Drinking water quality along the distribution network and associated antibiotic resistance in Maun, Botswana

By

Oaratwa Mashiqa
Student ID: 14100233

Department of Biological Sciences and Biotechnology
College Science,
Botswana International University of Science and Technology
Oaratwa.mashiqa@studentmail.biust.ac.bw

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Supervisor: Dr, Lesedi Lebogang
Department of Biological Sciences and Biotechnology
College of Science, BIUST
llebogang@biust.ac.bw

Signature:  Date: 19/03/2018

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The undersigned certifies that she has read and hereby recommends for acceptance by the College of Science a thesis titled: Drinking water quality along the distribution network and associated antibiotic resistance in Maun, Botswana, in fulfilment of the requirements for the degree of Master of Science in Biology and Biotechnology of the BIUST.

Dr: 

(Supervisor)

Date: 19/03/2018

DEDICATION

To my entire family and mostly my dear grandmother, Pauline Mashiqa her view of the world inspires me to be where I am.

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My heart felt gratitude to my supervisor Dr Lesedi Lebogang, I could not have asked for a more supportive adviser than I found in her. By her mentorship she showed me how to get there steering me into the right path and by example she showed me how to set and achieve high standards for research.

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ABSTRACT

Maintaining the water quality in the distribution system is crucial to supplying safe drinking water to consumers. The main aim of the study was to analyse physicochemical, bacteriological parameters and investigate prevalence of antibiotic resistant bacteria and their antibiotic resistance genes in the drinking water distribution system in Maun, Botswana. Forty-four water samples were collected at different season interval and were tested for chlorine and turbidity and the results showed that in May, September, January and March, turbidity and chlorine measured 1.0 NTU (0.2 mg/L), 1.1 NTU (0.5 mg/L), 1.0 NTU (0.2 mg/L), 2.7 NTU (0.1 mg/L) respectively. The indicator organisms recorded were heterotrophic bacteria (37.7 %), total coliforms (28 %), *Faecal streptococci* (18.9 %) while *Escherichia coli* was at 10.4 % and 5 % for unusual isolates. Antibiotic susceptibility tests were performed on 150 isolates, which were tested against 15 different antibiotics using the Kirby-Bauer diffusion test. Ninety-two percent of the isolates were susceptible to at least one antibiotic and only one isolate (121-S5; *E. coli*) showed resistance to all the antibiotics used. For multi-drug resistance (MAR) patterns, the most prevalent antibiotic resistance pattern observed was AMP-AML-MEL-C-W-RD-TET-PEN-STREP. In addition, 92 % of the isolates were susceptible to meropenem followed by gentamicin (88 %) and ciprofloxacin (81 %). The amplified 16S rDNA sequences were successfully used to identify the 10 selected isolates. The presence of targeted ARGs (*tet*A, *tet*B, *int*1, *strep*B and *sul*1) for both culture isolates and whole sample genome were established. Moreover, metagenomic analysis confirmed the presence of some pathogenic bacteria such as *Mycobacterium tuberculosis*, *Legonillela* sp, *Cholera* sp, *Staphylococcus* sp, *Sphingosinicella* sp. and *Pseudomonas* sp. In conclusion, the results indicated the occurrence of indicator bacteria and other non-culturable species in the drinking water as the water moves from the treatment plant to lines that feed the consumers. The results also signified the presence of antibiotic resistant bacteria and their resistance genes in the water samples. The data obtained may be thereof useful in monitoring the integrity of the water quality in the distribution network after treatment.

Key words: Drinking water quality, water distribution system, indicator microorganisms, metagenomics, antibiotic resistant bacteria and resistance genes.

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CHAPTER 1

1.0 INTRODUCTION

1.1 Background of the study

Safe drinking water is recognized as a basic human right and drinking water is supposed to be clean and should not cause complications after consumption. The health risks associated with consumption of contaminated water from naturally occurring microorganisms are of high interest to the world, more especially public health practitioners (Richards *et al*., 1992; Hunter 1993; Eckner, 1998; Pachepsky *et al*., 2011). These microorganisms are mostly of faecal origin and place the human population at risk of waterborne diseases such as cholera, hepatitis, typhoid and the predominant diarrhoea (Obi *et al*., 2002; Okeke *et al*., 2010). Diarrhoea has an estimated annual incidence of 4.6 billion and causes 2.2 million deaths every year worldwide (Walker *et al*., 2012). World Health Organization (WHO) guidelines for drinking water quality states that the water for drinking purposes should be free from any microorganism and must not cause any disease (WHO, 2002).

Water quality is a measure of how clean and safe the water is to the consumers. One of the most important factors influencing water quality is the distribution systems that deliver water to the end users (Brikke, 2002; Schoutern and Moriarty, 2003). Ideally, there should be no changes in the quality of treated water from the point it leaves the treatment plant until it reaches the consumer's tap. However, in reality extensive changes occur to treated water as a result of complex chemical, physical and biological reactions (Sim *et al*., 1987). Hence, it is imperative to monitor the quality of water throughout the distribution network, as safe drinking water should have limited physicochemical elements, colour and compounds that cause bad odour or taste and should be free from pathogenic microorganisms (Benignos, 2012).

Pathogenic microorganisms of concern can develop in drinking water networks by intrusion due to external contamination incidents at different stages of water treatment, storage and transmission through the pipe networks which include; cross connections, backflow events, pipe breaks, and negative pressure or due to improper flushing and disinfection procedures (Mceneill

and Edwards, 2001). Furthermore, the most disturbing consequences of contamination in drinking water distribution networks involve the presence, multiplication and dispersion of opportunistic pathogens (Pryor *et al*., 2004; Vaerewijck *et al*., 2005; Flemming and Wingender, 2010; Wang *et al*., 2012). Microorganisms present in water pipes may display two types of behaviour: 1. the planktonic state, where cells float in water as it moves and their physical and chemical activities are independent of neighbouring cells (Collins, 1957; Johnson and Swanson, 2000; Marshall, 2013) and 2. the biofilm, which is the attached state in which cells are adhered to each other and to the surface of the pipe walls (Costerton *et al*., 1987; Flemming, 2011). Characterization of water quality is ideally based on the detection of conventional indicator microorganisms. This conventional approach could exclude majority of microbial species found in the water since the methods use selective media to target specific bacterial species, while discriminating against many groups of microorganisms that could be present in the water, and are a potential health risk. However, with the development of new technologies, recent studies offer a more complete view of microbial communities in the environmental sample using molecular approaches (Gianoulis *et al*., 2009).

The use of antibiotics in everyday living is a concern globally since it is associated with the development and spread of antibiotics in the aquatic environment (Martinez, 2009; Kümmerer, 2009; Rizzoa *et al*., 2013). Various bacterial species, which carry resistance genes, are frequently released into the surface water systems through municipal sewage effluents, (Michael *et al*., 2013; Rizzoa *et al*., 2013). The occurrence and fate of resistant bacteria and their resistance genes in surface water is not desirable since high concentration of these resistant bacteria in surface water may enter drinking water supply (Jones *et al*., 2005). Subsequently, the water provided to the community could potentially cause untreatable diseases to consumers. Therefore, the determination of the resistant bacteria and their resistance genes is imperative.

The most widely accepted guidelines for water quality stipulate that there should be no detectable levels of pathogens at the point of distribution as a safe guard against microbial related waterborne disease (WHO/UNICEF, 2005). It is thus crucial to trace the water quality, especially bacteriological parameters from the treatment plant to the consumers and identify the points of re-contamination. The study investigates water quality of Maun distribution network by

examining the physicochemical, bacteriological parameters as well as determining antibiotic resistance and investigating the presence of antibiotic resistance genes at different distribution points. The results from the study will draw attention to deterioration of drinking water quality during transmission and relate it to causes of public health problems.

1.2 Statement of the problem

Adequate, good quality and safe supply of drinking water is a basic need for human life. Waterborne diseases due to unsafe drinking water is one of the leading causes of morbidity and mortality worldwide and have led to increased public awareness over the quality of drinking water (Fewtrell *et al*., 2005; Anadu and Harding, 2000; Mandomando *et al*., 2007; Yilgwan *et al*., 2012). In Botswana, water-related diarrhoeal incidences have been reported over the past years in the six major districts, with Ngamiland district recording the highest of such cases despite the efforts by the government to provide safe drinking water, through setting the national standard (BOS 32: 2009). The standard recommends that the drinking water should contain zero counts of pathogenic microbial indicators (BOS 32: 2009; Ngwenya and Kgathi, 2003; Mazvimavi and Mmopelwa, 2006; Swatuk and Kgomotso 2007; Kujinga *et al*., 2014; Tubatsi *et al*., 2015). The provision of adequate clean drinking water in the study area has been through challenges in the past years (Mmopelwa *et al*., 2005). The area experience unreliable water supply, increase poor quality of water (Mmopelwa *et al*., 2005) and increase in water demand as the population increases (Geoflux, 2002). Hospitals and clinics are mostly affected as they require consistent supply of good quality water since they deal mostly with patients who are immuno-compromised, and could easily get waterborne infections (Geoflux, 2002). A study by Mmopelwa (2005), in the suburbs of Maun revealed that about 50 % of residents were willing to pay for improved water quality as a coping strategy.

Numerous studies reported in Ngamiland focused on water contamination at the source point, overlooking the challenges in water quality changes that can occur in the distribution system (Masamba and Mazvimavi, 2008; Mmualefe *et al*., 2011; Kujinga *et al*., 2014; Tubatsi *et al*., 2014; West *et al*., 2015). The tendency to overlook these challenges after treatment is through the perception that the high quality water produced from water treatment works will not deteriorate to an unacceptable level, but in reality extensive changes occur to finished water as a

result of complex chemical, physical and biological reactions (Swain, 2004). The quality of tap water is said to be as good as the condition of the pipes it flows through (Martin *et al*., 2007; Liu *et al*., 2017), as such, the effect of physicochemical and microbiological contaminants can result in the deterioration of the water quality that reaches the customer's tap as compared to that at the treatment plant (Vreeburg and Boxall, 2007; Proctor and Hammes, 2015).

There is therefore the need for more research on water quality in the district to determine the extent of quality change that happens during transmission, more especially in communal arrangements such as healthcare facilities and schools, where mass infections could occur. Determination and identification of opportunistic pathogens in water is highly important in order to control and prevent waterborne infections and may be used to assess the degree of risks associated with the transmission of pathogens to humans through drinking water. Furthermore, constant exposure of bacterial population to antibiotics could result in bacteria acquiring resistance to antibiotics of clinical interest leading to compromised drug therapy. Increased exposure to pathogenic microorganisms that may have acquired resistance poses a health risks necessitating the need to study the trends of antibiotics resistance in water microorganisms.

1.3 Objectives

1.3.1 General objective

The main objective of the study is to investigate the deterioration of water quality as it is transmitted through the distribution network from treatment facility to consumers. This is done by determining the physical, microbial and antibiotic resistance properties of drinking water using both the culture dependent and culture independent methods.

1.3.2 Specific objectives

In line with the overall objective, the specific objectives are to:

1. Analyse the following physicochemical parameters; turbidity and free residual chlorine.
2. Evaluate bacteriological quality of water samples by isolating indicator microorganisms.
3. Determine antibiotic resistance of the isolates and identify selected bacterial strains using the 16S rDNA sequencing.
4. Use metagenomics to determine total microbial community of the water samples.

5. Investigate the presence of antibiotic resistance genes for both selected isolates and metagenomics samples.

1.4 Significance of the study

Microorganism regrowth and biofilm formation contribute significantly to water quality, and have often been associated with the deteriorating of water quality along the distribution networks as it travels to the consumers. The proposed study investigate whether the treatment process used is efficient and/or if there is any post treatment contamination as well as identifying the points of re-contamination. The study analysed the quality of drinking water along the distribution system from the treatment plant to consumer points to determine the level of re-contamination. In addition, the study established if the bacteria isolated at various points of the distribution system are pathogenic, or could be associated with occasional breakouts of water-related ailments in Maun. Moreover, this study established the treatability of isolated bacteria with antibiotics by testing their antibiotic resistance as well as detecting corresponding antibiotic resistance genes that are often held accountable for coding for resistance mechanisms of many bacteria.

This study would thus contribute to understanding the quality of water and its deterioration along the distribution system as well as factors that may be involved. The findings of this study will be used as recommendations to the water authorities to put corrective measures to their current operations for the purpose of providing adequate good quality water to the consumers. The method currently used at the study area involves the sole use of indicator organisms in routine water quality monitoring, which may exclude other pathogens present in the water. Furthermore, the findings are expected to be used as reference material for further studies focusing mostly on the methodology used for routine monitoring, thereby encouraging the use of molecular techniques to curb the limitations of indicator techniques. Metagenomics approach can also be introduced as complementary method for detection and identification of microorganisms, which may have entered a viable but not cultured stage together with those that are not associated with the coliform group.

CHAPTER 2

2.0 REVIEW OF LITERATURE

Water supports life in every way and thus all organisms on the planet need water to survive (Chaplin, 2008). The human body is made up of two-thirds water used for maintenance of our bodies and plays a key role in the prevention of diseases. As such consumption of safe and adequate amounts of water is believed to decrease the risks of disease occurrence (WHO, 2008; Hrudey and Hrudey, 2014; APEC, 2015). Water quality and quantity are the two critical dimensions to every aspect of life. Water quantity is defined by the satisfactory pressure service provided to the consumer while quality is commonly defined by its aesthetic, physical, chemical and biological characteristics. Water quality is also an important indicator of environmental health as a good environment is one in which the quality of water is in a state to protect the public against any water borne diseases (Ercumen *et al*., 2014; Ramírez-Castillo *et al*., 2015). Therefore, drinking water quality should be monitored from time to time to protect the public from water-associated health risks. There are two types of drinking water quality monitoring: 1. compliance monitoring, this determines whether water supplies comply with requirements from relevant standards (e.g. BOS 32:2009; National Water Policy, 2011) and 2. operational monitoring, which checks if treatment works and distribution networks are operating effectively in delivering the water that meets the standards (WHO, 2004). Operational monitoring should provide caution on water quality deterioration, failing of treatment process and detects problems in the distribution networks (Chapman, 1996; Aisopou *et al*., 2012; Van Blaricum *et al*., 2016).

2.1 Public health issues on drinking water

Potable water is water that is free from any harmful pathogens. It is used for human consumption and is supposed to not cause any complications. When drinking water is contaminated it could result in outbreaks of waterborne diseases. World health Organization (WHO) estimates that about 1.1 billion people globally drink water that is not safe, and as a result the increase in diarrhoeal disease is attributable to drinking contaminated water (Ohimain, 2017; Soleimani *et al*., 2017). Even though water authorities are committed to ensure that drinking water that leaves the treatment plant is safe for consumers' consumption, the quality of water may decline

dramatically from the time it leaves the plant to the time it reaches the consumer' tap (September *et al*., 2007). A significant number of disease outbreaks are attributed to contaminations that occur between the treatment works and the tap (Edberg *et al*., 2000; Blackburn *et al*., 2004; Enriquez *et al*., 2001).

Under epidemiology, many waterborne outbreaks are mainly due to bacterial species like *E. coli*, *Mycobacterium* species, *Salmonella* species, *Shigella* species, *Klebsiella* species, *Pseudomonas* species and *Legionella* species. (Szewzyk *et al*., 2000). Pathogens recovered from drinking water are mainly of feacal origin (Ashbolt *et al*., 2001; Hunter *et al*., 2009). Some of these microorganisms may be primary pathogens; those that cause disease or even death in healthy individuals while others may be opportunistic pathogens; those that cause disease in individuals with compromised immune systems conditions At high risk are infants under the age of 5 and individuals whose immune systems are compromised such as patients on chemotherapy (Centers for Disease Control, 2014; Simon *et al*., 2015). Even low level exposure to these microorganisms can cause discomfort or sickness (Villanueva, 2014).

The risk associated with the pathogenicity of microorganisms is intensified by the ability of microorganism to resist destruction by antibiotics, which are very important for management of infectious diseases caused by bacteria (Alighardashi *et al*., 2009; Turkdogan and Yetilmezsoy, 2009; Versporten *et al*., 2014). The phenomenon of bacteria to resist the effect of an antibiotic is called antibiotic resistance and it is one of the concerning public health issues worldwide (Huang *et al*., 2011; Deblonde *et al*., 2011). Antibiotic resistance is defined as a progression where bacteria have developed different mechanisms to reduce effectiveness of the antibiotics used against them (Lebeaux *et al*., 2014; Yang *et al*., 2014; Flores-Mireles *et al*., 2015; Brown and Wright, 2016). The resistance mechanism evolves from antibiotic resistance genes (ARGs) present in organisms producing antibiotics (Fatta-Kassinos *et al*., 2011). Genes encoding defense mechanisms of resistance are located on the bacterial chromosome and are transmitted to the next generation via horizontal gene transfer using mobile genetic elements such as plasmids, integrons and transposons (Zhang *et al*., 2009; Brown and Wright, 2016). Horizontal gene transfer via conjugation is common in nature, because the density of bacteria is high making it possible that the two suitable bacterial cells coming close to each other can share genetic

information (Brown and Wright, 2016). This gene transfer produces very dynamic genomes and has effectively changed the biological and pathogenic character of bacterial species (Ochman et *al*., 2000). The occurrence and spread of antibiotic resistant bacteria (ARB) and ARGs are pressing public health problems worldwide. This is mainly due to the fact that antibiotics are the first line of medication provided for most of the bacterial treatments before definite tests are made and if present in drinking water their genes can be disseminated to humans through ingestion (Baquero *et al*., 2008; Wright, 2010). Moreover, trace levels of antibiotics in water and chlorination of the water can contribute to the development of ARB and ARGs and (Armstrong *et al*., 1981; Schwartz *et al*., 2003; Xi *et al*., 2009). ARB are known to be tolerant to chlorine and some weaken the effect of chlorination (Armstrong *et al*., 1981; Shrivastava *et al*., 2004; Templeton *et al*., 2009). Studies have shown that chlorine disinfection can induce development of ARB (Armstrong *et al*., 1981; Bergeron *et al*., 2015) and spread of ARGs (Xi *et al*., 2009), as a result of increased expression of the multidrug efflux pumps leading to tolerance towards the disinfectant as well as antibiotics (Karumathil *et al*., 2014; Singh *et al*., 2017). Moreover, stress tolerant bacteria due to chlorination might be more resistant to antibiotics as compared to normal cells (Armstrong *et al*., 1981; Huang *et al*., 2011).

The ultimate residence of antibiotics excreted in animal and human wastes is the sewage treatment plants where they escape treatment and end up in the environmental drinking water sources. This is where antibiotics remain stable for months in the environment (Baquero *et al*., 2008; Khalaf *et al*., 2009; Kümmerer, 2009; Huang *et al*., 2011; Rizzo *et al*., 2013). Studies have shown the occurrence of antibiotics in surface waters and sewage effluents and different drinking water reservoirs (Mukherjee and Chakraborty, 2006; Castiglioni *et al*., 2008; Khalaf *et al*., 2009; Kümmerer, 2009; Thevenon *et al*., 2012). Certain bacteria can display resistance to one or more antibiotics (multi-drug resistance) where their genetic material confers selective advantages of surviving in the presence of multiple antibiotics (Modi *et al*., 2014; White *et al*., 2016).

The overabundance of genetic mechanisms for evolution and assortment of antibiotic resistance genes, extensive use of antibiotics in human and veterinary medicines encourage rapid dissemination of ARGs (Kümmerer *et al*., 2009; Jiang *et al*., 2011). Among all the ARGs, sulfonamide ARGs, tetracycline ARGs (Pruden *et al*., 2006; Auerbach *et al*., 2007; Jiang *et al*.,

2013), and streptomycin ARGs (Araújo *et al*., 2017) are commonly detected in the aquatic environments. Class 1 integrons are the common cause of antibiotic resistance as they can capture and express diverse resistance genes (Gilling *et al*., 2008). They are commonly embedded in plasmids and transposons and facilitate horizontal transfer into a wide range of pathogens hence considered to be potential mobile genetic element (Gilling *et al*., 2008). The mobility of integrons is of major concern in the spread of antibiotic resistance since it is associated with a variety of resistance gene cassettes among bacterial species, especially the Gram negative bacteria (Barlow *et al*., 2004; Labbate *et al*., 2009; Xu *et al*., 2011). Tetracycline resistance has been used as the key element in monitoring resistance genes in natural environments (Sandalli *et al*., 2010). Localization of *tet* genes on movable genetic structures (plasmids) is the main cause of tetracycline resistance among bacteria (Speer *et al*., 1992; Sandalli *et al*., 2010). *tet*A and *tet*B code for energy dependent efflux proteins linked to repressor proteins. The efflux proteins in the absence of tetracycline block transcription of the repressor and structural efflux genes (Roberts, 1996).

Streptomycin is an aminoglycoside with direct interaction with the small ribosomal subunit at the highly conserved region of transcription called ribosome accuracy center (Noller, 1991; Carter *et al*., 2000). The translational apparatus comprise of an rDNA domain and the ribosomal protein (S12), which generates resistance to streptomycin (Springer *et al*., 2001). Aminoglycosides genes primarily inhibit bacterial protein biosynthesis by binding to the 16S rDNA (Becker and Cooper, 2013). Sulfonamides are bacteriostatic inhibitors resulting from chromosomal point mutations in the dihydropteroate synthase (DHPS) gene (Hoa *et al*., 2008); an enzyme that is insensitive to drug inhibition (Skold, 2001). Sulfonamides interfere with the formation of dihydropteroic acid by blocking the enzymatic activity of the DHPS. It may also results from the acquisition of plasmid borne *sul*1 genes coding for resistant DHPS enzymes (Huovinen *et al*., 1995).

2.2 Drinking water treatment and supply

There are many impurities (physical, chemical, biological as well as antibiotics) in surface water that can harm human health if the water is consumed without treatment. Drinking water

authorities have put measures to ensure that the water that consumers take is free from impurities and pathogenic microorganisms in order to avoid waterborne disease outbreaks.

2.2.1 Water treatment process

Best drinking water production practices are necessary and mandatory in order to provide safe drinking water to the community. Water treatment involves pumping of raw water from either surface or underground source into the treatment facility to undergo a series of treatment stages. Water treatment involves different steps which are in place to remove any foreign material in the water including waterborne pathogens since its goal is to produce water that is biologically and chemically safe, free from colour, taste and odour (Momba *et al*., 2009). Water suppliers use different treatment processes to remove contaminants from drinking water and the choice depends on the quality and variability of the raw water sources and the treatment objectives, which may vary based on the end use (Blackburn *et al*., 2004).

Drinking water treatment processes differ from country to country and in Botswana the water sector uses the Actiflo conventional treatment involving various steps such as screening, coagulation, flocculation, sedimentation and maturation (Figure 1.1). Screening is the removal of floating debris, and coagulation involves addition of coagulant chemicals such as aluminum and iron salts (Bhatnagar and Sillanpää *et al*., 2017) to water to allow the naturally occurring particles to aggregate and form flocs that can be removed by flocculation. After flocculation, the water flows into the injection tank where flocculants and microsand are added to initiate floc formation. The water then passes through a clarifier tank where it is mixed with air under high pressure for removal of flocs making them rise to the surface as clear water moves to the maturation tank. The maturation tank is where microorganisms, fine particles and organic matter are removed. The clarified water exits the maturation tank and is collected in settler tanks where chorine is then added as a disinfectant for destruction of microorganisms present in the water. The final water leaving the plant is recommended to contain a certain amount of free chlorine concentration (0.6 - 1.0 mg/L) until it reaches the tap to avoid any possible contamination during transmission (BOS 32: 2009).

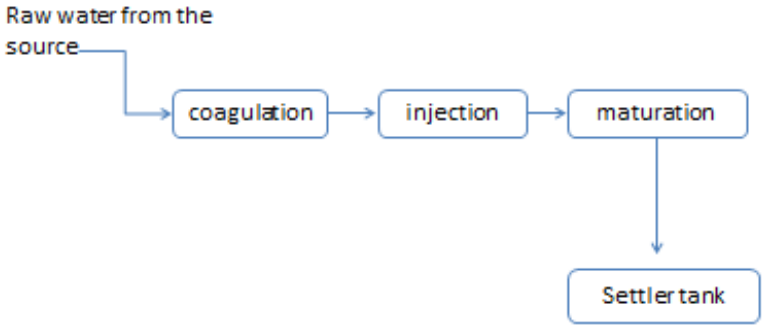

Figure 1.1: Schematic diagram of the Actiflo treatment process used for treatment of drinking water in Botswana**.**

2.2.2 Drinking water supply

Drinking water services providers have the responsibility of supplying water to customers and in Botswana the water sector comprises of several institutions. The Botswana Bureau of Standard (BOBS) under the Ministry of Trade and Industry, Department of Water Affairs (DWA) and Department of Geological Survey under the Ministry of minerals, energy and water resources, and Water Utilities Corporation (WUC) (Swatuk and Kgomotso, 2007). The nature of association ranges from formulation and implementation of policies, planning, management, supply and consumption. DWA is responsible for water sources and WUC is responsible for treatment and distribution of drinking water. The country depends on two water resources; surface water (rivers, dams, lakes) and groundwater from boreholes. Surface water resources are limited in the country, with most rivers flowing for only a few times per year, as such different parts of the country depend on different water sources. Maun village in Ngamiland District's main water source is the Thamalakane-Boteti River, which is an outflow of the Okavango delta (Gieske, 1997; Wolski and Savenije, 2006). Maun water provision is based on this perennial river that receives water from Okavango Delta. On the other hand, the river receives treated wastewater effluent from the village and run-offs from agricultural activities along the river coast (Palamuleni, 2002; Nhapi *et al*., 2002; Motsholapheko *et al*., 2011). Treated waste water from the Maun sewage treatment plant which is located upstream from the drinking water treatment

plant is discharged into the Thamalakane- Boteti River. The water is then treated to satisfactory levels as according to BOS 32: 2009 standard prior to distribution

2.3 Distribution system

A water distribution system is designed to deliver drinking water to people at sufficient pressure, quantity and quality (Walski and Male, 2000; Ministry of urban development, 2005). Adequate water delivery to consumers requires a full technologically manageable and functional system for easy and sustainable access of good quality water. Piped distribution is important to the safety and quality of drinking water as the treatment itself. The American Water Works Association (Kay, 1974) defines the water distribution system as all water utility components for the distribution of drinking water through pumping networks to consumers. To transport water, pumps must provide working pressures, convey sufficient water, holding tanks must store the water for fluctuations in water demand and stabilizing pressure in distribution systems (Longuevergne *et al*., 2013), and valves needs to open and close properly. The systems are generally comprises of pipes of varying sizes; distribution mains (75-254 mm), transmission mains (305-427 mm) and service pipes (20-50 mm), then the water enters household installation systems (Milzow *et al*., 2009).

2.3.1 Water quality in the distribution system

Current drinking water treatment and disinfection technologies produce safe water in treatment plants. Deterioration of water quality may however occur at various levels during transmission. The two broad categories of water contamination are point source where harmful substances are released directly into water body and nonpoint source which delivers pollutants indirectly through transport or environmental change in the distribution network (Table 1.1). After the treatment all the contamination is left to nonpoint sources. The residence time of the water, concentration of organic compounds, decreasing chlorine concentration, development of biofilms in the pipe surfaces, temperature and physicochemical characteristics of the pipe materials can influence growth of microorganisms and could lead to change in the water quality during distribution (Niquette *et al*., 2000). Moreover, water quality may also degrade during distribution

due to contamination from external sources such as improperly maintained storage facilities and other factors outlined in Table 1.1.

Table 1.1: Potential sources of contamination to potable water in the distribution system

Source of contamination	Mode of contamination
Treatment breakthrough	Pathogens may escape treatment and enter the distribution system.
Potable water storage holding tanks	Storage reservoirs can be susceptible to outside contamination if they are not adequately maintained by regular cleaning and disinfection, ultimately promoting the growth of bacteria.
Cross connections and backflow	Contamination can occur during negative pressure and backflow events.
Transient contamination	Intrusions may occur via leaky pipes, valves, joints and seals. As the pipes gets old, problems like leakage and break caused by corrosion.
Water main installation, breaks and repairs	The interior of pipes can be contaminated during installations and repairs.
Water age	Water aging leads to the deterioration of water quality especially in storage facilities.
Types of pipe material used	Pipe materials are crucial for maintenances of water quality during distribution and there are several materials and standard used globally which includes metallic (stainless steel galvanized steel, iron, and copper) and nonmetallic (chlorinated polyvinylchloride (CPVC), polyvinylchloride (PVC) and polyethylene (PE)) (Al-Jasser, 2007). The country uses mostly PVC and ductile ions which is also used in Maun with some parts of the country using stainless steel as their pipe material. PVC pipes contain traces of compounds that are harmful to the environment and solvents used to join PVC pipes and fittings together contain volatile organic compounds

Biofilm formation	Biofilm is a solid layer where microorganism cells are adherent to each other and to the surface and enclosed in a matrix of Extracellular polymeric substances (EPS) (Costerton *et al*., 1987; Flemming, 2011). The solid-liquid interface between a solid surface (pipes) and liquid (water) provides an ideal environment for the adherence and growth of microorganisms (Cunningham *et al*., 2008). After the attachment stage the microorganisms grow in the EPS and thereafter proliferate on the water as it flows by and impair the quality of water (Zacheus *et al*., 2001). Biofilms in the drinking water distribution systems pose a threat to human health as some of the microorganisms are pathogenic causing waterborne diseases. Westall *et al*., (2000) said that biofilms are robust and can remain unchanged for up to 3.5 billion years. The presence of biofilms in pipes may correlate with opportunistic pathogen including adherence, colonization and antimicrobial resistance (Van der Kooij *et al*., 2017).

2.3.2 Water quality parameters

Surface runoff, anthropogenic activities and discharge of wastewater can cause contamination of drinking water sources. This is in most cases controlled by the treatment process at the treatment plant, however like every other human built environment; nonpoint source contamination can occur (Table 1.1). Therefore, tracing the finished water quality of treated water becomes important to ascertain if there is re-contamination after treatment. Water quality is based on assessing the microbial, physical and chemical parameters. These parameters are linked to the safety of the drinking water and deliver important information about the fitness of a water body (Lenntech, 2005; Gupta *et al*., 2009). To assess threats to human health, the most important parameters considered are the physicochemical and bacteriological parameters, known to cause water quality deterioration (Chidya *et al*., 2011). In accordance with regulatory requirements, BOBS has put in place guidelines for drinking water quality to be adhered to (BOS 32, 2009; appendix 1).

383 *2.3.2.1 Physicochemical parameters*
384
385 The physicochemical parameters listed in BOS 32: 2009 include turbidity, temperature,
386 dissolved oxygen, residual chlorine and conductivity. Chemical and physical parameters of water
387 give an indication of potential contamination. Water temperature of 15 ºC has been reported as
388 critical for coliform growth and survival in water, biofilm formation and chemical reactions
389 (LeChevallier *et al*., 1996; Pritchard *et al*., 2009). BOS 32: 2009 standard states that turbidity
390 level for drinking water should be less than 1 NTU and WHO (2011) states that samples with
391 low values of less than 5 NTU should be accepted. Additionally, increase in turbidity during
392 distribution may indicate the presence of biofilms inside pipes and also outside contamination
393 entering pipes.
394
395 Free residual chlorine is used as a parameter to predict biological contamination events in the
396 distribution systems (Helbling and VanBriesen, 2008). Free residual chlorine measures the
397 portability of water and its presence correlates with the absence of pathogenic organisms. Free
398 chlorine concentration controls bacterial regrowth hence an important value in water quality
399 characterization (Lehtola, 2010). Regrowth of microbes in distribution systems is difficult to
400 prevent completely and water suppliers have resorted in adding excess chlorine to protect the
401 water from recontamination during storage (Lehtola, 2010). Ideal system supplies free chlorine at
402 a concentration of 0.6 - 1.0mg/L (BOS 32: 2009). Nonetheless, the concentration is most likely
403 to be inadequate to maintain the quality for 24 hours as chlorine decays with time and this could
404 negatively affects its ability to inhibit microbial growth (Figure 1.2) at the far ends network
405 (Niquette, 2001).
406

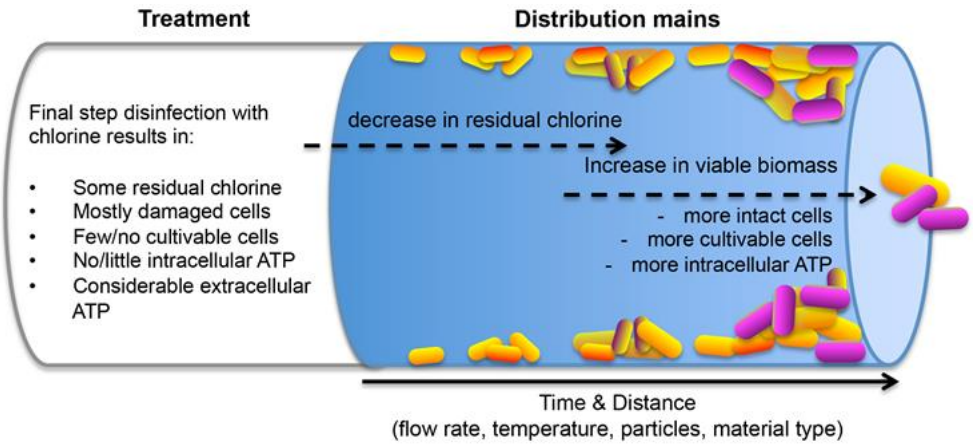

Figure 1.2: Unstable chlorinated distribution network (Nescerecka *et al*., 2014).

2.3.2.2 Bacteriological parameters

Bacteriological parameters assess the safety and quality of drinking water using indicator organisms (Figure 1.3) which include total coliforms, heterotrophic bacteria, *E. coli*, and *F. streptococci* (Whitlock *et al*., 2002; Pavlov *et al*., 2004; BOS 32: 2009; Okeke *et al*., 2011). Indicators microorganisms are mostly used as index of faecal pollution and the choice of which organisms to test for is mostly based on the presence of specific indicator pathogens and should be detectable by simple and inexpensive methods. Limitation of the indicators includes the different survival characteristics of pathogenic microorganisms which makes them not adequate indicators (WHO, 2000).

Indicator organisms are said not to be pathogenic but their presence indicates the presence of pathogenic microorganisms (Rompre´ *et al*., 2002). Coliforms are mostly found in different natural environments and their presence in drinking water is considered as an indicative of water quality deterioration due to microbial contamination. In addition, most coliforms are not harmful and do not cause diseases but come from the same sources as pathogenic bacteria and hence their presence could mean possible contamination and waterborne disease threat (Center for disease, 2013). When larger numbers of bacterial counts are detected in treated water, there is a high possibility that pathogenic bacteria exist, hence BOBS and WHO drinking water standards

require the absence of any indicator microbes in public drinking water supplies in order to declare the water safe to drink.

Deterioration of water quality is a major problem worldwide and to protect consumers from waterborne diseases, therefore the drinking water quality should be extremely monitored (Lehtola *et al*., 2004). The reference method used for bacteriological monitoring in drinking water is heterotrophic plate count (HPC), which measures the heterotrophic bacteria to indicate the general microbial quality of water. This group of bacteria is commonly present in all types of water and includes a broad range of bacteria that requires carbon source for growth. HPC is also used to measure the efficiency of water treatment, disinfection processes and integrity of distribution systems for regrowth of microorganisms in the distribution network. Under this broad group of heterotrophic bacteria, there are primary bacterial group of coliforms which are of faecal and non-faecal origin.

Total coliform include Gram negative, oxidase negative, non-spore forming rods bacteria that are influenced by surface water and nonpoint contamination (Medema *et al*., 2003; Payment *et al*., 2003; WHO, 2008). Total coliforms evaluate the purity and reliability of a distribution system as well as the presence of biofilm in the distribution network (Zamxaka *et al*., 2004). Under total coliform group is a group of *F. streptococci* which includes a large numbers of streptococcal Gram positive, catalase negative, non-spore forming cocci. The group indicates the presence of faecal contamination by warm blooded animals and nonpoint bacterial contamination in waterways (Santiago-Rodriguez *et al*., 2016; Taoufik *et al*., 2017). Lastly, in the group of coliforms is *E. coli* Gram negative, facultative anaerobic bacteria commonly found in the intestine of warm blooded organism. The presence of *E. coli* in drinking water signals the high likelihood of recent faecal contamination in the water (Edberg, 2000; Ashbolt *et al*., 2004; Meays *et al*., 2004). Moreover, on rare cases *E. coli* O157:H7 can be found in water and this is a strain that could cause serious illness (McQuigge *et al*., 2000; Olsen *et al*., 2002).

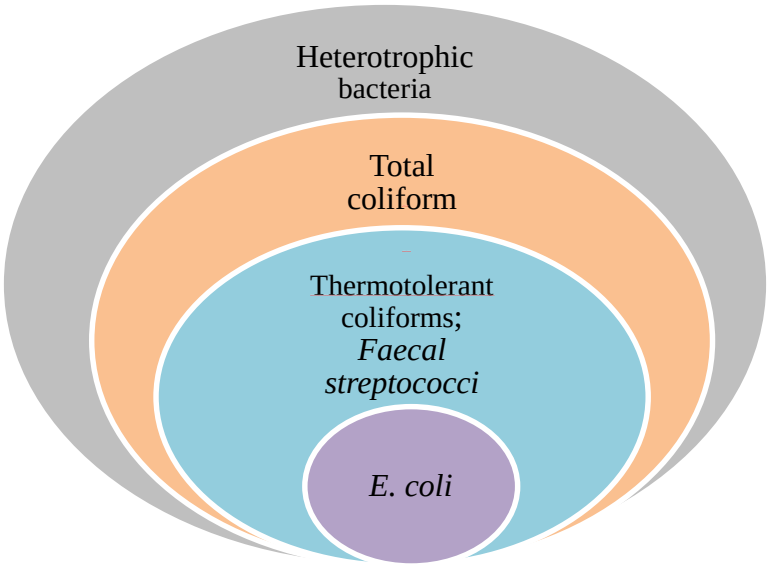

Figure 1.3: Diagram showing the relation of indicator bacteria.

2.4 Methods used to study microbiological quality of drinking water

Microbial analyses can be performed using culture-based approaches and non-culture methods (molecular approaches). Culture-based techniques are mostly used to detect, quantify and characterize bacteria in drinking water. They are currently used by water authority companies to routinely monitor microbial quality of drinking water (Rompré *et al*., 2002; Vaz-Moreira *et al*., 2017).

2.4.1 Culture-based techniques used to study microorganisms in drinking water

Traditionally, several methods have been used to determine the presence of indicator microorganisms in drinking water and they includes; multiple tube fermentation technique, spread plate, membrane filtration and Colilert (Maheux *et al*., 2014; Heredia *et al*., 2015).

Membrane filtration is a technique whereby water is filtered through a membrane filter to concentrate microbial cells. The filter is then removed and incubated on a specific medium for a given period of time and thereafter the developed colonies are enumerated (Douterelo *et al*., 2014). Examples of media are used include the plate count agar (PCA); a general media used to

monitor bacterial growth of a sample, Slanetz and Barkley; a very selective media for detection and numeration of *F. streptococci* (Slanetz and Bartley, 1957) and membrane lactose glucuronide agar, a medium for the differentiation and enumeration of *E. coli* and total coliforms. Membrane lactose glucuronide agar contains lauryl-sulphate to inhibit Gram positive organisms. One of the advantages of the filtration method is that large volumes of water sample can be used to increase the concentration of cells. Also, the filters can be easily placed on different media (Eckner, 1998; Scott *et al*., 2002, Berry *et al*., 2006). The drawbacks of the filtration method are that when the water turbidity level is high it becomes difficult to filter out the sample. Also, phenols can absorb to filters and inhibit growth and further testing are required for confirmation of the coliforms recovered (Ashbolt *et al*., 2001; Schraft and Watterworth, 2005; Yáñez *et al*., 2006). Moreover, there is a decrease in coliform recovery because of the presence of high numbers of non-coliform bacteria (Burlingame *et al*., 1984; Rompré *et al*., 2002). The predominant concern about membrane filtration is its failure to recover stressed cells during physical and chemical steps involved in water treatment as damaged cells are unable to grow on the media (LeChevallier, 1990; Douterelo *et al*., 2014; Metsämuuronen *et al*., 2014).

An alternative and sensitive method to microbial detection is the use of enzymatic reactions. The Colilert test is an example of the enzyme-based test that simultaneously detects total coliforms and *E. coli*. The test uses defined substrate technology, which contains two nutrient indicators, o-nitrophenyl-β-D-galactopyranoside and 4-methylumbelliferyl-β-D-glucuronide as carbon sources which can be metabolized by β-galactosidase and β-glucuronidase; enzymes found in coliforms and *E. coli*, respectively (Rompré *et al*., 2002; Chao, 2006). Unlike membrane filtration, Colilert can detect non culturable coliforms.

Culture-based techniques are simple to perform because some of the bacteria grow in nature as single cells making them easy to grow in culture medium. However, the culture dependent methods only show (<1 %) of the total diversity of the water samples (Riesenfeld *et al*., 2004). This could discriminate against viable but not cultivable (VBNC) microbes. These are living cells that have lost their ability to grow on microbiological media, which normally would allow their growth (Oliver *et al*., 2005). Moreover, some intact microorganisms are unable to grow on a

media plate because they are intracellular and live inside macrophages cells (Greub and Raoult, 2004; Kubica *et al*., 2008; Mahamed *et al*., 2017).

2.4.2 Molecular techniques used to study microorganisms in drinking water

To avoid the limitations of culture-based techniques in representing the actual microbial diversity (Douterelo *et al*., 2014), molecular methods are used to identify microbial isolates or study microorganisms within their environments, for example in soil and water (Fierer and Jackson, 2006; Janssen, 2006; Buée *et al*., 2009; Santiago *et al*., 2015).

2.4.2.1 Bacterial identification using 16s rDNA sequencing

The identification of bacterial isolates and diversity in water using biochemical methods are not always sufficient and do not provide the microbial species present in drinking water (Sanz and Köchling, 2007). The advent of a variety of molecular techniques combining polymerase chain reaction (PCR) and DNA sequencing has circumvented the problems associated with biochemical techniques in identifying isolates, determining microbial diversity as well as detection of specific genes (Rousselon *et al*., 2004).

Molecular analysis of isolates recovered from water samples involves the extraction and purification of DNA to provide genetic information of the isolate sample (Kahlisch *et al*., 2012). DNA extraction is followed by PCR amplification of marker genes which for bacteria is 16S ribosomal DNA (rDNA) gene to obtain taxonomic information. The steps involved initial denaturing of the DNA, annealing and finally extension of the new strand of DNA to produce multiple copies of DNA fragments. The 16S rDNA gene is highly conserved across most phylogenetic domains of bacteria and it allows understanding phylogenetic information from bacteria inhabiting different ecosystems (Prosser, 2002). In addition, 16S rDNA gene sequencing provides genus and species identification for isolates that are mostly broadly categorized under their family (Drancourt *et al*., 2000; Mignard and Flandrois, 2006). The DNA amplicons obtained from PCR form the basis for all the post PCR analysis including sequencing and

construction of phylogenetic trees. Various bacteria carry ARGs and once present in the environment, may be transferred among bacterial species (Davison, 1999; Agersø *et al*., 2007).

DNA sequencing is fundamental to research, defined as the process of determining the precise order of nucleotides within a DNA molecule (Ronaghi, 2001). It is important in disease discovery, forensics science, and identifying microorganisms in natural environments (Douterelo *et al*., 2014). There are several DNA sequencing techniques which involves, Maxam-Gilbert sequencing, Sanger sequencing and next generation sequencing. Next generation sequencing (NGS), involves the production of several gigabases of sequence data in a single run, at high speed, cost-effective and high-throughput nature (Mardis, 2008). Examples of NGS platforms include Roche/454, the Illumina, and ion torrent semiconductor sequencers (Metzker, 2010; Brocchieri, 2014).

Recent studies used Roche/454 pyrosequencing to characterize bacterial communities from samples from tap water; (Grahn *et al*., 2003; Douterelo *et al*., 2013), treatment plants (Jonasson *et al*., 2002; Gharizadeh *et al*., 2003) , assess the effect of disinfectants on microbial community (Grahn *et al*., 2003; Douterelo *et al*., 2013). Limitations of this method involve accuracy problems with homopolymeric repeats, emulsion PCR, its relatively expensive and the difficulty in differentiation between live and dead cells (Douterelo *et al*., 2014). Other technologies like the Illumina and ion torrent have advanced to try and solve the problems experienced with the Roche/454.

2.4.2.2 Metagenomics

Microorganisms are ubiquitous; despite their ubiquity the extensive efforts to study them have not been efficient due to current limitations in cultivation technologies (Chao *et al*., 2013). Culture-based techniques are often biased toward a small fraction of the inhabiting microbiota, while the use of DNA-based approaches, which targets phylogenetic genes provide limited information on health consequences of the microbial groups detected (Gomez *et al*., 2012). The field of metagenomics permitted the detection of culturable and non-culturable bacteria in a particular environmental sample. Metagenomics has proven to be successful in accessing the biosynthetic machinery of uncultured bacteria, thus bypassing traditional molecular methods that

rely on cultivation (Singh *et al*., 2009; Vaz-Moreira *et al*., 2014). Metagenomics is defined as the application of genomics to the mixed genetic material analysed and sequenced directly from environmental samples (Shokralla *et al*., 2012; Rashid and Stingl, 2015) and have high degree of sensitivity (Douterelo *et al*., 2014, Reddy *et al*., 2014; Salipante *et al*., 2015).

Metagenomics bypasses the need for isolation or cultivation of microorganisms by direct isolation and sequencing of community DNA (environmental DNA) (Lamendella *et al*., 2011; Gilbert and Dupont, 2011).Environmental DNA (eDNA) have the advantage of dealing with VBNC microorganisms. VBNC state is a critical adaptive mechanism found in non-spore forming bacteria having an intact cell membrane with undamaged genetic material (Heidelberg *et al*., 1997; Cook and Bolster, 2007), and bacteria enter the VBNC state as an adaptive strategy under nutritionally deficient conditions and low temperatures (Biosca *et al*., 1996; Ducret *et al*., 2014). Metagenomics is now providing significant insights into the sizes and metabolic functions of the genomes of a wide variety of microorganisms (Hasan *et al*., 2014; Jimenez-Infante *et al*., 2014; Reddy *et al*., 2014) and is a promising tool to detect the entire organismal DNA found in water.

CHAPTER 3

3.0 METHODOLOGY

3.1 Study Area

Botswana experiences water scarcity and rainfall varies widely throughout the country, averaging less than 250 mm/year in the southwest up to about 600 mm/year in the northeast. The country depends on two types of water resources; surface water and groundwater (Bauer, 2006). Maun village is situated in Northwest District of Botswana, and has a population of approximately 60 263 according to 2011 census (Statistics Botswana, 2011). The village's raw water source is mostly dependent on perennial Tlhamalakane river, which is one of the tributaries of the Okavango delta (McCarthy and Ellery, 1998; Masamba and Mazvimavi, 2008).

In this thesis drinking water quality of Maun distribution system was investigated to trace the integrity of the water along the distribution network.

Map of sampling points and the distance from the treatment plants

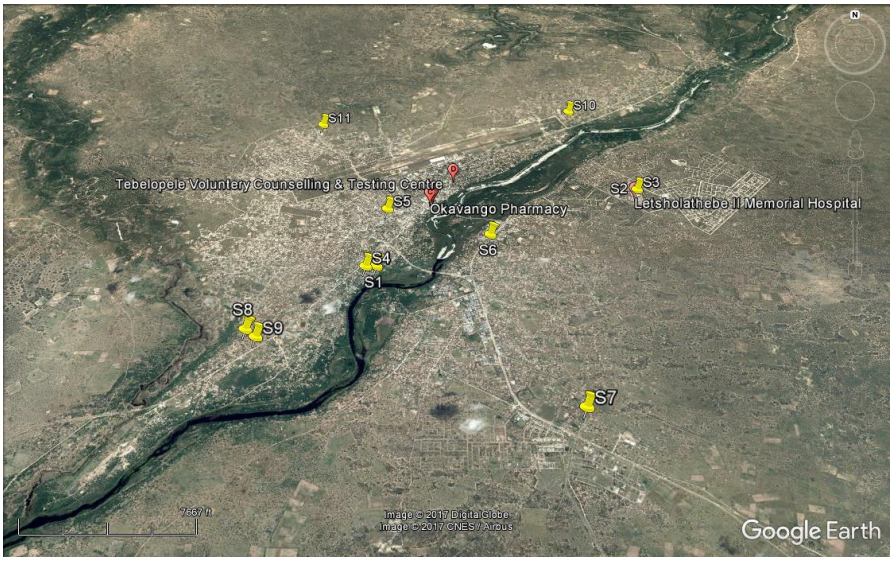

Figure 3.1: Goggle earth map of sampling sites in Maun, Botswana (by Oaratwa Mashiqa).S1, treatment plant; S2, pediatrics ward (Letsholathebe memorial hospital); S3, theater ward(Letsholathebe memorial hospital); S4, Maun general clinic; S5, Maun clinic; S6, Boseja clinic; S7, Matshwane clinic; S8, boaster plant; S9, Thito clinic; S10, Sedie clinic; S11, Moeti clinic.

3.1.1. Maun water distribution system overflow

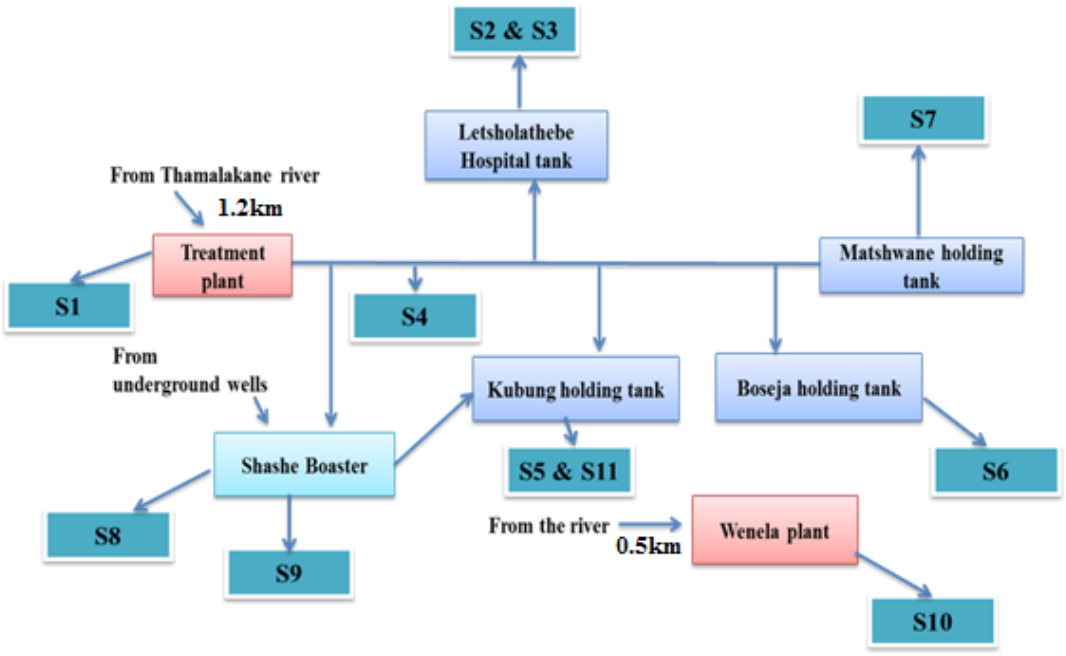

Figure 3.2: Schematic representation of the process of drinking water production and distribution analysed in the study. Samples were collected at the end of the treatment process at the treatment plant (S1) and at the different distribution points (S2- S11).

Table 3.1 Distance between the treatment plant and sampling sites

Treatment plant	Sampling point	Distance (km)
Borolong Treatment plant (S1)	S2 and S3	5.4
	S4	0.2
	S5	1.7
	S6	2.2
	S7	4.5
	S8	2.5
	S9	2.4
	S11	4.6
Wenela treatment plant	S10	2.5

3.2 Water Sampling

Water samples were collected from the main treatment plant, a boaster, main hospital, seven clinics (Figure 3.1, 3.2 and table 3.1). A 1 L water sample was taken once per site in May, September 2016 and in January, March 2017. Prior to sampling, the water taps were flushed for 3 minutes and then sterilized by heat flaming using a blow torch for 60 seconds, and then flushed again for additional 30 seconds. Water was collected aseptically in sterile 500 mL Duran Schott glass bottles from different sampling points. The samples were labeled and transported to Maun Department of Water Affairs laboratory for analysis following the BOS ISO 5667-5:2006 standard, and analyzed using standard microbiological procedures under BOS 32:2009 standard. A total of 44 water samples were collected throughout the study. All the water samples were first analyzed for free chorine levels (mg/L) using HI-96711C Free and Total Chlorine colorimetric (Hanna instruments, UK) and turbidity level measured in Nephelometric Turbidity Units (NTUs) using the Hach 2100Q Tubidimeter (Life Science, London).

3.3 Isolation and enumeration of bacteria

Microbiological parameters are used as indicators of the bacteriological quality of drinking water (Svagzdiene *et al*., 2010; Payment and Locas, 2011). These indicators include *F. streptococci*, total coliforms, *E. coli* and heterotrophic bacteria and their presence relates to possible presence of enteric pathogens in drinking water systems.

3.3.1 Isolation by membrane filtration and purification of isolates

The number of viable culturable bacteria in collected water samples was determined by membrane filtration method (Novo and Manaia, 2010; Lin *et al*., 2016). For all the samples volumes of 100 mL were filtered through 0.45 µm pore size filter which were aseptically placed on respective selective media ensuring that no air bubbles were trapped. The selective media used were Slanetz and Barkley (enterococcus agar) (HImedia Laboratories) for *F. streptococci*, plate count agar (PCA) (Acumedia, Lesher) for heterotrophic bacteria and membrane lactase gluconide agar (MLGA) (MRD; Oxoid CM733) for total coliforms and *E. coli.* All the media were prepared according to the manufacturers' instructions. Heterotrophic bacteria, total coliforms and *E. coli* plates were incubated at 37 °C for 24 hours and the incubation conditions

for *F. streptococci* were 44°C for 48 hours. The colonies formed after incubation periods were enumerated and characterized. The results were expressed as the number of colony forming units (CFU) in 100 mL of water. Isolated colonies were then purified by subculturing twice using streak plating method. Pure culture isolates were put in 50% glycerol and stored at -80 °C until further identification tests and analysis.

3.3.2 Enzymatic detection assay

Aliquots of 100 mL of water samples were used using the Quanti-Tray 2000 Colilert (IDEXX Laboratories, Portland, USA) to simultaneously detect and quantify total coliforms and *E. coli* in water samples. 100 mL of the sample was transferred into IDEXX water sampling bottles that contained sodium triphosphate and the pack of colisure reagent was poured into the samples. The reagent was allowed to dissolve before the solution was transferred into a 51-well Quanti-Tray. The tray was then sealed with a Quanti-Tray sealer, which automatically distributes the reagent mixture into the separate wells. The sealed trays were incubated at 35 °C for 24 hours. After incubation, the wells with the yellow-orange colour was read as positive for total coliforms and *E. coli* was determined by the number of wells that fluorescence at wavelength 540 fλ after incubation under UV light. The number of wells that presented a positive colour and fluorescence were subjected to the most probable number (MPN) table to obtain the MPN of total coliforms and *E. coli* per 100 mL of the sample.

3.4. Antimicrobial susceptibility testing

The antimicrobial susceptibility test determines the sensitivity or resistance of pathogenic bacteria with respect to different antimicrobial compounds (Begum *et al*., 2017). The ability of a microorganism to grow around the disk shows the inability of the antimicrobial compound in the disk to inhibit the organism (Darabpour *et al*., 2012; Begum *et al*., 2017). An antibiotic susceptibility test was performed using the Kirby-Bauer disk diffusion method using standard impregnated antibiotic disks (Oxoid, UK; Bauer 1966), as according to (Saffari *et al*., 2016; Gefen *et al*., 2017). Panels of 15 antibiotic discs (Oxoid, UK) (Table 3.2) representing antibiotics that are widely used in clinical diagnostics were tested against 150 bacterial isolates. Fresh bacterial suspensions were prepared from previously stored isolates in glycerol by picking and transferring single colonies into 3 mL of sterile distilled water. Aliquots of 100 µL of the

suspensions were spread plated on Mueller-Hinton (Franklin Lakes, NJ) agar plates. Different antibiotic discs were applied on top of the medium using an antibiotic discs dispenser (Oxoid, UK), and the plates were incubated at 37 °C for 24 hours (National Committee for Clinical Laboratory Standards, 2000). After incubation, the plates were determined for antibiotic susceptibility by measuring diameters zones of inhibition using digital vernier caliper. The inhibition zone results obtained were used to classify isolates as resistant, intermediate resistant or susceptible to a particular antibiotic using standard reference (Clinical and Laboratory Standards Institute; M100-S20 Vol.30, No.1). Multiple antibiotic resistance (MAR) defined as diminished susceptibility to >1 of the antibiotics used (Paterson, 2006; Abula and Kedir, 2017) and phenotypes were collated for isolates that showed resistance to >3 antibiotics.

Table 3.2: Antibiotics minimum inhibitory concentrations and their spectrum of activity

Antibiotics	Abbreviation	Concentration	Spectrum of activity
Ampicillin	AMP	10 µg	broad
Streptomycin	S	10 µg	broad
Chloramphenicol	C	30 µg	broad
Neomycin	N	30 µg	broad
Amoxicillin	AML	10 µg	broad
Ciprofloxacin	CIP	5 µg	broad
Gentamycin	CN	10 µg	broad
Rifampicin	RD	5ug	broad
Tetracycline	TET	30 µg	broad
Trimethoprim	W	5 µg	broad
Mecillinam	MEL	30 µg	broad
Kanamycin	K	30 µg	broad
Penicillin	PEN	16 µg	Narrow (Gram negative)
Meropenem	MERO	4 µg	broad
Sulfamethoxazole	SUL	512 µg	broad

National Committee for Clinical Laboratory Standards (2000)

3.5 Molecular identification of the isolates

Various molecular techniques; PCR, 16S rDNA analysis, DNA sequencing and phylogenetic analyses were used for definitive identification of the isolates. Microbial diversity of environmental samples was determined using a metagenomics approach.

3.5.1 Extraction of genomic DNA

Ten isolates were selected for molecular identification. Selection was based on their degree of resistance to the above mentioned antibiotics per sampling site. The DNA was isolated according to Mirmohammadsadeghi *et al*., (2013). Isolates cultured in nutrient broth overnight were pelleted by centrifugation at 14000 rpm for 10 seconds after which the supernatants were carefully removed to avoid disturbing the pellets at the bottom of the tube. 600 µL of the cell lysis buffer (10 mM Tris, and 1 mM EDTA and 0.1 M SDS) was added and gently mixed to re-suspend the isolate before incubating at 80 ^{o}C for 5 minutes. This was followed by cooling the mixtures to room temperature and adding of 3 µL of cell RNase solution to the tubes, then mixing by inverting the tubes 25 times. The mixtures were then incubated at 37 ^{o}C for 30 minutes. The samples were allowed to cool to room temperature before adding 200 µl of ammonium acetate and vortexing gently. The samples were thereafter centrifuged at 14000 rpm for 3 minutes to pellet the DNA. The supernatants were decanted and the pellets re-suspended in 600 µL of isopropanol. The mixtures were gently mixed by inverting the tubes 50 times and centrifuging again at 14000 rpm to pellet the DNA. The DNA pellets were washed twice with 600 µL of 70 % and inverted a few times, centrifuged at 14000 rpm for a minute and the supernatants discarded. The pellets were dried at 37 ^{o}C in an oven for 30 minutes. Lastly, 100 µL of TE buffer was added to hydrate the DNA, which was then ready for further analysis.

3.5.2 Polymerase Chain Reaction for amplification of 16S rDNA

Amplification of 16S rDNA was performed using the 16S universal bacterial primers E786F (5 -GATTAGATACCCTGGTAG- 3) and U926R (5 -CCGTCAATTCCTTTRAGTTT-3) (Soergel, 2010; Samarajeewa *et al*., 2015) to amplify the 140 bp region of the 16S rDNA gene (Yergeau *et al*., 2012). A 25 µL PCR reaction mixture contained 3 µL of 1.5 µmol/L of each primer, 7.5 µL

of sterile ddH_2O, and 12.5 µL of master mix to which 2 µL of template DNA was added. The master mix was made of an optimized buffer, Taq DNA Polymerase, dNTP mixture and gel loading dye. The thermal cycling protocol involved 5 minutes initial denaturation at 95 °C followed by 30 cycles of denaturation at 98 °C for 10 seconds, 30 seconds annealing at 55 °C and 45 seconds extension at 72 °C. The final extension was performed for 1 minute at 72 °C. The template DNA was amplified in a programmed thermocycler (Life Technologies, Singapore).

PCR amplicons were separated by electrophoresis on 1 % (w/v) agarose gel stained with 0.7 µg/mL ethidium bromide. Electrophoresis was conducted for 90 minutes at 120 V using 1X TBE buffer (Tris, 0.5 mM EDTA, and boric acid, pH 8.0). A 1 kb DNA molecular weight marker and tracking dye were used. The gels were visualized under UV light in the Gel-doc (Upland, CA, USA).

3.5.3 DNA sequencing and phylogenetic analysis

Sequencing of the amplified PCR amplicons was performed using pyro-sequencing next generation sequencing method by Inqaba Biotechnology laboratories, South Africa. PCR amplicons were purified with QIAquick PCR Purification Kit (Qiagen, Hilden, German) according to the manufacturer's recommendations. Single DNA strands were sequenced using Roche GS-FLX 454 Genome Sequencer and edited using BioEdit and analyzed by comparing with known 16S rDNA sequences using the NCBI-BLAST algorithm (Blastn) to find the closest match in GenBank (Altschul *et al*., 1990). Sequences obtained from GenBank were firstly aligned by multiple sequence alignment technique using CLUSTAL W (Larkin *et al*., 2007). Phylogenetic trees were constructed from the neighbor-joining analysis model (Saitou and Nei, 1987), using MEGA version 7.0 software (Quast *et al*., 2012; Yilmaz *et al*., 2014; Kumar *et al*., 2016).

3.5.4 Detection of antibiotic resistance genes

The ten bacterial isolates which were subjected to PCR identification and sequencing were further investigated for the presence of antibiotic resistance genes using primer specific for each of the targeted gene and the annealing temperature as described in Table 3. Genomic DNA was extracted from the isolates as template for PCR of the identified bacteria strains. PCR reaction

mixture contained 2 µL of template DNA, 1.5 µmol/L of each primer (Table 3.3), 7.5 µL of sterile ddH_2O, and 12.5 µL of master mix making a final volume of 25 µL. The amplification of all the genes was carried in a thermocycler (Life Technologies, Singapore). PCR amplicons were separated by electrophoresis on 1 % agarose stained in 0.7 µg/mL ethidium bromide. Thereafter, the gel was visualized under UV light to confirm amplification and visual band were considered positive for the targeted gene.

Table 3.3: Primers used for antibiotic resistance genes

Target gene	Resistance phenotype	Annealing temp (◦C)	Target Sequence (5´-3´)	Amplicon size (bp)	Reference
*int*1	Class 1integron	55	CAGTGGACATAAGCCTGT TC CCCGAGGCATAGACTGTA	461	Iyobe *et al*., (2000)
*strep*B	Streptomycin	46.1	ATACAAATTCTGCTGACTACG TTAAATCCTTCCTGACCATTCC	500	Sadaka *et al*., (2017)
*tet*A	Tetracycline	59	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	210	Selvam *et al*., (2001)
*tet*B	Tetracycline	54	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG	659	Selvam *et al*., (2001)
*sul*1	Sulfonamides	53.1	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	921	Peirano *et al*., (2005)

3.6 Metagenomics analysis

Metagenomics is a molecular method that by-pass the need for culture isolation of microorganism through direct extraction of environmental DNA (eDNA) in a sample, followed by construction of genetic libraries. The approach is to treat the collective genomes of a bacterial community as a single large genome. Four samples were chosen S1, S2, S5 and Sl1 to determine the trend of microbial contamination from the source (S1) and the health care facilities with the highest daily number of patients and the highest number of antibiotic resistance bacteria isolate (S2, S5 and Sl1).

3.6.1 DNA extraction and sequencing of eDNA

Planktonic microorganisms' cells were concentrated by filtration of 100 mL water samples through 0.2 µm pore size Supor-200 filters using a membrane filtration apparatus. The membranes were immediately cut into smaller pieces before adding to the lysis tube. DNA was extracted using zyco soil DNA isolation kit (Zymo Research, Germany) following manufacturer's instructions. 750 µL lysis solutions was added to the tube secured in a bead beater and the tubes were vortexed vigorously for 30 minutes to detach the bacteria cells from the membranes followed by centrifugation at 9500 rpm for 1 minute. The supernatants were transferred to spin filters and centrifuged at 9500 rpm for 1 minute followed by DNA wash at 9500 rpm for 1 minute. The extracted DNA were eluted and centrifuged at 9500 rpm for 30 seconds. DNA templates integrity was assessed by electrophoresis. 1 % (w/v) agarose gel stained with 0.5 µg/mL ethidium bromide. Electrophoresis was conducted for 90 minutes at 120 V using 1X TBE buffer. A 1 kb DNA molecular weight marker was used. Metagenomics sequencing and bioinformatics analysis were carried out at Inqaba biotechnology Laboratories, South Africa, targeting the 16S region using the next generation sequencing and ion torrent approach. After sequencing, the data were analyzed using the Ion 16S™ metagenome analyses module within the Ion Reporter™ to enabling a rapid and semi-quantitative assessment of complex microbial samples. The blast results were presented in krona chats to show the diversity of the microbial community.

3.6.2 Detection of antibiotic resistance genes in environmental DNA

The presence of genetic elements encoding antimicrobial resistance in the microbiota of drinking water was investigated through molecular analysis. The genetic determinants targeted are listed in Table 3.3: class 1 integrons (*int*1), the gene encoding resistance to sulphonamide (*sul*1), streptomycin (*strep*B) tetracycline efflux protein (*tet*A) and (*tet*B). As described earlier, the PCR reaction mixture contained 2µL of template DNA, 1.5 µmol/L of each primer, 7.5 µL of sterile ddH_2O, and 12.5 µL of master mix making a final volume of 25 µL. PCR products were separated by electrophoresis on 1 % agarose stained in 0.5 µg/mL ethidium bromide. Amplicons were visualized under UV light on the same GelDoc machine.

CHAPTER 4

4.0 RESULTS

Water quality was determined in 11 sampling points in Maun village. The sampling sites included mainly taps from the treatment plant and from the clinics. Different parameters including physicochemical parameters, microbiological quality and the antibiotic resistance profiling of isolates recovered from the water samples were analyzed over a 4 period's interval. WHO (2013) and BOS 32: (2009) drinking quality standards were used as reference to determine if the quality water meets the specified requirements.

4.1 Physicochemical parameters

For physicochemical parameters, turbidity and free residual chlorine levels were determined.

4.1.1 Turbidity

All the sampling points had turbidity levels ranging from 0.11 to 6 NTU (Figure 4.1). The variation within the sampling sites were not statistically significant (P=0.15) using one way ANOVA with S10 having the highest turbidity level with a mean value of 2.97 NTU while S1 had the lowest with 0.47 NTU. March 2017 samples recorded the highest mean value of 2.73 NTU and the lowest value was recorded in September 2016 (0.87 NTU).

4.1.2 Free chlorine

Residual chlorine recorded was in the range of 0.001 to 1 mg/L (Figure 4.2). The variation within the sampling sites using one way ANOVA were not statistically significant (P=0.18) where S1 had the highest mean of 0.68 mg/L and the lowest of 0.03 mg/L was recorded at S10. Variation between the months was statistically significant (P=0.001) having September recording the highest mean of 0.54 mg/L and the lowest was observed in March of 0.07 mg/L. The water samples for September were within the BOS 32: 2009 minimum recommended level of 0.6 mg/L except for water samples from S5, S6, S10 and S11 (Figure 4.2). In January and May, only S1

samples met the required level of residual chlorine while the other sites were below the recommended value 0.6 to 1 mg/L. Lastly in March, all the sampling points recorded lower than 0.6 mg/L.

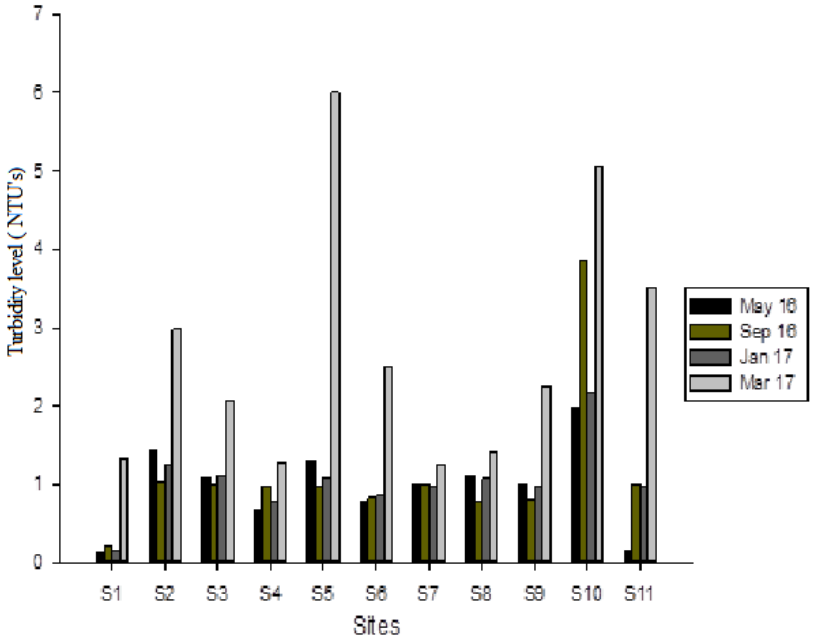

Figure 4.1: Turbidity level of the water samples collected at different sites

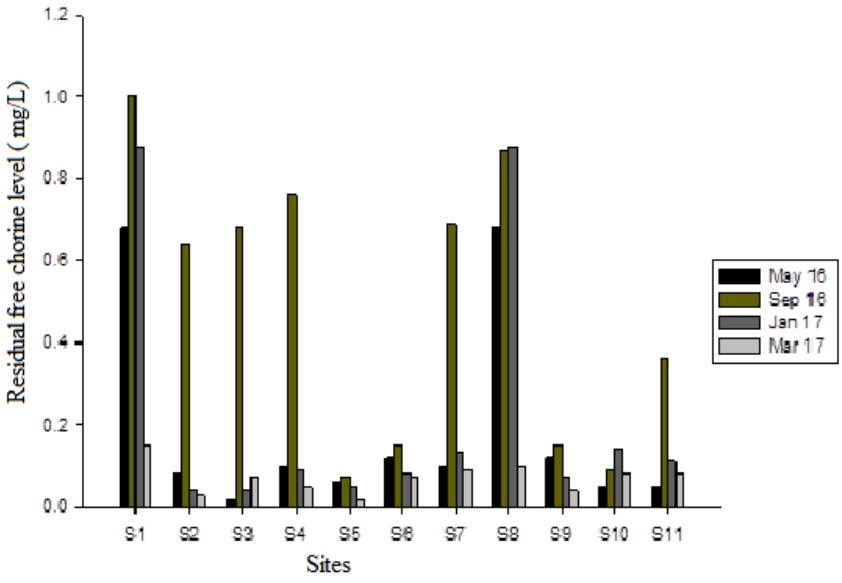

Figure 4.2: Residual free chorine level recorded in water samples from different sampling sites**.**

4.2 Microbial quality of drinking water

Microbial quality was determined by analyzing water samples for heterotrophic bacteria, total coliform, *F. streptococci* and *E. coli* and the results are summarized in Figure 4.3. The results indicate there was bacterial contamination in most of the sampling sites, with total of 6318 isolated bacterial colonies in 44 water samples and its statistically significant (P=0.002) using one way ANOVA. The data showed significant differences between months (P=0.001), the highest mean value (54 CFU/100mL) was recorded in March while the lowest (15 CFU/100mL) was recorded in September. Moreover, there was significant differences among the sampling sites (p=7.83E-15), the highest mean value (91 CFU/100mL) was reported in S5 (March) while the lowest (1 CFU/100mL) was reported in S1 (September).

The results showed positive for heterotrophic bacteria in March for all the sites. In the other months all indicator microorganisms were positive and unacceptable quality of water according to BOS 32: 2009 except for S1, which recorded zero counts (Figure 4.3). The highest mean counts for the indicator microorganisms tested were recorded in S5 (91 CFU/100mL), S6 (79 CFU/100mL), S11 (57 CFU/100mL), S10 (43 CFU/100mL), S2 (40 CFU/100mL), S3 (28 CFU/100mL) as compared to S9 (12 CFU/100mL), S7 and S4 (9 CFU/100mL), and S8 (3 CFU/100mL). The highest mean count for heterotrophic bacteria and total coliform was recorded in March having 52 CFU/100mL and 59 CFU/100mL respectively while the lowest mean count for heterotrophic bacteria and total coliforms was recorded in September as 27 CFU/100mL and 19 CFU/100mL respectively. For all the sites the highest mean was observed in S5 (126 CFU/100mL) heterotrophic bacteria, (97 CFU/100mL) total coliform, (81 CFU/100mL) *F. streptococci*, and (61 CFU/100mL) in *E. coli* while almost nothing was recorded in S1 (1 CFU/100mL), (0 CFU/100mL), (0 CFU/100mL), and (0 CFU/100mL) for heterotrophic bacteria, total coliform, *F. streptococci* and *E. coli* respectively.

For *F. streptococci* the lowest mean count were in September and May both recording 10 CFU/100mL while 44 CFU/100mL was recorded in March as the highest (Figure 4.3). The highest mean count for *E. coli* was recorded in January (32 CFU/100mL) and March (29

CFU/100mL) and the lowest was recorded in May (5 CFU/100mL) and September (3 CFU/100mL) respectively.

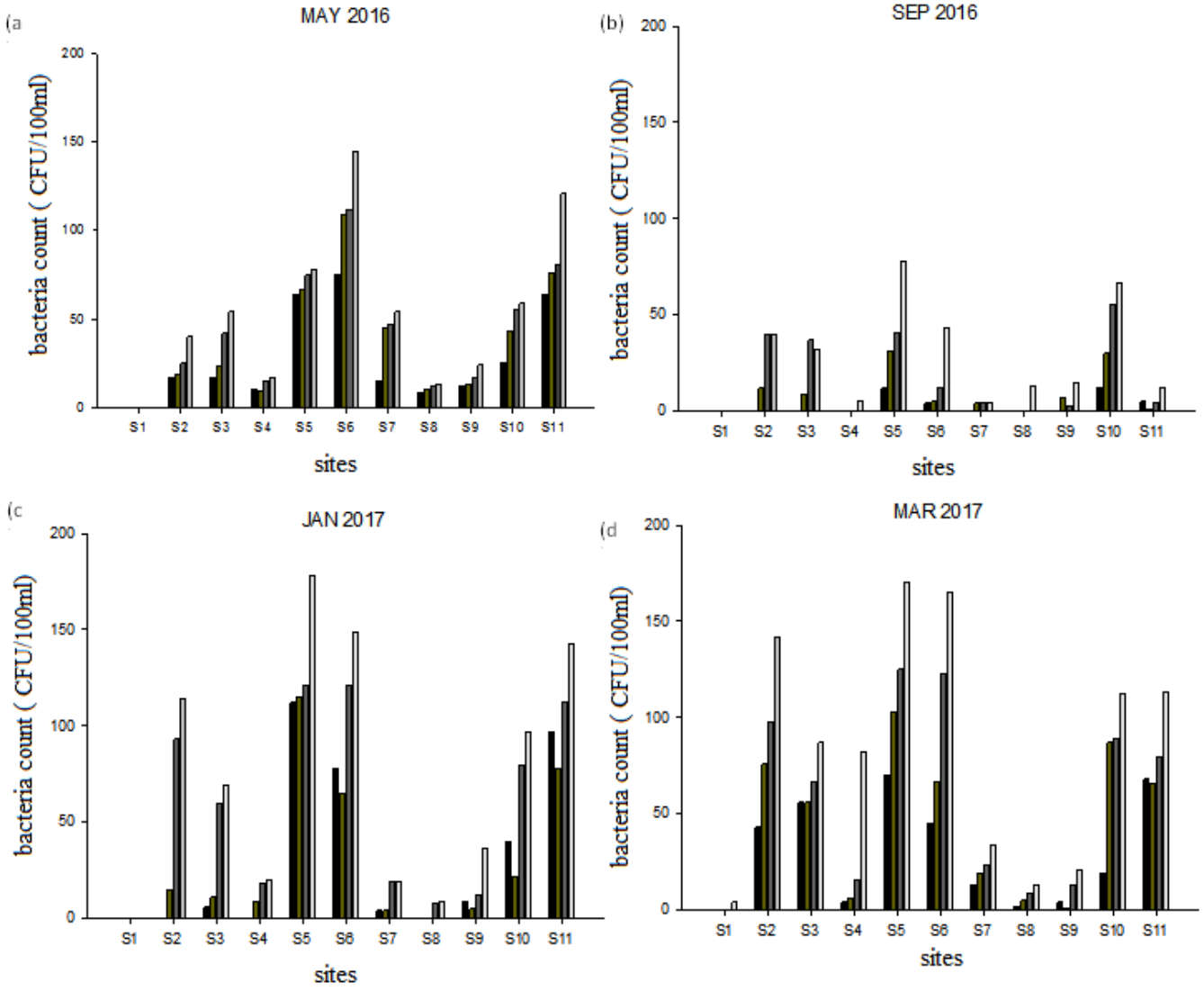

Figure 4.3: Graphical representation of bacterial colony counts in drinking water for different periods and different sampling sites.

4.2.1 Comparison of membrane filtration and Colilert for detection of total coliform

The distributions of total coliforms in the respective sampling sites were evaluated using membrane filtration (MF) and Idexx Colilert method (Figure 4.4). The results range between 0 and 170 CFU/100mL for membrane filtration and 0 to 200 MPN/100mL for Colilert (Figure 4.4). Statistical analysis by a two-factor ANOVA on total coliform counts by the type of test (Colilert and membrane filtration) shows no significant between the types (p=0.453). The variation within the sampling sites was statistically significant (p = 0.017).

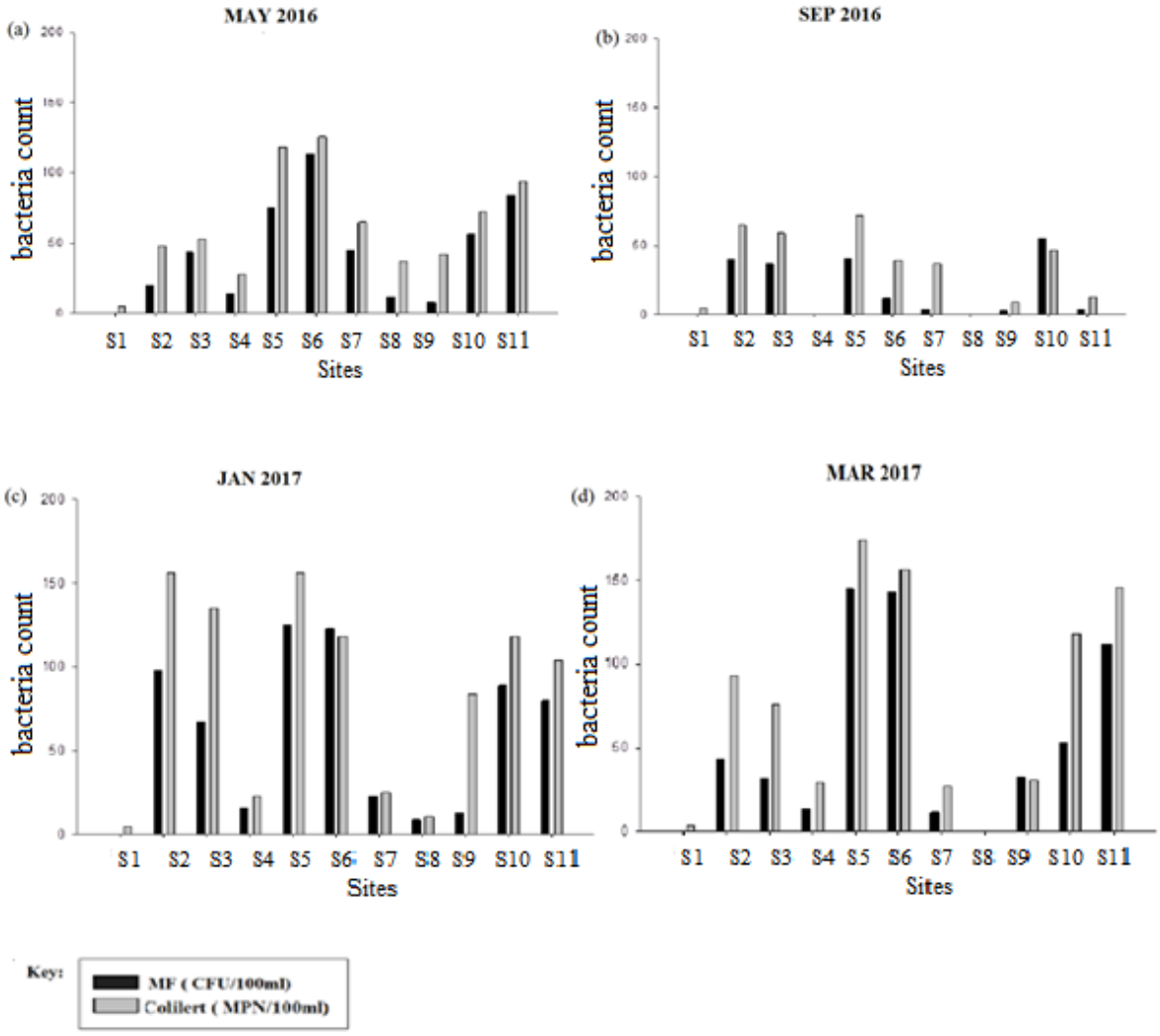

Figure 4.4: Total coliform bacterial counts with separate bar for Colilert (MPN/100ml) and MF (CFU/100ml) detection methods.

4.2.2 Comparison of membrane filtration and Colilert for detection of E. coli

E. coli counts ranged from 0 to 112 CFU/100mL for membrane filtration and 0 to 200 MPN/100mL for Colilert (Figure 4.5). Two-factor ANOVA was used to analyze *E. coli* counts to assess the type of test used and the results show significant difference (p=0.0242). The highest mean counts were recorded in March 2017 whilst the lowest was in September 2016. There was statistical variation among sites (p=0.0001) having the highest mean recorded in site 5 (105) and lowest in S1.

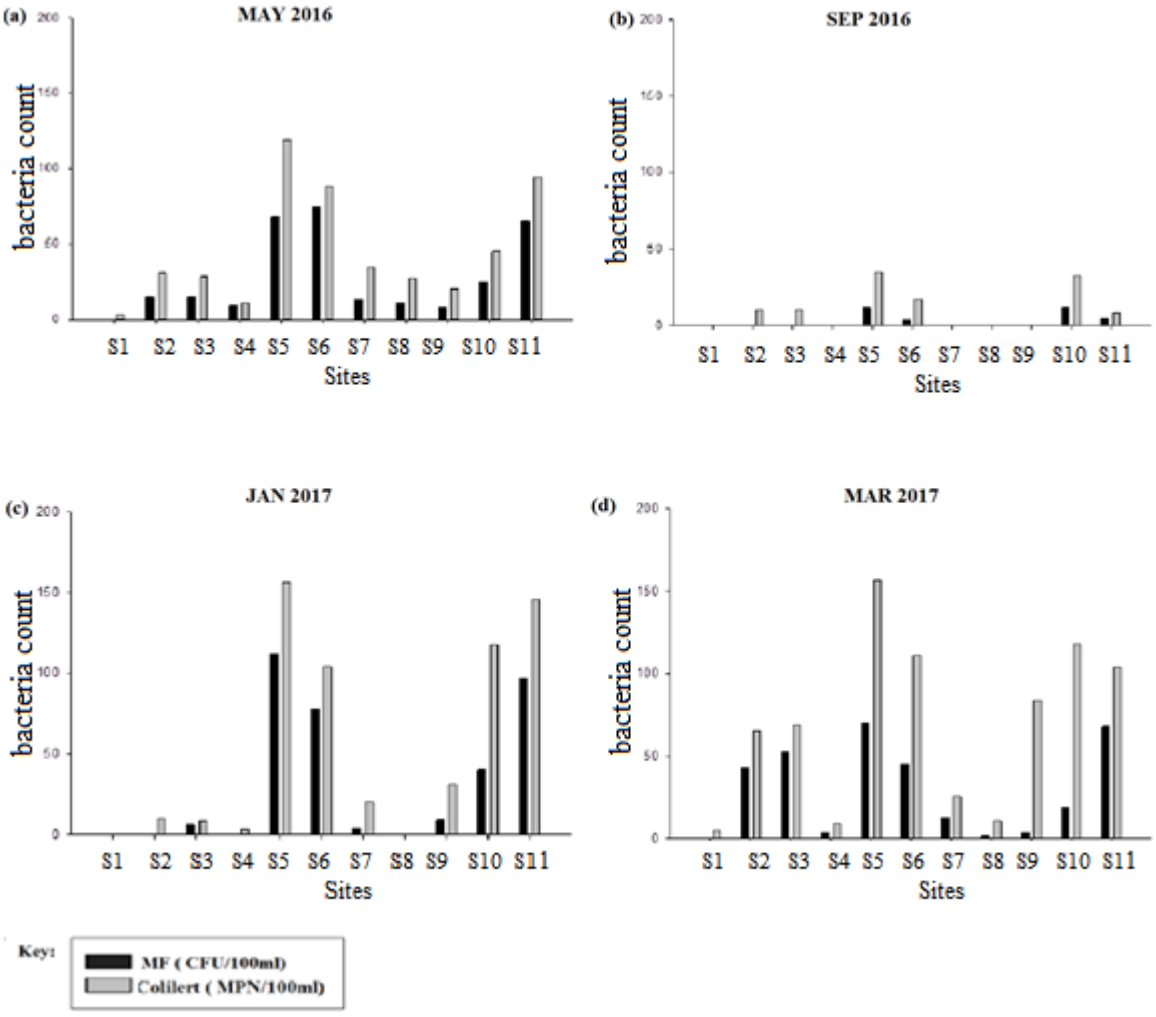

Figure 4.5: *E. coli* bacterial counts with separate bar for Colilert (MPN/100ml) and MF (CFU/100ml) detection methods.

4.3 Bacteria susceptibility to antibiotics

A total of 150 bacterial isolates were subjected to antibiotic susceptibility tests against 15 different antibiotics listed in Table 3.2. The antibiotic resistance profiles of the isolates were recorded as resistant, intermediate resistant and susceptible. Figure 4.6 shows that high antibiotic resistance was observed for ampicillin (98 %), amoxicillin (95 %), methicillin (94 %), chloramphenicol (93 %), trimethoprim (90 %), penicillin (87 %), tetracycline and rifampicin (88 %), streptomycin (65 %) and sulfamethoxazole (63 %) compared to kanamycin and neomycin with 45 % and 44 % respectively. Only 24 % of the isolates showed resistance to meropenem, 8 % to ciprofloxacin and 5 % to gentamicin. Generally, isolates were more resistant to older generation antibiotics than new antibiotics.

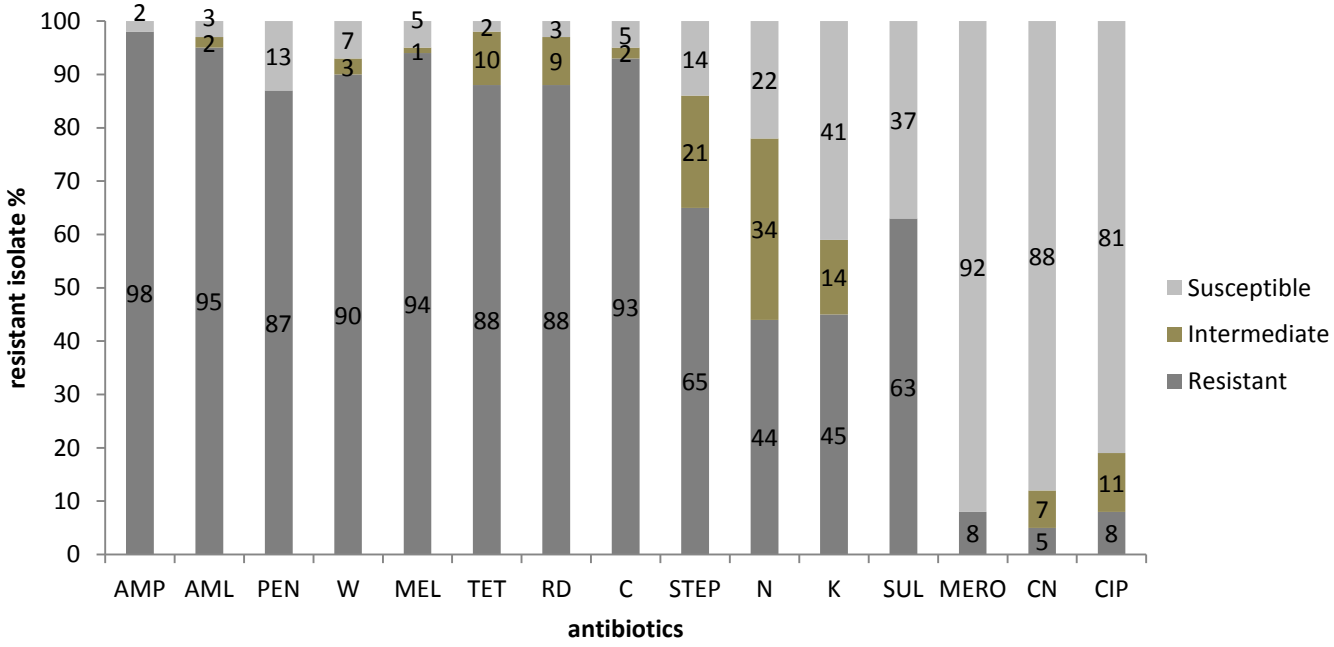

Figure 4.6: Antibiotic susceptibility of the isolates to different antibiotics.

Note: AMP ampicillin, AML amoxicillin, PEN penicillin, W trimethoprim, MEL mecillium, TET tetracycline, RD rifampicin, C chloramphenicol, STEP streptomycin,N neomycin, K kanamycin, SUL sulfamethoxazole, MERO meropenem, CN gentamicin, CIP ciprofloxacin.

4.3.1 Multiple drug resistance

Multiple drug resistance patterns indicate that 31 isolates showed resistance of over 65 % to 9 antibiotic being AMP-AML-MEL-C-W-RD-TET-PEN-STREP as shown in figure 4.7. Twenty-nine isolates were resistant to 11 antibiotics while 22 isolates were resistant to 12 antibiotics. Moreover, 15 isolates were resistant to 10 antibiotics, while 14 isolates were resistant to 8 antibiotics, followed by 9 isolates being resistant to 7 and 13 antibiotics. The lowest number was observed, with 8, 7, and 3 isolates being resistant to 6, 14 and 5 antibiotics respectively. Lastly one isolate was resistant to 3, 4 and 15 antibiotics (Figure 4.7).

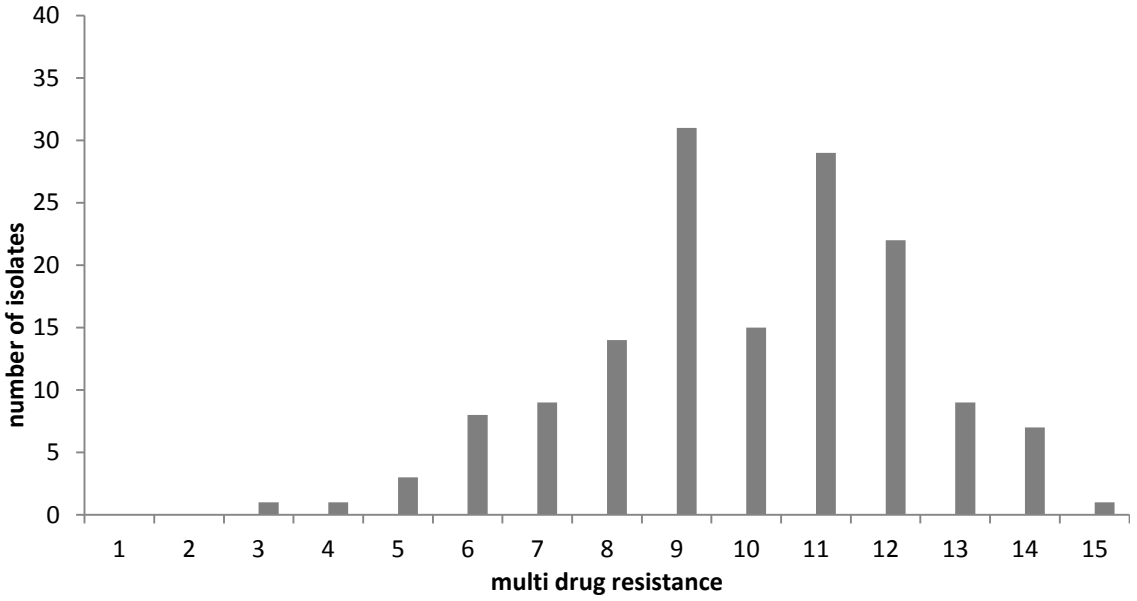

Figure 4.7: Multi-drug resistance of the isolated organisms.

4.4 Molecular identification on the basis of 16S rDNA sequence analysis

10 isolates were selected representing the different sampling sites for molecular identification based on their degree of resistance to the above mentioned antibiotics per sampling site. The isolates were selected looking at their antibiotic resistant pattern, those having the highest percentage of resistance at each site (Table 4.1). The blast results were selected using a limit of ≥90 % sequence similarity.

Table 4.1: Selected isolates bacterial sequence with their accession numbers identified using 16S rDNA sequence analysis.

Isolate-site	Identified species	Accession NO.	Identity %
107-S1	*Brevundimonas naejangsanensis*	KX223755.1	96
168-S2	*Klebsiella pneumonia*	KU711803.1	99
178-S3	*Enterobacter cloacae*	MF537624.1	95
124-S4	*Pseudomonas sp*	KY780232.1	91
121-S5	*Escherichia coli*	KF991482.1	94
180-S6	*Enterobacter hormaechei*	KX233851.1	100
045-S7	*Bacillis theringenesis*	KP137560.1	91
057-S9	*Serratia marcescens*	HQ834310.1	90
134-S10	*Bacillus cereus*	KF595063.1	91
198-S11	*Shigella sonnei*	KX082818.1	99

The isolates were denoted as isolate number (000) and sample site (S0).

4.4.1 Phylogenetic tree analysis

16S rDNA sequences of the 10 selected isolates were used to construct a phylogenetic tree. This was to show the relationship to the closest known relatives looking at their phylogenetic relationships. Most of the different isolates identified (6 isolates) belong to the same family of Proteobacteria and the class of Enterobacteriaceae (Figure 4.8). While *Brevundimonas naejangsanesis* belongs to Caulobacteraceae, *Bacillius* species belongs to a family of Bacillaceae and lastly *Pseudomonas* sp which belongs to a family of Pseudomonadaceae.

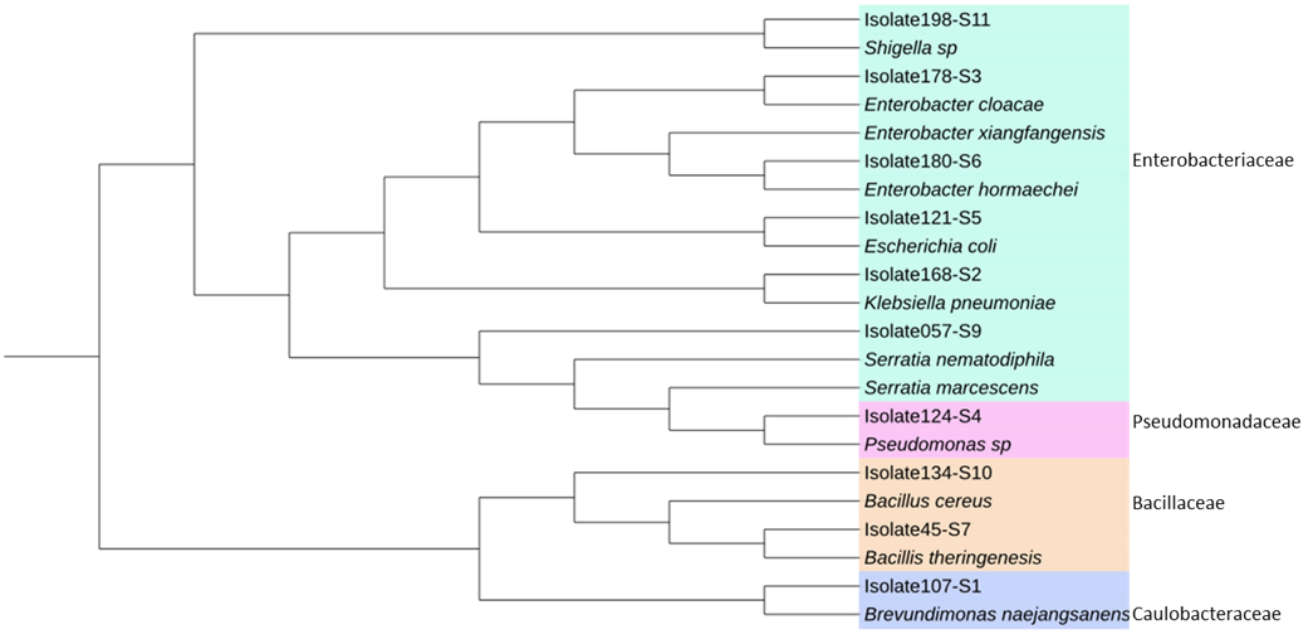

Figure 4.8: Phylogenetic relationship of 16S rDNA of the isolates. Phylogenetic trees were constructed from the neighbor-joining analysis model (Saitou and Nei, 1987), using MEGA version 7.0 software and iTOL (Letunic and Bork, 2016).

4.4.2 Detection of antibiotic resistance genes

The 10 identified isolates were further investigated for the presence of antibiotic resistance genes (Table 4.2; appendix 3). The *int*1 gene had the highest prevalence as it was present in all the isolates, followed by *strep*B gene which was detected in 5 of the isolates. *tet*B was present in only 3 isolates and *sul*1 in only 1 isolate (Table 4.2). All the 5 antibiotic resistance genes; *tet*A, *tet*B, *int*1, *strep*B and *sul*1 genes were detected in isolate 121-S5, which was identified as *E. coli*. 134-S10 (*B. cereus*) had 3 of the targeted genes (*tet*B, *int*1, *strep*B*)*. Two (*int*1 and *strep*B) out of the 5 targeted genes were detected in 168-S2 (*K. pneumonia*), S4 (*Pseudomonas* sp*)* and S9 (*S. marcescens*), while S1, S3, S7 and S11 had only 1 gene (*int*1*)* detected.

Table 4.2: Detection of antibiotic resistance genes of the identified isolates

Gene	Isolates									
	107-S1	**168-S2**	**178-S3**	**124-S4**	**121-S5**	**180-S6**	**045-S7**	**057-S9**	**134-S10**	**l98-S11**
Int1	+	+	+	+	+	+	+	+	+	+
tetA	-	-	-	-	+	-	-	-	-	-
tetB	-	-	-	-	+	-	-	-	+	-
strepB	-	+	-	+	+	-	-	+	+	-
Sul1	-	-	-	-	+	-	-	-	-	-

+; the presence of the targeted gene in isolate
- ; the absence of the targeted gene in the isolate

4.5 Metagenomics analysis

Metagenomics analysis was performed directly on four samples collected at S1, S2, S5 and S10 in March. Samples from March had recorded the highest bacterial count throughout the study and hence selected for metagenomics analysis. The results are presented at kingdom, order and species classification level.

4.5.1 Visualization at kingdom classification

Visualization at kingdom level showed that S1 bacterial proportion was at 99 %, S2 at 92 %, S5 at 74 % and S11 having 96 % (Figure 4.9). Besides bacteria, all the samples also contained DNA from kingdoms plantae, protozoa and archaea, and of interest is that as the water moved further away from the water treatment plant as other classes such as fungi and viruses which were not present at S1 were found (Figure 4.9). The results showed the presence of fungi and viruses in

S2, S5 and S11. Ten percent of the organisms in sample S5 were viruses, which were also observed in S11 at 3 %. Traces of fungal DNA were observed in all the samples (Figure 4.9).

Figure 4.9: Krona chart visualization at kingdom level.

4.5.2 Visualization at order classification

From Figure 4.9 bacterial DNA was the most predominant in all the samples and hence were analysed at order level. Taxonomical composition revealed that DNA for the order Pseudomonadales (0.2% S1, 23% S2, 20% S5 and 50% S11), Actinomycetes (21% S1, 0.9% S2, 13% S5 and 0.6% S11) and Sphingomonadales (0% S1, 40% S2, 13% S5 and 0.4% S11) were the most prevalent among the samples (Figure 4.10). The least bacterial diversity at the order level was Enterobacteriaceae having 0.1% S1, 0.05% S2, 0.05% S5 and 0.2% S11. Legionellales was present in S2 (0.1%), S5 (0.2%) in the water samples but not detected in the S1 and S11 sample (Figure 4.10). Within these dominant orders, the bacterial families with the highest

relative abundance across most samples were Streptomycetaceae, Comamonadaceae, Bacillaceae, Vibrionaceae, Bacillales, Enterobacteriaceae.

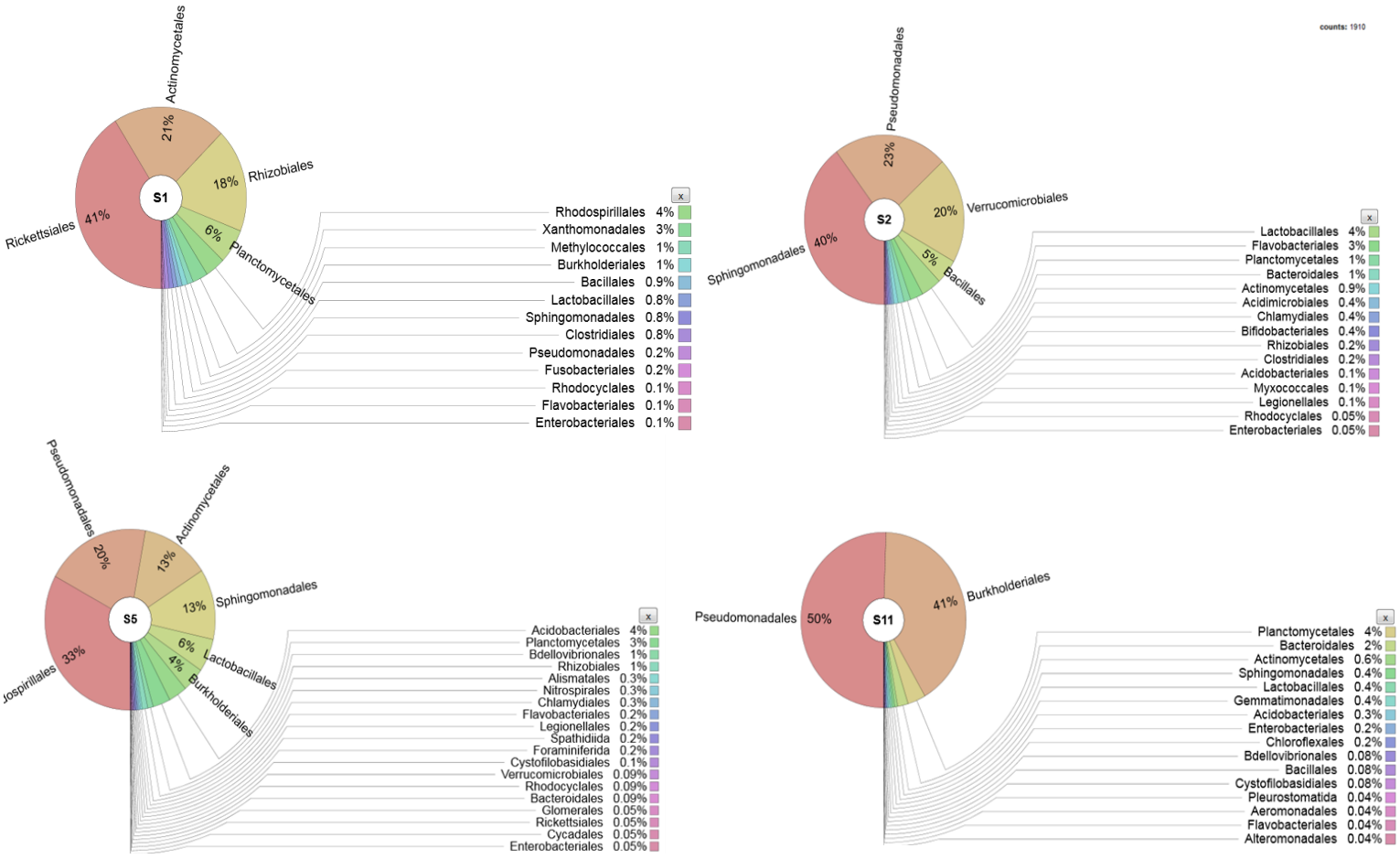

Figure 4.10: Visualisation of the samples at order level

4.5.2 Visualization at genus level

The relative abundance and bacterial diversity at the genus level was identified using metagenomonics. The most prevalent bacteria genus identified across the sampling sites eDNA (Figure 4.11 -4.14) were *Mycobacterium sp* (0.1% S1, 0.01% S2, 0.04% S5 and 0.03% S11) with *M. tuberculosis* (0.04%) being found at S5 (Figure 4.13). Other bacterial genera such as *Bacillus* (S1,S2,S5 and S11), *Clostridium sp* (S1,S5 and S11), *Enterobacter sp* (S2 and S5), *Staphylococcus sp* (S1 and S5), *Acinetobacter sp* (S1,S2,S5 and S11), and *cholera sp* (S1,S5 and S11) were detected (Figure 4.11- 4.14).

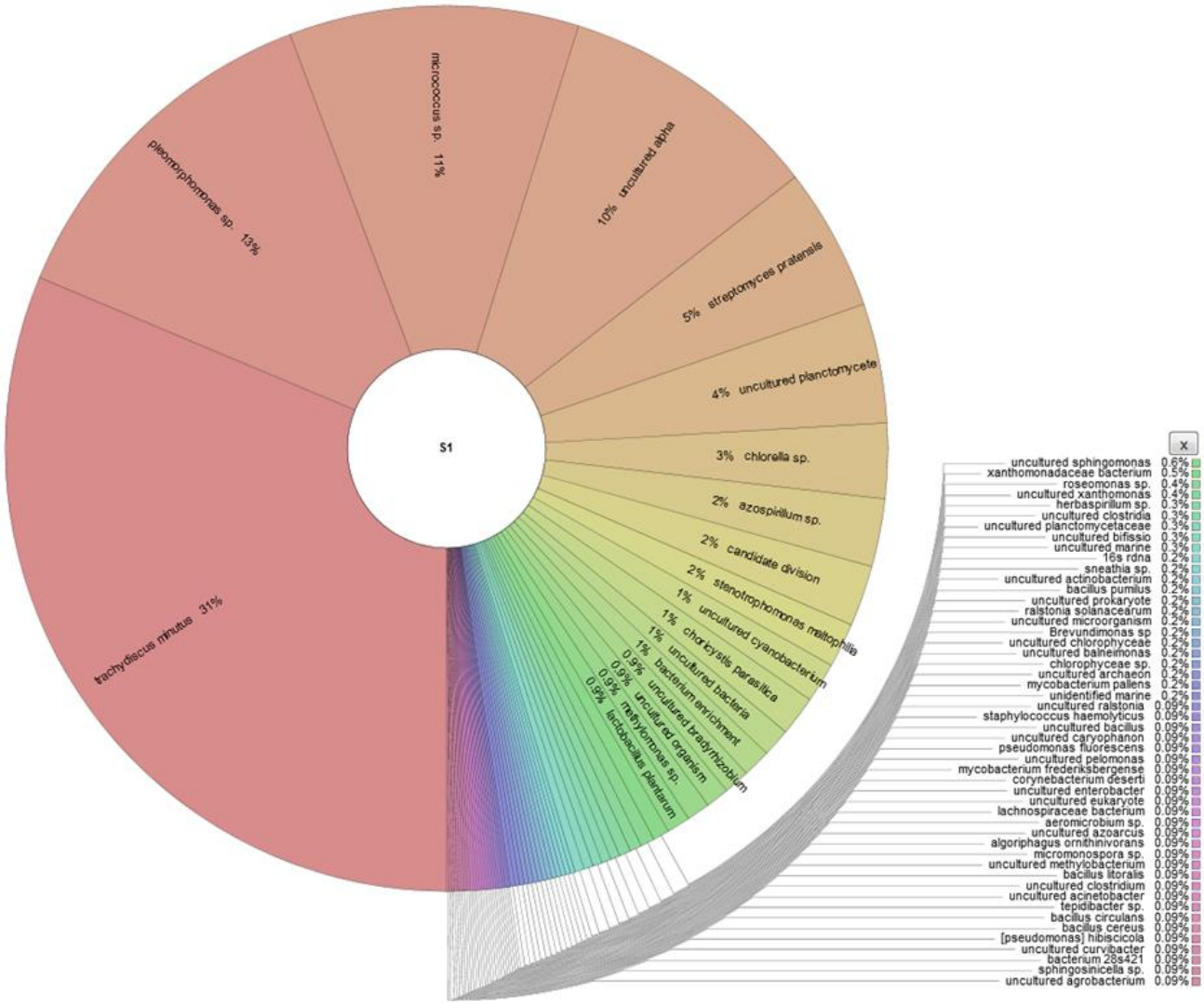

Figure 4.11: Visualisation of the sample obtain at S1 at genus level

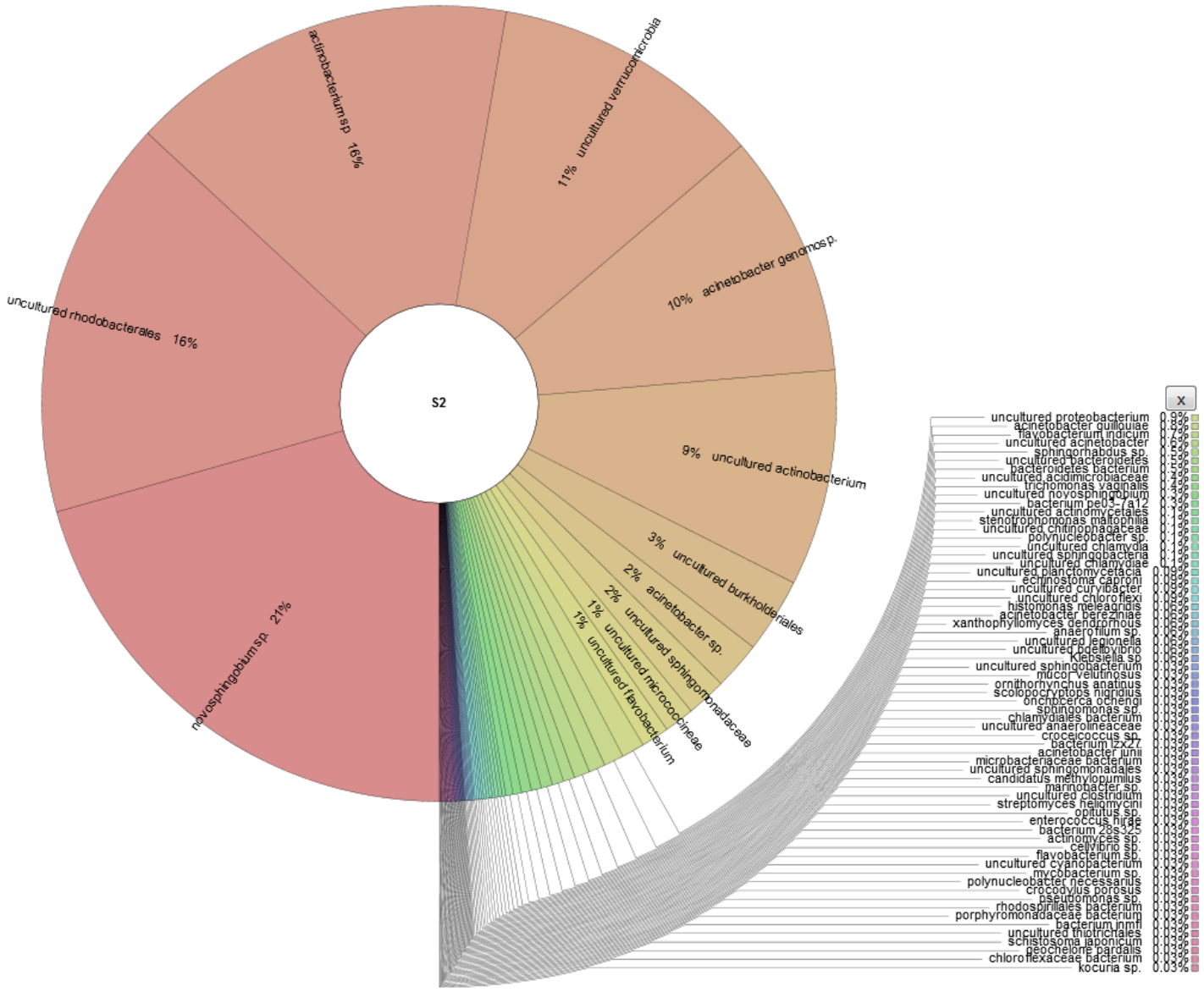

Figure 4.12: Visualisation of the sample obtain at S2 at genus level

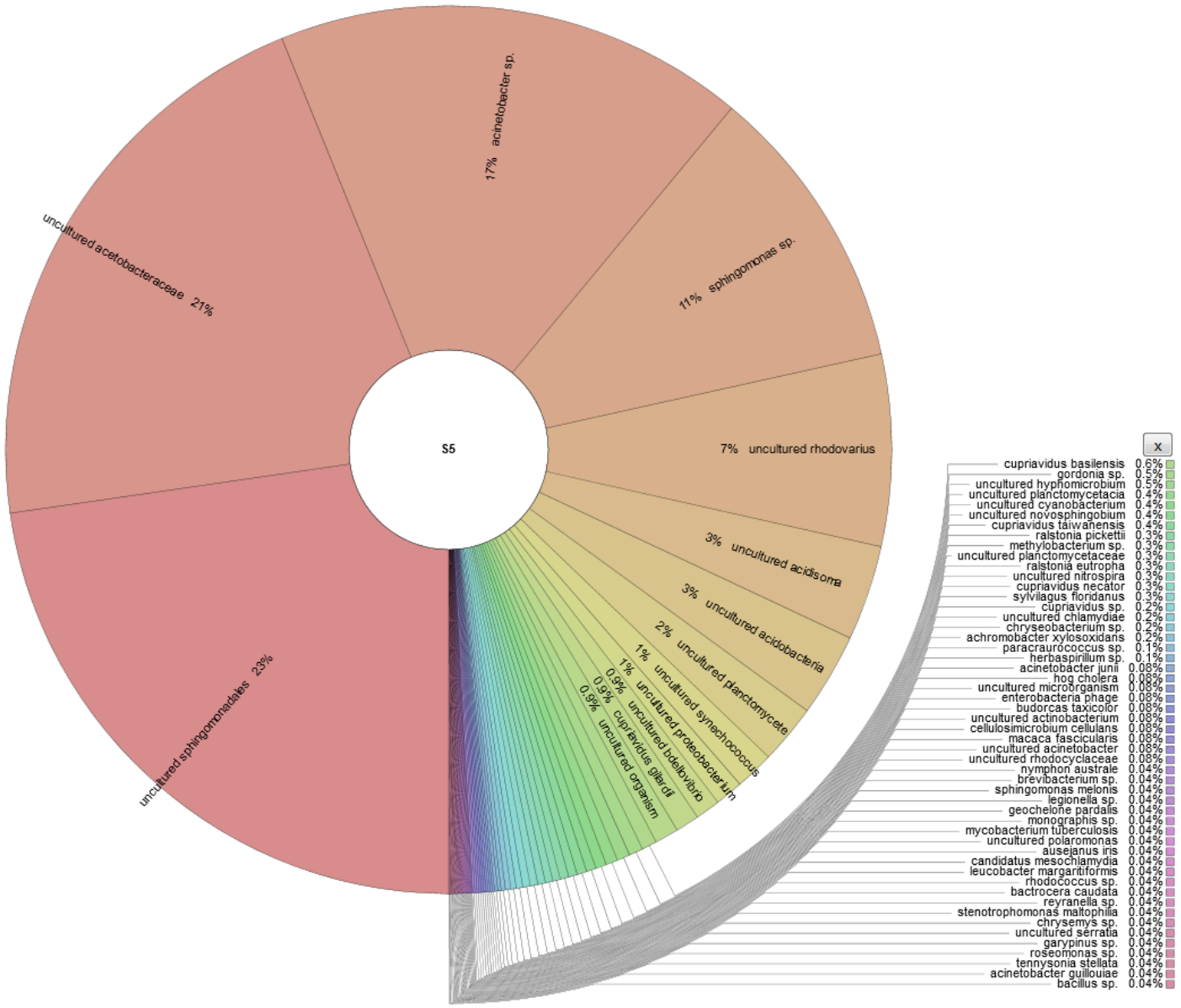

Figure 4.13: Visualisation of the sample obtain at S5 at genus level

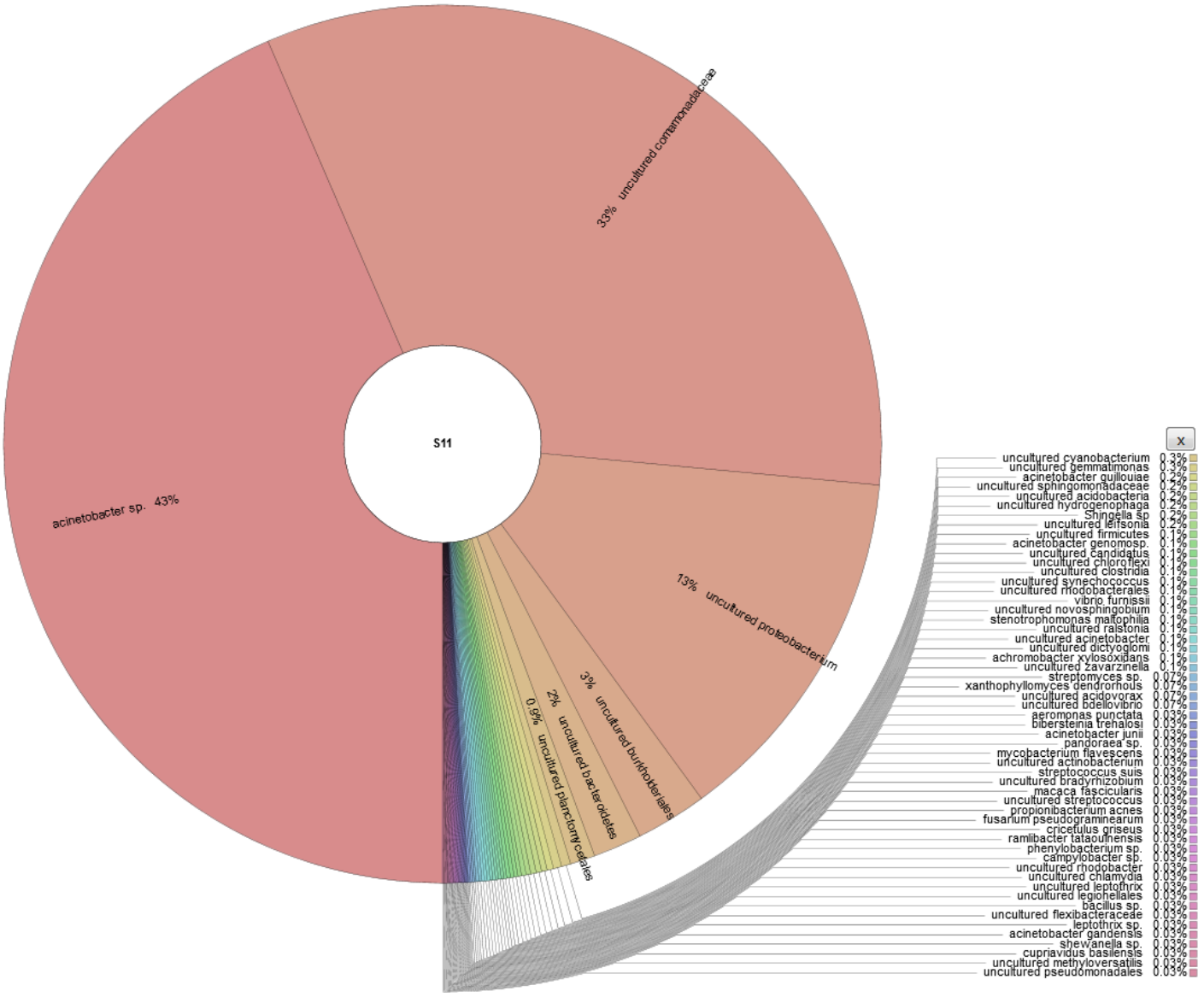

Figure 4.14: Visualisation of the sample obtain at S11 at genus level

4.6 Detection of antibiotic resistance genes in metagenomics samples

eDNA samples were tested for 5 ARGs (*sul*1, *int*1, *tet*A, *tet*B *and strep*B) using PCR (appendix 4). Four sampling sites we selected to determine the trend of microbial contamination from the source (S1) and the health care facilities with the highest daily number of patients and the highest number of antibiotic resistance bacteria isolates. The results showed that *sul*1, and *int1* were present in all the samples (Table 4.3). However, 3 of the targeted genes (*tet*A, *tet*B and *strep*B) were not detectable in S1 but were found to be present in S2, S10, and S5.

Table 4.3: Detection of antibiotic resistance genes from metagenomics (eDNA) samples

Gene	eDNA sample			
	S1	S5	S2	S10
*Int*1	+	+	+	+
*tet*A	-	+	+	+
*tet*B	-	+	+	+
*strep*B	+	+	+	+
*Sul*1	+	+	+	+

+; the presence of the targeted gene in sample

- ; the absence of the targeted gene in the sample

5.0 DISCUSSION

5.1 Physicochemical quality of water

The physicochemical properties of water are directly related to the safety of the drinking water and are contributed by climatological, geological and hydrological factors. They affect mostly the bacteriological component of water (Bhandari *et al*., 2008).

The physicochemical parameters tested were water turbidity and residual chlorine. Measurement of turbidity reflects the clarity in water and is mostly caused by substances present in the water including organic matter and microscopic organisms (Yao *et al*., 2014). As such turbidity is used to indicate water quality and filtration effectiveness of the treatment process (Pollock *et al*., 2014). The turbidity levels in most (64 %) of the sampling sites exceeded the maximum permissible concentration of 1 NTU set by BOS 32: 2009 standard (Figure 4.1). The S1 sample for March exceeded the allowable turbidity limit, measuring 1.32 NTU and even higher turbidity levels were observed at successive sites. This could indicate ineffective treatment in clarifying the water as well as treating microbial contamination. Turbidity level for S5 (6 NTU) and S10 (5.07 NTU) were higher than the WHO (2011) limit of 5 NTU, which is much higher than the BOBS limit. Since turbidity implies the presence of foreign materials in the water, this suggests that bacterial cells can attach themselves to the suspended particles in turbid water, which could then interfere with disinfection by protecting microbial contaminants from the disinfectant (Lantagne *et al*., 2008; Kotlarz *et al*., 2009). Therefore, highly turbid water at the treatment works leads to increased chlorine demand and hence an increase in the cost of water treatment (LeChevallier *et al*., 1981).

Another parameter tested was residual chlorine. Chlorine is a chemical that is used as a disinfectant in drinking water prior to its discharge into the distribution system. Chlorination ensures that the water quality is maintained from source to the point of consumption. Consequently, a detectable amount of residual chlorine should remain in the water in order to minimize pathogens and biofilm formation, thus preventing recontamination throughout distribution (Clark and Coyle, 1990). BOS 32: 2009 standard states that residual chlorine level in drinking water should be between 1-0.6 mg/L to meet the recommendation for human

consumption. In September, (54.5 %) and in May and January, (91.9 %) of the samples were not within the BOS 32: 2009 recommended limit. In March 100 % of the samples were below the limit of 0.6 mg/L (Figure 4.2). The absence or low level of residual chlorine in the distribution system may lead to the possibility of post treatment contamination (Ercumen *et al*., 2014). This suggests that additional chlorine is needed at the treatment plant (S1) to ensure that residual chlorine reaches the end of the pipe lines for maintaining the quality of drinking water prior to distribution. Generally, chlorine decreases in concentration with distance from source (S1), until it reaches levels as very low as 0.01 mg/L observed in S10 in March. Furthermore, the challenge with chlorine levels in drinking water is that residual chlorine should not exceed 1mg/L since high levels can affect human health. Additionally, recommended chlorine concentration should not be exceeded to control any by product formation associated with taste and odour. High chlorine doses potentially increase haloacetic acid leading to increased risk of getting cancer (Nieuwenhuijsen *et al*., 2000; Pressman *et al*., 2010; Doñaque *et al*., 2015; Xie, 2016).

5.2 Bacteriological indicators

The analysis of microbiological quality of water ensures that consumers are protected from pathogenic bacteria and this is done through the detection of indicator microorganisms. Heterotrophic bacteria, total coliform, *F. streptococci* and *E. coli* are indicator microorganisms that are generally recommended for assessment of the microbiological safety and the potential occurrence of pathogens in potable water (Yáñez *et al*., 2006; Okeke *et al*., 2011). BOS 32:2009 and Rouse (2016) guidelines recommend the complete absence of microbial indicators in any 100 mL of drinking water. WHO (2014) characterize detection of 1-10 CFU as low risk, 10-100 CFU intermediate risk, 100-1000 CFU as high risk and over 1000 as very high risk human health. The counts for the indicator microorganisms ranged between the low and high risk categories in this study. Generally, samples from the treatment plant were categorized as low risk for all the samples, whereas as the water moved further away from the treatment plant, counts classified as high risk category were observed. The observed trend could be explained as the result of post contamination as the water moved along the distribution network. Higher bacterial counts were observed in March (2394 CFU/100mL) with detection of indicator microorganisms from the treatment plant (S1) to successive sites and these could correlate with the low residual

chlorine levels that were observed during the same month. Chen and Stewart, (1996) observed that free chlorine is gradually consumed before it can act on bacterial components. Heterotrophic microorganisms are derived from diverse pollutant sources and are typically described as opportunistic pathogens (Hambsch and Werner, 1993; APHA, 1999). In March S1 recorded 4 CFU/100mL of heterotrophic bacteria. The presence of these bacteria at the treatment plant may suggest that treatment may have been ineffective in killing microorganisms compared to the other months where there was zero detection at the plant (Figure 4.3). In May, September and January, there was no detection of heterotrophic bacteria in S1, however in subsequent sampling sites along the distribution lines heterotrophic bacteria were detected in the samples. In May, Jan and March increased counts were recorded in S6, S5 and S11 recording 459, 426 and 377 CFU/100mL respectively, which could mean post contamination of the treated water. Ainsworth (2004) also reported that an increase in heterotrophic bacteria could be attributed to several factors such as an ineffective treatment, installations and repairs, presence biofilms or microbial growth in the distribution system. According to Prévost (1998) identification of heterotrophic bacteria is important in the assessment of drinking water quality in distribution networks and storage tanks as their presence can be associated with an increased risk of gastroenteritis. Studies have shown that a high density of heterotrophic bacteria found in treated drinking water is of great concerns due to the fact that a wide range of water associated opportunistic pathogens that are of human health concern are represented by this group of bacteria (Lye and Dufour, 1991; Chowdhury, 2012).

Total coliform counts were present in all the sampling sites except for treatment plant (S1) for all the sampling periods. The absence of total coliform could be a good indication of effective treatment. However, safe drinking water from the treatment plant does not guarantee safe water at the consumer point, since the water is subjected to possible contamination during transmission through pipes. This was observed in Figure 4.3 that total coliforms were detected along the transmission lines. The highest mean counts were recorded at S11 (277), S2 (186), S6 (360), and S5 (362) CFU/100mL in May, September, January and March sampling periods respectively. Total coliforms counts were very high in wet seasons March (362 CFU/100mL) and January (360 CFU/100mL) and compared to dry seasons May (277 CFU/100mL) and September (186 CFU/100mL). This could be explained by the fact that in wet season runoffs from rainfall

introduces contaminants into the water, the observation that was reported in Okavango delta in Botswana (Masamba and Mazvimavi, 2008). The presence of total coliforms can be seen as an indication of deteriorating water quality and ineffective water disinfection (APHA, 1999; Rompré *et al*., 2002; WHO, 2006). Moreover, presence of total coliforms in drinking water indicates that disease causing organisms could be in the water system (Emmanuel *et al*., 2009).

Furthermore, the results showed positive for presence of *F. streptococci* after treatment in most of the sampling sites, posing concern about the potential incidences of enteric pathogens that could cause illness to exposed individuals (Dufour, 1984; Ashbolt *et al*., 2001; Abdelzaher *et al*., 2010; Cabral *et al*., 2010). *F. streptococci* was not detected at the treatment plant (S1) in all the months sampled, however after the treatment process, down the distribution network high counts were detected especially in March and January. Of concern was that S5 in January recorded the highest number of *F. streptococci* (155 CFU/100mL), which is categorized as high risk (WHO, 2014) and could cause diseases if consumed, declaring the water unsafe to drink. The March samples showed that after treatment all the successive sites were positive for *F. streptococci* (Figure 4.3). These high numbers may be attributed to prolonged water stagnation in holding tanks, disinfectant residuals dissipating after some time in the distribution pipes (Berry *et al*., 2006).

Several studies have suggested *E. coli* to be the best indicator for the assessment of faecal contamination and indicator for possible presence of enteric pathogens (Clark *et al*., 1990; Edberg *et al*., 2000; Geissler *et al*., 2000; US EPA, 2001). The results show generally low counts for *E. coli* recorded compared to other indicator organisms targeted. In September only 36.4 % of the sampling sites were positive for *E. coli* as compared to total coliform and *F. streptococci* where 72.7 % of the sites were positive and heterotrophic bacteria where 90.2 % of the sites were positive. January, May and March had positive counts for *E. coli* except at the treatment plant. Studies show that even after effective treatment, high microbial counts can be detected in drinking water (Chidya *et al*., 2011; Schraft and Watterworth, 2005). S5 recorded the highest bacterial counts in all the sites, which could be attributed to the pipes in the area been slogged with biofilms as the clinic tank is one of the oldest holding tank for the area.

Drinking water contaminated with these bacteria can cause gastroenteritis characterized by diarrhoea, vomiting and nausea (Schraft and Watterworth, 2005; Tubatsi *et al*., 2015). The effects may possibly be life threatening for children under the age of 5, the elderly or people with immune deficiencies (EPA, 2004; Swatuk and Kgomotso 2007; Kujinga *et al*., 2014).

5.2.1 Comparison of membrane filtration and Colilert for detection of total coliform and E. coli

The choice of the correct method for enumeration of indicator microorganisms is very important when aiming to achieve the accurate conclusions. The most common method used for total coliform and *E. coli* detection in drinking water is membrane filtration technique. There are also some enzymatic assays used to enumerate total coliform and *E. coli* (Rompré *et al*., 2002; Chao, 2006) eventhough they are not commonly used for routine monitoring. MF and Colilert methods were compared in the detection of total coliform and *E. coli* indicator microorganisms.

The results (Figures 4.4 and 4.5) indicate that when all parameters of collection, preservation, and testing are constant, Colilert exhibited higher mean counts of bacterial coliforms detected than MF. The average counts for Colilert and MF are 532 and 499 for total coliforms and 464 and 251 for *E. coli*, respectively. The statistical analysis (two-way ANOVA) showed that there was no significant difference between the two methods for testing total coliforms. The results are further supported by Rompré *et al*., (2002) and Chao, (2006) who also did not find any statistical difference between the two methods. Even though the statistics by a two-way ANOVA shows no difference, Colilert has been reported to perform better than MF (Niemela *et al*., 2003; Eccles *et al*., 2004). The enzymatic method has been developed to curb the limitations of decrease in coliform recovery and the inability to recover stressed and injured coliforms by the MF approach and it offers sensitivity, rapidity and specificity to substrates (George *et al*., 2000; Murphy *et al*., 2017). The results for the Colilert method showed that almost all the samples representing the four sampling periods recorded high counts compared to MF. Total coliforms were detected at S1 during May, January and March while there were no coliform detections when using the membrane filtration for the same periods. This implies that Colilert assay is much more sensitive than MF. The Colilert enzymatic assay is based on the hydrolysis of chromogenic substrates by

β-galactosidase and β-glucuronidase activity, which are enzymes found in total coliforms and *E. coli* respectively (Brenner *et al*., 1993; Berger, 1994; George *et al*., 2000; Rompre *et al*., 2002; Bitton, 2005).

When the two methods were compared for enumeration of *E. coli*, the statistical analysis showed a statistical difference as $p < 0.05$ and higher counts were observed for the Colilert test (Figure 5.5). In addition, the Colilert test can recover stressed cells (McFeters *et al*., 1993; Niemela *et al*., 2003; Jiang *et al*., 2002; Eccles *et al*., 2004). Colilert sensitivity was also reported by Cowburn *et al*., (1994); Fricker *et al*., (1997); Niemela *et al*., (2003) were testing potable water, as more counts were detected over other traditional methods. For both total coliform and *E. coli*, the trend between the two methods is similar showing high counts at S5, S6, S9, and S10 for all the sampling periods, with September recording the lowest counts. September is recorded as the dry season in the area hence no runoffs from the riparian zones (Masamba and Mazvimavi, 2008). Notably S4, which immediately follows S1, also recorded the low counts for all sampling periods in both methods (Figures 4.4 and 4.5).

5.3 Antibiotic resistant bacteria

Antibiotic resistant is a trait acquired by previously antibiotic susceptible bacteria via horizontal gene transfer or by occurrence of mutations within chromosomal genes (Rysz and Alvarez 2006; Pruden *et al*., 2006; Zhang *et al*., 2009). Over 90 % of the 150 isolates investigated were resistant mostly to ampicillin followed by amoxicillin, chloramphenicol, mecillinam and trimethoprim (Figure 4.6). Currently, it has been recognized that many bacteria species have developed resistance to different classes of antibiotics resistance trend towards, macrolide, phenicols and β-lactam (Obi *et al*., 2004; Messi *et al*., 2005; Cox and Wright, 2013). The families of Enterobacteriaceae are especially resistant to ampicillin, chloramphenicol, amoxicillin, aminoglycosides and tetracycline (Leclercq *et al*., 2013).

Multiple antibiotic resistances were observed in all the isolates targeted, with some strains showing resistance to nine different antibiotics (Figure 4.7). The predominant antibiotic resistant pattern that was obtained for the isolates from different sites was AMP-AML-MEL-C-W-RD-

TET-PEN-STREP (Figure 4.7). From the results, it is evident that gentamycin, ciprofloxacin and meropenem were the most effective antibiotics as large proportion of the isolates were susceptible to all of them. Unexpectedly, some isolates were resistant to meropenem, an antibiotic that belongs to the carbapenem; new generation antimicrobial agents and are only used as a fallback for serious or complex hospital infections (Babic *et al*., 2006). Moreover, the results indicate that since most of the isolates targeted were isolated from sites after the treatment plant, the distribution systems may act as reservoir for antibiotic resistant organisms. Prevalence and antibiotic resistance patterns of various microbial species isolated from distribution system have been reported in other studies (Koksal *et al*., 2009; Ram *et al*., 2005). Forsberg *et al*., (2012) have found that there was an exchange of genetic information encoding antibiotic resistance genes between environmental and pathogenic bacteria. The antibiotic resistant phenomenon comes as a health concern predominantly to infants, immunocompromised and old individuals in the community.

5.4 Molecular identification of isolates

Ten bacterial isolates were selected and successfully identified through 16S rDNA gene sequencing. Among the identified species were some *Enterobacter* species, *Bacillius* species, *Pseudomonas* sp. *Klebsiella pneumonia*, *Brevundimonas naejangsanesis, Escherichia. coli*, *Serratia marcescens*, as well as *Shigella sonnei* (Table 4.1). Six of the 10 isolates belonged to the family of Enterobacteriaceae while *Brevundimonas naejangsanesis* belongs to Caulobacteraceae, *Bacillius* species to Bacillaceae and lastly *Pseudomonas* sp. to Pseudomonadaceae. The prevalence of this species could have been influenced mainly by the media used, which targeted certain bacterial species. The occurrence of this species in drinking water is of concern since they are a major cause of morbidity and mortality as most are waterborne opportunistic pathogen associated with causing diarrhoea and fatal dysentery (Kotloff *et al*., 2013). Sequence 198-S11 was closely related to *Shigella* species, a common cause of bacterial diarrhoea worldwide and can survive in the human body as the bacteria is less susceptible to acid than other bacteria (Figure 4.8). Symptoms of shigellosis include abdominal pain, watery diarrhoea, abdominal tenderness, fever, vomiting and dehydration and later progresses to dysentery (Thompson *et al*.,

2005; Niyoji, 2005; Emch *et al*., 2008). Shigellosis involves bacterial invasion of the colonic epithelium and inflammatory colitis which is associated with malabsorption, and results in characteristic sign of diarrhoea. It is noted that water supply networks are important route of transmission. Incidences of shigellosis are highest among children less than five years and increase on the immune compromised people (Niyogi, 2005; Von Seidlein *et al*., 2006). Studies have shown that despite gradual improvements in water supply, shigellosis continues to be endemic among young children and the elderly. *Shigella* species can also exist in environmental waters in a viable but non-culturable state such as vibrios (Hale, 1991; Faruque *et al*., 2002; Todar, 2009).

Other species that belong to Enterobacteriaceae family were *E. coli, K. pneumonia, S. marcescens* and two *Enterobacter* species. *E. coli* is mostly natural and essential part of the bacterial flora in the gut of humans and most strains are nonpathogenic and reside harmlessly in the colon. However, certain serotypes do play a role in diseases such as urinary tract infections and diarrhoea (Herzog *et al*., 2014). *K. pneumonia* is a pathogen that causes severe diseases such as pneumonia, urinary tract infections, and associated with hospital acquired infections (Podschun and Ullman, 1998). Isolate 168-S2, was related to *K. pneumoniae* (Figure 4.8), is the second most common Gram-negative bacteria after *E. coli* that causes disease outbreaks in adult population (Zhou *et al*., 2015). Old age, nosocomial infections, intensive care interventions are reported to present attractive host factor to *K. pneumoniae* contributing to the increased mortality rates of the disease (Meatherall *et al*., 2009; Hasan *et al*., 2010). Strains of *K. pneumoniae* are mostly opportunistic pathogens and are ubitiquous as some have been discovered from aquatic environments receiving wastewaters (Nordmann *et al*., 2009; Percival *et al*., 2013).

Enterobacter cloacae and *Enterobacter aerogenes* are the most frequently isolated species, causing infections in hospitalized and immunocompromised patients (Cabral, 2010; Chen *et al*., 2014). Environmental waters are a natural habitat for *Enterobacter* species and their presence is considered the result of faecal pollution. *Enterobacter* species and their increasingly encounter in drinking water are now found as pathogens causing nosocomial infections and gastroenteritis causing outbreaks which are often found to occur among intensive care unit patients (Jarvis *et*

al., 1984). Lastly in the Enterobacteriaceae family was *S. marcescens*, which is now recognized as an important opportunistic pathogen associated with healthcare infection and antimicrobial resistance (Coque *et al*., 1995; Mahlen, 2011). *S. marcescens* is the most common cause of pneumonia and with an increased incidence (Jones, 2010). The organism gains access to a suitably compromised host and at most risk those with immuno-compromising disorders and it's shown to be closely related to isolate 057-S9 (Figure 4.8). *S. marcescens* is implicated in a wide range of serious infections which includes pneumonia, meningitis and urinary tract infection (Naas *et al*., 1995; Jones, 2010; Kawecki *et al*., 2011; Merkier *et al*., 2013).

Another isolate 124-S4 was closely related to *Pseudomonas* sp. (Figure 4.8), an emerging waterborne pathogen (Bert *et al*., 1998). *Pseudomonas* sp., an opportunistic pathogen often associated with infections of the urinary tract, respiratory system and gastrointestinal infections particularly in patients with immunocompromised systems (Pirnay *et al*., 2005). *Pseudomonas* species have been implicated in a number of waterborne outbreaks which may have impacts on human health and if present in high levels in water may cause taste, odour and turbidity problems (Trautmann *et al*., 2005; Da Silva *et al*., 2008; Waszczuk *et al*., 2010; Fricks-Lima *et al*., 2011; Wang *et al*., 2012). The emergence of this waterborne pathogen is particularly worrisome to public health sector as environmental forms of the bacteria may serve as a store of genetic material, if transferred to other bacterial strains, may transfer new virulence capabilities. Moreover, the prevalence of multi-drug resistance is usually high in *Pseudomonas* species (Sharma *et al*., 2003).

Anaerobic *Bacillus* species was identified from the drinking water closely related to isolate 134-S10 and 045-S7 (Figure 4.8). The species were also identified from the drinking water by other studies (Penna *et al*., 2002; Pindi *et al*., 2013; Kansole *et al*., 2016). *Bacillus* species are mostly known to cause gastrointestinal infections in humans and in increased radiation resistance. The organism is ubiquitous and if ingested in small numbers can cause harm mostly to the immunocompromised (Jensen *et al*., 2003; Ozkocaman *et al*., 2006; Ribeiro *et al*., 2009). Lastly isolate 107-S1, closely related to *Brevundimonas naejangsanensis* (Figure 4.8), and the species is rarely associated with human infections and its clinical significance remains undetermined (Scotta *et al*., 2011).

5.4.1 Molecular determination of antibiotic resistance genes

In order to confirm antibiotic resistance in selected isolates, the presence of antibiotic resistance genes that could have been mediating resistance ability of the isolates were determined, and the results are presented in Table 5.2. In this study, *int*1, *strep*B, *tet*A, *tet*B *and sul*1 were targeted. These genes are known to occur in the aquatic environment (Pruden *et al*., 2006; Araújo *et al*., 2017). Class 1 integron (*int*1) was present in all the samples followed by *strep*B where it was detected in only 5 isolates and their frequencies than other ARGs in the bacteria. Integrons contain collections of genes that impart the recombination function by having the ability to enhance stability in bacteria DNAs particularly for drug resistance (Mazel, 2006; Alekshun and Levy, 2007). The mobility of ARGs usually depends on integrons. *Tet*A, was only positive in isolate 121-S5 and this could be due to resistance of *tet*A which is widely disseminated in *E. coli* organism (Thea, *et al*., 2006). *Tet*B was only detected in 2 isolates (121-S5 and 134-S10). It was observed only in *E. coli* (isolate 121-S5). *Sul*1 was also observed in *E. coli* by Kozak *et al*., (2009).

5.5 Metagenomics

The methods that are used in water quality monitoring mostly involve the use of culture-based approaches utilizing selective media. These approaches have a huge limitation since only indicator microorganisms are targeted, hence the evaluation and complementation of these methods with metagenomics. Different other microorganisms, which are not related to total coliforms or *E. coli* were, detected (Figures 4.9- 4.14). Archaeal and protozoan reads were detected in all the sampling sites with most of the reads related to the bacteria domain. In addition, fungal and viral reads were not detected at S1 but detected in all other sites that succeeded S1 (Figure 4.9). While identification of isolates from MF and Colilert was mostly limited to a few orders especially the Enterobacteriaceae, metagenomics analysis shows that other orders such as Pseudomonadales, Bacillales and Lactobacillales (Figures 4.10) were also present in the water samples.

Metagenomic results of the 4 water samples performed thus show that the method complements the more traditional methods and shows the intrinsic complexity of the water. The taxonomic analysis revealed an increase in the microbial community structure as several dominant orders were detected after the treatment plant with Pseudomonadales having the largest increase (Figure 4.10). This trend was also observed by Miao *et al*., (2015).

The emerging pathogenic bacteria of concern that were found in the study included *Legionella* sp., *Cholera* sp., *Mycobacterium tuberculosis*, *Staphylococcus* sp., and *Pseudomonas* sp. (figure 4.11-4.14). In S1 *Stenotrophomonas maltophilia*; a pathogenic bacterium that causes human infections that are difficult to treat (Looney *et al*., 2009; Brooke, 2012) was present. Moreover, in S5 (figure 4.13) one of the species found was the *M. tuberculosis* an obligate pathogenic bacterium that causes tuberculosis while at S11 (figure 4.14) *Chryseobacterium indologenes* was recorded in the water sample. *Chryseobacterium indologenes* causes infections such as bacteremia, pneumonia and meningitis (Lin *et al*., 2010; Sudharani *et al*., 2011) and is found to be resistant to most antimicrobial agents commonly used to treat Gram negative bacteria (Hsueh *et al*., 2005; Bishara *et al*., 2006). *Pseudomonas* sp. is one of the organisms known to cause meningitis in children under the age of 5 and its presence at S2 (paediatric ward) is of great concern. The results further show detection of Legionellales, regarded as the most opportunistic bacterial pathogens linked to presence of biofilms in the distribution systems (Wingender and Flemming, 2011). S1 (figure 4.11) was free from *Legionella* species and *Mycobacteria* species but there were detected in subsequent sampling points. This could be an indication of biofilms formation in the pipes, where normally the water will reach when the disinfectant has dissipated.

5.5.1 eDNA characterization of antibiotic resistance genes

Comprehensive characterization of ARGs in nature using culture dependent methods remains a challenge since most of environmental bacteria have entered the non-culturable state. The use of metagenome DNA extraction to get comprehensive species composition becomes advantageous as more AGRs could be determined to characterize general trends in their proliferation (Schmieder and Edwards, 2012). *int*1, *strep*B and *sul*1 had the highest occurrence of 100 % followed by *tet*A and *tet*B at 75 % (Table 4.3). These show more gene detection in the site when

using the whole genome as compared comparison to culture-based using PCR isolation may be due to them failing to grow in laboratory culture (table 5.2) (Mao *et al*., 2015). Metagenomics analysis showed that the relative abundance of ARGs in S1 sample, with a gradual increased from 20 % that was detected while using the isolated DNA (107-S1) to 60 % on the whole genome. S2, S5, S1 whole sample genome showed detection of all the genes (100 %) while the respective isolates DNA had detection of 40 %, 100 % and 20 % of the ARGs respectively (Table 4.3). These shows that the use of whole genome with variant bacterial species gives off better results.

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study demonstrated that the water relatively met the requirements for drinking immediately after treatment. However, as the water moved along the distribution system there was recontamination, which could be attributed to the many changes happening in the distribution network. Recontamination was confirmed by the presence of various phylogenetic bacterial communities. When the culture-dependent method was used, the results showed selectivity towards the major bacterial phyla; Proteobacteria (Enterobacteriaece, Caulobacteraceae, Pseudomonadaceae) and Firmicutes (Bacillaceae). The traditional culture techniques were certainly limited as they only revealed relatively fewer microbial communities than the metagenomics results. In metagenomics analysis, bacterial communities were found to be from the major bacterial phyla, Fusobacteria, Firmicutes, Cyanobacteria, Bacteroidetes, Proteobacteria and Actinobacteria. The less abundant phyla included Chlamydiae, Acidobacteria and Gemmatimonadetes, as well as some unidentified microbial sequences. The presence of sequences from other kingdoms like fungi, archaea and 'viruses' at subsequent points could suggest that water carried potential waterborne disease-causing microorganisms that were omitted by the culture-dependent methods. The presence of unmapped microbial sequences emphasizes the extent of the water quality challenges and the need for paradigm shift from traditional to modern techniques. Moreover, using 16S rDNA sequencing proved to be a useful confirmatory tool for the definite identification of the isolated bacteria at genus level and 10 selected isolates were successfully identified.

It has been demonstrated through this study that most bacteria have developed mechanisms to reduce effectiveness of the antibiotics used against them, with some targeted bacteria showing multiple antibiotic resistance of up to 9 antibiotics. In addition, most bacteria have been found to harbour antibiotic resistances genes that were targeted. Only 5 resistance genes were targeted; however there could have been many more resistance genes present in the samples. This

necessitates more studies to investigate all the possible resistant genes in the drinking water systems.

6.2 Recommendation

1. Adequate treatment by chlorine to prevent microbial post contamination is needed together with intermittent chlorine dosing at some points in the distribution network. In addition, holding tanks should get daily chlorine boost to reduce microbial concentration. Also, water supply authorities should find an alternative disinfectant that does not dissipate rapidly and will stay in the system until the last point.

2. Regular cleaning and disinfection of the storage tanks to guarantee cleanliness and suitability of water for drinking as well as replacement of old pipes of the network.

3. The bacterium *Clostridium perfringens,* is considered a good indicator of faecal contamination and should be used as recommended by BOS 32; 2009. *C. perfringens* is a better indicator than coliforms because viruses and protozoa can survive under similar condition with regularly testing.

4. By comparing the effectiveness of different methods and using molecular method should be employed especially in big population water supplies to guard against non-culturable pathogens.

5. The quality of water at source point can further be influenced by hygiene practices during transportation, storage, and handling leading to microbiological contamination, hence uptake of water treatment techniques at household level could improve drinking water quality and significantly reduce diarrhoea outbreaks.

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APPENDIX

APPENDIX 1

BOBS 32:2009-Physical requirements

Determinands	Risk category	Units	Upper limit and ranges	
			Class I (Acceptable)	Class II (Max. Allowable)
Turbidity	aesthetic / operational	NTU	1	5
Chlorine residual	aesthetic	mg/L	0.6 – 1.0	1.0

BOBS 32:2009- Microbiological safety determinants' requirements

Determinants	Units	Acceptable limits [a)]
Total coliform	Count/ 100mL	Not detected
E.coli [b)]	Count/ 100mL	Not detected
Thermotolerant (faecal) coliform bacteria [c)]	Count/ 100mL	Not detected
Faecal streptococci	Count/100 mL	Not detected
Clostridium perfringens [d)]	Count/100 mL	Not detected
Cryptosporidium [e)]	Count/volume sampled [f)]	Not detected
Giardia [e)]	Count/volume sampled [f)]	Not detected

[a)] The allowable annual compliance contribution shall be atleast 95% to the limits in column 3.

[b)] Definitive, preferred indicator of faecal pollution

[c)] Indicator unacceptable microbial water quality could be tested instead of e.coli but is not the preferred indicator of faecal pollution.

[d)] Analysis of Clostridium perfringens should be done biannually. However, during raining season or outbreaks of waterborne diseases, analyses should be carried more often.

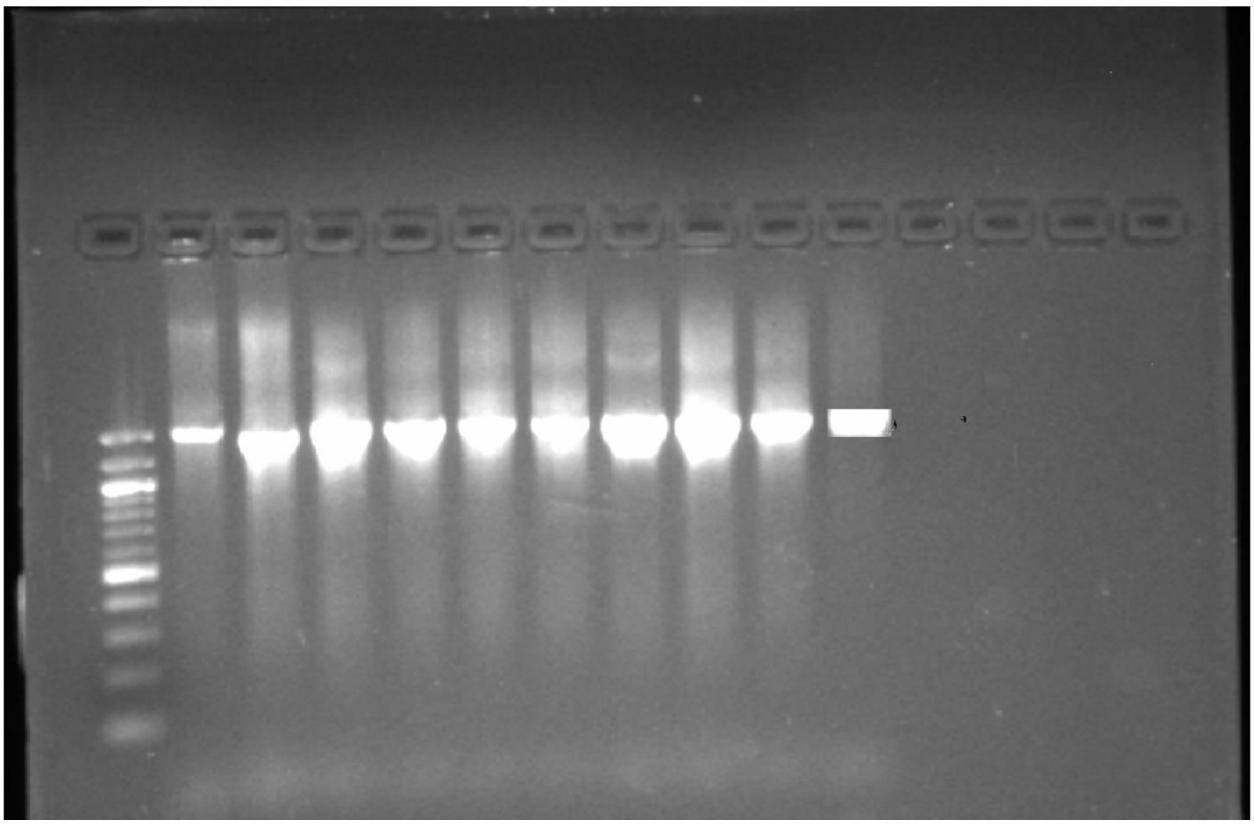
[e)] If *Clostridium perfringens* is detected, then *Cryptosporidium* and *Giardia* should be analysed for.

[f)] standard volume usually used is 10l to 1000l

The presence of any pathogen renders water unacceptable for drinking.

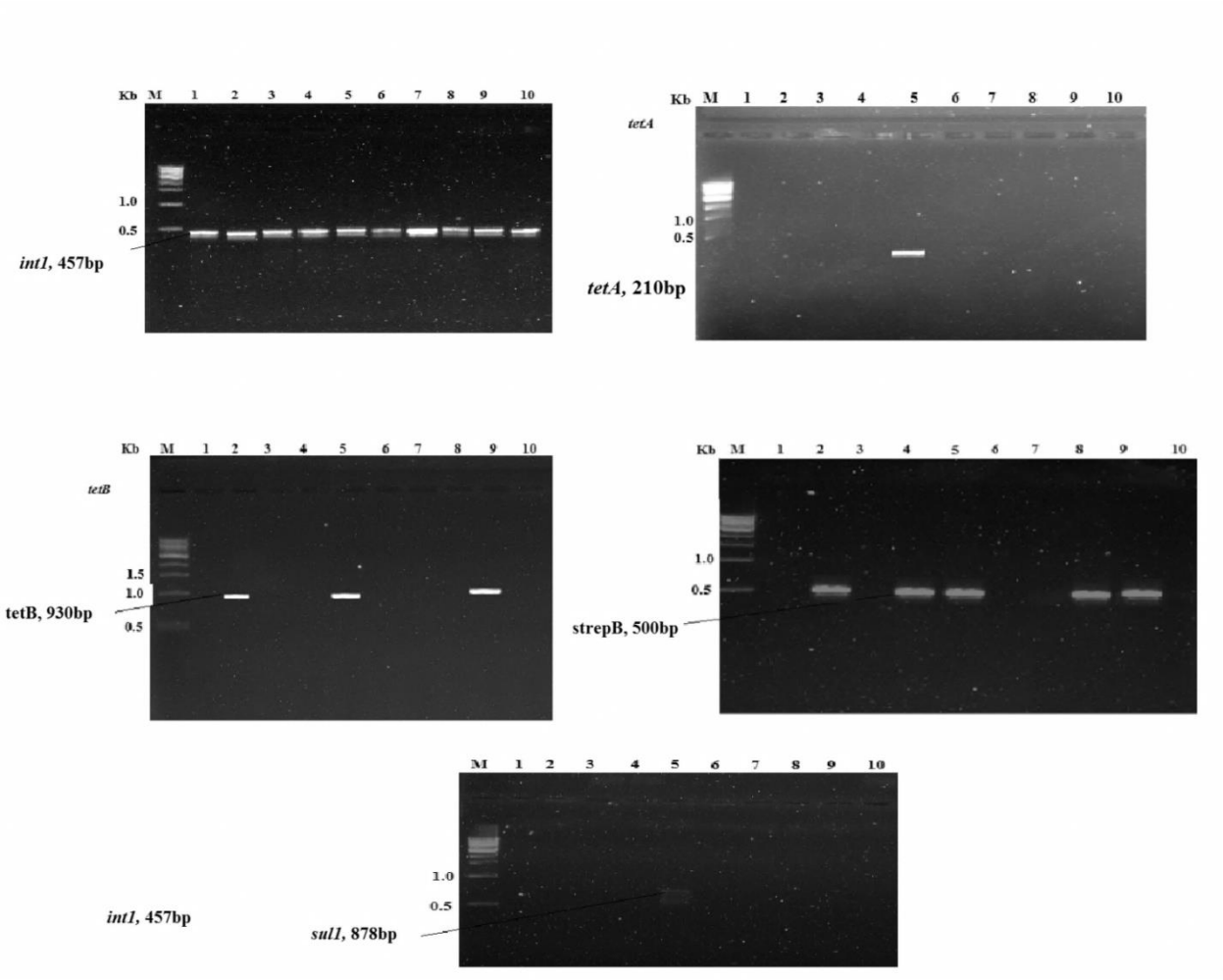
APPENDIX 2

16S rDNA gel electrophoresis image



APPENDIX 3

Resistant genes gel electrophoresis visual imaging for the isolated bacteria DNA



APPENDIX 4

Resistant genes gel electrophoresis visual imaging for the isolated bacteria DNA