEF, 2008). According to SANS 241 (2015), the standard limit is ≤ 1200 mg/L.

1.8.1.4. Salinity

As with electrical conductivity, salinity is also a principle component of TDS as it forms part of the dissolved material in the water sample. An increase in either sodium or chloride can produce an unpleasantly salty taste when concentrations exceed 200-300 mg/L (UNICEF, 2008). Although hypertension patients can be sensitive for sodium, no standardized value have been allocated to sodium or chloride (UNICEF, 2008).

1.8.1.5. Turbidity

Suspended particles in water cause turbidity. The ineffective treatment and filtration of water can attribute to increased turbidity. Furthermore, the mobilization of sediments,

minerals or biomass within the distribution system, also increases the turbidity (UNICEF, 2008). Turbidity affects the treatment of drinking water by increasing the demand for chlorination and it can stimulate growth of bacteria. This is because pathogens can be shielded from disinfectants with increased suspended particles (UNICEF, 2008; WHO, 2011). Therefore, effective disinfection of pathogens in water, requires the turbidity to be less than 1 NTU (SANS 241: 2015).

1.8.2. Chemical properties

1.8.2.1. Free chlorine

Chlorine is most commonly used as the disinfectant of drinking water (UNICEF, 2008). When dosed correctly, the chlorine leaves a residual or free chlorine in the water that serves as protection against any added contamination after treatment, in the distribution system and in the reservoirs where water is stored (UNICEF, 2008). The effectiveness of chemical treatment such as chlorine, depend on the dosage, contact time, temperature and pH of the water (WHO, 2003). A residual concentration of free chlorine should be ≥ 0.5 mg/L after 30 min of contact time at $\text{pH} < 8.0$. SANS 241 (2015) has set a maximum guideline value of ≤ 5 mg/L for free chlorine which is well above the taste and odour threshold for most consumers (UNICEF, 2008). Such high dosage is to ensure that the free chlorine concentrations at the point of delivery, are 0.2 mg/L (UNICEF, 2008).

1.8.2.2. Phosphorus

Phosphorus does not pose any threats to human health. The SANS 241 (2015) also does not have any standards established for phosphorus concentrations in drinking water (Dissmeyer, 2000). However, phosphorus can affect the colour and odour of the water. It is also an indication of organic pollution as phosphorus is an important source of nutrient for photosynthetic organisms. In freshwaters, phosphorus often is the limiting nutrient and the main source of eutrophication. Such excessive growth of algae and aquatic vegetation, can cause the formation of biofilms in the distribution systems and deteriorate municipal supplies (Dissmeyer, 2000).

1.8.2.3. Nitrites and Nitrates

The different forms of nitrogen serve as nutrients for plants and microorganisms and are essential for their growth and reproduction (Dallas and Day, 2004; UNICEF, 2008). This is because they convert inorganic nitrogen to organic forms, which include nitrate (NO_3^-) and nitrites (NO_2^-) and form part of the complex nitrogen cycle (Chapman and Kimstach, 1996). Nitrates are the products of the oxidation process of organic nitrogen (Dallas and Day, 2004). Nitrates can often be converted to nitrites in anaerobic conditions, and nitrites can easily be oxidised to nitrates (Chapman and Kimstach, 1996; UNICEF, 2008). Without any anthropogenic activities, nitrates and nitrites are seldom abundant in the natural environment. However, most of the time, nitrates and nitrites are found in large concentrations in water sources, and this is due to the disposal of human and animal wastes, the use of fertilizers and pesticides, the effluents of municipal and industrial wastewater; all of which eventually run off into our water sources (Chapman and Kimstach, 1996; Dallas and Day, 2004). According to Dallas and Day (2004) and WHO (2012), the health burden from nitrates and nitrites are often considered to be insignificant. The SANS 241 (2015) standard for nitrates and nitrites are ≤ 11 mg/L and ≤ 0.9 mg/L respectively.

1.8.2.4. Sulphides and Sulphates

Sulphur is an important compound of proteins (Dallas and Day, 2004) and plays important roles in biological processes of organisms. Sulphur largely occurs in the form of sulphate (SO_4^{2-}) in the environment, and is often used by bacteria as a source to obtain oxygen and form sulphide compounds (Chapman and Kimstach, 1996; Dallas and Day, 2004). Normally sulphate concentrations are lower than those of bicarbonates and chloride ions and naturally enter water bodies through the breakdown of sedimentary rocks, sulphate minerals or through atmospheric deposition. However, industrial effluents and mines; and acid rain contribute hugely to the addition of high concentrations of sulphates in surface waters (Chapman and Kimstach, 1996). Concentrations above 400 mg/L, make water unpleasant to drink. The SANS 241 (2015) standard for sulphates is ≤ 500 mg/L. Sulphide (S^{2-}) enters water bodies through the decomposition of sulphurous minerals, anaerobic decay of organic substances by bacteria and the presence of sewage. Under aerobic

conditions, sulphide can easily be converted to sulphur and sulphates. No standard for sulphides is set by SANS 241.

1.8.2.5. Chemical oxygen demand (COD)

The measurement of the COD is equivalent to and an indication of the amount of organic and inorganic material present in the water, which are susceptible to oxidization. This measurement is an efficient way to rapidly assess organic and inorganic pollution from industrial wastes and effluents (Chapman and Kimstach, 1996). Low concentrations of COD can range from between 20 mg/L O₂ to 200 mg/L O₂ in natural conditions. However, high concentrations of organic effluents can rise from 100 mg/L O₂ to 60 000 mg/L O₂ (Chapman and Kimstach, 1996). There is no SANS 241 standard set for COD.

1.9. Microorganisms in drinking water

The potential for drinking water to transport and facilitate microbiological pathogens to all consumers, is a reality and has been shown through several illnesses and waterborne disease outbreaks (OECD and WHO, 2003). The most commonly used indicator organisms are total coliforms, faecal coliforms and *Escherichia coli*. Total coliforms occur in the gut of animals including humans and is therefore suitable indicator organisms. However, they also occur widely in the environment and is not necessarily an indication of human pollution. Faecal coliforms and *E. coli* are subsets of total coliforms and therefore are better indicators for recent faecal pollution (Schutte, 2006). Although the faecal derived coliforms and *E. coli* have several drawbacks, they have been very effective and therefore have been adopted in all drinking water quality standards (OECD and WHO, 2003, 2005). Water intended for the human use and consumption must be free of any faecal indicator organisms (WHO, 2017).

1.10. Heterotrophic plate counts (HPCs)

Heterotrophic bacteria include all bacteria that use organic nutrients for growth (Allen *et al.*, 2004; WHO, 2003). These bacteria are universally present in all types of water systems. Heterotrophic plate count (HPC) bacteria represent microbes that have been isolated from water by a specific method. The number of HPC bacteria in drinking

water varies and depend on a wide range of variables such as the quality of the water source, the physico-chemical properties, the type of water treatment, the age and condition of the distribution system and the HPC method used such as media composition, time of incubation, temperature incubation and medium inoculation (Allen *et al.*, 2004). The SANS 241 (2015) standard for HPC levels in drinking water is ≤ 1000 CFU/mL. However, important to note that only a fraction of the total bacterial population in a sample can be grown and isolated (Allen *et al.*, 2004; WHO, 2003). R2A is a media most often used for heterotrophic bacteria found in aquatic systems because of their low-nutrient and low-ionic strength which is more suitable for water-based lifestyles. Incubation at low temperatures between 20°C and 28°C and incubation times of 5 - 7 days are also more favourable for the growth of water-based bacteria.

HPC values in drinking water do not correlate directly to health risks (Allen *et al.*, 2004). Some pathogens can be isolated from drinking water by HPC methods, but most are not human pathogens (Bartram *et al.*, 2004). Aesthetic quality is also very important, as increases in HPC bacteria can undermine the taste and odour and may indicate the presence of nutrients and biofilms in the drinking water (Bartram *et al.*, 2004; WHO 2003). An increase in HPC bacteria concentrations after treatment can indicate a problem with the treatment process within the plant itself or a change in the quality of the source water being treated (WHO, 2003).

1.11. Potential pathogenicity of HPC bacteria

Bacteria colonise the drinking water distribution systems and in general, these HPC bacteria are considered to be harmless to human health which has its merit arguments (WHO, 2003; Allen *et al.*, 2004; Vaz-moreira *et al.*, 2014). However, Pavlov *et al.* (2004), indicates that epidemiological studies have been conducted that suggest the potential health risks associated with HPC bacteria in drinking water. These bacteria can potentially produce virulence factors that would make them opportunistic pathogens. It is the young children and the elderly with weak and underdeveloped immune systems as well as the immunocompromised patients that are at risk of HPC infections (Pavlov *et al.*, 2004). Another study done by Horn *et al.* (2016), on drinking water boreholes, found that several HPC isolates were alpha- or beta-haemolytic,

produced two or more extracellular enzymes, and showed resistance against selected antibiotics. Antibiotic resistance in HPC bacteria was also demonstrated in a study done by Carstens *et al.*, (2014).

1.12. Extracellular enzymes

There are several extracellular enzymes that can be used as indicators for potential pathogenicity in HPCs (Tropeano *et al.*, 2014). These enzymes include haemolysin, DNase, proteinase and lipase. Haemolysin is responsible for the lysis of erythrocytes (Dhaliwal *et al.*, 2004). Bacteria can be grown on blood containing agar to test whether they lyse the erythrocytes. There is a distinction that must be made between alpha- and beta-haemolysins. Alpha haemolysins only partially break down erythrocytes whereas beta haemolysins break down erythrocytes completely. According to Horn *et al.* (2016), previous studies have shown that HPC bacteria that test positive for alpha- or beta-haemolysins can produce two or more other extracellular enzymes that can be potentially pathogenic (Pavlov *et al.*, 2004). DNase is responsible for the breakdown and degradation of nucleic acids. Pathogens can utilise the broken down nucleic acids as a source of energy. DNase is a standard method for the identification and differentiation of several pathogens (Pavlov *et al.*, 2004; Horn *et al.*, 2016). Proteinase, also known as protease, is an enzyme and virulence factor when secreted by bacteria. Proteinase is responsible for protein catabolism that degrade peptide bonds of long protein chains. Proteinase can therefore break through its host defence mechanisms through protein catabolism and gain access to its host. Lecithinase breaks down fatty substances within the membrane of cells and also gain access to the host cell. Lipase enzymes are responsible for the hydrolysis of triacylglycerols into diacylglycerols, monoacylglycerols, fatty acids and glycerol. Once HPC bacteria produce these extracellular enzymes, then they have the potential to be invasive which makes them more prone to be or become pathogenic (Tropeano *et al.*, 2014; Dhaliwal *et al.*, 2004).

1.13. The production of safe drinking water

The best way of consistently providing safe and potable drinking water, is to have a comprehensive and integrative risk assessment and management approach that entails all steps and factors of water supply from catchment to the consumer (WHO

and IWA, 2009; WHO, 2017). As can be seen in Figure 2, in order to provide safe drinking water to consumers, a framework consisting of health-based targets, a water safety plan (WSP) and independent surveillance must be set up and managed. Health-based targets provide the basis for the application of guidelines. It provides information against which to evaluate the effectiveness of the treatment process and the quality of the water produced. The water quality parameters and its possible influences mentioned above, are based on these health-based targets. Independent surveillance plays a part that allow for legal redress in the pursuit for producing safe drinking water. An example of such surveillance would be the Blue Drop certification. According to WHO (2005), surveillance is “the continuous and vigilant public health assessment and overview of the safety and acceptability of drinking water supplies”.

The WSP is part of, and integrated into drinking water production and not an independent process. It is essential that the WSP team has adequate experience and expertise to understand as well as recognise the possible risks and hazards that go with drinking water production. Although the water utility should take responsibility for the WSP approach, it should not be done in isolation. Part of the WSP approach is to identify the responsibilities of other role players as well which can include agriculture and forestry, industries, transportation, local government and consumers. These role players do not necessary need to be part of the WSP team, but must be part of the communication network and be aware of the impacts of their contributions (WHO and IWA, 2009).

Broadly speaking a WSP should entail a system assessment, effective operational monitoring, and management and communication plans. A system assessment is related to the functionality of the drinking water supply chain and whether the system can deliver drinking water that meet specified targets. Operational monitoring is a set of routine activities that are used to determine and monitor specific identified control measures. These control measures such as water quality parameters already mentioned, are monitored in a timely manner for effective systematic management and will ensure that any deviation from required performances is rapidly detected. Management, documentation and communication entails actions to be taken during normal and incident conditions. Documentation is essential which include the

description and assessment of the drinking water system, programmes to upgrade and improve water delivery, plans for operational monitoring, water safety management

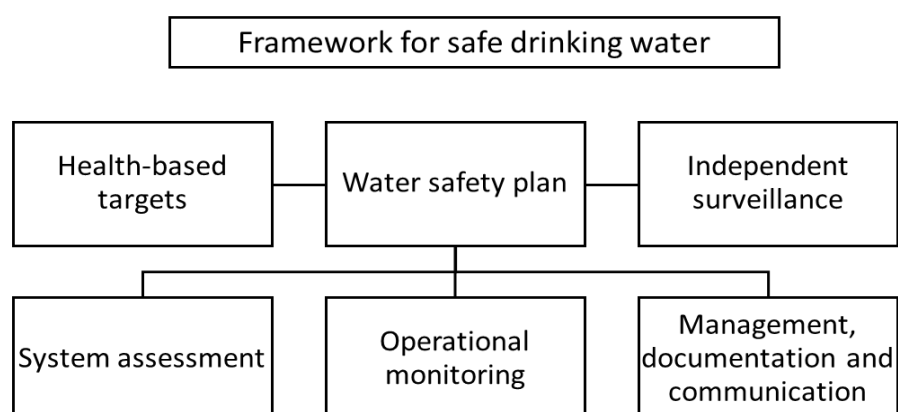


Figure 2: A framework that will produce potable and safe drinking water and consists of health-based targets, a water safety plan and independent surveillance (WHO, 2005).

procedures during normal circumstances as well as incidents, and description of supporting programmes. The right of consumers to health-related information regarding the drinking water supplied to them for domestic use is fundamental. Therefore, procedures should be in place for any significant incidents taking place in the drinking water supply; summary information must be made available to consumers on a regular basis such as annual reports and on their websites; there must be mechanisms in place to receive and actively address community complaints (WHO, 2017).

1.14. Antibiotics

The discovery of penicillin in 1929 brought the “golden age” of antibiotics to light when this product was mass produced and commercialised to treat infections in 1941 (Hardman *et al.*, 1996; Madigan, 2012). Penicillin and sulphonamides were initially scarce and very expensive and were reserved for use by the military during World War II. Shortly after, penicillin resistance became a substantial clinical problem. In response, new beta-lactams as well as the production of streptomycin, chloramphenicol and tetracycline were the temporal solution that restored some confidence. However, in the same decade, the first case of methicillin-resistant *Staphylococcus aureus* (MRSA) was detected in the United Kingdom in 1962 and in the United States in 1968 (Ventola, 2015). Eventually resistance to most antibiotics

were observed and vancomycin was introduced into clinical practice in 1972 for the treatment of methicillin-resistance in *S. aureus* and coagulase-negative staphylococci. As it was difficult to induce vancomycin resistance, it was believed that it would be unlikely to happen in clinical settings. Then in 1979 and 1983, vancomycin resistance was reported in coagulase-negative staphylococci. Since the 1960s to the early 1980s, many new antibiotics were introduced to solve the resistance problem. But after that the production of new antibiotics became increasingly difficult and less drugs were introduced (Ventola, 2015). Today we can see that the evolution of antibiotic resistance has rendered these original antibiotics and most of their successors ineffective (Clardy *et al.*, 2009).

Antibiotics are distinctly different from one another based on physical, chemical and pharmacological properties; antibacterial spectra; and mechanism of action (Hardman *et al.*, 1996). Despite the very complex molecular and chemical structure of antibiotics, the therapeutic use of antibiotics can also be very complicated. In order for antibiotics to exert their bactericidal or bacteriostatic effects, there are a few factors that need to be considered and include: the source of illness must be diagnosed; the correct antibiotic must be prescribed (that would have the best and most sufficient outcome); the correct concentration of the antibiotic at the site of infection must be achieved (which is sufficient to prevent further bacterial growth); and the antibiotic cycle must be completed (Hardman *et al.*, 1996; CDCP, 2013; Laxminarayan *et al.*, 2013; Hiltunen *et al.*, 2017).

Table 1: Classification of antibiotics

Class	Antibiotic	Active against	Effect	Mechanism
Beta-lactams	Penicillin G	+/-	Bactericidal	Inhibition of cell wall synthesis
	Ampicillin			
	Cephalothin			
Glycopeptides	Vancomycin	+	Bactericidal	Inhibition of cell wall synthesis
Chloramphenicol		+/-	Bacteriostatic	Affect the functionality of 30S or 50S ribosomal subunits
Tetracyclines	Oxytetracycline	+		
Macrolides	Erythromycin	+/-		
Aminoglycosides	Neomycin, Streptomycin	-	Bactericidal	Affect the functionality of 30S or 50S ribosomal subunits
Quinolones	Ciprofloxacin	+/-	Bactericidal	Affect nucleic acid metabolism and inhibit the activity of DNA gyrase
Trimethoprim (often used together with sulfamethoxazole)		+/-	Bacteriostatic and Bactericidal	Block metabolic pathways, also called antimetabolites

+ = Gram positive; - = Gram negative (Berkow *et al.*, 1992; Hardman *et al.*, 1996)

1.14.1. Classes of antibiotics

Antibiotics used for humans can be divided into the following groups according to Berkow *et al.* (1992): Beta-lactams, aminoglycosides, quinolones, tetracyclines, tigecycline, macrolides, ketolides, clindamycin, chloramphenicol, glycopeptides, oxazolidinones, fosfomycin, cotrimoxazole and polymixins. However, for this project only the following groups and subsequent antibiotics were used and classified on their mechanism of action and chemical structure and can be observed in Table 1.

1.14.2. A perspective on antibiotic resistance

Antibiotic resistance is indeed a problem that the WHO stated in 2014 is no longer a prediction for the future, but that it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country (DoH, 2014). The phenomena of antibiotic resistance is therefore the ability of bacteria to become resistant to the toxic effects of antibiotics through specific adaptive abilities and mechanisms. However, the problem becomes more complicated when multiple resistance across several antibiotics emerge, which is already happening (Alanis, 2005; Madigan, 2012). Globally the emergence of multidrug-resistant *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Acinetobacter* and *Pseudomonas aeruginosa* is evident as well as worrying (CDCP, 2013; WHO, 2014; Ventola, 2015) and concerning according to ECDC and EMEA (2009), is that there are very few antibacterial agents with new mechanisms of action under development to meet the challenges of multidrug-resistance.

Bacteria have sufficient ways to become resistant as they can lack the structure that antibiotics inhibit and thus make it naturally resistant; they can acquire this ability through the mutation of resistant genes; the cell can be impermeable to antibiotics; it might develop a resistant biochemical pathway; bacteria might be able to pump the antibiotics out of the cell through efflux; or they can acquire resistant genes through horizontal transmission such as conjugation, transformation or transduction to the same species or even different species. Such foreign DNA can also be extracellular DNA suspended in water or imbedded in soil and biofilms which may be parts of genes, complete genes or even defined elements. The integration of new forms of a gene

creates a mosaic gene composed of the host's gene and foreign DNA which has the potential to reduce antibiotic susceptibility of the host bacteria. The most common way bacteria develop resistance is through genes that are associated with mobile elements such as plasmids, transposons and integrons. These mobile elements can carry genes for metal resistance, use of alternative carbon sources, and/or classical virulence genes as well as a variety of antibiotic resistance genes (Keen and Montrofts, 2012; Madigan, 2012; Cox and Wright, 2013; Vaz-moreira *et al.*, 2014; Hiltunen *et al.*, 2017).

1.14.3. Development of antibiotic resistance

Antibiotic resistance has been present before the introduction of antibiotics, but was mostly found in natural antibiotic producing microorganisms. This underlines the fact that the acquisition of resistant mechanisms against antibiotics, is a natural process of evolution which forms part of complex microbial communities and should in fact not be surprising (Alanis, 2005; Cox and Wright, 2013; Vaz-moreira *et al.*, 2014; Holmes *et al.*, 2016; Hiltunen *et al.*, 2017). According to Baquero *et al.*, (2008), there are four main genetic reactors in which antibiotic resistance evolves and can be seen in the Figure 3 below.

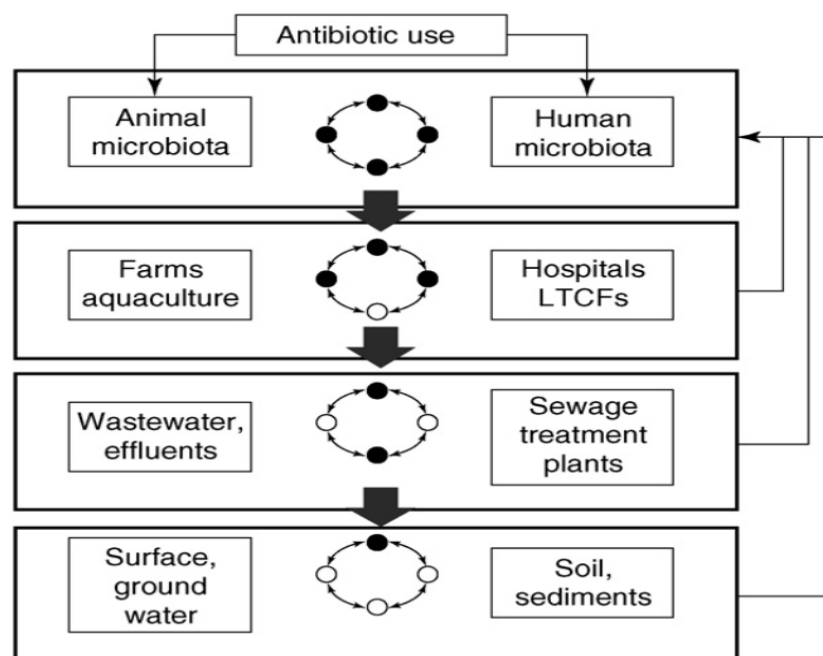


Figure 3: The four main genetic reactors in which antibiotic resistance evolves (Baquero *et al.*, 2008)

The primary reactor is human and animal microbiota that are being influenced by therapeutic or preventive antibiotics. The secondary reactor involves hospitals, long-term care facilities, farms, or any other place where susceptible individuals are crowded and exposed to bacterial exchange. The tertiary reactor constitutes wastewater and all other places where biological residues originated in the secondary reactor. Examples include lagoons, wastewater treatment plants and all places where bacterial communities from different people have the opportunity to genetically interact. The fourth reactor is where the bacterial communities from the above mentioned reactors mix and interact with environmental organisms. During all these reactors, water plays an integral part as transfer medium especially in the fourth reactor. The possibility of reducing the development and evolvability of antibiotic resistance surely depends on the control and use of active antibiotics at each of these reactors (Baquero *et al.*, 2008).

However, as Barbosa and Levy, (2000) indicated (Figure 4), the control and influences humans have on antibiotic resistance is very complex and not straight forward. There are a lot of factors that influence the forward resistance reaction to antibiotics. Factors that are difficult to quantify can include education, poverty, hygiene, socioeconomic factors, the travel of people and foodstuffs, patient movement within and between medical institutions, the appropriate use of antibiotics, infection control, and many others (Barbosa and Levy, 2000). Prolonged low-dose antibiotics, which are typical for non-therapeutic uses of antibiotics in food animals, increase the selective pressure for the development of antibiotic resistance in bacteria (Barbosa and Levy, 2000; Laxminarayan *et al.*, 2013; Moyane *et al.*, 2013; So *et al.*, 2015). Such concentrations can also increase bacterial mutation rates, increase phenotypic and genotypic variability, affect biofilm formation and also affect multispecies communities where small differences can have cascading effects on a community level (Hiltunen *et al.*, 2017). It is known that the frequency of resistance can be decreased by the reduction of antibiotics consumption. However, according to Holmes *et al.* (2016), the reversibility of antibiotic resistance after the withdrawal of antibiotic selective pressures is not clear cut. Resistant genes are very stable and the development thereof can increase quite drastically, but the decrease of resistance is much slower. This means

that once the antibiotics are reintroduced after its reduction, the resistance will increase drastically (Barbosa and Levy, 2000).

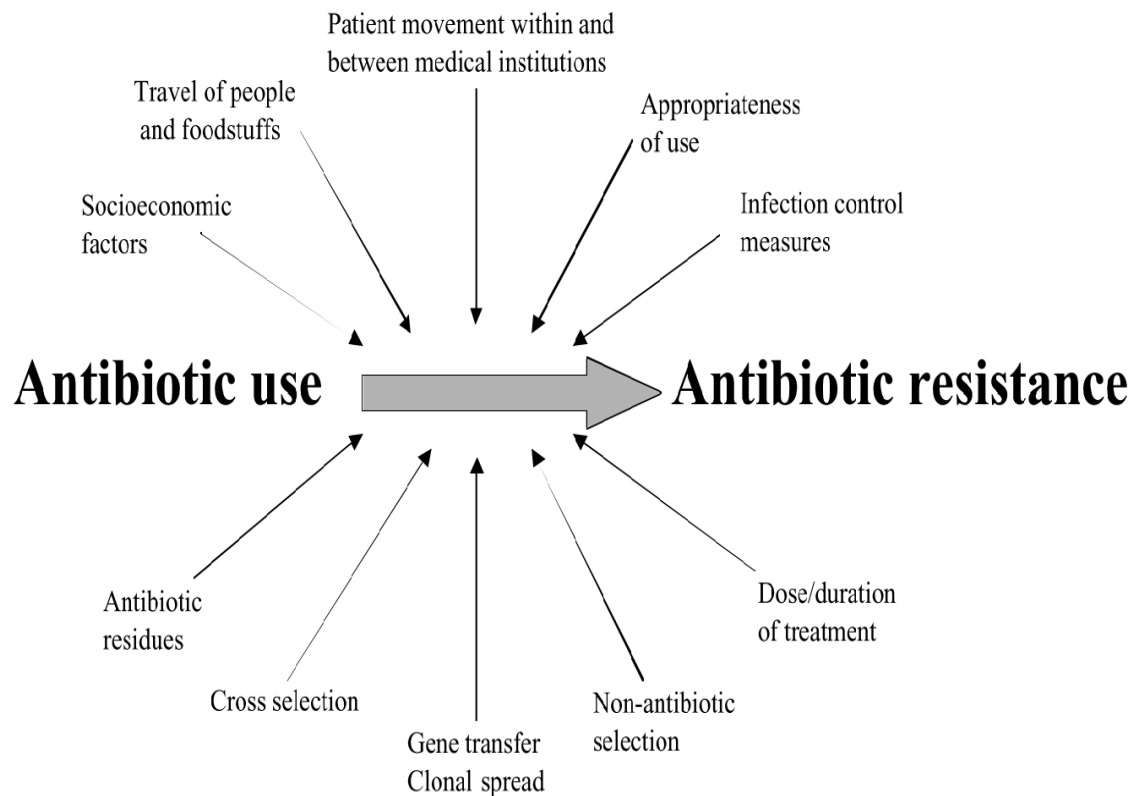


Figure 4: The complex interactions of human induced and management factors that influence the process of forward resistance (above the horizontal arrow) as well as factors that are related to antibiotics itself and their mechanisms (below the horizontal arrow) (Barbosa and Levy, 2000).

1.14.4. Antibiotic resistance genes

ARGs are the sequences of DNA that confer and prescribe for the resistance of bacteria against antibiotics. As mentioned above, there are various ways in which these ARGs play a role in resistance. Intracellular ARGs are harboured inside bacterial cells' genomes and are subject and bound to the fundamental laws of biology (Keen and Montrofts, 2012). Extracellular ARGs are DNA fragments that are not found inside bacterial cells at a specific moment. These ARGs are therefore freely suspended in their environment. However, soil components such as clay, have shown to bind to DNA fragments that protect it from DNase while still maintaining the integrity to transform

and interact with bacterial cells (Keen and Montrofts, 2012). It is these intercellular and extracellular ARG fragments that give rise to the antibiotic resistance patterns observed in the environment. However, the correlation between the resistance patterns and the intracellular and extracellular ARGs are not yet well understood, especially in drinking water environments. A summary of ARGs in source water and drinking water can be observed in table 2.

1.14.5. Antibiotic resistance and drinking water production

Water sources always contain bacteria that are primarily part of the natural composition of water environments (WHO, 2003; Vaz-moreira *et al.*, 2014). Antibiotics, ARB and ARGs are present in our wastewater treatment plants, livestock and soil which eventually runoff into our surface waters (Kinge *et al.*, 2010; Proia *et al.*, 2016). Although different wastewater treatment plants across the globe use different treatment processes, they are responsible for the discharge of approximately one billion culturable coliforms per minute into the environment (Vaz-moreira *et al.*, 2014). Once the antibiotics, ARB and ARGs are present in our water sources, they have the potential to enter drinking water distribution systems and form selective pressures for ARB (Xi *et al.*, 2009; Guo *et al.*, 2014; Bai *et al.*, 2015; Titilawo *et al.*, 2015).

“Thus far research is mainly focused on ARB and ARGs on wastewater treatment plants. There is a lack of knowledge regarding the prevalence, dynamics and function of ARB and ARGs in drinking water (Xu *et al.*, 2016). A study done by Bai *et al.* (2015), showed that chloramine disinfection as well as biological activated carbon filtration can increase the antibiotic resistance of bacteria in drinking water treatment. The study done by Xu *et al.* (2016), showed that there was an elevated abundance of ARGs in the tap water of consumers. This indicates that the distribution system could be an important reservoir for ARGs and ARB. Biofilms also play important roles in the distribution system of drinking water. According to Wingender and Flemming, (2011), about 95% of bacteria in the distribution systems are located on the surfaces. Only 5% are found suspended in the water and is the fraction that is being sampled during quality control (Wingender and Flemming, 2011). Biofilm communities are diverse and have shown to contain ARB and ARGs (Xu *et al.*, 2016).

This study therefore forms part of this developing knowledge of water from the perspective of microbiology and genetics with the focus and application on antibiotic resistance. With sustainability as foundational perspective, we can identify and understand the challenges and then find solutions in which we can approach and solve it so that the people and all other life after us can find delight in our natural environment.

Table 2: A summary of ARGs present in water sources and their possible influences on antibiotic resistance

ARG	Antibiotics resistant to	Associated bacterial isolates	Water source	Reference
<i>ampC</i>	Cephalothin, ceafazolin, cefoxitin and penicillins	<i>Citrobacter</i> , <i>Enterobacter</i> , <i>E. coli</i> , viable but non-cultivable bacteria	R, D	Schwartz <i>et al.</i> , 2003; Volkmann <i>et al.</i> , 2004; Zhang, <i>et al.</i> , 2009; Xu <i>et al.</i> , 2016
<i>TEM1</i>	Cephalothin and penicillins	<i>E. coli</i> , Heterotrophic plate count bacteria	R, T, D	Alpay-Karaoglu <i>et al.</i> , 2007; Xi <i>et al.</i> , 2009; Zhang, Zhang, <i>et al.</i> , 2009; Xu <i>et al.</i> , 2016
<i>ermB</i>, <i>ermF</i>	Macrolides, lincosamide and streptogramin	<i>Bacillus</i> , <i>Enterococcus</i>	R, T, D	Zhang, <i>et al.</i> , 2009; Xu <i>et al.</i> , 2016
<i>tetM</i>	Tetracycline	<i>Aeromonas</i> , <i>Bacillus</i> , <i>Escherichia</i> , <i>Lactococcus</i> , <i>Pseudoalteromonas</i> , <i>Vibrio</i>	R	Zhang, <i>et al.</i> , 2009
<i>IntI</i>	Depends on genes present in cassettes	<i>E. coli</i> , <i>Vibrio</i>	R, T, D	Ozgumus <i>et al.</i> , 2007; Taviani <i>et al.</i> , 2008; Xu <i>et al.</i> , 2016

ARG – antibiotic resistant gene; R – raw water; T – treated water; D – drinking water.

CHAPTER 2

MATERIALS AND METHODS

2.1. Sample collection

2.1.1. Conventional sampling

“Sampling was conducted in March, May and August of 2016; and during May and November of 2017. Collected samples for each drinking water treatment plant included: 1L raw water, 1L treated water and 1L distribution water. The water was sampled using sterile glass bottles and were stored in cooler boxes filled with ice. The samples were transported to the laboratories at the North-West University immediately after sampling. Sampling was done according to the DWAF Sampling Guide (DWAF 2000).

2.1.2. eDNA

In order to extract environmental DNA (eDNA) a lot of water (specifically treated- and drinking water) needed to be filtered to increase the concentration of eDNA onto filters for extraction. In order to achieve this, the methodology used by Ma *et al.*, (2017) was followed. However, modifications were made to this protocol as follows: A LifeStraw filter consisting of a hollow fibre filter (Vestergaard Frandsen, Switzerland) was modified so that it could be fixed onto a tap. Approximately 500L - 1400L of water (raw water, treated water and distribution water) was filtered through the hollow fibre filter. After filtration, the hollow fibre filter was cut up and inserted into a schott bottle containing 300mL of distilled water that was autoclaved. The schott bottle was then sonicated for 10min. The sonicated water was then filtered onto a 0.45µm, 47 mm grid PALL Corporation sterilised filter membrane [(PALL Life Sciences, Mexico) (CAT No: GN-6 Metrice Membrane 66191)]. The membrane was used for DNA extraction (as described in DNA extraction section 2.11.1). The Figure below illustrates the process:

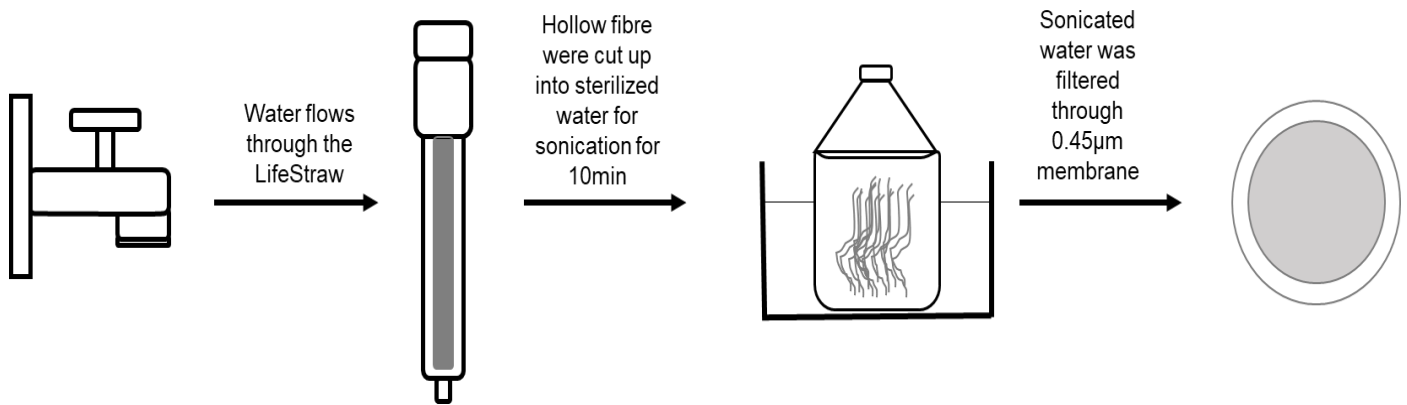


Figure 5: Demonstration of the filtering system for eDNA.

2.2. NW-C treatment plant

Raw water was sampled directly from a canal; the treated water was sampled directly after chlorination from a tap whereas water in the distribution system was sampled at three different sites. The local municipality (LM) of the NW-C treatment plant is situated in the Dr Kenneth Kaunda District in the North-West Province. It is an area with few urban development and industries that can pollute the environment and fresh water abstracted for drinking water. The LM has a population size of approximately 56 702 people and 14 562 households according to the 2011 census. The capacity of the filtration plant is 350m³/h (Diedericks, 2013). The eye of the water source originates 6 km from the treatment plant and serves as a potable water source with constant levels even during dry seasons. This water flows into the drinking water production facility to be treated for drinking water (Diedericks, 2013).

The raw water is considered to be clean because the origin of the river is nearby. The treatment process is therefore elemental and consists only of: physical separation, sand filtration and chlorination as depicted in Figure 6. Physical separation is the removal of large objects in the raw water that enters the facility. Such objects include sticks, leaves, litter or sometimes fish. Raw water is then pumped into the bottom of sand filters which allows for a coarse-to-fine filtration process to take place. The filtered water flows out at the top of the filters into a sump where water is collected. The final treatment process is disinfection or chlorination whereby bacteria and possible pathogens are being killed or inhibited. At this facility chlorine is being used as the disinfectant (Schutte, 2006; NW-C Local Municipality, 2013).

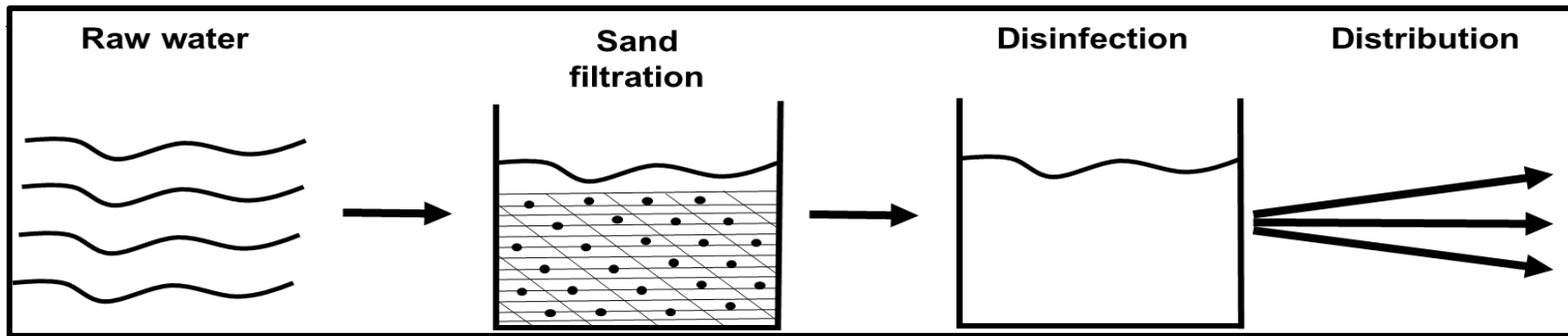


Figure 7: Demonstration of the treatment process taking place at NW-C.

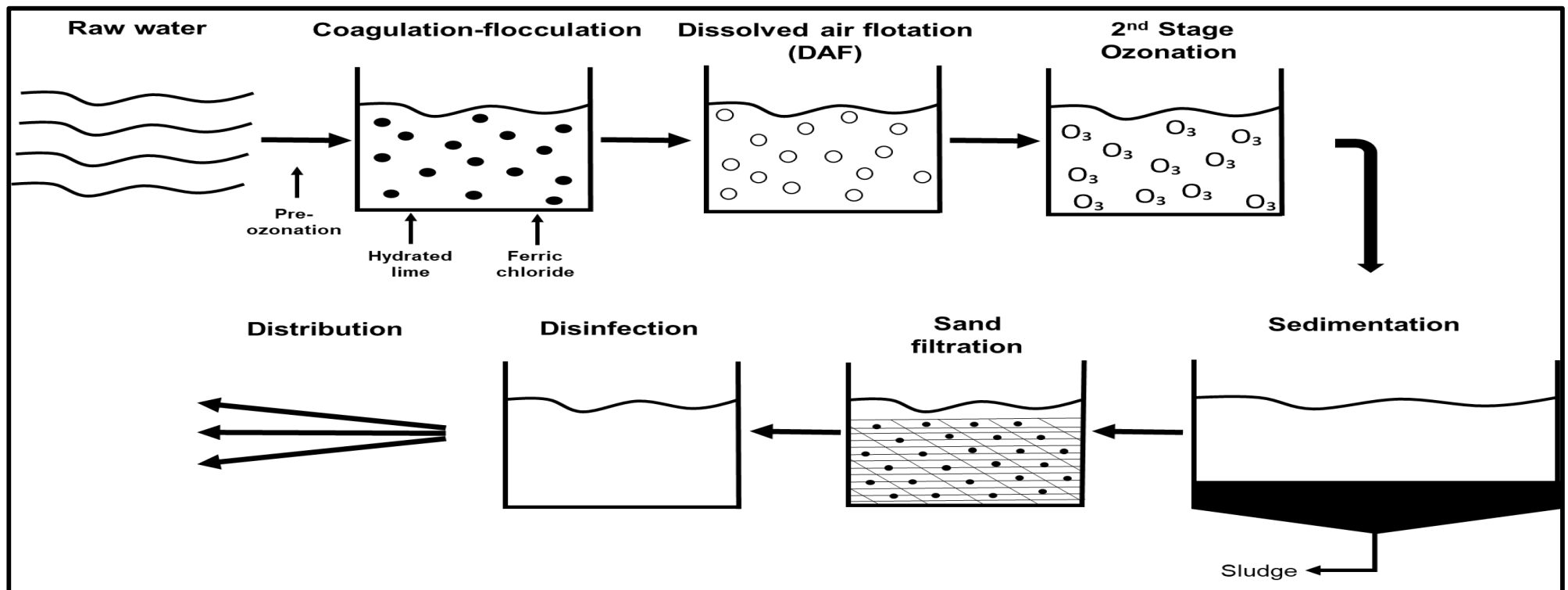


Figure 6: Demonstration of the treatment process taking place at NW-G.

2.3. NW-G treatment plant

The raw water and the treated water were collected from a tap at the NW-G treatment plant, and water in the distribution systems were sampled at three different sites. NW-G is an organisation that operates as a Section 21 of the Companies Act, non-profit organisation and has a successful track record in delivering and generating enough funds to sustain the production of potable drinking water. NW-G purchases raw water from the Department of Water and Sanitation (DWS) and sells purified water to the local municipality which serves as the company's Water Service Authority. The municipality then distributes the water to consumers that span an area of approximately 900 square kilometres. This municipality is categorized as a B1 local municipality which is a secondary city with a large budget (Brettigny and Sharp, 2016). NW-G has constituted a joint technical forum with the local municipality in which they promote the planning of infrastructure, strive to achieve and maintain the Blue Drop Certification status and provide technical support and security of water supply to consumers and the surrounding areas (NW-G annual report, 2015).

According to NW-G annual report (2015), NW-G has abstraction rights from the DWS for 238 megalitres of raw water per day . The company has the capacity to supply and distribute 250 megalitres of potable treated water per day and has an installed capacity of 320 megalitres of water per day. The river that serves as raw water is an extensively utilized source and is therefore constantly exposed to a variety of pollutants that affect its quality. It is therefore essential that the raw water quality is assessed regularly to ensure that the treatment processes are adequate to produce good quality drinking water. NW-G monitors the drinking water quality from source to tap to ensure that health risks can be detected early enough once they occur. NW-G is participating in catchment management activities in order to influence strategies and plans to improve the resource quality and quantity of the river (NW-G annual report, 2015).

At the NW-G treatment plant pre-ozonation takes place at low concentrations to deal with algae in the raw water. Ferric chloride is then used to coagulate suspended particles into bigger particles that eventually form flocs. Air bubbles are formed and attach to the light weighted flocs which rise to the surface forming a froth which is then removed. Dissolved air floatation then removes relatively light types of particles such

as organic material and algae. Heavier particles settle out of suspension as sludge which is also removed. The second stage of ozone is then added at higher concentrations after most of the algae and suspended particles were removed to deal with more complex constituents. Small doses of flocculants are added to coagulate oxidised particles into flocs. These flocs are removed in specialised sedimentation units as they settle to the bottom of the tanks. Silica sand is used in rapid gravity filters to remove remaining floc particles. Chlorine gas is then added as a disinfectant by which bacteria and other microorganisms are oxidised.

2.4. Physico-chemical analysis

The physical analysis was performed on-site by measuring the temperature, pH, total dissolved solids (TDS), salinity, and turbidity with a multi-meter (PCSTestr 35, Eutech Instruments Pte Ltd, Singapore). The multi-meter as well as the measurement beaker were cleaned with distilled water and rinsed with the specific water sample before the measurements were taken. The chemical analysis was done in the laboratory at the North-West University. The concentrations of free chlorine (method 8021), phosphorous (method 8178), nitrite (method 8153), nitrate (method 8039), sulphide (method 8131), sulphate (method 8051), and COD (method 8000) were measured using the Hach Lange DR 2800 spectrophotometer as per the manufacturer's instructions (Hach Company, 2007).

2.5. Microbiological

Membrane filtration was used in order to enumerate the total coliforms, faecal coliforms and *E. coli* in all water samples according to APHA *et al.* (1998). Water samples were filtered through 0.45 m diameter, 47 mm grid PALL Corporation sterilised filter membranes [(PALL Life Sciences, Mexico) (CAT No: GN-6 Metrice Membrane 66191)] and placed on selective media: m-FC agar (Merck, Germany) and MLG agar (Oxoid, UK). The filter funnels were washed and cleansed with 70% ethanol prior to use. The m-FC agar plates were incubated at 44.5°C for 24 hours while all MLG agar plates were incubated at 37°C for 24 hours. Blue and yellow colonies on m-FC agar represented total coliforms; only blue colonies on m-FC agar represented faecal coliforms; and green colonies on MLG agar represented *E. coli*. Results were

recorded as colony forming units (cfu) per 100mL. It needs to be noted that microbiological tests were only done for the May 2017 and November 2017 sampling runs for both NW-C and NW-G.

2.6. Statistical analysis

Statistical calculations of data such as the averages and standard errors were done using Microsoft Office Excel. Correlations were made between the physico-chemical parameters and HPC levels for all the sampling runs by a Principal component analysis (PCA) using Canoco software version 4.5. Three PCA plots were constructed for the correlation between (1) the raw water from NW-C and NW-G (Figure 8); (2) the raw water and drinking water from NW-C (Figure 9); and (3) the raw water and drinking water from NW-G (Figure 10). Correlations in the PCAs were determined by the size of the angles between the plots. The smaller the angle between the parameter, the closer the correlation.

2.7. Heterotrophic plate count (HPC)

The enumeration of HPC bacteria was done according to Carstens *et al.* (2014). A dilution series up to 10^{-5} for each water sample was made and spread onto R2A agar plates. The plates were incubated at room temperature ($\pm 25^{\circ}\text{C}$) for 5-7 days. The total number of colony-forming units (cfu) were counted on the plates and converted to cfu/mL. Morphologically distinct colonies were recorded according to their colour, elevation, surface appearance and shape. These colonies were purified by the streak-plate method onto R2A agar plates. Streaking was done twice and the plates were incubated at room temperature ($\pm 25^{\circ}\text{C}$) for 5-7 days each time.

2.8. Gram staining

The Gram staining procedure was based on the methods described by Claus (1992). The colonies that were streaked out twice were used for Gram staining to determine whether they were Gram-positive or Gram-negative. The colonies were heat fixed onto a glass slide with the help of an inoculating loop and were left to air dry. The slide was covered with crystal violet stain for one minute followed by a distilled water wash. After drying Grams iodine was added for one minute. This was consecutively washed with

acetone-ethanol and then covered with safranin for one minute. The safranin was washed off with water and left to air dry. The bacterial smears were observed under the microscope. The cell walls of Gram-positive bacteria appeared purple while those of Gram-negative bacteria appeared pink. The shape of the bacterial cells was also noted and observed.

2.9. Identification of HPC isolates

2.9.1. DNA isolation

The DNA of each pure isolate was extracted using the Chemagic DNA Bacteria Kit (PerkinElmer, Germany). Instructions were followed as prescribed by the manufacturer. DNA samples were then centrifuged at 4°C for 3min to get rid of remaining impurities. eDNA was extracted using the DNeasy PowerWater Kit (Qiagen, Netherlands). Instructions were followed as prescribed by the manufacturer. The purity, concentration and quality of extracted DNA were measured using a NanoDrop™ 1000 Spectrophotometer v3.5.2 (Thermo Scientific, US) and a 1.5% agarose gel electrophoresis in 1x TAE buffer [20 mM Acetic acid, 40 mM Tris and 1 mM EDTA at pH 8.0]. The gel contained ethidium bromide (Bio-Rad, UK) and was run at 100V for 30min.

2.9.2. PCR

All PCR reactions were done using the 1000Cycler (BioRad, UK) thermal cycler. All the samples consisted of a total volume of 25µL. This volume consisted of 1 µL DNA (20-60 ng/µL), 12.5µL Dream Taq PCR commercial master mix (2 mM MgCl₂, 0.2 mM of each dNTP, 0.025 U/µL *Taq*-polymerase) (Thermo Scientific, US); 1µL of the forward primer (conc) and 1µL of the reverse primer (conc) (Table 3); and nuclease free water. All primer sets used in this study are listed in Table 3. The PCR amplicons were confirmed by the same electrophoresis process described in section 2.9.1

2.9.3. 16S rRNA

The 16S rRNA gene from all the isolates as well as eDNA was amplified (Jiang *et al.*, 2006). The following thermal cycling conditions were used: initial denaturation at 95°C for 2min; 30 cycles of denaturation at 95°C for 30 seconds; annealing at 54°C for 30 seconds; extension at 72°C for 1min; and the final extension at 72°C for 5min.

2.9.4. Sequencing

The 16S amplicons from the 2017 sampling runs were sent to Inqaba Biotechnical Industries (Pty) Ltd for the sequencing of these genes. However, in-house sequencing was done for isolates from the 2016 sampling runs and therefore the following preparations were done for sequencing:

2.9.5. First PCR clean-up for sequencing

In order to sequence and identify the unknown isolates, the PCR products had to be purified and amplified once more. The first purification of the PCR amplicons was done using a silica matrix method according to Li *et al.* (2010). The PCR products were mixed with 150 µL of 6M sodium iodide (Sigma-Aldrich). The DNA was bound to 10 µL of a 100 mg/mL silica matrix (silicone dioxide - Siga-Aldrich) and incubated at room temperature for 5 minutes. The tubes were centrifuged for 30 seconds at maximum speed (13 000 rpm) and the supernatant was removed using a micropipette. The pellet was washed and vortexed with 200 µL of wash buffer (50mM NaCl; 10mM Tris pH 7.5; 2.5mM EDTA; 70% ethanol; and sterile ultra-pure water). The tubes were centrifuged for 30 seconds and the process was repeated two more times. After the third time, the supernatant wash buffer was removed and the pellet centrifuged for 1 minute. The last trace of liquid was removed. The tubes were left open to air dry for 5 minutes. The matrix was resuspended in 20 µL of ultra-pure water. The tubes were centrifuged for 5 minutes and the quality of the products were determined by using a NanoDrop™ 1000 Spectrophotometer v3.5.2 (Thermo Scientific, US). Gel electrophoresis was done once again to confirm the presence of the 16S rRNA amplicon product.

2.9.6. Sequencing PCR

The purified products underwent a second round of PCR amplification. The BigDye Terminator v3.1 cycle sequencing kit from Life Technologies was used. Each PCR tube contained 4 µL of Ready Reaction mix (0.25X), 2 µL of BigDye sequencing buffer (5X), 1 µL of a 3.2 pmol/µL forward (27F) primer (Fermentas Life Sciences, US), 3 µL of template DNA and 10 µL of Nuclease free water to make up a total volume of 20 µL. The thermal cycling conditions were as follows: initial denaturation at 96°C for 1 minute; 25 cycles of denaturation at 96°C for 10 seconds; and annealing at 50°C for 5 seconds; extension at 60°C for 4 minutes. PCR amplification was done using the 1000Cycler (BioRad, UK) thermal cycler.

2.9.7. Second clean-up of PCR amplicons

The final purifying process was done using a ZR (Zymo Research) Sequencing Clean-up Kit (The Epigenetics Company, US). Instructions were followed as prescribed by the manufacturer.

2.9.8. Sequencing and phylogenetic analysis

After purification, 10 µL of ultra-pure products were loaded and mixed with 5 µL of HiDi Formamide in the appropriate plate ready for sequencing. Sequencing was done using the ABI 3130 Genetic Analyser (Applied Biosystems, UK). Geospiza Finch TV (version 1.4) was used to observe the chromatogram results. EzTaxon (www.ezbiocloud.net/eztaxon/identify) was then used to identify the sequences. Reference sequences were obtained from the EZTaxon database and imported into MEGA X within which neighbor-joining trees were constructed using the Jukes Cantor model and bootstrap values with 1000 replications (Saitou and Nei, 1987).

2.10. Pathogenicity

For all the pathogenicity test illustrated below (2.11.1 – 2.11.5), the isolates were not grown at 37°C as the literature indicate but rather at 28°C - 30°C. This is because most the HPC isolates could not grow at 37°C. The incubation time of some of the enzyme

tests such as proteinase, lipase and lecithinase was adapted to prevent over growth of plates.

2.10.1. Haemolysin

5% sheep blood agar (BioMérieux, SA) was used to select isolates capable of lysing blood cells and were incubated at 28°C - 30°C for 24 hours (Xiao *et al.*, 2009). The isolates were distinguished between alpha (α), beta (β) or gamma (γ) based on the type of growth observed on the blood agar plate. A green-black ring surrounding the bacterial growth was referred to as α – haemolytic activity. A clear and colourless zone surrounding bacterial growth were referred to as β – haemolytic activity. When there was only bacterial growth there was an absence of haemolysis and was considered γ – haemolysis.

2.10.2. Proteinase

The isolates were tested for proteolytic enzyme production on skimmed milk agar plates (Sen *et al.*, 2010). The plates contained equal volumes of 3% (w/v) skimmed milk (Oxoid, England) and Brain Heart Infusion Broth (BHIB) (Merck, Germany) with the addition of 3g agar (Merck, Germany) per 100mL (Pavlov *et al.*, 2004). The ingredients were prepared separately, autoclaved and 20mL of the culture medium was poured into plates and left to solidify (Saran *et al.*, 2007). Isolates were inoculated and the plates incubated at 28°C - 30°C for 24 hours. The development of transparent zones around the colonies indicated proteolytic activity (Boominadhan *et al.*, 2009).

2.10.3. Lipase

A medium containing tryptone soy agar (prepared according to manufacturer's instructions) (Merck, Germany) was supplemented with 1% Tween-80 (Sigma, Germany) and served as the substrate for lipase production (Israil *et al.*, 2011; Kumar *et al.*, 2012). A positive reaction for lipase is the appearance of a turbid halo around the inoculation spot after a 48 hour incubation period at 28°C - 30°C (Pavlov *et al.*, 2004).

2.10.4. DNase

DNase agar (Merck, RSA) was used and prepared according to manufacturer instructions. Toluidine blue (Sigma, Germany) was added as an indicator that binds to hydrolyzed DNA indicating positive DNase activity (Sen *et al.*, 2010). The plates were incubated for 48 hours at 28°C - 30°C. After incubation the plates were scanned for the presence of a clearing zone around the colony, which indicated DNase activity (Gündoğan *et al.*, 2006).

2.10.5. Lecithinase

The secretion of lecithinase by bacteria was determined using McClung-Toabe egg yolk agar (Steffen and Hentges, 1981). Briefly, McClung-Toabe agar (Difco, France) was prepared according to the manufacturer instructions and sterilized. After cooling 1 part of the 50% egg yolk mix (Merck, RSA) was added to 9 parts of agar and plates were prepared. Plates were examined for evidence of egg yolk degradation after 24 hours of incubation at 28°C - 30°C. A distinct zone of opacity around or beneath the inoculum spot on the egg yolk agar indicated the production of lecithinase (Jula *et al.*, 2011).

2.11. Measuring antibiotics in water samples

Quantification of the selected antibiotics, antiseptics and other antimicrobials were carried out for samples collected following the analytical methodology of the analytical facility at the North-West University. Glass containers (1L) were used for sampling. Contaminants from previously used glassware were removed using the procedure following the USEPA, 2007 method.

2.11.1. Extraction

The extraction of target compounds from water was based on methods used by Ferrer *et al.* (2010) for pharmaceuticals. Target compounds were concentrated 2 000 times by automated SPE using the SPE-DEX system (Horizon Technology, Salem, NH, USA). Oasis HLB disks were used because they are efficient at extracting analytes with various polarities and acid/base characteristics at different pH levels (Pedrouzo *et al.*, 2011). HLB-L were the best fit for this study's purpose because the samples are

of a low organic type. US EPA method 1694 for pharmaceuticals and personal care product analysis makes use of these disks. The HLB extraction disks (47 mm, Horizon Technology) were done according to the application note. The eluent was concentrated to near dryness using a gentle stream of nitrogen gas. The samples were reconstituted in methanol and subjected to UPLC-QTOF for analysis.

2.11.2. Analysis

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

2.12. Determining antibiotic resistance

The antibiotic susceptibility test was done using the Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966; Carstens *et al.*, 2014; Jorgensen and Ferraro 2009). The pure colonies were transferred from R2A agar plates to R2A broths and incubated at room temperature ($\pm 25^{\circ}\text{C}$) for 5-7 days. After incubation, 100 μL of the R2A broth was used to make spread plates onto Mueller-Hinton agar plates. The antibiotic discs were immediately placed onto the Mueller-Hinton agar plates and were incubated at 37°C for 24 hours. Twelve antibiotic discs with the following concentrations (Mast

Diagnostics, (UK) were used: ampicillin (10 µg); cephalothin (30 µg); chloramphenicol (30 µg); ciprofloxacin (5 µg); erythromycin (15 µg); kanamycin (30 µg); neomycin (30 µg); oxytetracycline (30 µg); penicillin-G (10 units); streptomycin (25 µg); trimethoprim (5 µg); vancomycin (30 µg). After incubation, the inhibition zones were measured (in mm) to characterize the isolate as either sensitive (S), resistant (R) or intermediate resistant (I). The interpretation this methodology was done according to (CLSI, 2014).

2.13. Determining multiple antibiotic resistance (MAR)

The multiple antibiotic resistance (MAR) index is based on the detection of antibiotic resistance to a panel of antibiotics (Guan *et al.*, 2002). The MAR is an excellent tool that enables scientists to attach a value to multiple resistance patterns observed in water samples. It is therefore a representation of multiple antibiotic resistance in a given population at a specified location (Titilawo *et al.*, 2015). The way in which the MAR values are determined per sampling site are illustrated below:

MAR index = $a / (b \times c)$ per sample

- a = total amount of resistance to antibiotics
- b = amount of antibiotics used
- c = number of isolates in sample

As indicated in section 2.12, isolates were classified as sensitive (S), resistant (R) or intermediate resistant (I). (I) and (R) isolates were used in the calculation as being resistant to antibiotics.

2.14. Detection of antibiotic resistance genes

All the samples contained the reaction mix described in 2.9.2.

2.14.1. *BlaTEM*

The following thermal cycling conditions were used: initial denaturation at 94°C for 5min; 35 cycles of denaturation at 94°C for 1min; annealing at 63°C for 30 seconds; extension at 72°C for 1min; and final extension at 72°C for 10min.

2.14.2. *ermB* and *ermF*

The *ermF* and *ermB* genes were successfully amplified by Chung *et al.* (1999). The PCR conditions for *ermF* were: 35 cycles of denaturation at 94°C for 30 seconds; annealing at 50°C for 1min; extension at 72°C for 2min. This amplification delivered an expected amplicon size of 466bp. The PCR conditions were the same for *ermB* except for the annealing temperature that was at 48°C for 30 seconds. This delivered an expected amplicon size of 639bp.

2.14.3. *Int1*

The *Int1* gene has previously been amplified by (Ozgumus *et al.*, 2007). The following thermal cycling conditions was used: initial denaturation at 95°C for 5min; 30 cycles of denaturation at 94°C for 30 seconds; annealing at 64°C for 30 seconds; extension at 72°C for 1min; and the final extension at 72°C for 5min.

2.14.4. *ampC*

The *ampC* gene has previously been amplified by (Schwartz *et al.*, 2003). The following thermal cycling conditions were used: initial denaturation at 94°C for 5min; 33 cycles of denaturation at 94°C for 30 seconds; annealing at 49°C for 30 seconds; extension at 72°C for 1min; and the final extension at 72°C for 7min.

2.14.5. *tetM*

The following thermal cycling conditions were used: initial denaturation at 95°C for 5min; 35 cycles of denaturation at 95°C for 1min; annealing at 50°C for 1min; extension at 72 °C for 30 seconds; and final extension at 72°C for 5min.

2.15. Whole genome sequencing

Whole genome sequencing (WGS) was done on six *Bacillus spp.* in order to identify possible antibiotic resistant genes (ARGs) as well as virulence genes. The *Bacillus spp.* were isolated from NW-C. Of the six isolates, two represented the raw water, treated water and distribution water each. DNA was isolated and used for 250-bp paired-end sequencing (North-West University, Potchefstroom campus, South Africa) on a MiSeq sequencer (Illumina). The raw reads were assessed for quality-based

trimming and filtering in Trimmomatic (v.0.36) (Bolger *et al.*, 2014). Low quality value fragments from each end (lower than 15) were trimmed off as well as ambiguous coding extending for more than 2 nucleotides. Short reads, less than 50 nucleotides were removed from further analysis. The remaining read pairs were assembled using SPAdes version 3.9.0 (Bankevich *et al.*, 2012) and each genome was assembled independently. Open reading frames and RNA genes were identified by Prokka (Seemann, 2014). Prokka is a software tool for the rapid annotation of prokaryotic genomes. It produces GFF3, GBK and SQN files that are ready for editing in Sequin and ultimately submitted to Genbank/DDJB/ENA. Also it allows appointing Open Reading Frames (ORFs) on each contig and annotating them accordingly to specified database (in this case it was Bacterial database). Antibiotic resistant genes as well as virulence genes were identified with DeepARG (Arango-Argoty *et al.*, 2018).

Table 3: The forward and reverse primer sequences used to amplify the ARGs

Target gene	Name	Sequence (5' > 3')	Size (bp)	Reference
16S rDNA	27F	AGA GTT TGA TCM TGG CTC AG	1 465	Jiang <i>et al.</i> , 2006
	1492R	GG TTA CCT TGT TAC GAC TT		
blaTEM	TEM-F	ATT CTT GAA GAC GAA AGG GC	1 150	Briñas <i>et al.</i> , 2002; Dolejska <i>et al.</i> , 2007; Costa <i>et al.</i> , 2008
	TEM-R	ACG CTC AGT GGA ACG AAA AC		
ermF	ermF1	CGG GTC AGC ACT TTA CTA TTG	466	Chung <i>et al.</i> , 1999
	ermF2	GGA CCT ACC TCA TAG ACA AG		
ermB	ermB-F	GAA AAG GTA CTC AAC CAA ATA	638	Chung <i>et al.</i> , 1999
	ermB-R	AGT AAC GGT ACT TAA ATT GTT TAC		
Int1	HS463A	CTG GAT TTC GAT CAC GGC ACG	473	Koenig <i>et al.</i> , 2009
	HS464	ACA TGC GTG TAA ATC ATC GTC G		
ampC	ampC-F	TTC TAT CAA MAC TGG CAR CC	550	Schwartz <i>et al.</i> , 2003; Coertze and Bezuidenhout, 2018
	ampC-R	CCY TTT TAT GTA CCC AYG A		
tetM	tetM-FW	ACA GAA AGC TTA TTA TAT AAC	171	Aminov <i>et al.</i> , 2001
	tetM-RV	TGG CGT GTG TCT ATT GAT GTT CAC		

CHAPTER 3

RESULTS

The following chapter reveals the results that were obtained for the sampling runs that were done in March, May and August 2016, as well as May and November 2017. The results are presented in the order described in Chapter 2. The use of the term “drinking water” in this chapter refers to treated water as well as distribution water that are combined in some of the results.

3.1. Physico-chemical

The physical results for the sampling runs at the NW-C and NW-G treatment plants are represented in Table 4 and Table 5 respectively and included the following parameters: temperature, pH, TDS, salinity and turbidity. The chemical results for the sampling runs at the NW-C and NW-G treatment plants are represented in Table 6 and Table 7 respectively and included the following parameters: free chlorine, phosphorus, nitrites, nitrates, sulphides, sulphates and COD.

3.1.1. Physical results

3.1.1.1. Temperature

The temperatures for NW-C were consistent with seasonal changes as the March 2016 and November 2017 sampling runs showed the highest temperatures in the raw, treated and distribution water (Table 4). The temperatures for NW-G also showed consistency with the seasonal changes during March 2016 and November 2017 showing the highest temperatures for raw, treated and distribution water (Table 5). However, the differences between summer and winter temperatures are considerably higher compared to NW-C. For both NW-C and NW-G, temperatures in the distribution system were most of the times higher than the raw and treated water.

3.1.1.2. pH

The pH values in the drinking water for NW-C complied to SANS 241 and were very consistent across all the sampling runs as can be seen in Table 4. Values ranged from 8.50 – 8.80 between the raw, treated and distribution water. The pH values in the drinking water for NW-G all complied with the SANS 241. Values for drinking water ranged from 8.00 – 8.90 whereas pH values for the raw water ranged from 9.20 – 9.70 in the raw water from May 2016 – November 2017 (Table 5).

3.1.1.3. Total dissolved solids (TDS)

The total dissolved solids (TDS) levels for drinking water at NW-C were well within the limits of the SANS 241 of ≤ 1200 mg/L (Table 4). Across all the sampling runs the TDS values between the raw water and drinking water would not differ with more than 5 mg/L. For all the sampling runs the TDS levels ranged from 366.00 mg/L – 385.7 mg/L for raw and drinking water except for the sampling run in November 2017 which showed decreased TDS levels that ranged from 331.1 mg/L – 334.0 mg/L for raw and drinking water. TDS values in the drinking water at NW-G complied with SANS 241. The TDS values between raw, treated and distribution water during each sampling run were more variable. During most sampling runs except for August 2016 and November 2017, distribution water differed with treated water with 26.3 mg/L, 18.7 mg/L and 72.7 mg/L for March 2016, May 2016 and May 2017 respectively (Table 5).

3.1.1.4. Salinity

There is no SANS 241 set for salinity. The salinity levels at NW-C ranged from 202.6 mg/L – 233.3 mg/L for all the sampling runs and all the sites shown in Table 4. Salinity levels stayed consistent through the treatment process as the biggest difference between raw water and drinking water were only 3.3 mg/L. At NW-G the salinity values in the drinking water ranged from 279.0 mg/L – 361.7 mg/L. Salinity in the drinking water were always more than that found in the raw water (Table 5). The raw water and treated water could not be measured in the May 2017 sampling run.

3.1.1.5. Turbidity

The turbidity of the drinking water at NW-C did not comply to SANS 241 of ≤ 1 NTU during the March 2016, May 2016 and August 2016 sampling runs. Turbidity levels ranged from 0.34 NTU – 3.30 NTU in the drinking water (Table 4). For all the 2016 sampling runs, the turbidity levels in the drinking water were higher than that of the raw water. However, turbidity levels in the drinking water complied to the SANS 241 during the May 2017 and November 2017 sampling runs. Still there was an increase of turbidity from the raw water to the drinking water. At NW-G the turbidity of the drinking water for all the sampling runs except August 2016 complied with SANS 241. These levels ranged from 0.30 NTU – 1.00 NTU. During the August 2016 sampling run, the turbidity in the treated water was 3.85 NTU and in the distribution water 3.26 NTU which is almost 3 to 4 times higher than the standard. The turbidity in the raw water ranged from 11.67 NTU – 19.00 NTU across all the sampling runs (Table 5).

3.1.2. Chemical results

3.1.2.1. Free chlorine

The free chlorine levels in the drinking water for NW-C were acceptable and complied to the SANS 241 of ≤ 5 mg/L as can be seen in Table 6. Levels in the drinking water ranged from 0.02 mg/L – 1.60 mg/L. During the March 2016 sampling run, the free chlorine levels could not be measured. The free chlorine levels in the drinking water at NW-G complied to the SANS 241 (2015). Levels ranged from 0.03 mg/L – 0.80 mg/L in the drinking water (Table 7). Free chlorine could not be measured in the March 2016 and May 2016 sampling runs.

3.1.2.2. Phosphorus

There is no SANS 241 for phosphorus. Levels were not very high in both the drinking water and raw water at NW-C. However, in all three sampling runs, the phosphorus levels were higher in the treatment water compared to the raw water. In the distribution water the levels were consistently low that ranged from 0.15 mg/L – 0.66 mg/L except for the May 2016 sampling run that showed levels of 1.10 mg/L and 1.33 mg/L in the treated water and distribution water respectively (Table 6). At NW-G drinking water

levels ranged from 0.22 mg/L – 3.09 mg/L. In March 2016 and May 2016 there was an increase of phosphorus levels from the raw water to the distribution water. But in the August 2016, May 2017 and November 2017 sampling runs the phosphorus levels decreased from raw water to distribution water (Table 7).

3.1.2.3. Nitrites and Nitrates

The nitrite levels in the drinking water at NW-C did not comply to SANS 241 at any stage except for only the treated water in the May 2017 sampling run (0.67 mg/L). Nitrite levels in the raw water ranged from 3.00 mg/L – 5.00 mg/L showed in Table 6. Nitrite levels in the treated water ranged from 0.67 mg/L – 5.00 mg/L. Nitrite levels in the distribution water ranged from 1.67 mg/L – 5.00 mg/L. These levels in drinking water were as much as five times higher than the expected SANS 241 (2015) of ≤ 0.9 mg/L. Nitrite levels at NW-G were also very high and did not comply to SANS 241 (2015) (Table 7). Nitrite levels in the treated water ranged from 4.00 mg/L – 7.67 mg/L and in the distribution water from 2.17 mg/L – 10.50 mg/L. These levels are more than ten times higher than the limit set by SANS 241 (2015). In the March 2016 and May 2017 and November 2017 sampling runs, the nitrite levels increased in the drinking water compared to the raw water with more than 6 times. NW-C complied to SANS 241 (2015) for nitrate levels (≤ 11 mg/L) for all the sampling runs. Nitrate levels in the drinking water ranged from 1.35 mg/L – 2.97 mg/L (Table 6). Nitrate levels at NW-G also complied to SANS 241 (2015) that ranged from 1.30 mg/L – 4.27 mg/L in the drinking water (Table 7). However, during all the sampling runs, the nitrate levels increased from the raw water to the drinking water.

3.1.2.4. Sulphides and Sulphates

At NW-C the sulphide levels were consistent during individual sampling runs. For instance, if the raw water showed low sulphide values then the drinking water also had low sulphide levels. Low sulphide levels in the drinking water were observed (0 mg/L – 23 mg/L) during all the sampling runs except for August 2016 where the treated water and distribution water showed sulphide levels of 58 mg/L and 46 mg/L respectively (Table 6). At NW-G the sulphide levels decreased in the drinking water compared to the raw water during all the sampling runs except for May 2016 and

November 2017. In May 2016 sulphide levels increased from 41 mg/L in the raw water to 208 mg/L and 161 mg/L in treated water and distribution water respectively. In November 2017 sulphide levels increased from 17 mg/L in the raw water to 41 mg/L and 43 mg/L in the treated water and distribution water respectively (Table 7).

Sulphate levels at NW-C were very low and complied to the SANS 241 (2015) of ≤ 500 mg/L for all the sampling runs (Table 6). Sulphate levels in the raw water ranged from 0.00 mg/L – 6.00 mg/L and in the drinking water they ranged from 0.00 mg/L – 2.00 mg/L. At NW-G the sulphate levels were much higher but the drinking water still complied to SANS 241 (2015) during all the sampling runs. Slight increases in sulphate levels occurred from the raw water into the drinking water during the May 2016, August 2016 and November 2017 sampling runs (Table 7).

3.1.2.5. Chemical oxygen demand (COD)

COD levels were not detected in the raw water during any of the sampling runs at NW-C. However, the May 2016 and August 2016 sampling runs had high levels of COD in the treated water (63 mg/L and 32 mg/L respectively) which decreased into the distribution water again. Therefore, the COD levels in the drinking water across all the sampling runs were 0 mg/L – 63 mg/L (Table 6). During all the sampling runs at NW-G, the COD levels decreased from the raw water into the drinking water. COD levels in the raw water ranged from 19 mg/L – 63 mg/L. The COD levels in the drinking water in March 2016, May 2016, May 2017 and November 2017 were between 8 mg/L – 58 mg/L (Table 7).

Table 4: Physical results for 2016 and 2017 sampling runs at NW-C

Sampling date	Site	Temperature (°C)	pH	TDS (mg/L)	Salinity (mg/L)	Turbidity (NTU)
SANS 241:2015		-	≥ 5 to ≤ 9.7	≤ 1200	-	≤ 1
March 2016	R	18.4	8.56	374.1	227.0	0.20
	T	17.5	8.54	373.0	227.0	2.33
	D	23.0	8.50	374.7	228.3	1.50
May 2016	R	15.7	8.74	382.0	231.0	1.74
	T	15.9	8.60	384.0	232.0	0.34
	D	17.4	8.60	380.0	231.3	1.90
August 2016	R	15.9	8.62	367.0	222.0	0.30
	T	16.2	8.50	366.0	222.0	1.90
	D	15.8	8.70	372.7	225.3	3.30
May 2017	R	16.2 ± 0.06	8.80 ± 0.04	381.7 ± 0.58	231.0 ± 0.00	0.31 ± 0.05
	T	15.5 ± 0.15	8.70 ± 0.01	385.7 ± 1.15	233.3 ± 0.58	0.89 ± 0.11
	D	18.6 ± 0.75	8.70 ± 0.01	380.3 ± 0.58	231.6 ± 0.38	0.69 ± 0.29
November 2017	R	20.5 ± 0.75	8.60 ± 0.01	332.7 ± 0.58	202.7 ± 0.58	0.39 ± 0.13
	T	18.7 ± 0.15	8.54 ± 0.01	334.0 ± 0.53	203.0 ± 1.00	0.59 ± 0.13
	D	23.2 ± 2.86	8.50 ± 0.07	331.1 ± 8.76	202.6 ± 5.70	0.55 ± 0.13

R – raw water; T – treated water; D – drinking water.

*Note that for the 2016 sampling runs there are no standard error deviation due to the fact that measurements were not done in replicates.

Table 5: Physical results for 2016 and 2017 sampling runs at NW-G

Sampling date	Site	Temperature (°C)	pH	TDS (mg/L)	Salinity (mg/L)	Turbidity (NTU)
SANS 241:2015		-	≥ 5 to ≤ 9.7	≤ 1200	-	≤ 1
March 2016	R	23.3	8.40	452.0	276.0	17.50
	T	23.2	8.45	453.0	279.0	0.31
	D	26.5	8.40	479.3	296.0	0.30
May 2016	R	15.9	9.30	456.0	277.0	11.67
	T	16.3	8.51	468.0	285.0	0.33
	D	18.6	8.30	449.3	284.7	0.34
August 2016	R	12.2	9.70	447.0	287.0	14.23
	T	11.3	8.34	511.0	307.0	3.85
	D	16.4	8.00	518.7	316.3	3.26
May 2017	R	13.0 ± 0.06	9.20 ± 0.01	465.7 ± 0.58	309.5 ± 0.00	18.4 ± 2.11
	T	14.5 ± 0.06	8.70 ± 0.01	464.3 ± 1.15	319.8 ± 0.01	0.83 ± 0.28
	D	15.4 ± 0.77	8.90 ± 0.28	537.0 ± 9.84	324.7 ± 9.60	0.41 ± 0.16
November 2017	R	22.4 ± 0.06	9.53 ± 0.02	563.0 ± 2.52	348.0 ± 2.00	19.00 ± 0.35
	T	24.4 ± 0.06	8.17 ± 0.01	583.0 ± 4.16	361.0 ± 3.06	0.43 ± 0.15
	D	23.8 ± 0.88	8.30 ± 0.18	580.1 ± 5.72	359.0 ± 3.61	1.00 ± 0.88

R – raw water; T – treated water; D – drinking water.

*Note that for the 2016 sampling runs there are no standard error deviation due to the fact that measurements were not done in replicates.

Table 6: Chemical results for 2016 and 2017 sampling runs at NW-C

Sampling date	Site	Free chlorine (mg/L)	Phosphorus (mg/L)	Nitrites (mg/L)	Nitrates (mg/L)	Sulphides (mg/L)	Sulphates (mg/L)	COD (mg/L)
SANS 241:2015		≤ 5	-	≤ 0.9	≤ 11	-	≤ 500	-
March 2016	R	-	0.00 ±0.00	3.00 ±2.83	1.15 ±0.21	12 ±1.41	0.00 ±0.00	0 ±0.00
	T	-	0.50 ±0.14	1.00 ±0.00	1.55 ±0.07	14 ±0.71	0.00 ±0.00	0 ±0.00
	D	-	0.28 ±0.12	1.67 ±1.03	1.57 ±0.66	14 ±2.64	0.00 ±0.00	11 ±10.88
May 2016	R	0.03 ±0.01	0.55 ±0.10	5.00 ±0.00	2.00 ±0.00	11 ±2.12	4.00 ±5.66	0 ±0.00
	T	0.99 ±0.23	1.10 ±0.14	5.00 ±1.41	2.30 ±0.28	23 ±2.83	0.00 ±0.00	63 ±9.19
	D	0.41 ±0.27	1.33 ±0.78	4.50 ±1.05	2.03 ±0.45	21 ±3.50	0.00 ±0.41	6 ±9.17
August 2016	R	0.05 ±0.01	0.13 ±0.04	3.50 ±0.71	2.75 ±0.49	50 ±1.41	1.00 ±0.71	0 ±0.00
	T	0.02 ±0.00	0.22 ±0.01	3.50 ±2.12	1.35 ±1.49	58 ±10.61	2.00 ±2.12	32 ±1.41
	D	0.60 ±0.13	0.15 ±0.07	5.00 ±3.03	2.27 ±0.96	46 ±15.20	1.00 ±0.52	19 ±25.83
May 2017	R	0.06 ±0.08	0.22 ±0.09	3.33 ±1.15	1.77 ±0.40	5 ±4.58	6.00 ±4.93	0 ±0.00
	T	1.60 ±0.05	0.23 ±0.12	0,67 ±0.58	1.90 ±0.56	0 ±0.00	0.00 ±0.00	3 ±5.20
	D	0.19 ±0.10	0.21 ±0.10	1,56 ±1.24	1.79 ±0.28	1 ±1.94	0.00 ±0.33	1 ±1.96
November 2017	R	0.01 ±0.02	2.48 ±1.15	3,33 ±1.53	3.63 ±1.05	13 ±5.20	0.33 ±0.58	0 ±0.00
	T	1.05 ±0.02	3.17 ±1.76	4,00 ±1.00	2.97 ±0.38	12 ±5.29	0.33 ±0.58	0 ±0.00
	D	0.35 ±0.17	0.66 ±0.71	2,89 ±1.27	2.50 ±0.56	16 ±4.65	0.33 ±0.50	0 ±5.02

R – raw water; T – treated water; D – drinking water; COD – chemical oxygen demand.

Table 7: Chemical results for 2016 and 2017 sampling runs at NW-G

Sampling date	Site	Free chlorine (mg/L)	Phosphorus (mg/L)	Nitrites (mg/L)	Nitrates (mg/L)	Sulphides (mg/L)	Sulphates (mg/L)	COD (mg/L)
SANS 241:2015		≤ 5	-	≤ 0.9	≤ 11	-	≤ 500	-
March 2016	R	-	1.02 ±0.00	2.00 ±0.00	0.25 ±0.21	47 ±0.71	615 ±35.36	25 ±1.41
	T	-	0.85 ±0.02	4.00 ±0.00	3.75 ±0.07	14 ±0.71	195 ±7.07	12 ±0.00
	D	-	1.06 ±0.1	2.17 ±0.41	2.20 ±0.67	16 ±12.97	330 ±219.18	10 ±3.21
May 2016	R	-	0.31 ±0.01	36.00 ±25.46	0.30 ±0.28	41 ±4.24	115 ±21.21	51 ±2.83
	T	-	0.58 ±0.39	4.50 ±0.71	1.35 ±0.07	208 ±198.70	120 ±14.14	8 ±0.71
	D	-	0.44 ±0.23	10.50 ±9.09	1.30 ±0.60	161 ±153.19	133 ±30.11	16 ±15.32
August 2016	R	0.05 ±0.01	6.70 ±4.95	10.00 ±1.41	1.15 ±0.21	82 ±2.12	135 ±21.21	63 ±6.36
	T	0.03 ±0.01	2.70 ±0.42	5.00 ±0.00	2.00 ±0.14	51 ±0.71	465 ±21.21	58 ±10.61
	D	0.40 ±0.61	2.72 ±1.67	9.50 ±8.69	1.98 ±0.65	45 ±4.98	145 ±18.71	28 ±10.20
May 2017	R	0.11 ±0.01	4.23 ±2.19	1.33 ±0.58	0.97 ±0.67	45 ±4.36	93 ±1.00	19 ±17.01
	T	0.80 ±0.05	0.28 ±0.09	7.67 ±5.03	4.27 ±0.32	36 ±46.13	90 ±2.08	8 ±13.86
	D	0.24 ±0.31	0.22 ±0.05	3.78 ±0.97	2.22 ±0.65	9 ±6.33	95 ±4.72	19 ±16.03
November 2017	R	0.15 ±0.02	3.62 ±1.66	1.33 ±0.58	0.00 ±0.20	17 ±5.51	92 ±4.36	33 ±2.83
	T	0.59 ±0.03	3.09 ±1.61	6.33 ±2.08	2.30 ±0.36	41 ±13.61	102 ±2.65	19 ±2.83
	D	0.07 ±0.05	1.34 ±1.20	4.22 ±0.83	1.86 ±0.76	43 ±7.95	103 ±2.50	19 ±9.83

R – raw water; T – treated water; D – drinking water; COD – chemical oxygen demand.

3.2. Microorganisms n drinking water

Indicator organisms used in general water quality testing were only detected in the raw waters for both NW-C and NW-G during May 2017 and November 2017 sampling runs (microbiological tests were only done for May 2017 and November 2017 sampling runs). During the May 2017 sampling run at NW-C *E. coli*, faecal- and total coliform levels were 32 CFU/100mL, 4 CFU/100mL and 36 CFU/100mL respectively. During the November 2017 sampling run these levels were 46 CFU/100mL, 33 CFU/100mL and 79 CFU/100mL respectively. During the May sampling run at NW-G *E. coli*, faecal- and total coliform levels were 8 CFU/100mL, 0 CFU/100mL and 8 CFU/100mL respectively. During the November 2017 sampling run these levels were 23 CFU/100mL, 12 CFU/100mL and 35 CFU/100mL respectively.

HPC bacteria were detected in the raw water, treated water and the distribution water. Due to statistical incoherency with HPCs specifically in drinking water, the HPC levels are not indicated. However, more important was that it could be observed that HPC levels decreased from the raw water to the drinking water during all the sampling runs at NW-C and NW-G.

3.3. Statistical analysis

A principle component analysis (PCA) was used to indicate possible correlations between the physico-chemical properties and the HPCs for NW-C and NW-G during all the sampling runs. Correlations were made between the raw water sources of NW-C and NW-G (Figure 8) as well as between the raw water and drinking water of NW-C and NW-G respectively (Figure 9 and Figure 10). Average values of the parameters were used for these analysis.

In Figure 8 the raw water quality (physico-chemical and HPC levels) of NW-C and NW-G were compared. It can be observed that the raw water for NW-C and NW-G has a negative correlation to each other in terms of water quality. Nitrites were the only parameter closely associated with the raw water at NW-C. It is clear that turbidity and temperature were associated. Strangely the HPCs were negatively correlated to turbidity and temperature. HPCs were closely related to nitrates, sulphates, pH, COD and phosphates and were more closely related to NW-G.

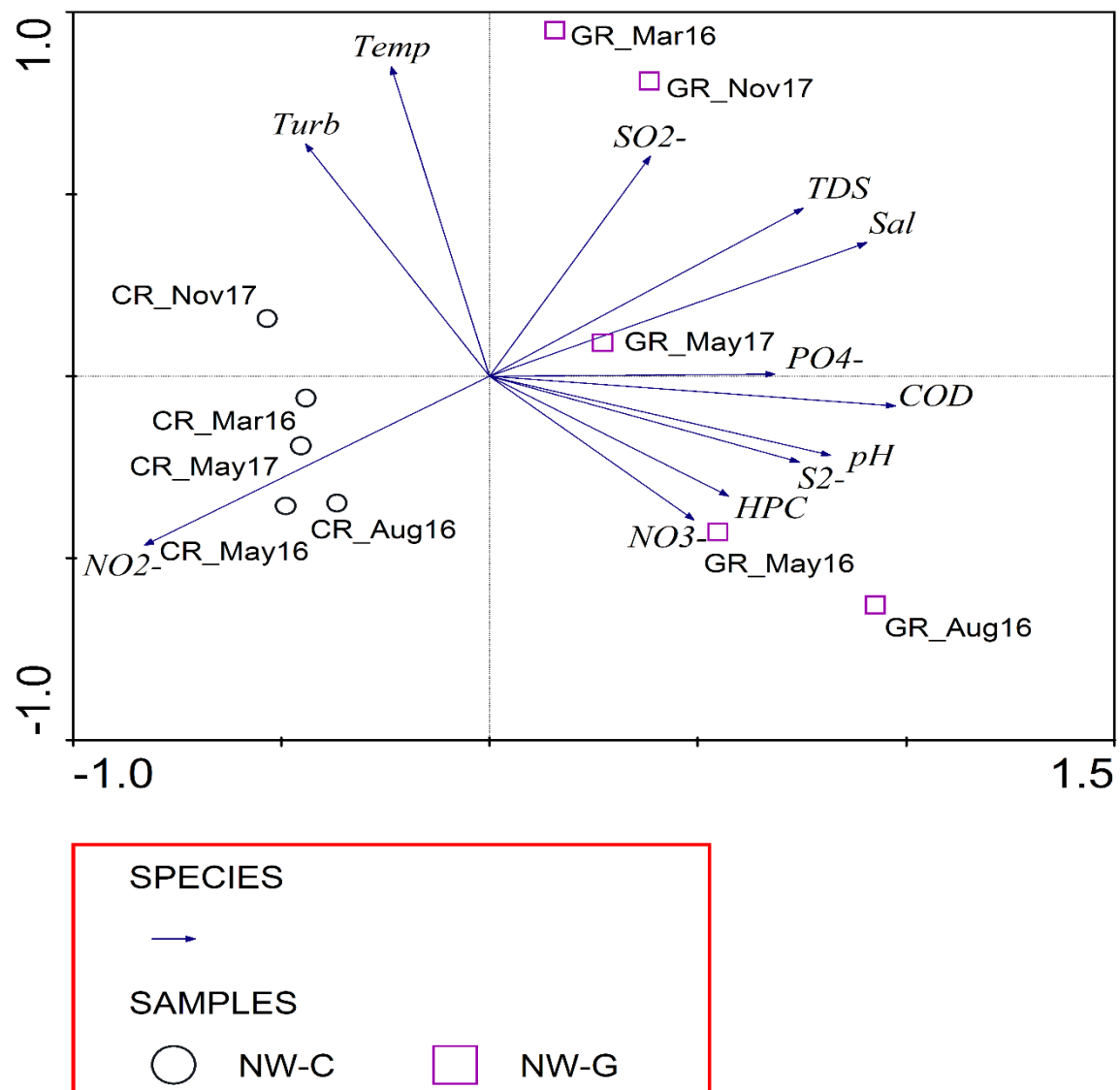


Figure 8: PCA plot comparing the water quality (physico-chemical and HPC levels) between the raw waters at NW-C and NW-G. Temp – temperature; TDS – total dissolved solids; Sal – salinity; Turb – turbidity; PO_4^- – phosphate; NO_3^- – Nitrates; NO_2^- – nitrites; S_2^- – sulphides; SO_2^- – sulphates; COD – chemical oxygen demand.

In Figure 9 the raw water was compared to the drinking water at NW-C. It can be seen that the raw water is closely related to HPCs, nitrites, sulphates and pH. The November sampling run 2017 (raw, treated and distribution) showed to be closely related to temperature and phosphates. The treated water and distribution water in the 2016 sampling runs showed correlations with each other as well as with turbidity, COD, sulphides and nitrates. Negative correlations were observed between the raw

water and drinking water (treated and distribution) except for the May 2017 sampling run when the drinking water was closely related to the raw water quality.

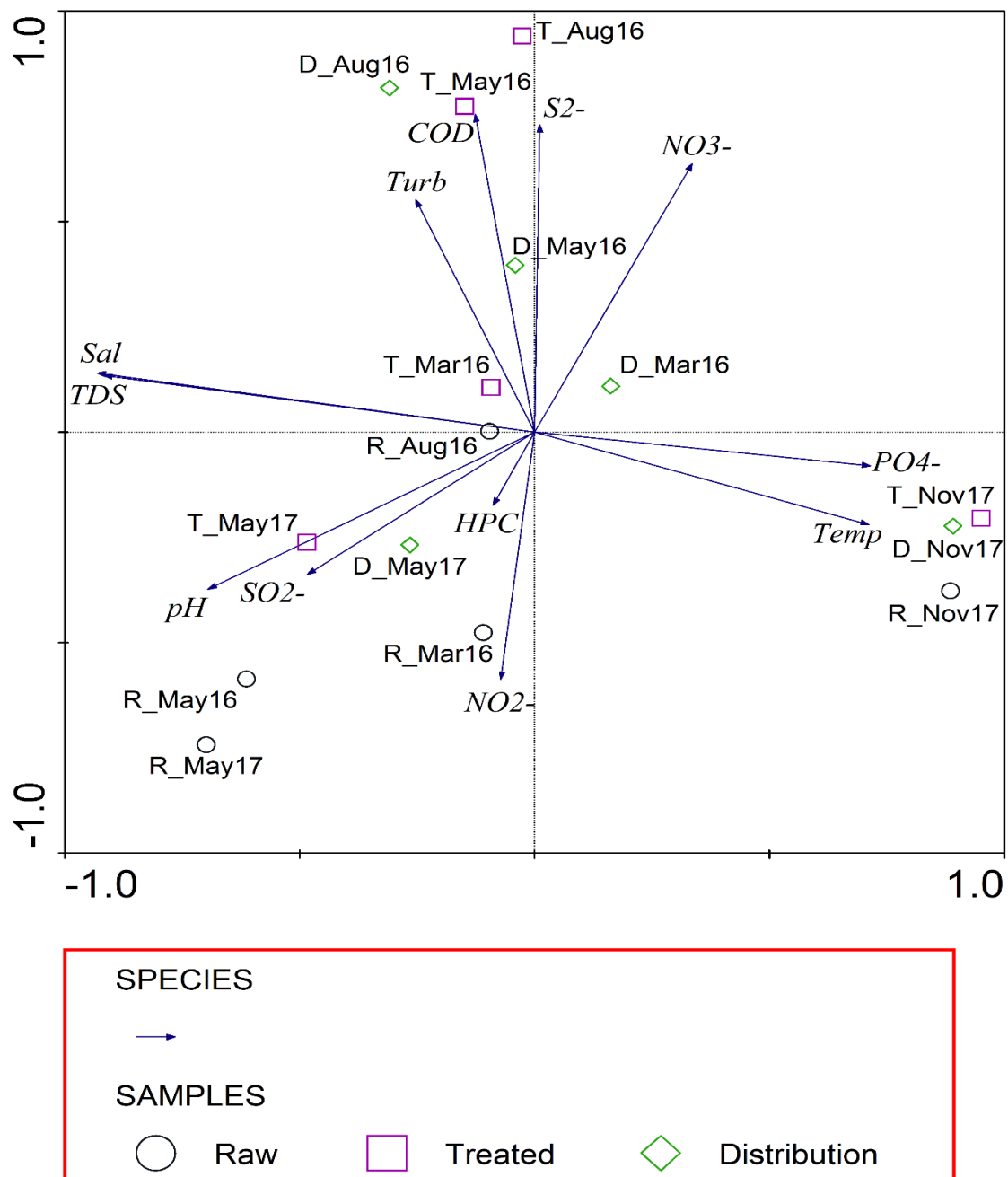


Figure 9: PCA plot comparing the water quality (physico-chemical and HPC levels) between the raw water and drinking water at NW-C. Temp – temperature; TDS – total dissolved solids; Sal – salinity; Turb – turbidity; PO_4^{4-} – phosphate; NO_3^- – Nitrates; NO_2^- – nitrites; S_2^{2-} – sulphides; SO_4^{2-} – sulphates; COD – chemical oxygen demand.

Figure 10 illustrates that the drinking water is negatively correlated with the raw water at NW-G. Turbidity, pH, COD and HPCs are closely correlated whereas nitrites and sulphates were closely related. All these parameters were associated with the raw water whereas the TDS, temperature and nitrates were associated with the drinking water.

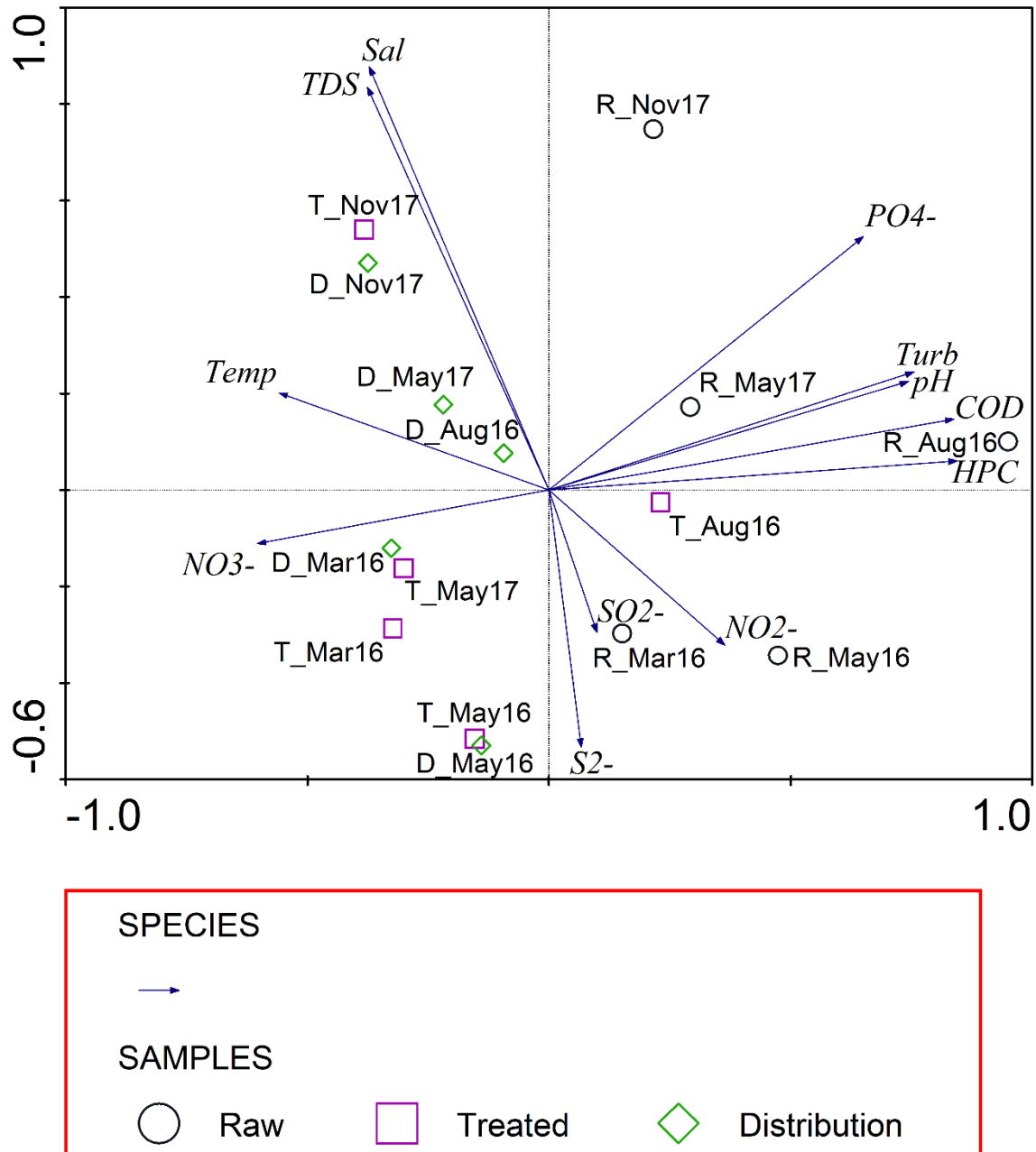
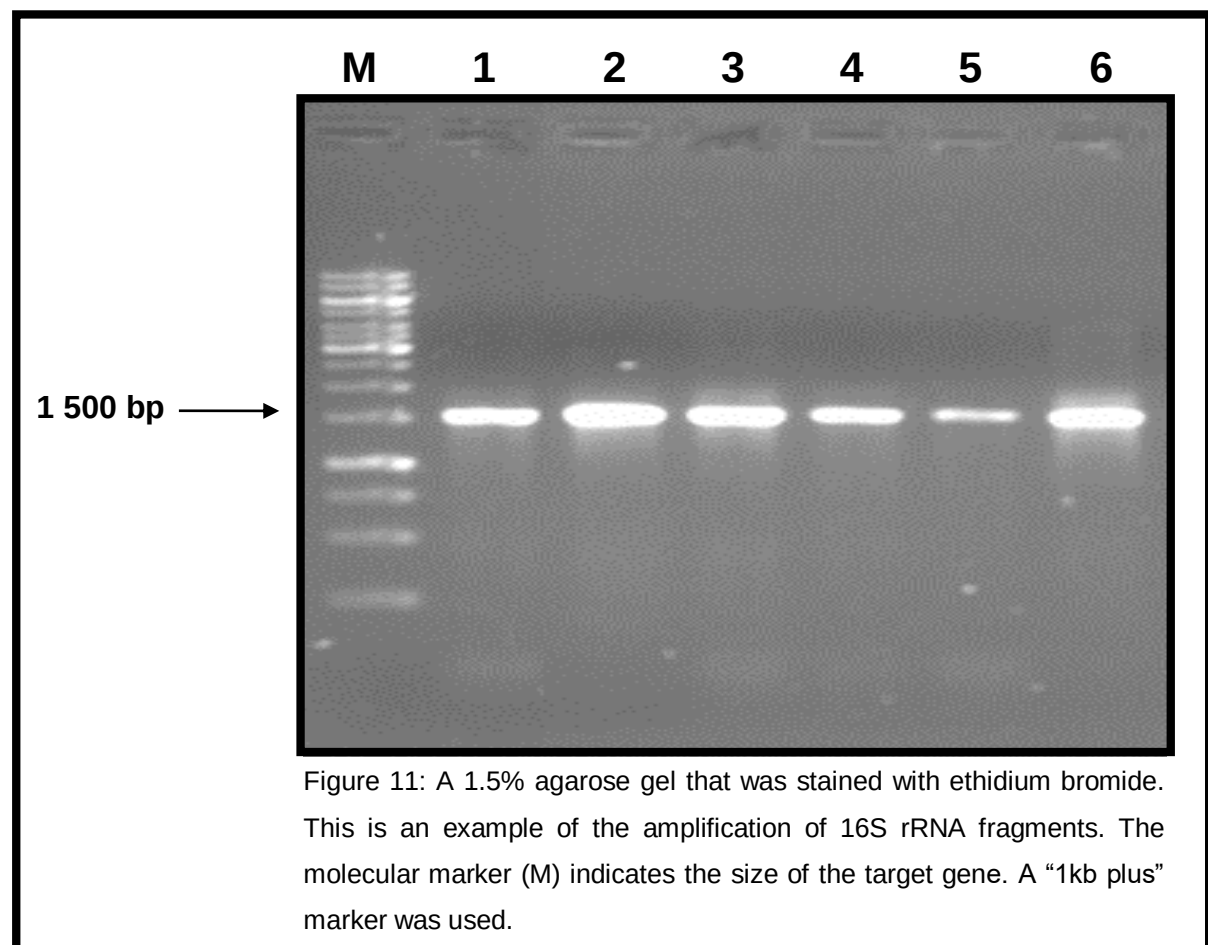


Figure 10: PCA plot comparing the water quality (physico-chemical and HPC levels) between the raw water and drinking water at NW-G. Temp – temperature; TDS – total dissolved solids; Sal – salinity; Turb – turbidity; PO₄⁻ – phosphate; NO₃⁻ – Nitrates; NO₂⁻ – nitrites; S₂⁻ – sulphides; SO₄²⁻ – sulphates; COD – chemical oxygen demand.

3.4. Identification of HPC isolates

3.4.1. 16S rRNA amplification

In total, 135 HPC isolates (from NW-C and NW-G including all the sampling runs) were subjected to DNA isolation. The 16S rRNA gene fragment was then amplified which is needed in order to sequence and identify the specific isolate. Figure 11 illustrates the amplification of the 16S rRNA gene.



3.4.2. Sequencing

The 16S rRNA genes of the HPC isolates were used for sequencing in order to identify the bacteria up to species level. Figures 12 and 13 illustrate the total number of isolates that were identified in the raw water and drinking water respectively at NW-C for all the sampling runs. Figures 14 and 15 illustrate the total number of isolates that were identified in the raw water and drinking water respectively at NW-G for all the sampling

runs. The identified organisms presented in figures 12 - 15 are grouped to genus level and not to species level to make the illustration more presentable.

3.4.3. HPC bacteria in raw water and drinking water at NW-C

Although there are only 13 genera illustrated in the raw water (Figure 12), a total of 34 isolates (n) were identified and grouped under these genera. In the drinking water (Figure 13), 47 isolates were grouped under 11 genera. The diversity of HPC bacteria was higher in the raw water compared to the drinking water when observing the genera. The difference in diversity is however only by two genera which is not considerable. The total amount of bacteria in the drinking water (47) is higher compared to the raw water (34) as a consequence of combining the treated water and distribution water to make “drinking water”. In both the raw water and drinking water, *Bacillus* were by far the most abundant species group. However, in the drinking water, *Bacillus* represented 73% of HPC bacteria and more than double compared to the amount of *Bacillus* in the raw water (35%). In the raw water, *Flavobacterium* and *Roseateles* made up 14% and 12% of HPC bacteria respectively where the rest of the genera represented 6% and less. In the drinking water, *Arthrobacter* represented 7% of HPC bacteria while the rest of the genera represented 4% and less.

3.4.4. HPC bacteria in raw water and drinking water at NW-G

In the raw water, 26 isolates were identified and made up 7 genera (Figure 14). In the drinking water 28 HPC bacteria were identified (Figure 15) but once again it must be noted that it is treated water and distribution water combined. However, opposite to what was observed at NW-C, the diversity in the drinking water (15 genera) at NW-G was more than twice higher compared to the raw water. The same trend was observed with *Bacillus* representing most of the HPC bacteria in the raw water (31%) as well as the drinking water (39%). Once again the representation of *Bacillus* in the drinking water was higher, compared to the raw water, but the difference was much less compared to NW-C. In the raw water *Pseudomonas* represented 23% whereas *Aeromonas* and *Flavobacterium* made up 15% of HPC bacteria. The rest of the genera represented 8% and less. In the drinking water, the highest representation of HPC

bacteria following *Bacillus* were *Pseudomonas* with 11% and *Rheinheimera* with 7%. The rest of the HPC bacteria represented 4% or less.

3.4.5. Comparing identified HPC bacteria between NW-C and NW-G

It is clear that *Bacillus* dominates the raw water and drinking water at both NW-C and NW-G. Also, the representation of *Bacillus* increased from the raw water into the drinking water at NW-C and NW-G. Among the rest of the bacteria, *Flavobacterium*, *Aeromonas* and *Pseudomonas* were present in both the raw water sources and then decreased in the drinking water. Opposite to NW-C, NW-G had higher diversity in the drinking water compared to the raw water.

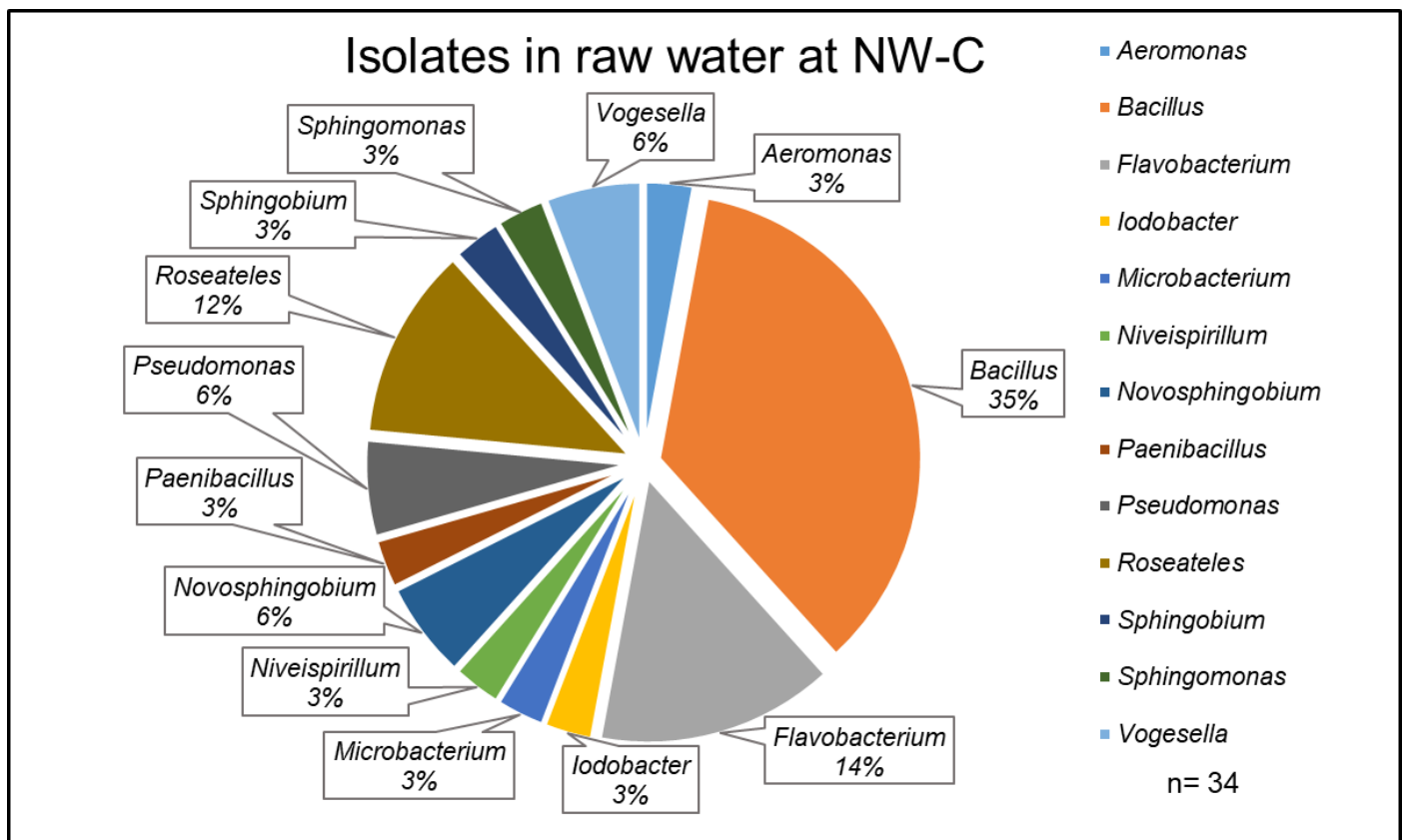


Figure 12: The representation of HPC bacteria that were identified in the raw water at NW-C.

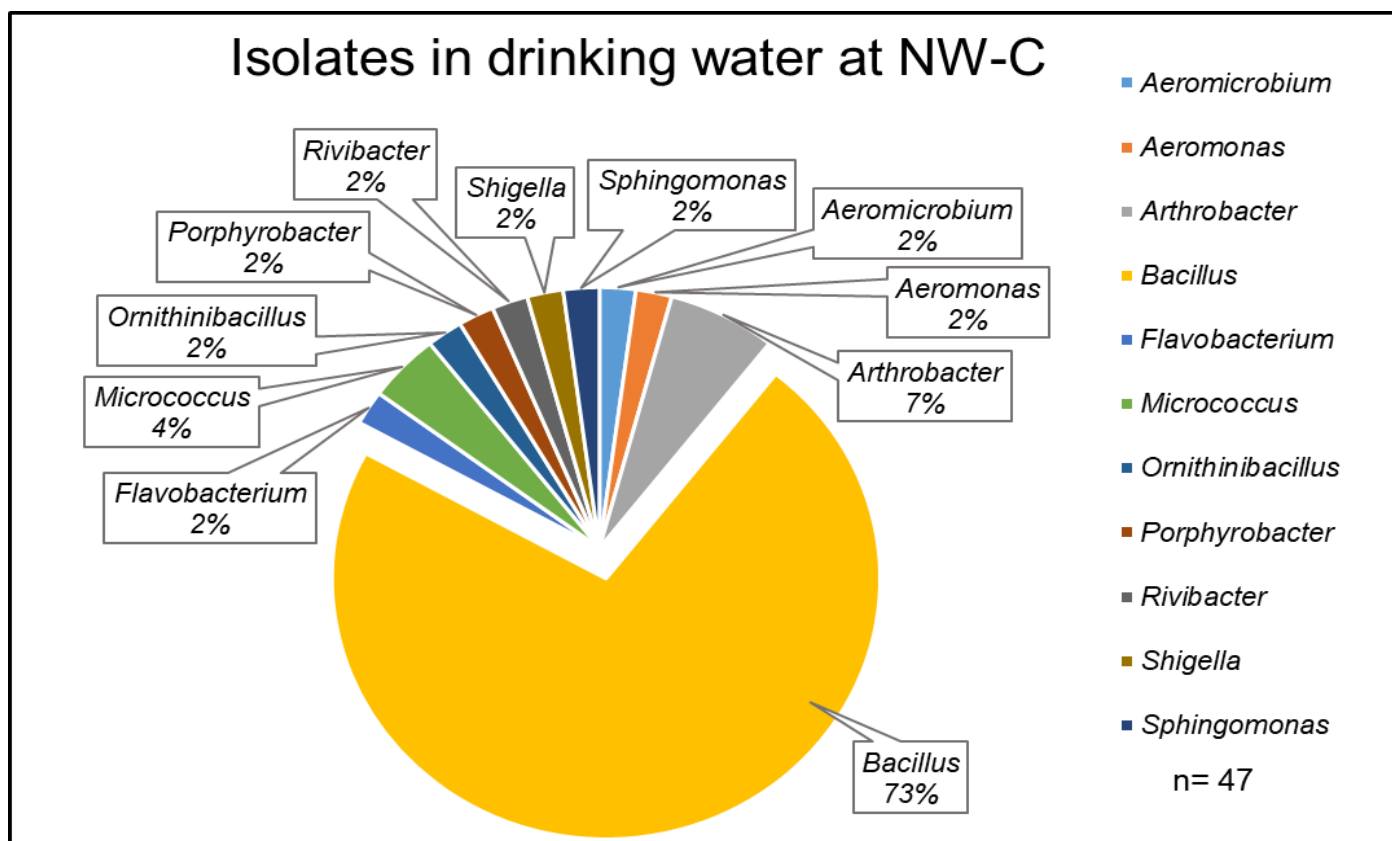


Figure13: The representation of HPC bacteria that were identified in the drinking water at NW-C.

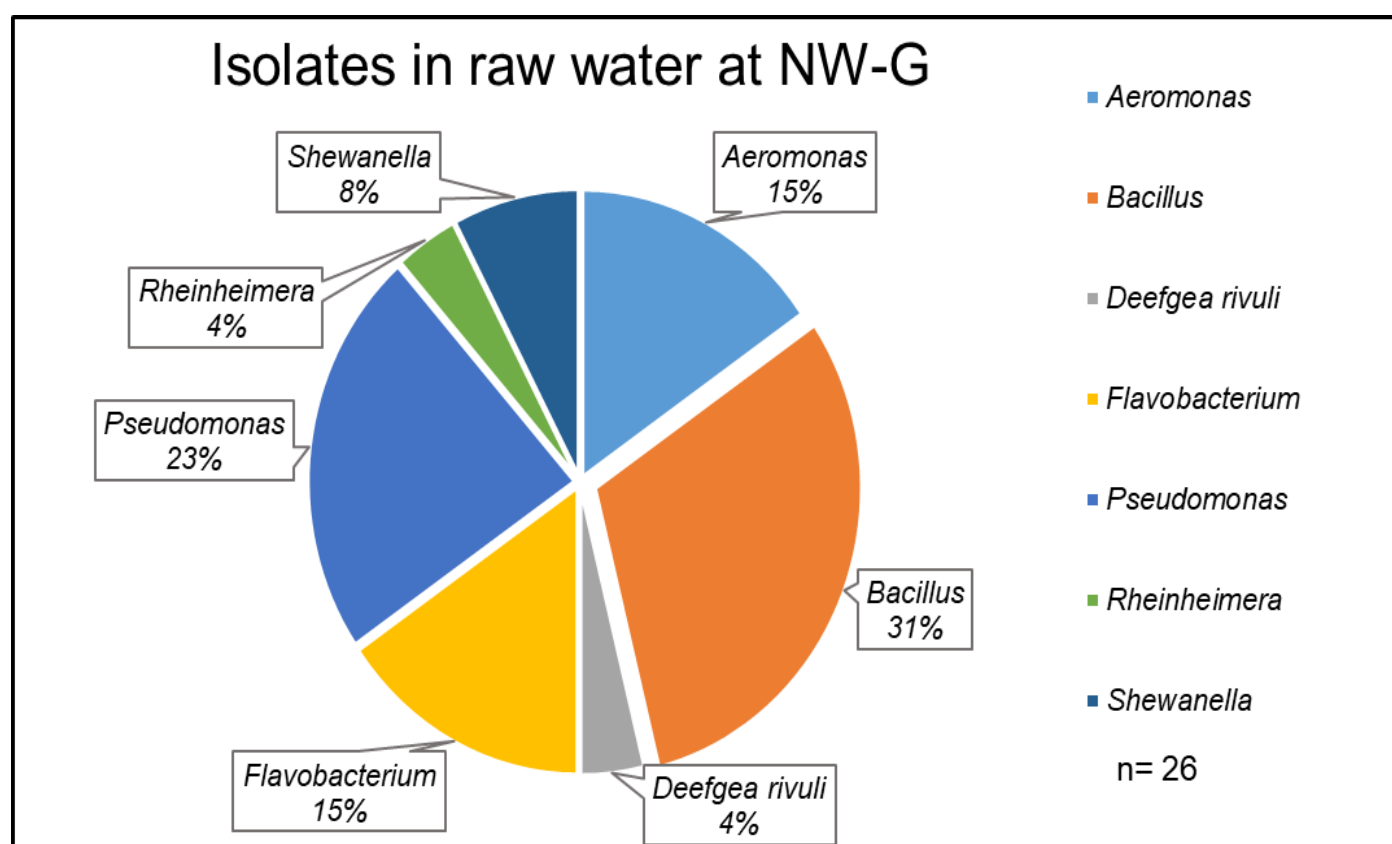


Figure14: The representation of HPC bacteria that were identified in the raw water at NW-G.

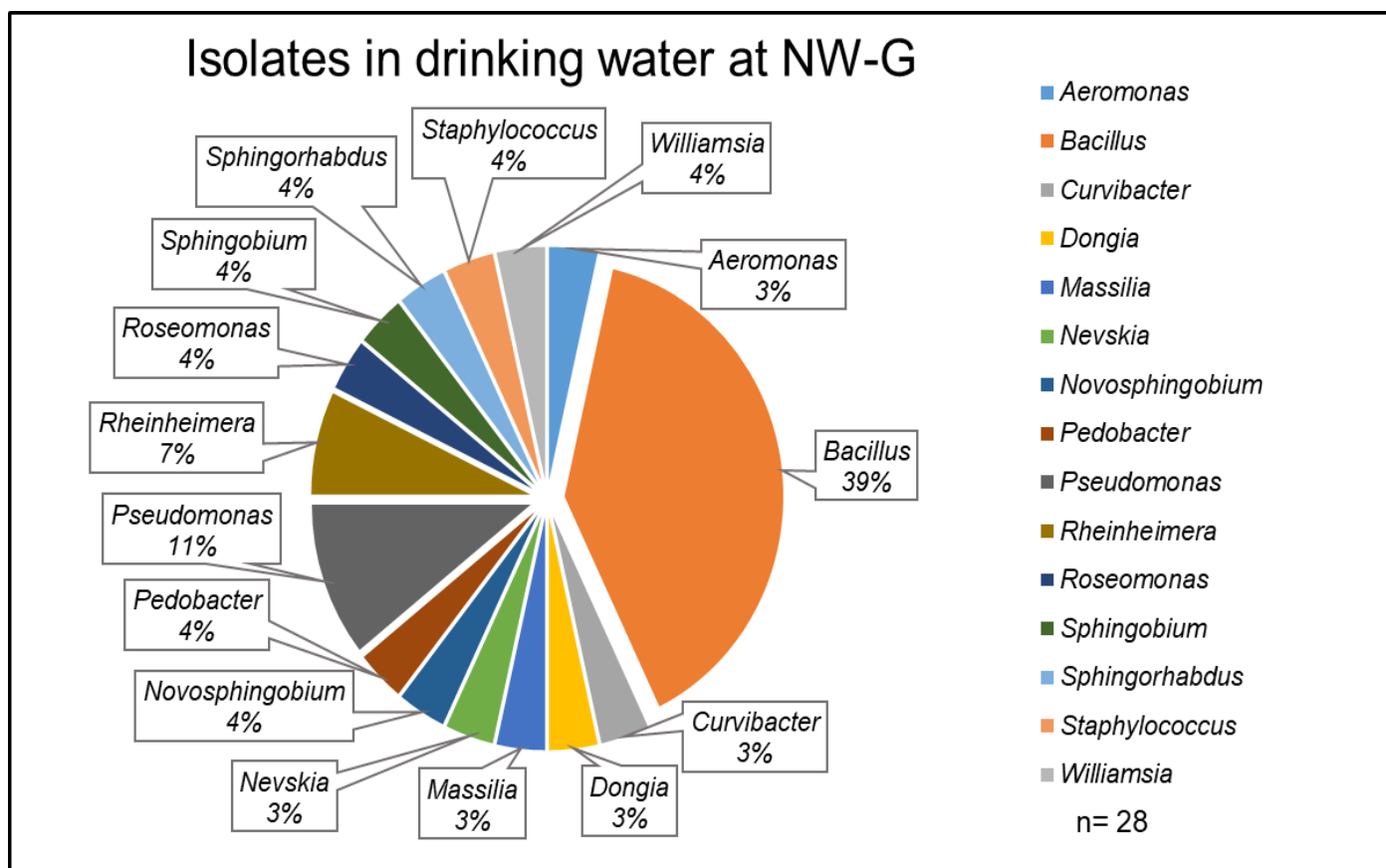


Figure 13: The representation of HPC bacteria that were identified in the drinking water at NW-G.

3.4.6. Evolutionary relationships

Tables 13 and 14 in the appendix illustrates the identified isolates together with the amount of isolates (n) and where the species have been isolated (raw water, treated water or distribution water) for NW-C and NW-G respectively. Figure 16 and 17 illustrates the evolutionary relationships between all the identified isolates (raw water, treated water and distribution water) from NW-C and NW-G respectively by the reconstruction of a phylogenetic tree. The isolates clustered into four phyla called Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes. The majority of the isolates (60.61%) belonged to the Firmicutes, followed by the Proteobacteria (24.24%), Actinobacteria (9.09%) and lastly the Bacteroidetes (6.06%). The Firmicutes phylum was dominated with *Bacillus spp.* The Actinobacteria phylum consisted of *Aeromicrobium*, *Microbacterium*, *Micrococcus* and *Arthrobacter* species. The Proteobacteria phylum consisted of *Shingobium*, *Shingomonas*, *Niveispirillum*,

Aeromonas, *Shigella*, *Pseudomonas*, *Iodobacter*, *Vogesella*, *Rivibacter* and *Roseateles* species. The Bacteroidetes only consisted of *Flavobacterium* species.

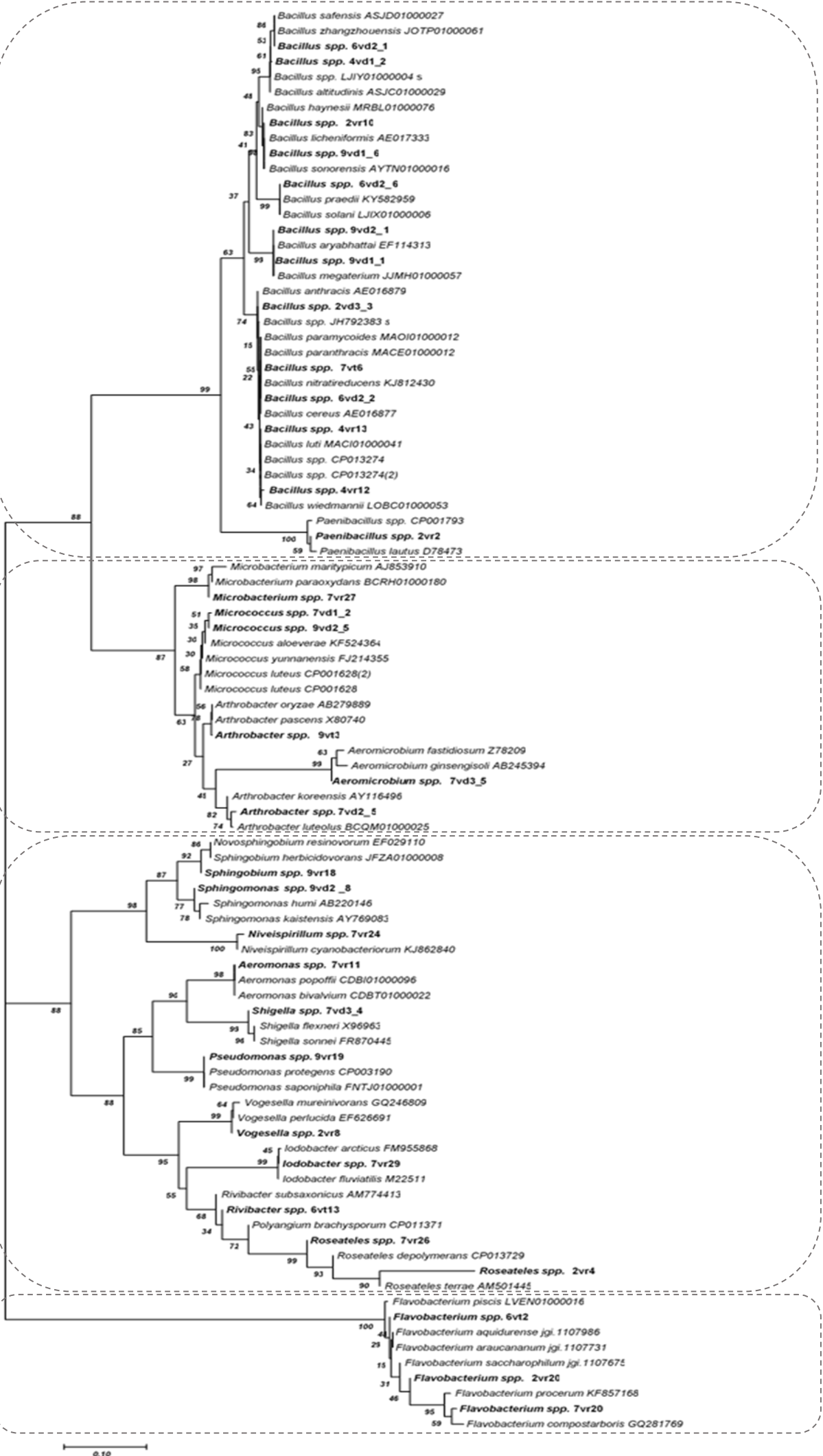
Figure 17 illustrates that all the isolates at NW-G clustered into the same four phyla as NW-C. However, other than NW-C, the majority of isolates (62.86%) belonged to Proteobacteria, followed by the Firmicutes (22.86%), Bacteroidetes (11.43%) and Actinobacteria (2.86%). The Proteobacteria phylum consisted of *Aeromonas*, *Shewanella*, *Rheinheimera*, *Pseudomonas*, *Nevskia*, *Deefgea*, *Curvibacter*, *Masilia*, *Roseomonas*, *Dongia*, *Sphingobium*, *Novosphingobium*, *Sphingohabdus* species. Only *Williamsia spongiae* formed part of the Actinobacteria phylum. The Firmicutes consisted mostly of *Bacillus* species and one *Staphylococcus* species. The Bacteroidetes phylum consisted of *Pedobacter* and *Flavobacterium* species.

Firmicutes

Actinobacteria

Proteobacteria

Bacteroidete



Firmicutes

Actinobacteri

Proteobacteria

Bacteroidete

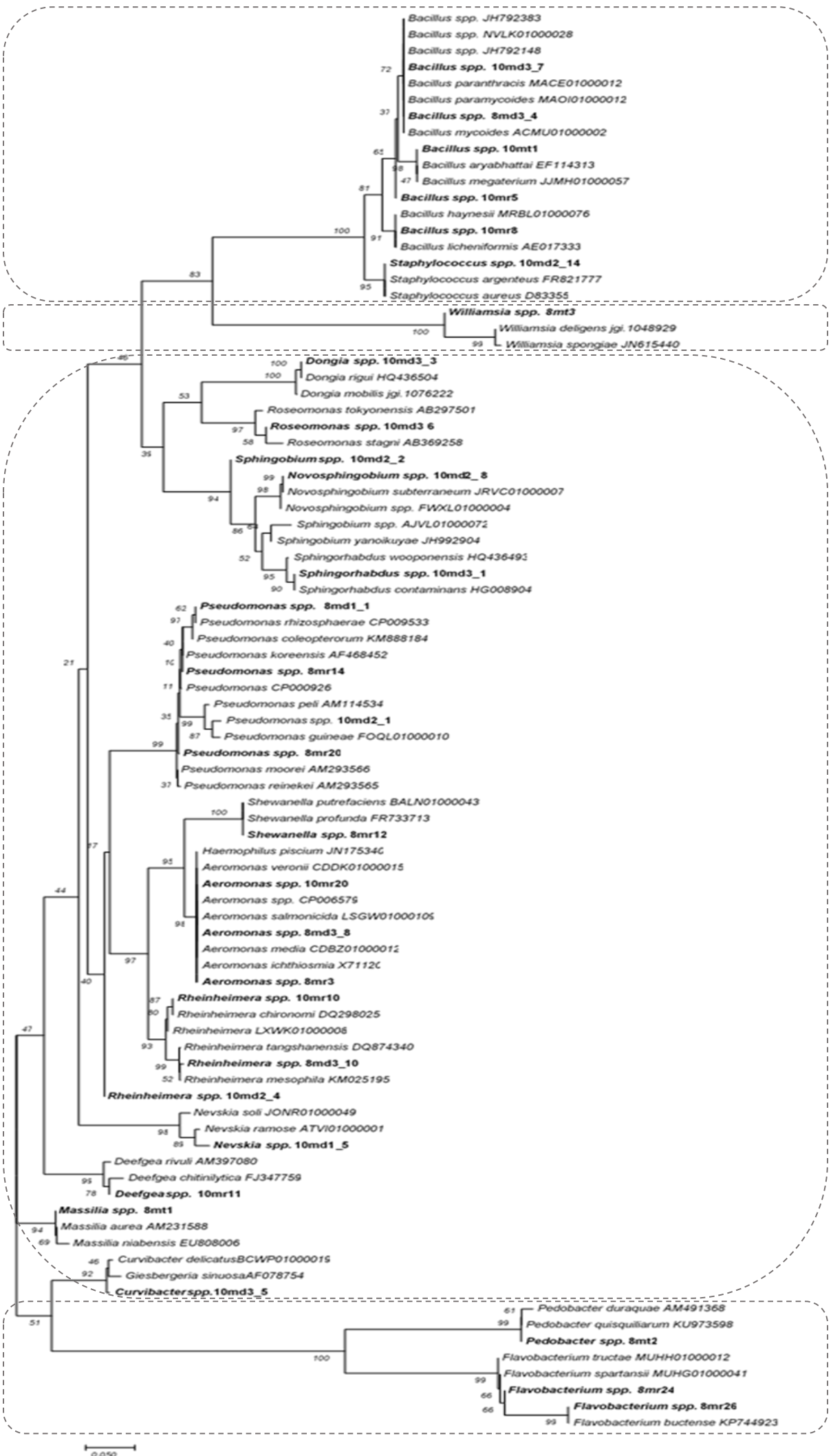


Figure 15: A Neighbor-Joining tree constructed using partial 16S rRNA gene sequences from HPC isolates at NW-G. The Bootstrap confidence values (1000 replicates) are given at the branch point. The entries in **Bold (Genus spp.)** are isolated HPC bacteria with their codenames (eg. **10mr5**).

3.5. Pathogenicity

The potential pathogenicity of the HPC bacteria was determined by first using the haemolysis test. All the isolates that tested positive for haemolysin, underwent further extracellular tests. At NW-C a total of 63 isolates were subjected to the haemolysin test of which 23 were representatives from raw water and 40 from the drinking water. As can be seen in Table 8, a staggering 86.95% and 82.50% of isolates tested positive for haemolysis. Of all the haemolysin positive isolates at NW-C, only one in the raw water and one in the drinking water showed α -haemolytic activity whereas the rest showed β -haemolytic activity. At NW-C, the rest of the enzymatic tests were performed on 20 isolates in the raw water and 33 in the drinking water. Proteinase and lecithinase showed the highest percentages with 85.00% and 90.00% in the raw water respectively. Then for both proteinase and lecithinase there was an increase into the drinking water with 93.94% and 96.97% respectively. Lipase showed percentages of 45.00% and 39.39% in the raw water and drinking water respectively. DNase increased from the raw water into the drinking water from 40.00% to 51.51%.

Table 8: The extracellular enzyme tests for HPC isolates from NW-C and NW-G

Plant	Site	Haemolysis %	Proteinase %	Lecithinase %	Lipase %	DNase %
NW-C	Raw	86.95	85.00	90.00	45.00	40.00
		n = 23	n = 20	n = 20	n = 20	n = 20
	Drinking	82.50	93.94	96.97	39.39	51.51
		n = 40	n = 33	n = 33	n = 33	n = 33
NW-G	Raw	76.47	96.15	96.15	38.46	42.31
		n = 34	n = 26	n = 26	n = 26	n = 26
	Drinking	86.67	96.15	96.15	19.23	50.00
		n = 30	n = 26	n = 26	n = 26	n = 26

n – number of isolates tested

A total of 34 HPC isolates in the raw water and 30 in the drinking water were subjected to the haemolysin test at NW-G. In the raw water 76.47% tested positive and in the drinking water 86.67% were tested positive. The haemolysin positive isolates were subjected to further tests and therefore in the raw water as well as the drinking water 26 isolates were tested. For proteinase and lecithinase 96.15% of the isolates tested

positive in the raw water as well as the drinking water. For lipase 38.46% of isolates were positive in the raw water and 19.23% were positive in the drinking water. Regarding DNase, 42.31% of isolates were positive in the raw water and 50.00% were positive in the drinking water.

Table 9: The HPC isolates that tested positive for all the extracellular enzymes from NW-C and NW-G

Treatment plant	Sampling site	Isolate	Number of antibiotics resistant to	ID
NW-C	Drinking water	4d2_5	5	<i>Bacillus species</i>
	Drinking water	7vd3_7	6	<i>Bacillus paramycoides</i>
	Drinking water	9vt3	1	<i>Arthrobacter oryzae</i>
	Drinking water	9vt6	1	<i>Arthrobacter species</i>
	Drinking water	9vd1_6	6	<i>Bacillus sonorensis</i>
NW-G	Raw water	8mr23	2	<i>Shewanella species</i>
	Drinking water	8md3_8	2	<i>Aeromonas salmonicida</i>
	Raw water	10mr8	1	<i>Bacillus licheniformis</i>

ID – identification of isolate

Among the 127 HPC isolates from NW-C and NW-G combined, there were only three isolates (two *Pseudomonas protegens* and one *Sphingomonas kaistensis*) that tested positive for only two enzyme tests which was haemolysin and lipase. The rest of the 125 isolates tested positive for three or more of the enzymes. Also, among the 127 isolates, nine tested positive for all the enzymes (Table 19). Five of the nine isolates were from NW-C and were only found in the drinking water. These isolates included *Bacillus spp.*, *Bacillus paramycoides*, *Arthrobacter oryzae*, *Arthrobacter spp.* and *Bacillus sonorensis*. The other four isolates were from NW-G of which three were

isolated from the raw water and one isolated from the drinking water. These isolates included *Shewanella spp.*, *Aeromonas salmonicida*, *Bacillus licheniformis* and

Deefgea rivuli. The *Bacillus spp.* (4d2_5; 7vd3_7; 9vd1_6) in the drinking water at NW-C showed multiple resistance against ampicillin, cephalothin, penicillin, trimethoprim and vancomycin. Isolate 7vd3_7 showed resistance against ampicillin, cephalothin, penicillin, trimethoprim, erythromycin and kanamycin. Isolate 9vd1_6 were resistant to ampicillin, cephalothin, penicillin, trimethoprim, kanamycin and oxytetracycline. The *Arthrobacter spp.* (9vt3 and 9vt6) did not show multiple resistance patterns. At NW-G, a different pattern was observed. The isolates showed resistance to only 2 antibiotics at the most and *Bacillus licheniformis* to one antibiotic.

3.6. Antibiotics in water samples

Among all the antibiotics mentioned in section 2.11, ciprofloxacin concentrations were found in the raw water, treated water as well as the distribution water at NW-C and NW-G. Neomycin concentrations were found only at NW-C in the treated water and distribution water. Benzyl-penicillin (Penicillin G) were found only at NW-G in the raw water and distribution water. Penicillin concentrations were only detected at NW-C in the raw water, treated water as well as the distribution water. Finally, streptomycin concentrations were only detected at NW-G in the raw water and treated water.

3.7. Antibiotic susceptibility

Table 10 represents the average amount of isolated bacteria that were resistant to each antibiotic for NW-C and NW-G. Table 11 represents the average inhibition zone sizes (IZS) that were measured against each antibiotic using the Kirby-Bauer disc diffusion method.

In Table 10 it is apparent that the average resistance is high against the beta-lactams (ampicillin, cephalothin and penicillin) and trimethoprim. The average percentages in the raw water and drinking water for these four antibiotics at both NW-C and NW-G ranged from 47% - 100%. At NW-C, average resistance against ampicillin and cephalothin were closely related. In the raw water it was 63% and 66% respectively, and ranged from 56% - 66% in the drinking water. However, for penicillin and trimethoprim average resistance increased. For penicillin, the average resistance in the raw water was 67% and increased in the treated water to a shocking 100%

resistance (among 23 isolates), and decreased again to 76% resistance in the distribution water which is still very high. For trimethoprim average resistance in the raw water were 83% and decreased to 73% and 76% in the treated water and distribution water respectively.

At NW-G, average resistance against ampicillin and cephalothin were 63% and 56% in the raw water respectively. In the treated water the average resistance was 47% for both ampicillin and cephalothin but increased in the drinking water to 72% and 67% respectively. The average resistance against penicillin at NW-G were lower compared to NW-C and ranged from 58% - 61% in raw water and drinking water. For trimethoprim, resistance was also lower compared to NW-C with 62% in the raw water, 56% in the treated water and 72% in the distribution water.

A general trend that can be observed for all the antibiotics is the increase of average resistance from the raw water into the drinking water at both NW-C and NW-G. The average resistance percentages for the antibiotics excluding ampicillin, cephalothin, penicillin and trimethoprim ranged from 0% to 42% for both NW-C and NW-G. Comparing NW-C with NW-G, it seems as if higher resistance percentages are associated with NW-C.

In Table 11 the same trend can be observed as seen in Table 10 regarding the average IZS. As one would expect, the average IZS were most of the times smaller than the standard zone size that indicates whether that isolate is resistant to the specific antibiotic. However, in the raw water at NW-G, the average IZS for ampicillin, cephalothin and trimethoprim were bigger than the standard ($\leq 13\text{mm}$, $\leq 15\text{mm}$ and $\leq 10\text{mm}$ respectively) that indicates resistance. For treated water the average IZS for ampicillin at NW-G were also higher than the standard indicating resistance. For the beta-lactams (ampicillin, cephalothin and penicillin) the IZS were not substantially smaller than the standards indicating resistance. It was only the average IZS with trimethoprim that were significantly lower than the standard for resistance except for the raw water at NW-G. The average IZS for the rest of the antibiotics were comfortably above the standard that indicates sensitivity towards the specific antibiotic.

Table 10: The average percentages of isolates resistant to the selected antibiotics during all the sampling runs at NW-C and NW-G

Plant	Site	n	Amp%	KF%	C%	Cip%	E%	K%	Ne%	Ot%	P%	S%	W%	Va%
NW-C	R	47	63 ±23.24	66 ±182.82	26 ±30.94	17 ±16.46	40 ±24.44	17 ±9.76	29 ±27.60	22 ±25.42	67 ±31.18	23 ±27.37	83 ±13.75	42 ±35.36
	T	23	60 ±43.46	61 ±38.90	10 ±22.36	20 ±44.72	13 ±21.65	7 ±14.91	11 ±23.85	0 ±0.00	100 ±0.00	0 ±0.00	73 ±43.46	25 ±50.00
	D	42	64 ±13.72	56 ±14.01	8 ±12.44	14 ±17.56	24 ±20.25	22 ±21.03	18 ±21.53	31 ±24.49	76 ±16.58	10 ±22.36	75 ±23.29	30 ±28.28
NW-G	R	66	63 ±32.12	56 ±29.48	12 ±7.64	4 ±5.48	38 ±21.72	9 ±12.45	8 ±10.31	7 ±8.21	59 ±36.12	9 ±9.36	62 ±25.38	10 ±22.36
	T	8	47 ±41.11	47 ±41.11	11 ±19.24	0 ±0.00	11 ±19.24	22 ±38.49	11 ±19.24	0 ±0.00	58 ±52.04	11 ±19.24	56 ±50.92	0 ±0.00
	D	50	72 ±25.55	67 ±22.55	18 ±20.54	6 ±8.52	50 ±38.72	23 ±35.14	12 ±26.83	7 ±10.11	61 ±47.28	22 ±25.49	72 ±21.73	23 ±43.46

AMP – Ampicillin (10 µg); KF – Cephalothin (30 µg); C – Chloramphenicol (30 µg); CIP – Ciprofloxacin (5 µg); E – Erythromycin (15 µg); K – Kanamycin (30 µg); NE – Neomycin (30 µg); OT – Oxytetracycline (30 µg); P – Penicillin-G (10 units); ST – Streptomycin (25 µg); W – Trimethoprim (5 µg); Va – Vancomycin (30 µg); R – raw; T – treated; D – distribution; n – number of isolates.

Table 11: The average sizes of the inhibition zones during all the sampling runs at NW-C and NW-G

Plant	Site	n	Amp (mm)	KF (mm)	C (mm)	Cip (mm)	E (mm)	K (mm)	Ne (mm)	Ot (mm)	P (mm)	S (mm)	W (mm)	Va (mm)
NW-C	R	47	11 ±12.42	15 ±14.03	23 ±9.38	28 ±10.58	24 ±9.77	21 ±8.63	20 ±6.31	20 ±6.89	13 ±16.13	20 ±5.74	4 ±10.27	19 ±3.68
	T	23	9 ±9.55	14 ±10.95	27 ±4.88	29 ±5.94	28 ±4.98	22 ±3.39	20 ±4.01	20 ±4.85	13 ±19.18	23 ±3.80	5 ±9.47	23 ±7.31
	D	42	12 ±12.23	15 ±12.54	24 ±5.05	25 ±6.25	26 ±6.46	20 ±4.46	21 ±3.26	19 ±5.64	8 ±12.65	20 ±4.26	5 ±9.97	18 ±3.10
NW-G	R	66	15 ±8.36	18 ±9.83	24 ±1.42	31 ±2.72	25 ±4.46	23 ±3.72	23 ±3.16	22 ±1.42	13 ±10.60	23 ±3.41	12 ±8.40	19 ±4.34
	T	8	15 ±4.82	14 ±8.03	24 ±6.56	28 ±3.11	26 ±6.71	21 ±10.36	21 ±5.12	23 ±3.50	11 ±6.77	23 ±6.16	6 ±10.69	20 ±2.51
	D	50	12 ±9.27	14 ±12.07	24 ±9.13	30 ±8.06	26 ±8.65	22 ±7.08	23 ±5.38	23 ±4.95	14 ±11.07	22 ±7.75	6 ±12.37	18 ±4.56

AMP – Ampicillin (10 µg); KF – Cephalothin (30 µg); C – Chloramphenicol (30 µg); CIP – Ciprofloxacin (5 µg); E – Erythromycin (15 µg); K – Kanamycin (30 µg); NE – Neomycin (30 µg); OT – Oxytetracycline (30 µg); P – Penicillin-G (10 units); ST – Streptomycin (25 µg); W – Trimethoprim (5 µg); Va – Vancomycin (30 µg); R – raw; T – treated; D – distribution; n – number of isolates.

3.8. Multiple antibiotic resistance (MAR) index

Table 12 illustrates the MAR index calculated for both NW-C and NW-G for all the sampling runs.

At NW-C, MAR levels in the raw water ranged from 0.29 – 0.47 with the highest value in August 2016. In the treated water, MAR values ranged from 0.12 – 0.38 with the highest value in August 2016 and May 2017. In the distribution water the MAR values ranged from 0.24 – 0.38 with the highest value in August 2016. It is clearly illustrated that the August 2016 sampling run had the highest MAR values compared to the other sampling runs. Additionally, it is difficult to find another trend other than the August 2016 sampling run showing the highest MAR values. Therefore, the average MAR value for raw water, treated water and distribution water was calculated respectively across all the sampling runs. This clearly indicates that the raw water has the highest value of 0.39. The average MAR value in the treated water dropped to 0.29 and increased again to 0.34 in the distribution water. Once again there was an increase in values of some physico-chemical parameters from the raw water to the distribution water.

At NW-G, MAR values in the raw water ranged from 0.15 – 0.35 with the highest value during the August 2016 and November 2017 sampling runs. In the treated water, MAR values ranged from 0.08 – 0.35. However, it needs to be noted that only one isolate was isolated at the treated water in the November 2017 sampling run that showed the MAR value of 0.08. Statistically that value is not representative. For the March 2016 and May 2016 sampling runs no HPC bacteria were observed in the treated water. In the distribution water values ranged between 0.13 – 0.56. The March 2016 and May 2017 sampling runs showed values of 0.51 and 0.56 respectively which is the highest among all the sampling runs. The average MAR value across all the sampling runs for the raw water was 0.28, in the treated water 0.23 and 0.37 in the distribution water. Once again there is an increase of values into the distribution water. Comparing NW-C and NW-G, it is clear that the average MAR values in the raw water and treated water were higher at NW-C. However, in the distribution water, NW-G showed a higher MAR average than NW-C with 0.37 and 0.34 respectively.

Despite the trends described above, the most important observation regarding the MAR index is that most of the values (with a few exceptions) during all the sampling runs at NW-C and NW-G were above 0.20. Values above 0.20 indicate contamination of antibiotics within samples.

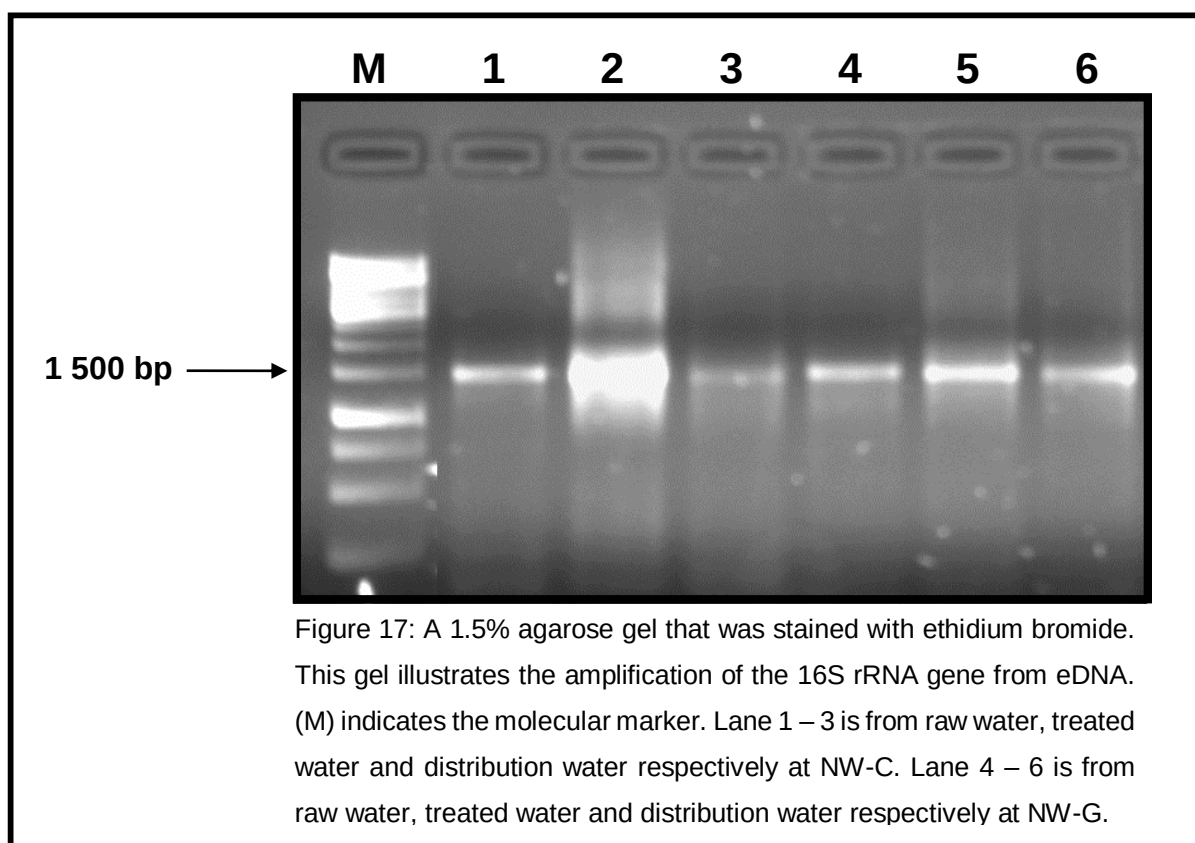
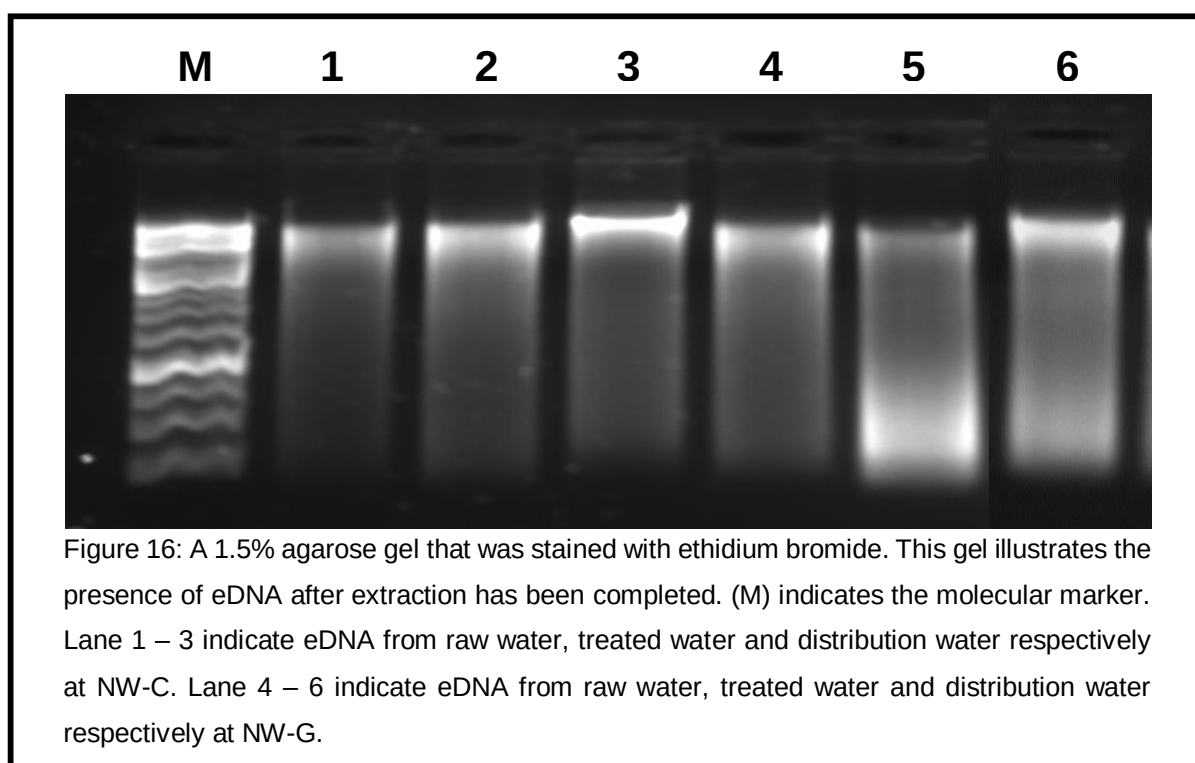
Table 12: MAR indices for 2016/2017 sampling runs for NW-C and NW-G

Sampling date	Raw		Treated		Distribution	
Treatment plant	NW-C	NW-G	NW-C	NW-G	NW-C	NW-G
March 2016	0.32	0.28	0.25	-	0.44	0.51
May 2016	0.29	0.15	0.31	-	0.24	0.13
August 2016	0.47	0.35	0.37	0.27	0.38	0.28
May 2017	0.39	0.28	0.38	0.35	0.28	0.56
November 2017	0.46	0.35	0.12	0.08	0.35	0.35
Average	0,39	0,28	0,29	0,23	0,34	0,37

3.9. Detection of antibiotic resistance genes

Among the 135 HPC isolates, only the multidrug-resistant (MR) isolates were chosen for the detection of resistant genes. “Between NW-C and NW-G only 34 MR isolates were chosen. No amplification was observed for the *bla-TEM*, *ermB*, *ermF*, *int1* and *tetM* genes. PCRs were run with positive controls that amplified; the concentration of DNA was adapted and changed; different annealing temperatures were used; and different master mix reagents were used. For the *ampC* gene, one positive amplification was observed from an isolate in the distribution water at NW-C. The rest of the samples were run several times with the adaptations mentioned above and amplification was still not achieved. The eDNA samples included raw water, treated water and distribution water which were subjected to 16S rRNA amplification after DNA extraction. Figure 18 illustrates the result of eDNA extraction and Figure 19 illustrates the 16S rRNA gene amplification of eDNA samples. Regarding the eDNA, only the *int1* gene was detected in both the raw water and distribution water at NW-G.

The *ampC*, *Bla-TEM*, *ermB*, *ermF* and *tetM* genes were not detected in any of the samples.



3.10. Whole genome sequencing

The total genome sizes of the six isolates varied between 3.85 Mbp and 6.03 Mbp. As illustrated in Figure 20, DeepARG identified 36 and 41 different genes involved with antibiotic resistance in the raw water. In the treated water 47 and 92 genes were identified whereas in the distribution water 92 and 91 different ARGs were identified. These ARGs belonged to 10 groups which include multidrug resistant gene groups, macrolide-lincosamide-streptogramin, glycopeptide, tetracycline, aminoglycoside, sulphonamide, quinolone, beta-lactam, chloramphenicol, trimethoprim. The amount of genes of each group can be observed in Figure 20. For all six isolates, the multidrug resistant gene group contained the most identified genes. This was followed by glycopeptide and tetracycline. The rest of the groups showed less identified groups. A clear trend can be observed in Figure 20 where the amount of identified resistance genes increases from the raw water going into the drinking water. The amount of genes identified in the treated water (2T2) and distribution water (2D25 and 2D33) increased to more than double the amount identified in the raw water.

DeepARG also predicted up to 103 possible new ARGs in the raw water, 162 in the treated water as well as the distribution water. An addition of three extra groups were added within this prediction and include bacitracin, fosfomycin, polymyxin. It needs to be noted that this prediction had less than 30% amino acid similarity with the database.

Virulence genes were also identified and associated with specific characteristics such as immune, adherence, enzyme production, invasion, metal uptake, regulation, secretion, toxin and other. Illustrated in Figure 21, it is clear that virulence genes identified under the group "other", were the most prevalent by reaching 102 genes in the raw water and 187 genes in the treated water as well as the distribution water. Genes in the adherence group ranged from 45 in the raw water to 61 in the drinking water. The amount of genes in the immune group ranged from 23 in the raw water to 44 in the drinking water. The regulation group had 20 genes from the raw water and increased to 50 in the drinking water. The metal uptake group had 25 genes in raw water and increased to 43 in the drinking water. Once again it is observable that with most of the virulence genes, there was an increase from the raw water to the drinking water except for the invasion group genes which were mostly present in the raw water.

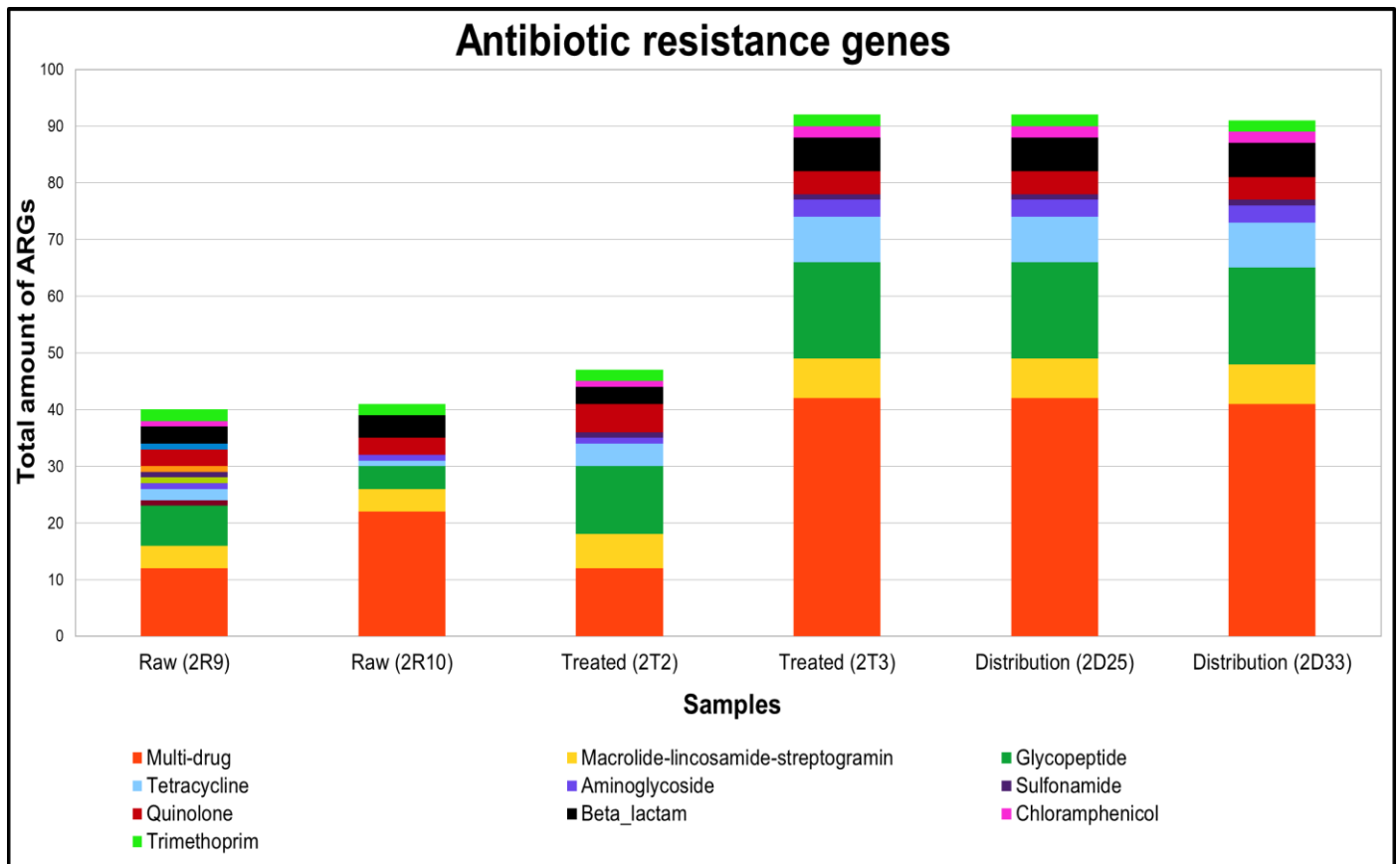


Figure 19: An illustration of the amount of identified antibiotic resistance genes in raw water, treated water and distribution water obtained from whole genome sequencing.

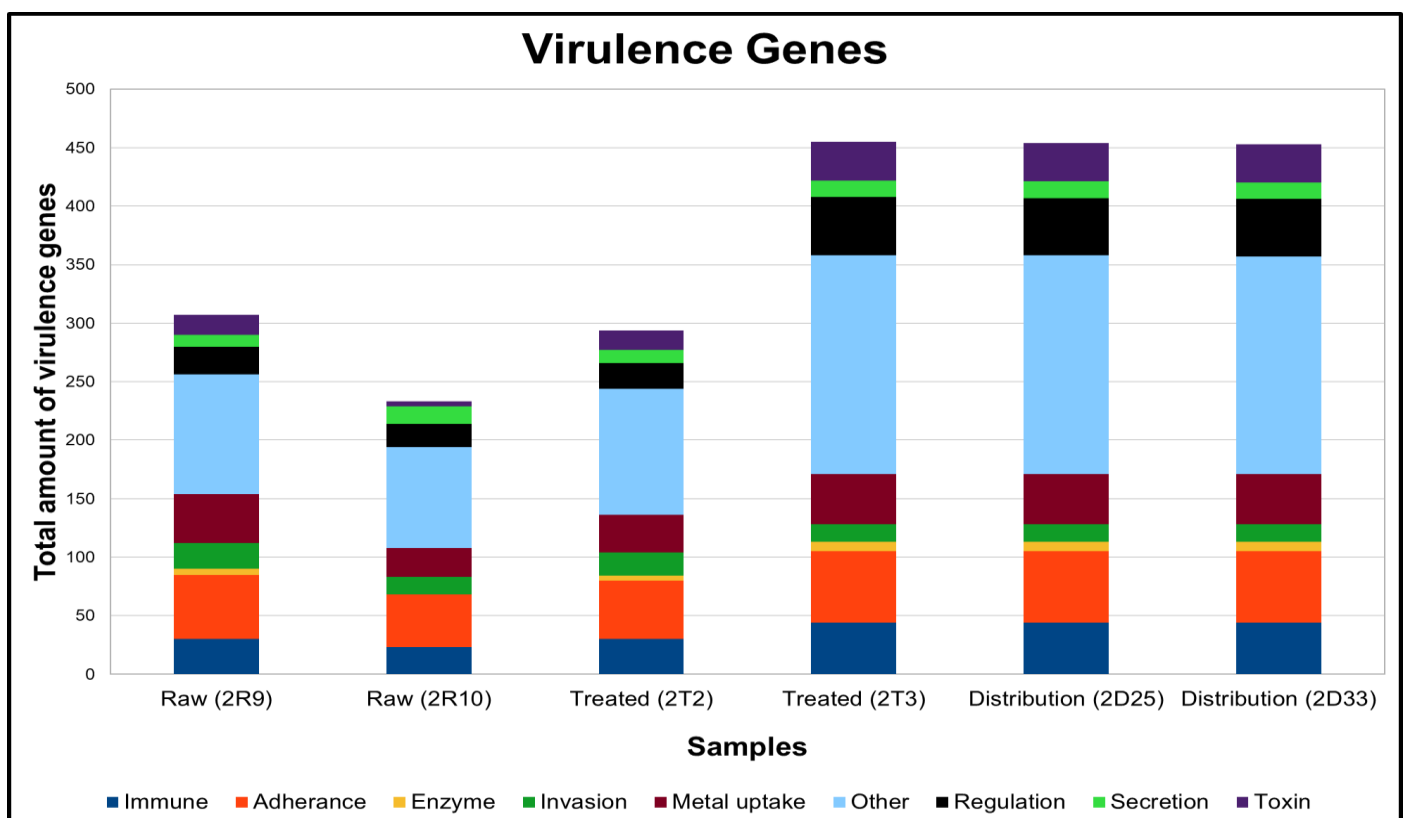


Figure 18: An illustration of the amount of identified virulence genes in raw water, treated water and distribution water obtained from whole genome sequencing.

CHAPTER 4

DISCUSSION

4.1. Physical properties

4.1.1. Temperature

The temperatures for both NW-C and NW-G showed correlations with seasonal changes as the March 2016 and November 2017 sampling runs showed the highest temperatures for raw water, treated water and distribution water. The distribution water showed higher temperatures than the raw water for both NW-C and NW-G during all the sampling runs. At NW-G during the March 2016 sampling run the water temperature in the distribution was 26.5°C which falls in the range of growing temperatures for HPC bacteria. Temperature is intrinsically linked to the physico-chemical and biological reactions in water. A rise in temperature would generally increase the chemical reactions, metabolic- and growth rates of microorganisms which can also increase the turbidity (Chapman and Kimstach, 1996; Mulamattathil *et al.*, 2015). However, the correlation between temperature and turbidity in this study proved difficult as turbidity levels in the drinking water for both NW-C and NW-G during the November 2017 sampling run (high temperatures) all complied with the SANS 241 (2015). Also, very high turbidity levels during the August 2016 sampling run (low temperatures) were observed. However, according to Mulamattathil *et al.* (2015), temperatures in the range of 18.3°C and 25.3°C can encourage biofilm formation and regrowth in distribution systems.

4.1.2. pH

The pH levels in the drinking water for both NW-C and NW-G complied to SANS 241 (2015) across all sampling runs. pH levels were very consistent at NW-C for raw water, treated as well as distribution water. pH levels in the raw water for NW-G ranged from 9.20 – 9.70 during the May 2016, August 2016, May 2017 and November 2017 sampling runs.

Most unpolluted waters have a pH that ranges from 6.5 – 8.5 (Mulamattathil *et al.*, 2015). Many reports and incidences showcase that the raw water source at NW-G is being polluted with high amounts of raw sewage that flows into it. For successful disinfection of drinking water, the pH must be regulated and therefore controlled which costs money and effort especially if the raw water is contaminated (WHO, 2011). The raw water source of NW-G is highly utilized and exposed to industrial and sewage pollution regularly. According to NW-G annual report (2015), there was an increase in the deterioration of raw water quality in the river system during 2014. The aesthetic quality of the raw water was affected due to low rainfall, high pollution load and high algal growth. Despite these raw water quality challenges, the company still produced drinking water that complied to the SANS 241 (2015). Although the company's Blue Drop Certification status was lost in 2014, it still received the highest inspection score of 98% in its province as the performances were rated to be excellent (NW-G annual report, 2015).

4.1.3. Total dissolved solids (TDS) and salinity

The TDS levels in drinking water for both NW-C and NW-G were well within the range of SANS 241 (2015) of ≤ 1200 mg/L. NW-C showed very consistent TDS levels from raw water through to distribution water and ranged from 331.1 mg/L – 385.7 mg/L across all sampling runs. At NW-G TDS levels varied more between raw water and drinking water which can once again be due to the state and pollution taking place in the raw water.

Salinity is also a component of TDS that forms part of the dissolved material in water. But, TDS is often the preferred parameter to use. There is no SANS 241 (2015) for salinity but NW-C showed levels that ranged from 202.6 mg/L – 233.3 mg/L for all the sites and across all the sampling runs. Salinity was very consistent per sampling run as there was only a 3.3 mg/L difference between the raw water and drinking water for each sampling run. At NW-G salinity levels in the drinking water were always higher than that of the raw water and ranged from 279.0 mg/L – 361.7 mg/L.

According to UNICEF (2008), TDS levels below 600 mg/L in drinking water is considered to be good. However, when TDS levels exceed 1000 mg/L it becomes

aesthetically unpalatable. The TDS levels were well below 600 mg/L and therefore should not be a concern. No health risks are associated with these concentrations (UNICEF, 2008). The more important parameter to consider is TDS for which NW-C and NW-G complied to.

4.1.4. Turbidity

High levels of turbidity were observed at NW-C for the March 2016, May 2016 and August 2016 sampling runs (0.34 NTU – 3.30 NTU) and did not comply to SANS 241 (2015). Although turbidity levels complied with SANS 241 (2015) during the May 2017 and November 2017 sampling runs, there was still an increase in turbidity from the raw water to drinking water. At NW-G turbidity levels in the drinking water complied for all the sampling runs except during August 2016 where the turbidity in the treated water was 3.85 NTU and in distribution water 3.26 NTU.

The increase in turbidity from raw water to drinking water at NW-C is concerning and is an indication of the efficiency of the treatment process. It seems as if the filtration process is not effective as the turbidity is high after filtration. Sand filters maybe need to be backwashed or the sand needs to be changed. High turbidity levels can also be an indication of biofilm formation within the distribution system, on the filters or in the treatment process. But treatment plants using filtration as part of its treatment process should be able to limit turbidity levels from rising above 0.5 NTU according to (OECD and WHO, 2003). The raw water at NW-G showcased very high turbidity levels (11.67 NTU – 19.00 NTU) which can be expected due to the extensive usage and pollution taking place in this resource as well as rainfall and algal growth. Turbidity in water can affect the disinfection with chlorine-based chemicals as microorganisms and pathogens can shield themselves from disinfectants (OECD and WHO, 2003; WHO, 2011). It is therefore important to know the turbidity levelsof the raw water so that the treatment processes can be managed correctly to achieve compliance.

4.2. Chemical properties

4.2.1. Free chlorine

Free chlorine levels complied to SANS 241 (2015) of ≤ 5 mg/L at both NW-C and NW-G during all the sampling runs. Although free chlorine levels in the treated water during the August 2016 sampling run at both NW-C and NW-G were very low (0.02 mg/L and 0.03 mg/L), concentrations increased in the distribution systems. During the November 2017 sampling run at NW-G in the distribution system free chlorine levels were very low (0.07 mg/L).

Although free chlorine levels complied to SNAS 241 (2015), there is still a concern regarding the turbidity levels at both NW-C and NW-G. With high turbidity levels as observed in March 2016, May 2016 and August 2016 microorganisms can get shielded from chemical disinfectants (WHO, 2011). Also, many studies found that there were positive correlations between turbidity and HPCs in drinking water. An increase in HPC levels will mean that more of the free chlorine in the water will be used up and no residue will be left (Chowdhury, 2012).

4.2.2. Phosphorus

Phosphorus levels were not very high in both NW-C and NW-G. However, in most cases concentrations increased from the raw water to the drinking water at NW-C and in some cases at NW-G. Phosphorus in drinking water does not pose health risks and therefore there is no set standard in SANS 241 (2015). However, studies showed that exceptionally low increases in phosphorus levels with as little as 1 $\mu\text{g/L}$ can results in an increased microbial growth in treated water as well as in distribution water (Miettinen *et al.*, 1997; Glasser, 2000; Lehtola *et al.*, 2002). This off course promotes and increases biofilm formation in distribution systems. The addition of polyphosphates to drinking water treatment plants can limit and decrease corrosion effects on the distribution system pipes. This might be the reason for the increase of phosphorus levels in the drinking water (Cantor *et al.*, 2000).

4.2.3. Nitrites and nitrates

Nitrite levels for both NW-C and NW-G did not comply with SANS 241 of ≤ 0.9 mg/L in the drinking water. Nitrite levels in the drinking water ranged from 0.67 mg/L – 5.00 mg/L which was as high as five times higher than the set standard. At NW-G nitrite levels ranged from 2.17 mg/L – 10.50 mg/L in the drinking water which was ten times higher than the set standard. Nitrate levels in the drinking water complied to SANS 241 of ≤ 11 mg/L for both NW-C and NW-G. In the drinking water nitrate levels ranged from 1.30 mg/L – 4.27 mg/L for both NW-C and NW-G. Despite the low levels of nitrates, there was still an increase in concentration in the drinking water compared to the raw water for all the sampling runs.

According to Dissmeyer, (2000) and Weyer *et al.* (2001), high nitrate levels in drinking water is a health concern because nitrate can endogenously be reduced to nitrite that can be dangerous for human health. Nitrites and nitrates are related nitrogen compounds that occur naturally in the environment. The principal sources of nitrite and nitrate contamination can include commercial fertilizer, sewage disposal systems, industrial wastes and livestock manure (Alemdar *et al.*, 2009). In a study done by Alemdar *et al.* (2009), nitrate levels in the drinking water (ranged from 2.40 mg/L – 2.80 mg/L) from Turkey were not very high, however, the nitrite levels posed a concern. Nitrates can be converted to nitrites which might explain the low levels of nitrates and high levels of nitrites in the drinking water (Chapman and Kimstach, 1996; Dallas and Day, 2004). Nitrate is more commonly found in water than nitrite. The presence of both nitrite and nitrate in drinking water might be an indication of biofilm formation and is a health risk and concern for infants younger than 6 months of age and sensitive individuals. The nitrite levels were higher than compared to the study done by Alemdar *et al.* (2009).

4.2.4. Sulphides and Sulphates

At NW-C the sulphide levels in the drinking water were not considerably high (0 mg/L – 23 mg/L) except for during the August 2016 sampling run when levels rose to 58 mg/L and 46 mg/L in treated water and drinking water respectively. Sulphide levels at NW-G in the drinking water ranged from 9 mg/L – 51 mg/L except for during the May

2016 sampling run when sulphide levels in the drinking water rose to 208 mg/L and 161 mg/L in the treated water and distribution water respectively from the raw water with a concentration of 41 mg/L. There is no SANS 241 standard for sulphides. Sulphate levels at NW-C were very low during all the sampling runs as well as at all the sampling sites. Raw water, treated water and distribution water levels ranged from 0 mg/L – 6 mg/L. At NW-G sulphate levels were much higher in the drinking water (90 mg/L – 465 mg/L) but still complied to SANS 241 of ≤ 500 mg/L. Most of the times sulphate levels increased in the drinking water compared to the raw water levels.

Sulphur compounds usually occur in the form of sulphates. Sulphate reducing bacteria often use sulphates as a source to obtain oxygen and then form sulphide compounds (Chapman and Kimstach, 1996; Dallas and Day, 2004). During all three sampling runs it was observed that the sulphate levels in the drinking water were very low and the sulphide levels very high. Therefore sulphate reducing bacteria might be responsible for this occurrence (Chapman and Kimstach, 1996; Dallas and Day, 2004). According to Kristiana *et al.* (2010), sulphides have a great affinity for metal ions and therefore play a role in the formation of sulphide minerals. The presence of sulphides in drinking water distribution systems is a concern because they can indicate the presence of biofilms in the distribution system. Additionally, sulphides can consume disinfectants and dissolved oxygen resulting in taste and odour problems (Kristiana *et al.*, 2010). According to (WHO, 2011), there is no existing data that identifies sulphate levels in drinking water that will have adverse human health effects. However, sulphate levels higher than 400 mg/L can make water unpleasant to drink. It was only at NW-G during the August 2016 sampling run at the treated water site that sulphate levels exceeded 400 mg/L. The distribution water that reach consumers had a lowered concentration of 145 mg/L.

4.2.5. Chemical oxygen demand (COD)

At NW-C there was no detection of COD in the raw water during all the sampling runs. But concerning is that during the March 2016, May 2016, August 2016 and May 2017 sampling runs COD levels were detected in the drinking water. For the May 2016 and August 2016 sampling runs COD levels in the drinking water ranged from 6 mg/L – 63 mg/L. At NW-G COD levels were detected in the raw water for all the sampling runs.

For all the sampling runs the COD levels decreased from the raw water into the drinking water. COD levels in the drinking water ranged from 8 mg/L – 58 mg/L.

Although there is no COD limit in SANS 241 (2015) for drinking water, the DWAF, (1996) general wastewater effluents standard indicates that COD levels of 0 – 30 mg/L are satisfactory for domestic use. The COD levels were at some cases higher than 30 mg/L for both NW-C and NW-G. Mamba *et al.* (2009) assessed the effects of water treatment techniques at the Sedibeng- and the Midvaal water treatment plants. The COD levels at the Sedibeng water treatment plant were very high (110.73 mg/L). Despite these high levels in the raw water, the treatment processes were able to decrease the COD levels in the distribution systems of Bothaville to 13.28 mg/L (Mamba *et al.*, 2009). A sharp decrease in COD levels was observed directly after sand filtration which might indicate that the sand filtration process was effective in removing the organic matter (Mamba *et al.*, 2009). The fact that the COD levels in the raw water were lower than those found in some of the drinking water sites is concerning. According to Yin *et al.* (2011), high levels of COD is usually an indication of serious water pollution.

4.3. Microorganisms in drinking water

General microbiological quality of water samples includes the testing for the presence of total coliforms, faecal coliforms and *E. coli*. These are indicator organisms that show possible faecal contamination in the water source and therefore can contain dangerous pathogens. Water intended for human use and consumption must be free of any faecal indicator organisms (WHO, 2017). There were no indicator organisms found in the treated water or drinking water of NW-C and NW-G. Although indicator organisms were present in the raw waters, they did not enter the distribution system at both plants. HPC bacteria are always present and form part of water environments even in drinking water. However, it is very important that the HPC levels in drinking water comply to SANS 241 (2015) with ≤ 1000 CFU/mL as an increase in HPCs in drinking water can indicate ineffective treatment of the water (WHO, 2003). HPC levels at NW-C and NW-G were always higher in the raw water and decreased into the treated water and distribution water. As indicated by Figure 9 and Figure 10 it is also clear that HPC levels are closely related to the raw water sources. It is expected that

the total amount, and diversity of bacteria be more in raw water sources, as drinking water treatment would generally reduce the colony counts as well as select for lower range of bacterial diversity (Allen *et al.*, 2004; Chowdhury, 2012).

4.4. The correlations between physico-chemical, HPC bacteria, raw water and drinking water

Figure 8 illustrates the correlations of raw water quality (physico-chemical and HPC levels) between NW-C and NW-G. It is immediately noticeable that sampling runs at NW-C were negatively correlated with sampling runs at NW-G. All the parameters showed close correlations with NW-G except for the nitrites that were closely correlated with NW-C. Raw water quality between NW-C and NW-G differed as one would expect. The origins, pollution inputs, usage, capacity etc. of the two raw sources are completely different. As the origin of the raw water source at NW-C is only 6 km away from the treatment plant, and with no industrial and very little agricultural influences to pollute the water. The raw water at NW-G is the opposite. In 2014 NW-G lost its Blue Drop Certification status in assessment by the Department of Water and Sanitation. According to NW-G annual report (2015), there was an increase in the deterioration of raw water quality in the river system during 2014 and 2015. The aesthetic quality of the raw water was affected due to low rainfall and pollution upstream of NW-G such as the discharge of inadequately treated sewage effluents and agricultural and industrial effluents. This caused growth of high levels of taste and odour producing algae. According to NW-G annual report (2015), this resulted in a lot of consumer complaints to the aesthetic quality of the drinking water. Despite these raw water quality challenges, the company still produced drinking water that complied to the South African National Standards (SANS 241). The most prevalent contaminants that affect the raw water quality are total dissolved solids (TDS), organic carbon, nutrients such as nitrogen and phosphorus, and microorganisms (NW-G annual report, 2015). Due to the raw water quality at NW-G, it is no surprise to find that the HPCs were correlated with the raw water (Allen *et al.*, 2004; Chowdhury, 2012). However, despite the clear and clean raw water at NW-C, nitrite levels (3.00 mg/L – 5.00 mg/L) are associated with this plant and can possibly be due to the

disposal of human and animal wastes or the use of fertilizers and pesticides (Chapman and Kimstach, 1996; Dallas and Day, 2004).

Figure 9 illustrates the comparison between the raw water and drinking water quality (physico-chemical and HPC levels) at NW-C. The raw water was closely related to HPCs, nitrites, sulphates and pH. As indicated in section 3.2, the HPC levels decreased from the raw water into the drinking water as the treatment processes were applied. One would rightly expect HPCs to be more correlated to the raw water. As seen in Figure 8, nitrites were correlated to the raw water. Nitrites can be an indication of pesticide pollution in surface waters (Chapman and Kimstach, 1996; Dallas and Day, 2004), however, it must be noted that during all the sampling runs, the nitrite levels in the drinking water did not comply to the SANS 241 (2015). Nitrite levels were not sufficiently removed during the treatment process or the nitrite levels increased from the treated water into the distribution water. Sulphur components in surface waters are usually in the form of sulphates and are usually used by bacteria as a source to obtain oxygen. Sulphates enter surface waters through the breakdown of sedimentary rocks, sulphate minerals or through atmospheric deposition (Chapman and Kimstach, 1996; Dallas and Day, 2004). However, although the sulphate levels are correlated with the raw water, it was still very low (0.00 mg/L – 6.00 mg/L) considering the SANS 241 (2015) limit of ≤ 500 mg/L. To the contrary, nitrate levels were closely related to the drinking water as was the sulphide levels. Both of which are actively involved in nitrate-nitrite interactions as well as sulphate-sulphide interactions. The reason for the high increases and especially the non-compliance to SANS 241 (2015) from nitrites in the drinking water can be due to the sump where water is collected after filtration. After filtration water is not allowed to stand long enough for sedimentation to take place which can add to the increase of organic constituents. The fact that there are increases from the raw water to the drinking water can also mean that the filtration process is contaminated or not working efficiently. In a study done by (Alemdar *et al.*, 2009), they also found that the nitrate levels in the drinking water in Turkey were not very high, however, the nitrite levels posed a concern and ranged from 2.40 mg/L – 2.80 mg/L. Nitrates can be converted to nitrites which might explain the low levels of nitrates and high levels of nitrites in the drinking water. The high turbidity levels that are associated with the drinking water are also associated

with the nitrites and rarely complied to the SANS 241 (2015). Turbidity affects the treatment of drinking water by increasing the demand for chlorination and it can stimulate growth of bacteria. This is because pathogens can be shielded from disinfectants with increased suspended particles (WHO, 2011; UNICEF, 2008). Therefore, effective disinfection of pathogens in water, requires the turbidity to be less than 1 NTU. Sulphate reducing bacteria might be responsible for the increase of sulphides in the drinking water. According to Kristiana *et al.* (2010), sulphides have a great affinity for metal ions and therefore play a role in the formation of sulphide minerals. The presence of sulphides in drinking water distribution systems are a concern because it consumes disinfectants and dissolved oxygen, it can cause taste and odour problems, and it might be an indication of biofilms present in the distribution system. The consumption of dissolved oxygen by sulphate reducing bacteria can contribute to the COD levels that are also positively correlated with the drinking water.

Figure 10 illustrates the comparison between the raw water and drinking water quality (physico-chemical and HPC levels) at NW-G. Once again it is clear that the raw water and the drinking water are negatively correlated and that most of the physico-chemical parameters are associated with the raw water except for the temperature, TDS, salinity and nitrates. Sulphates and sulphides were closely correlated where the sulphate levels in the raw water were very high. During some sampling runs (May 2016 and November 2017) the sulphide levels increased from the raw water into the drinking water. This once again might be the presence of sulphate reducing bacteria that oxidize sulphate to produce sulphides (Kristiana *et al.*, 2010). Nitrite levels were very high in the raw water considering the SANS 241 (2015) limit of ≤ 0.9 mg/L and did not comply to the limit in the drinking water. Nitrate levels were not high and complied with ease to the SANS 241 (2015). It might be that nitrite levels are not removed from water during the treatment processes and then proliferates and increases in the distribution system. HPCs were closely correlated with turbidity, COD, pH and phosphorus levels. Phosphorus is an essential element for living organisms that can proliferate HPCs in water environments. In freshwaters, phosphorus often is the limiting nutrient and the main source of eutrophication. Such excessive growth of algae and aquatic vegetation, can cause the formation of biofilms in the distribution systems and deteriorate

municipal supplies (Dissmeyer, 2000). The proliferation of HPC bacteria in the drinking water can also contribute to the increase and positive correlation with COD levels.

In conclusion regarding the statistical analysis of physico-chemical and HPC levels it is evident that the raw water sources from NW-C and NW-G differ. The drinking water has a close correlation with nitrites and can be seen at both NW-C and NW-G (Figure 9 and 10). Despite the high nitrite levels in the raw waters, there were often increases of nitrite concentrations in the drinking waters and very rarely complied to SANS 241 (2015). The same trend was observed for turbidity levels especially at NW-C. It might be possible that the treatment processes at both NW-C and NW-G are not effective enough to remove nitrites from the water which then enters the distribution system where concentrations can increase with complex biochemical interactions. The high turbidity levels especially at NW-C can support the notion of the inability of reliable filtration. Increases in sulphide levels in the drinking water were observed at NW-C and NW-G which can indicate biofilm formation in the distribution system. HPC levels were strongly correlated with the raw waters at both NW-C and NW-G but decrease into the drinking water.

4.5. Identified HPC bacteria in the raw water and the drinking water

It was clear that *Bacillus* dominated the raw water as well as the drinking water at NW-C and NW-G. However, the increase of *Bacillus* representation in the drinking water compared to the raw water is striking. Water treatment processes select for specific bacteria. These bacteria that are being selected are the ones that can withstand the treatment process and have sufficient adaptations to finally enter the distribution systems (Guo *et al.*, 2014). The ability of *Bacillus species* to form spores during nutrition deficiency or excessive heat or dryness is characteristic of Gram-positive bacteria and enable them to withstand the treatment process and pose resistance to disinfection. It might be due to this ability that *Bacillus species* were observed mostly in the drinking water (WHO, 2003). According to WHO, (2011), *Bacillus species* are often detected in drinking water even when acceptable treatment and disinfection procedures are being used. Although water treatment processes select for fitter bacteria that are able to resist disinfection, the methods used for the detection and isolation of HPC bacteria most probably play a bigger role. The culturing methods also

select for specific bacteria of which *Bacillus* forms part of. As Allen *et al.* (2004) describes, “heterotrophic plate count (HPC) bacteria represent those microbes isolated by a particular method, whose variables include media composition, time of incubation, temperature of incubation, and means of medium inoculation”. Thus, there are a lot of factors that can influence the representation and isolation of heterotrophic bacteria. Bacteria isolated with using the HPC method represent less than 1% of the total bacteria in the water (Chowdhury, 2012).

Aeromonas, *Flavobacterium* and *Pseudomonas* were detected consistently in raw water as well as drinking water. This is expected as Allen *et al.* (2004) indicated that these genera among others are commonly found in drinking water. Less often detected were *Shingomonas*, *Micrococcus* and *Staphylococcus* which are, according to Allen *et al.* (2004), also supposed to be commonly found.

4.6. Evolutionary relationships

Figures 16 and 17 illustrates the evolutionary relationships of all the isolates (raw water and drinking water combined) from NW-C and NW-G respectively. At both treatment plants the isolates were divided into the four phyla called Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes. At NW-C, most of the isolates belonged to the Firmicutes (60.61%) that were mostly represented by *Bacillus* species. At NW-G it was the Proteobacteria that represented most of the isolates with 62.86%. Many studies found these four phyla present in raw water and drinking water together with other phyla (Eichler *et al.*, 2006; Vaz-Moreira *et al.*, 2013; Lautenschlager *et al.*, 2014). In these studies, Proteobacteria were shown to predominate in the raw water as well as the drinking water. This strongly correlates with the finding at NW-G. Never the less, most studies found the relative abundance of these four phyla as follows: Proteobacteria > Actinobacteria > Bacteroidetes > Firmicutes, where the Firmicutes represent a small fraction in correlation with the other three phyla (Martiny *et al.*, 2005; Eichler *et al.*, 2006; Pinto *et al.*, 2012; Vaz-Moreira *et al.*, 2013; Lautenschlager *et al.*, 2014; Proctor and Hammes, 2015). However, according to Proctor and Hammes (2015), the relative abundance of Proteobacteria is inconsistent within drinking water over time as well as between stages within one treatment system. This might be a reason why Firmicutes predominated isolates found at NW-C and not Proteobacteria.

It must be noted that at NW-G, the Firmicutes still represented a considerable fraction (22.86%) of all the isolates when compared to literature. It seems that at both NW-C and NW-G, the Firmicutes played a bigger role compared to other studies. It is also very important to consider the selective pressures that cultivated methods play when isolating heterotrophic bacteria from the water. In this study it was clear that the culturing methods were very selective towards *Bacillus* species. Non culturing methods such as the isolation of eDNA will be a more representable way of determining what the real composition and representation of bacteria in these systems are.

4.7. Pathogenicity

Results in Table 8 show that more than 76% of HPC isolates from the raw water and 82% from the drinking water were positive for α - and β -haemolysis at NW-C and NW-G. Compared to other studies in literature, the haemolytic results in this study were very high. Pavlov *et al.* (2004) showed that 55.5% of their HPC isolates were α - and β -haemolytic. A study done by Prinsloo *et al.* (2014) reported that 27% of their HPC isolates were α - and β -haemolytic. It must be noted that in this study all the isolates were grown at 28°C - 30°C for pathogenicity tests as most of the isolates could not grow at 37°C. This might be a reason for the high percentages of haemolytic positive isolates compared to other literature. A trend seen in most literature, is that a greater amount of HPC isolates that are haemolytic positive are β -haemolytic. Prinsloo *et al.* (2014) found that 63% of haemolytic HPC isolates were in fact β -haemolytic and the remaining 37% were α -haemolytic. In the study done by Pavlov *et al.* (2004), they found that 74% of their HPC isolates were β -haemolytic and 26% were α -haemolytic. It was also observed this trend but with more extreme values. Of the 127 isolates from NW-C and NW-G combined that were haemolytic, only two isolates tested α -haemolytic (1.6%). The remaining 98.4% were β -haemolytic. Haemolytic bacteria have the ability to break down erythrocytes in order to gain access to iron for growth. It is usually the first screening test for potential pathogenicity (Dhaliwal *et al.*, 2004).

The isolates that tested positive for haemolysis, were then further subjected to enzymatic tests to determine their potential pathogenicity. As can be seen in Table 8, more than 85% of isolates in the raw water and drinking water at both NW-C and NW-

G tested positive for proteinase as well as lecithinase. As with the haemolysis results, these results were very high compared to literature. Pavlov *et al.* (2004) found that 64.4% of isolates were positive for proteinase and 47.9% were positive for lecithinase. Prinsloo *et al.* (2014) showed that 40% of the isolates were positive for proteinase and 28% were positive for lecthinase. Once again, it might be due to the growth conditions that more of the HPC isolates tested positive for these tests compare to literature. Proteinase and lecithinase enzymes secreted by bacteria enable them to penetrate and gain access to the host cell. Proteinase can break through its host defence mechanisms by breaking up peptide bonds that link different amino acids (Pavlov *et al.*, 2002). Lecithin is one of the fatty substances that are present in animal and plant tissue. Lecithinase can destroy lecithin in the plasma membranes of cells (Willey *et al.*, 2008).

In Table 8 it can be observed that 45% and 38% of isolates in the raw water at NW-C and NW-G respectively tested positive for lipase production. In the drinking water 39% and 19% of isolates tested positive for lipase at NW-C and NW-G respectively. Pavlov *et al.* (2004) and Prinsloo *et al.* (2014) found that 54.8% and 30% of their HPC isolates produced lipase. Lipase production in the current study were similar to trends reported in the latter studies. Lipase breaks down and catalysis the hydrolysis of triacylglycerols, monoacylglycerols, free fatty acids and glycerol (Kumar *et al.*, 2012). Phospholipids are also constituents of cellular membranes which lipase production by bacteria can possibly break down and gain access to the host cell.

Regarding DNase production, 40% and 42% of the isolates in the raw water at NW-C and NW-G respectively produced DNase. In the drinking water at NW-C and NW-G, 51% and 50% of the isolates produced DNase. Pavlov *et al.* (2004) found that 60.6% of their isolates produced DNase whereas Prinsloo *et al.* (2014) found that 72% of their isolates tested positive for DNase production. The results for NW-C and NW-G were pretty much the same and increased from the raw water into the drinking water with almost 10%. Although the results showed lower percentages compared to these two studies, it is still more than 50% of isolates in the drinking water that produce this enzyme. DNase is also a standard method for the detection and identification of

several pathogens as this enzyme breaks down DNA and nucleic acids of host cells and can potentially use it as an energy source (Pavlov *et al.*, 2002).

Three out of 127 isolate produced only two enzymes which were haemolysis and lipase. The other 125 isolates produced more than two enzymes. The purpose of the pathogenicity tests on HPC bacteria is to indicate that although they are considered to be non-pathogenic and that they do not pose health risks, they can produce enzymes that allow them to be invasive and enter host cells which is the first step towards infection. Although it is known that high amounts of water need to be consumed in order for such bacteria to have real effects on general health, they still have the potential to be or become pathogenic which can become dangerous especially for the immunocompromised, elderly and young children.

There were nine isolates that produced all the enzymes tested for. Five of these isolates were from NW-C and isolated from drinking water (Table 9). They included *Bacillus spp.*, *Bacillus paramycoides*, *Arthrobacter oryzae*, *Arthrobacter spp.* and *Bacillus sonorensis*. The other four isolates were from NW-G where *Shewanella spp.*, *Bacillus licheniformis* and *Deefgea rivuli* were isolated from raw water and *Aeromonas salmonicida* from the drinking water. According to Allen *et al.*, (2004), microorganisms most often called opportunistic pathogens in drinking water include *Pseudomonas*, *Klebsiella*, and *Aeromonas*. As can be seen in figures 13 and 15, *Aeromonas* were present in the drinking water at NW-C (3%) and at NW-G (3%). *Pseudomonas* were present in the drinking water at NW-G and represented 11% of HPC isolates. No *Klebsiella* were present.

As illustrated in Figures 14 – 17, it was clear that *Bacillus* dominated with its presence of HPC isolates especially in the drinking water. The study done by Prinsloo *et al.* (2014), also reported *Bacillus spp.* that produced proteinase, lecithinase, lipase and DNase. According to WHO (2011), *Bacillus spp.* are often detected in drinking water even when acceptable treatment and disinfection procedures are being used. A study done by Celandroni *et al.* (2016) found that the isolation of bacteria that belong to *Bacillus spp.* or *Paenibacillus* genera, should never be underestimated with regards to their potential pathogenicity. *Bacillus spp.* are not associated with waterborne pathogens (WHO, 2011), but according to Celandroni *et al.* (2016), these bacteria are

increasingly isolated from patients in hospitals and seem to be equipped with virulence factors. This poses dangers to immunocompromised patients especially within clinical environments.

A study done by Moore *et al.* (2017), reported the first case of *Aeromonas salmonicida* present in the blood of a diabetic patient that consumed water chronically from a well. Its virulence factors allow for the colonization of surface waters such as salt water, beaches, and fresh water wells (Moore *et al.*, 2017). In the current study *Aeromonas salmonicida* was found in the drinking water at NW-G and showed resistance to ampicillin, cephalothin, trimethoprim and erythromycin.

Yamamoto *et al.* (2017) described a case where an 87-year old diabetic man with a fever and elevated hepatobiliary enzyme levels, was diagnosed with bacteraemia (bacteria present in the blood) due to *Arthrobacter spp.* infection. But *Arthrobacter spp.* was isolated from the raw water and only showed resistance against two antibiotics. Still it has pathogenicity potential.

According to Vignier *et al.* (2013), *Shewanella spp.* infections are being increasingly reported and there is no comprehensive review and literature describing these infections. Srinivas *et al.* (2015) also states that *Shewanella spp.* are emerging human pathogens and that skin and soft tissue infections are more commonly seen in immunocompromised patients who are associated with exposure to marine environments. However, *Shewanella spp.* was isolated from the raw water and only showed resistance to two antibiotics. But due to their production of enzymes, they can always have the potential first to enter drinking water, become pathogenic and also develop multiple resistance.

A study done by Bai *et al.* (2015) showed that drinking water treatment processes can select for antibiotic resistance bacteria. As can be seen in Table 9 all the potential pathogens in the drinking water at NW-C and the one at NW-G showed multiple antibiotic resistance patterns. The implication is that HPC bacteria can produce enzymes that can make them potential pathogens together with the increasing development of antibiotic resistance as these organisms enter drinking water systems.

4.8. Antibiotics in water samples

Apart from the physico-chemical and microbiological properties of water that indicate water quality, the presence of pharmaceuticals and personal care products in water sources are also contaminants that influence water quality as well as the prevalence of antibiotic resistance (Ferrer *et al.*, 2010). The occurrence of antibiotics in the natural environment raises concern as these chemical substances find their way through treatment processes into the drinking water (Ye *et al.*, 2007). The current study confirmed this hypothesis with ciprofloxacin, neomycin, Benzyl-penicillin (Penicillin G), penicillin and streptomycin being found in the drinking water. In a study done by Ye *et al.*, (2007), levels of erythromycin, sulfamethoxazole and four other antibiotics not used in the current study were detected.

4.9. Antibiotic susceptibility

As illustrated in Tables 10 and 11, the resistance against beta-lactams (ampicillin, cephalothin and penicillin) and trimethoprim was apparent at both NW-C and NW-G. In a study done by Boeckel *et al.* (2014), trends for consumption of standard units of antibiotics were reviewed between 2000 and 2010 for 71 countries. They found that the consumption of antibiotics increased by 36% in this time span. Cephalosporins and broad-spectrum penicillins accounted for 55% of total antibiotic units consumed in 2010. The largest increase in consumption between 2000 and 2010 were observed for cephalosporins, broad-spectrum penicillins and fluoroquinolones. Although fluoroquinolones like ciprofloxacin, macrolides like erythromycin and the tetracyclines did not show real resistance trends in this study, the wide and frequent use of beta-lactams in practice can be correlated with the high resistance patterns observed against beta-lactam antibiotics. Furthermore, Boeckel *et al.* (2014) also found that antibiotic consumption increased significantly in developing countries with the highest rates shown in BRICS countries (Brazil, India, China and South Africa) (Laxminarayan *et al.*, 2013; Boeckel *et al.*, 2014). According to Laxminarayan *et al.* (2013), precise estimates are scarce, but roughly estimated 100 000 – 200 000 tonnes of antibiotics manufactured worldwide every year mostly goes to agriculture, aquaculture and veterinary sectors. The raw water at NW-G is exposed to a lot of agricultural influences. In a study done by Eagar *et al.* (2012), it was reported that in the years

2002 – 2004, 234 registered antibiotics were available for food animals in South Africa. In total 64 antibiotics were registered as in-feed mixes for growth promotion. The majority of antibiotics consumed were from the macrolide class followed by the tetracycline class, the sulphonamide class and lastly the penicillin class (Eagar *et al.*, 2012). Although there is still systematic information lacking regarding the exact volume and patterns of antibiotics used in livestock, some studies indicate that South Africa does use antibiotics extensively, the latter includes antibiotics that have been banned for use in other countries (Moyane *et al.*, 2013).

Resistance against trimethoprim is also no surprise. In clinical practice, trimethoprim is used in combination with a sulphonamide called sulfamethoxazole to treat urinary tract infections. Resistance to both drugs can be transferable and therefore resistance traits can be linked to one another. The study by Eagar *et al.* (2012), where sulphonamides are one of the classes that are extensively used in agriculture and veterinary medicine, can possibly prove the high resistance against trimethoprim that were observed. According to Eliopoulos *et al.* (2001), the clinical use of sulfamethoxazole-trimethoprim and only trimethoprim has declined primarily due to the development and rapid spread of resistance against these antibiotics. These studies mentioned above are a few of many that indicate the rapid use of antibiotics especially the beta-lactams and sulphonamides. The result is an increase in resistance patterns as can be seen in the results.

According to Xi *et al.* (2009), beta-lactamases are a major cause of resistance to beta-lactams such as ampicillin, cephalothin and penicillin G, and they are increasingly being found in different environments worldwide. In their study, Xi *et al.* (2009) detected the genes that code for beta-lactamases in most of their drinking water samples and therefore concluded that it is evident that such genes are now widely spread in drinking water systems. A study done by Shi *et al.* (2013) assessed the chlorination effects on the microbial resistance patterns in drinking water. They found that after chlorination, the relative abundance of the chloramphenicol, trimethoprim and cephalothin resistant bacteria was enhanced by 4.1 fold, 1.5 fold and 3.0 fold respectively. This pattern of relative abundance continued and even increased with the transportation of water in the distribution systems (Xi *et al.*, 2009; Shi *et al.*, 2013).

The same pattern was observed in this study except for chloramphenicol that did not show significant resistance patterns. Despite the resistance patterns observed against beta-lactams and trimethoprim, the overall resistance increased against all the antibiotics increased from the raw water into the drinking water. This is due to the fact that treatment processes select for bacteria that have the ability to survive in these conditions and also be able to pick up and transfer resistant genes in the distribution systems (Guo *et al.*, 2014).

4.10. MAR index

As can be seen in Table 12, the average MAR values for NW-C and NW-G ranged from 0.29 – 0.39 and 0.23 – 0.37 respectively. The MAR index offers a way in which scientists can assign a number to multiple antibiotic resistance within a sample. MAR values above the threshold of 0.2 suggest that the isolates have an origin of a high risk source of contamination where antibiotics are used (Paul *et al.*, 1997; Titilawo *et al.*, 2015). The average MAR values for both NW-C and NW-G are above 0.2 which is concerning. It is especially concerning for the fact that there are a few studies that determined the MAR index for clinical isolates from hospital environments and water environments of high risk faecal contamination. MAR values for these studies ranged from 0.40 – 0.80 (Subramani and Vignesh, 2012; Osundiya *et al.*, 2013). It is expected that the MAR values for the latter studies be higher compared to the findings of this study and it certainly was the case. However, it was not dramatically lower, especially keeping in mind that those environments are contaminated with antibiotics and pathogens. The MAR values in this study were derived from presumed non-pathogenic HPC bacteria in drinking water environments, however, MAR values almost edging 0.40 were observed.

Once again (as seen with physico-chemical and antibiotic susceptibility results) a trend can be observed where there is an increase of MAR values either from the raw water into the distribution water or from the treated water into the distribution water. No study determined the MAR index for HPC bacteria in raw water, treated water and distribution water comparatively. However, compared to studies that focused on raw water sources (dams, rivers, boreholes etc.) only, there were MAR values even as low as 0.15 (Guan *et al.*, 2002; Chitanand *et al.*, 2010; Kumar *et al.*, 2013; Titilawo *et al.*,

2015; Odonkor and Addo, 2018). The results in Table 12 therefore showcases concerning information regarding resistance of HPC bacteria in drinking water.

4.11. Detection of antibiotic resistance genes

Detecting only one positive amplification of the *ampC* gene among 122 isolates is quite surprising. A study done by Knapp *et al.* (2010), analysed archived soils from the Netherlands that spanned from 1940 to 2008, which encapsulates the whole of the antibiotic era. DNA was extracted from these samples and 18 ARGs from different antibiotic classes were quantified. Results showed that ARGs from all the classes tested have increased drastically since 1940, especially tetracycline, erythromycin and the beta-lactam ARGs (Knapp *et al.*, 2010). Based on the increasing resistance patterns observed worldwide regarding bet-lactams, one would have suspected to find more positive amplicons for the *ampC* gene. Also, the *ampC* gene confers resistance to cephalothin, ceafazolin, cefoxitin and most penicillins and according to Jacoby (2009); and Keen and Montrofts, (2012), overexpression of the *ampC* gene confers resistance to broad-spectrum cephalosporins. All together this is a pattern that was observed in this study. However, a study done by Schwartz *et al.* (2003), the *ampC* gene was detected and amplified from drinking water distribution biofilms whereas enterococci and Enterobacteriaceae which are said to carry this gene originally, were not detected. Heterotrophic bacteria that were isolated from the drinking water distribution systems, also didn't show any amplicons for the *ampC* gene. It therefore seems that the *ampC* genes that were detected can be part of viable but non-cultivable aquatic bacteria.

The non-detection of *bla-TEM*, *ermB*, *ermF*, *int1* and the *tetM* genes as well as the detection of only one *ampC* amplicon in HPC bacteria does not necessarily mean that those genes are not present. It is also known that the detection of a gene does not necessarily mean it confers direct resistance to a specific antibiotic. There are many factors that influence the molecular functioning of genes. However, based on the observed phenotypic results and antibiotic resistance patterns, it was expected that amplicons for these genes would be identified as reported in other studies (Xi *et al.*, 2009; Zhang *et al.*, 2009). However, one class of genes can have many different variations for which primers used in this study were not able to amplify. Also, the

primers used were obtained from studies that detected clinical genes while this study focused on environmental bacteria and eDNA.

For eDNA samples in the raw water and distribution water at NW-C only the *int1* gene was amplified. Bacterial integrons are gene capturing systems that consist of a specific recombination site that entails the *intI* gene and encodes for the recombination enzyme called integrase. Integrase is responsible for the insertion and integration of resistance genes at a specific site as well as the mobilisation thereof. More than one gene can be recombined by the integrase and is then called a gene cassette (Ozgumus *et al.*, 2007; Bennett, 2008; Zhang *et al.*, 2009). Integrons can carry as many as nine ARGs but typically four or five ARGs are frequently found in clinical environments, agricultural wastewaters, urban wastewaters and even in the waters not recently exposed to antibiotics (Zhang *et al.*, 2009). Most of the resistance integrons conform to a specific structure known as class 1 integrons. In a study done by Taviani *et al.* (2008), *Vibrio* strains showed to have the class 1 integrons and integrating conjugative elements that confer resistance to trimethoprim, aminoglycoside and beta-lactam from surface urban water in Mozambique. In a study done by Ozgumus *et al.* (2007), 117 antibiotic resistant *E. coli* strains were isolated from public tap and spring waters that were polluted with faecal coliforms. Only three of these 117 isolates carried the class 1 integrons. Once again, the lack of detection of the other resistance genes in eDNA might be described to the specificity of the primers as they cannot necessarily amplify environmental DNA

4.12. Whole genome sequencing

Up to 41 ARGs were identified within the raw water samples which increased to 92 ARGs in the drinking water. Of these, the multidrug resistant gene group contained the most identified ARGs which correlates with the antibiotic susceptibility results reported in this study, and might be the reason for the multidrug resistance patterns observed in section 4.9 and 4.10. According to Wang *et al.* (2012), multi-resistance genes such as the *cfr* gene has been identified with a number of staphylococcal species. However, recent studies showed that this multi-resistant gene was identified within other Gram positive bacteria such as *Bacillus spp.* According to the latter authors, it is plasmids that mediate the important role of the transfer of such genes.

One would suspect that among the genes identified that belong to the multidrug resistance group in this study, are also plasmid related as a lot of these genes were detected in this group. However, efflux pumps play important roles in multidrug resistance and are found in almost all bacterial species (Zhang *et al.*, 2007; Sun *et al.*, 2014). These efflux pumps can be chromosomal or plasmid mediated. The rest of the identified genes in this study can certainly also play a role in the phenotypic results observed. The strong trend observed with antibiotic susceptibility regarding the beta-lactams as well as trimethoprim might also be attributed to these identified genes found with the whole genomes. A study done by Liu *et al.* (2017) sequenced the genome of a *Bacillus thuringiensis* strain and identified only nine antibiotic resistant genes of which some conferred resistance to penicillin and encoded Class A beta-lactamase. They identified vancomycin resistance genes which confer resistance to glycopeptides. In this study glycopeptide resistance genes were identified the second highest after the multidrug resistant group.

The presence of these identified genes using the WGS confirmed something that conventional PCR could not achieve. Phenotypic antibiotic resistant patterns were observed in this study from which it was expected that resistant genes from the isolates could be amplified, however only one *ampC* amplification was achieved. Nevertheless, the WGS data reveals that there are even more genes present than expected as well as a greater variety. Thus, it can be deduced that the primers used for conventional PCR in this study were not specific enough to amplify these genes.

As seen in all the other sections, there was an increase in the amount of identified genes from the raw water into the drinking water. It is clear that the treatment processes carry out a selection process for greater antibiotic resistance as well as the amount of ARGs. The exact same trend can be seen regarding the virulence genes that can contribute to the potential pathogenicity of these isolates. Additionally, more than 80% of HPC isolates produced more than two enzymes. This is indicative that heterotrophic bacteria contain within their genetic composition these ARGs as well as virulence genes. A study done by Liu *et al.* (2017) sequenced the genome of a *Bacillus thuringiensis* strain and identified 21 virulence genes. However, the expression of these genes is complex and does not necessarily play an immediate role in antibiotic

resistance (Davies and Davies, 2010). But the presence of these entities means that these bacteria can actively participate and play a role in the development and spread of antibiotic resistance.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

1.1. Conclusion

To determine the antibiotic resistant profiles of heterotrophic bacteria and to correlate it with antibiotic resistant genes, the following objectives were set. The findings of each objective are briefly discussed below.

i. To analyse the selected physico-chemical properties of drinking water

The physico-chemical properties that were selected showed compliance to SANS 241 (2015) for all the sampling runs at NW_C and NW-G, except for the turbidity and nitrites. The high turbidity levels can prevent effective disinfection to take place because bacteria can shield themselves using the suspended particles. Furthermore, the turbidity levels in the drinking water were higher than those found in the raw water, which indicates biological activity such as biofilm formation taking place in the distribution system or inefficient treatment of water. The presence of high levels of nitrites which also increased from the raw water into the drinking water, is a concern and may pose health risks to consumers. Nitrites in the distribution system might also be an indication of biofilm formation and nitrate reducing bacteria present in the drinking water. Although most of the physico-chemical properties did comply to SANS 241 (2015), in most cases the levels in the raw water were lower than the levels tested in the drinking water. It can be assumed that the latter indicates some biological activity taking place within the drinking water is most likely biofilm formation.

ii. Identify the HPC bacteria

A clear trend could be observed where *Bacillus* species represented the biggest fraction of the total identified isolates within the raw water as well as the drinking water. Once again the representation of *Bacillus* species in the drinking water was higher as compared to that of the raw water. It therefore seems as if the treatment processes select for *Bacillus* species mostly because of their ability

to form endospores which enable them to survive the oligotrophic conditions as well as disinfection. However, it must be noted that isolation methods and growing conditions of this study also allow for selection pressures on specific heterotrophic bacteria being isolated. The isolated bacteria only represent 1% of the total amount of heterotrophic bacteria present in the water. Furthermore, the evolutionary relationships between all the isolated bacteria showed that there were four distinctive phyla called the Firmicutes, Protobacteria, Actinobacteria and the Bacteroidetes. Of these phyla the Firmicutes and the Proteobacteria represented the biggest fraction of all the isolated HPC bacteria.

iii. Determine the potential pathogenicity of isolated bacteria

HPC bacteria are often regarded as not posing any health risks within drinking water. However, this study showed that more than 80% of the isolated heterotrophic bacteria tested positive for haemolysis and that most of them produced at least more than two extracellular enzymes. Furthermore, nine isolates were able to produce all the extracellular enzymes screened. It must be noted that these heterotrophic bacteria are not pathogens but clearly produce enzymes that allow them to be invasive and enter host cells which is the first step towards infection.

iv. Determine the antibiotic resistance profiles of HPC bacteria and detect the presence of antibiotic resistance genes (ARGs)

The antibiotic resistance profiles showed a clear trend across all the sampling runs at both NW-C and NW-G. High levels of resistance is shown against the beta-lactams (ampicillin, cephalothin and penicillin) and trimethoprim. The average percentage of all the isolates from NW-C and NW-G showing resistance against these antibiotics ranged from 47% to 100% which is considerable. The second trend observed is the increase in resistance from the raw water into the drinking water for all the antibiotics at NW-C and NW-G. It seems as if the treatment processes select for more resistant bacteria. The multiple antibiotic resistance (MAR) index showed that all the average values

exceeded the threshold of 0.2 which means that the isolates have an origin of a high risk source of contamination where antibiotics are used.

The presence of the selected antibiotic resistant genes was not identified in any of the isolated samples. Only one *ampC* gene was detected in the drinking water at NW-C. For the eDNA, only the *int1* gene was detected from the raw water and distribution water at NW-C. This however does not mean that these genes are not present (as the WGS indicated). It seems as if the primers used in this study might not have been specific enough as they were designed for clinical related rather than environmental isolates.

v. Do whole genome sequencing on *Bacillus* species

The analysis of whole genome sequencing (WGS) off six *Bacillus* species from NW-C, showed that these bacteria contain a lot of antibiotic resistance genes and virulence factors. Of the ten groups of antibiotic resistant genes the multidrug resistant genes were the most identified. This can be correlated with the resistance patterns observed as well as with the MAR results. The WGS data also clearly shows an increase in the amount of antibiotic resistant genes as well as virulence genes from the raw water into the drinking water. This trend has been seen in all the objectives. It seems as if water treatment plays an important role in the selection, distribution as well as the prevalence of resistance and antibiotic resistant bacteria.

5.2. Recommendations

Regarding this study, a few recommendations are described:

1. As seen in this study, the nitrite levels in the drinking water exceeded the SANS 241 (2015) regularly. This trend is observed at other drinking water treatment plants in South Africa as well. However, it is not clear what the specific causes of this observation are. It is recommended that further studies be conducted regarding the nitrate-nitrite reactions within drinking water distribution systems. High nitrite levels in drinking water can pose health risks for consumers.
2. The Department of Water Affairs and Forestry (DWAF) initiated the development of a Drinking Water Quality Framework for South Africa in 2005 in order to combat the difficulties faced by the water service authorities (WSAs). However, no newer documents can be found on the website of the Department of Water and Sanitation (DWS) regarding the Drinking Water Quality Framework as well as any other document relating to drinking water quality. This is concerning as the generation of such information as well as its availability play an important part in the process of developing more sustainable water management and infrastructures in the country. Such information must be more readily available and access must be easier.
3. eDNA was isolated from water samples and conventional PCR was used to search for antibiotic resistant genes (ARGs). However, qPCR should also be conducted on these samples in order to quantify the ARGs. The identification of bacterial eDNA through sequencing must also be conducted as this will give a better representation of the composition of bacterial communities in the water samples.
4. The search for ARGs in environmental bacteria might present some challenges. Considering the current study, very little of the genes were detected through conventional PCR. It seems the primers used were not specific enough. This is due to the fact that these primers are designed to amplify ARGs found in clinical environments. Clinical ARGs might be different to ARGs found in the

environment. Therefore, it is recommended that primers and cycling conditions specific for environmental situations be used.

5. This study focused only on the isolation of genomic DNA in order to search for the presence of ARGs that might confer resistance antibiotics. However, plasmids play very important roles in the capturing and spread of ARGs. It might be informative to investigate antibiotic resistance based on the genomes as well as the plasmids of HPC bacteria.
6. Gathering the information from this study, it is important to start paying attention to making contributions for reducing antibiotic resistance. Although it is a multidisciplinary issue, environmental scientists; engineers; city planners; etc. can try to minimize these effects. This can be achieved by adapting and capacitating wastewater treatment plants as well as drinking water production facilities enough so that the effective removal of antibiotics and ARGs can occur. Further studies can be conducted with regard to the microbiome of the drinking water distribution systems as well as the microbiomes within treatment plants. One of the many solutions is to manage and operate the microbiome of drinking water distribution systems more effectively.

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APPENDIX

Table 13: All the identified HPC isolates from NW-C

Identification	n	Site	Similarity (%)
<i>Aeromicrobium fastidiosum</i>	1	D	98
<i>Aeromonas popoffii</i>	1	R	100
<i>Arthrobacter koreensis</i>	1	D	100
<i>Arthrobacter oryzae</i>	1	T	100
<i>Bacillus licheniformis</i>	1	R	99
<i>Bacillus safensis</i>	5	T, D	99
<i>Bacillus nitratreducens</i>	11	T, D	99
<i>Bacillus praedii</i>	1	D	99
<i>Bacillus anthracis</i>	2	D	100
<i>Bacillus altitudinis</i>	7	R, T, D	100
<i>Bacillus luti</i>	2	R	100
<i>Bacillus wiedmannii</i>	1	R	98
<i>Bacillus megaterium</i>	2	T, D	100
<i>Bacillus aryabhatai</i>	1	D	100
<i>Bacillus sonorensis</i>	1	D	100
<i>Bacillus paramycoides</i>	5	T, D	100
<i>Flavobacterium compostarboris</i>	1	R	97
<i>Flavobacterium araucanum</i>	1	T	99
<i>Flavobacterium saccharophilum</i>	2	R	97
<i>Iodobacter fluvialis</i>	1	R	99
<i>Microbacterium paraoxydans</i>	1	R	100
<i>Micrococcus aloeverae</i>	1	D	99
<i>Micrococcus luteus</i>	1	D	100
<i>Niveispirillum cyanobacteriorum</i>	1	R	99
<i>Paenibacillus lautus</i>	1	R	100
<i>Pseudomonas protegens</i>	2	R	100
<i>Rivibacter subsaxonicus</i>	2	T	99
<i>Roseateles depolymerans</i>	3	R	99
<i>Roseateles terrae</i>	1	R	99

<i>Shigella flexneri</i>	1	D	100
<i>Sphingobium herbicidovorans</i>	1	R	100
<i>Vogesella perlucida</i>	1	R	100
<i>Sphingomonas kaistensis</i>	1	D	100
<i>Vogesella perlucida</i>	1	R	100
Total	66		

n – number of isolates

Table 14: All the identified HPC isolates from NW-G

Identification	n	Site	Similarity (%)
<i>Aeromonas media</i>	1	R	100
<i>Aeromonas salmonicida</i>	1	D	100
<i>Aeromonas veronii</i>	2	R	100
<i>Bacillus licheniformis</i>	1	R	100
<i>Bacillus megaterium</i>	1	T	100
<i>Bacillus mycoides</i>	1	R	100
<i>Bacillus paramycoides</i>	2	D	100
<i>Bacillus species</i>	2	D	100
<i>Curvibacter delicatus</i>	1	D	98
<i>Deefgea rivuli</i>	1	R	98
<i>Dongia rigui</i>	1	D	99
<i>Flavobacterium buctense</i>	1	R	98
<i>Flavobacterium tructae</i>	2	R	99
<i>Massilia aurea</i>	1	T	99
<i>Nevskia ramosa</i>	1	D	97
<i>Novosphingobium subterraneum</i>	1	D	100
<i>Pedobacter quisquiliarum</i>	1	T	99
<i>Pseudomonas coleopterorum</i>	1	D	99
<i>Pseudomonas guineae</i>	1	D	97
<i>Pseudomonas koreensis</i>	1	R	100
<i>Pseudomonas moorei</i>	2	R	99
<i>Rheinheimera chironomi</i>	1	R	99

<i>Rheinheimera mesophila</i>	1	D	98
<i>Rheinheimera tangshanensis</i>	1	D	98
<i>Roseomonas stagni</i>	1	D	98
<i>Shewanella profunda</i>	1	R	99
<i>Sphingobium yanoikuyae</i>	1	D	99
<i>Sphingorhabdus contaminans</i>	1	D	99
<i>Staphylococcus argenteus</i>	1	D	100
<i>Williamsia spongiae</i>	1	T	99
Total	35		

n – number of isolates