Analysis of physico-chemical characteristics of drinking water, biofilm formation and occurrence of antibiotic resistant bacteria

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Thesis submitted for the degree Philosophiae Doctor in Microbiology at the Potchefstroom Campus of the North-West University

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ABSTRACT

The main aim of the study was to analyse the impact of physico-chemical parameters on drinking water quality, biofilm formation and antibiotic resistant bacteria in the drinking water distribution system in Mafikeng, North West Province, South Africa. Another objective was to isolate and characterise *Pseudomonas* and *Aeromonas* species from drinking water distribution system and detect the virulence gene determinants in the isolates by PCR analysis. The physico-chemical data obtained were subjected to statistical analysis using Excel 2007 (Microsoft) and SPSS (version 14.0) programmes. Pearson's correlation product of the moment was used to determine the correlation between EC, TDS, pH and temperature. The two tailed test of significance (p<0.05) was used in order to determine the significance of the result. Antibiotic susceptibility tests were performed using Kirby-Bauer disk diffusion method. Cluster analysis based on the antibiotic inhibition zone diameter data of different organisms isolated from different sites was determined and was expressed as dendograms using Wards algorithm and Euclidean distance of Statistica version 7. Specific PCR was used to determine the identities of presumptive *Pseudomonas* and *Aeromonas* species through amplification of the *gyrB*, *toxA* and the *ecfX* gene fragments. Virulence gene determinants for the confirmed *Pseudomonas* and *Aeromonas* species were detected by amplifying the *exoA*, *exoS* and *exoT* genes and the *aerA* and *hylH* gene fragments, respectively. A Gene Genius Bio imaging system (Syngene, Synoptics; UK) was used to capture the image using GeneSnap (version 3.07.01) software (Syngene, Synoptics; UK) to determine the relative size of amplicons.

Physico-chemical parameters were monitored from three drinking water sources three times a week and bacteriological quality was monitored weekly for four months from raw and treated drinking water. Water samples were analysed for pH, temperature, total dissolved solids (TDS) and electric conductivity (EC). Bacterial consortia from drinking water samples were isolated using selective media and enumerated. The results revealed a good chemical quality of water. However, the microbial quality of the water is not acceptable for human consumption due to the presence of *Pseudomonas*, *Aeromonas*, faecal coliforms (FC), total coliforms (TC) and Heterotrophic bacteria. The results showed that the drinking water is slightly
alkaline with pH value ranging between 7.7 to 8.32. What is of concern was the microbial quality of the water. *Pseudomonas* sp., faecal coliforms (FC), total coliforms (TC) and heterotrophic bacteria were present in some of the treated water samples. The most significant finding of this study is that all drinking water samples were positive for *Pseudomonas* sp. (>100/100ml), but also that when one considers the TDS it demonstrates that water from the Modimola Dam has an impact on the quality of the mixed water.

The prevalence and antibiotic resistance profiles of planktonic and biofilm bacteria isolated from drinking water were determined. The susceptibility of these isolates was tested against 11 antibiotics of clinical interest and the multiple antibiotic resistance (MAR) patterns were compiled. The most prevalent antibiotic resistance phenotype observed was KF-AP-C-E-OT-K-TM-A. All isolates from all samples were susceptible to ciprofloxacin. However, all faecal coliforms and *Pseudomonas* spp. were susceptible to neomycin and streptomycin. On the contrary all organisms tested were resistant to erythromycin (100%) trimethoprim and amoxycillin. Cluster analysis based on inhibition zone diameter data could not differentiate the various isolated into sample types. The highest prevalence of antibiotic resistant isolates was observed in Modimola Dam and Molopo eye.

Biofilms were investigated in both raw water and treated drinking water sources for the presence of faecal coliforms, total coliforms, *Pseudomonas* spp., *Aeromonas* spp. and heterotrophic bacteria based on conventional microbiology and molecular methods. Drinking water biofilms were grown twice and the biofilm developing device containing copper and galvanized steel coupons were utilized.

The Mini Tap filter, a home water treatment device which can be used at a single faucet, under constant flow was used during the second collection of treated water samples from cold water taps. Scanning electron micrograph revealed the existence of biofilms in all the sites investigated and the highest density was obtained on galvanized steel coupons.

Isolates were tested against the antibiotics ampicillin (10µg), cephalothin (5µg), streptomycin (10µg), erythromycin (15µg), chloramphenicol (30µg), neomycin (30
µg), amoxycillin (10 µg), ciprofloxacin (5 µg), trimethoprim (25 µg), kanamycin (30 µg), and oxytetracycline (30 µg). The multiple antibiotic resistance profiles and the presence of virulence related genes were determined. Various types of drug resistance and presence of virulence genes were observed. The most prevalent resistance phenotype observed was KF-AP-C-E-OT-TM-A.

In conclusion, the results indicated the occurrence of faecal indicator bacteria in the drinking water destined for human consumption. Faecal indicator bacteria are the major contributors of poor drinking water quality and may harbour opportunistic pathogens. This highlighted survival of organisms to treatment procedures and the possible regrowth as biofilms in plumbing materials. The detection of large proportion of MAR Aeromonas and Pseudomonas species which possessed virulent genes was a cause of concern as these could pose health risks to humans. The data obtained herein may be useful in assessing the health risks associated with the consumption of contaminated water.

Key words: Aeromonas, Antibiotic resistance, Biofilm, Drinking water distribution system, Physico-chemical parameters, Pseudomonas, Surface water, Total coliforms.
ACKNOWLEDGEMENTS

This research work would not have been possible or even imaginable without the help and cooperation of many people and organisations.

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I am greatly indebted to my husband George Mulamattathil and my family for their constant encouragement and support.
DECLARATION

I declare that, the dissertation for the degree of Doctor of Philosophy in Microbiology at the North-West University – Potchefstroom Campus hereby submitted by me for a degree at this university, that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

30/04/2014

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Mulamattathil Suma George  
Date
THESIS STATEMENT

A study was conducted to analyse the physico-chemical characteristics of drinking water in Mafikeng, South Africa, ability of microorganisms to form biofilm and occurrence of antibiotic resistant bacteria. Based on the research conducted a thesis was compiled. This thesis consists of five chapters.

Chapter 1 is the Introduction, problem statement, aims and objectives.

Chapters 2, 3 and 4 are three different papers submitted to various journals for publication.

Chapter 2 will give an account of the physico-chemical and bacteriological quality of drinking water

Chapter 3 describes the antibiotic resistance profiles of environmental bacteria from surface and drinking water

Chapter 4 demonstrates the ability of organisms in surface and drinking water distribution systems to form biofilms.

Chapter 5 constitute general discussion, conclusion and recommendations.

While writing the different topics there has been an overlap of some of the aspects in the different chapters which could not be avoided.
DEDICATION

This work is dedicated to my late father Mr. Idiculla Mathew Paranickal.
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LIST OF ABBREVIATIONS

The following abbreviations have been used throughout this thesis

ADA : Ampicillin dextrin agar
ADP : Adenosine diphosphate
APHA : American Public Health Association
API : Analytical profile index
ASBA : Ampicillin sheep blood agar
AWWA : American Water Works Association
BIBG : Bile salts irgasan brilliant green agar
BOM : Biodegradable organic matter
bp : Base pair
DFS : Dextrinfuchsinsulphite agar
DNA : Deoxyribo nucleic acid
DWAF : Department of Water Affairs and Forestry
EC : Electric conductivity
EPA : Environmental Protection Agency
FC : Faecal coliform
FEMS : Federation of European Microbiological Societies
HIV : Human immunodeficiency virus
HPC : Heterotrophic plate count bacteria
ICU : Intensive care unit
IFOWAHB : International Forum on Water Hygiene in Buildings
IWA : International Water Association
IWRM : Integrated Water Resources Management
MAR : Multiple antibiotic resistance
MIX: Ampicillin bile salts inositol xylose agar
MPN: Most probable number
MUG: 4-methylumbelliferyl- β-D-glucuronide
NCCLS: National Committee for Clinical Laboratory Standards
NNIS: National Nosocomial Infections Surveillance System
NWP: North West Province
ONPG: o-nitrophenyl-β-D-galactopyranoside
PVC: Polyvinylchloride.
RNA: Ribonucleic acid
SADC: Southern African Development Community
SANS: South African National Standards
SEM: Scanning electron micrograph
SER: State of the Environment report
SGAP-10C: Starch glutamate ampicillin penicillin C-glucose agar
SSA: Starch ampicillin agar
T2SS: Type II secretion system
TC: Total coliform
TDS: Total dissolved salts
TSI: Triple sugar iron
TSS: Toxic shock syndrome
UV: Ultraviolet light
VBNC: Viable but non-culturable
w/v: Weight per volume
WEF: The World Economic Forum
WHO: World Health Organisation
WWTP: Waste water treatment plant
ZID: Zone inhibition diameter
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CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 General introduction
Water is consumed in large quantities, for domestic purposes, including personal hygiene and is also used for recreational purposes. Hence the health risks associated with consumption of contaminated water are of great interest (Eckner, 1998) particularly in a water stressed country such as South Africa. Access to safe drinking water is a basic concern for human health and health protection (Völker et al., 2010). Providing populations with safe drinking water is recognized as a basic human right and this right is enshrined in the Bill of Rights of South Africa (Constitution of the Republic of South Africa Act, No.108 of 1996 (ss27) Date of commencement: 4 February 1996). Pollution of water resources by microorganisms of faecal origin is a current world-wide public health concern (Okeke et al., 2011) and this places the human population at high risk of contracting water related diseases such as typhoid, cholera, bacterial and amoebic dysentery, infectious hepatitis and gastroenteritis (Obi et al., 2002). Drinking water should be suitable for human consumption and for all usual domestic purposes including personal hygiene (WHO, 2002).

1.2 Water availability in South Africa and in particular the North West Province (NWP)
Water is a scarce and unevenly distributed national resource (National Water Act, Act No 36 of 1998). More than one-third of world’s population lives in water stressed regions and this number is expected to rise (DWAF, 2012, National Water Resource Strategy 2). South Africa is a semi-arid country with low levels of rainfall and has limited water resource (Germs et al., 2004) and it is the 30th driest country in the world with less water available per person than countries widely considered to be much drier (DWAF, 2012, National Water Resource Strategy 2). The quantity of water available for direct human use or to support aquatic ecosystems depends on the availability and sustainability of the resource. Water situation in the country is characterised by highly variable rainfall, erratic runoff, high levels of evaporation due
to high temperatures and shallow dam basins as well as sedimentation problems and large scale inter-basin water transfers (DWAF, 2012, National Water Resource Strategy 2). Due to the scarcity of water in South Africa, extensive exploitation of water resources for domestic and other water uses is common in the rural areas (Younes and Bartram, 2001). Groundwater is also used extensively, particularly in rural and arid areas where surface water is inadequate (Mukheirbir, 2005). This is particularly the case in the North West Province (Momba et al., 2009). Most of South Africa’s water requirements are provided by surface water supplies (DWAF, 2004). Generally, the surface water resources are highly developed over the country, with about 320 major dams having a total capacity of more than 32 400 million m$^3$, which is some 66% of the total mean annual runoff of about 49 000 m$^3$/annum. This includes about 4 800 million m$^3$/annum draining from Lesotho into South Africa and a further 500 million m$^3$/annum draining from Swaziland to South Africa (DWAF 2004). A portion of this runoff (typically about 20%) needs to remain in rivers and estuaries to support the ecological component of the reserve. Only part of the remainder can be derived effectively as a usable yield. The usable yield may be further constrained by sources of pollution, such as irrigation, return flows, urban drainage, and industrial and mining activities (DWAF, 2012, National Water Resource Strategy 2).

The North West Province of South Africa is a dry province with surface, ground, imported water and reusable effluent being the Province’s four major water sources together with a few rivers (State of Environmental Report (SER), 2002). There are four water management areas which manage five river catchments exist in the province. The catchment areas include the Crocodile and Elands, Marico and Hex, Marico and Molopo, Mooi and Vaal and the Harts (SER, 2002). The major rivers in these catchment areas include Crocodile, Elands, Hex, Groot Marico, Molopo, Mooi, Skoonspruit and Vaal, as well as the Marico Bosveld, Molatedi, Boskop, Vaalkop, Hartebeesport, Rooikopjes, Potchefstroom, Bloemhof and Modimola dams (DWAF, 2007). The water resources of the North West Province are becoming increasingly stressed, largely due to population growth, development, agriculture and mining (DWAF, Blue Drop Report, 2011). Aquatic systems in the Province are susceptible to a wide range of extreme climatic conditions (e.g. droughts and floods) (DWAF, 2004). Most of the surface waters in the Province are polluted to varying extent from mining (through acid mine drainage), sewage effluent discharges, urban and
agricultural runoff and from sources outside the Province (particularly Gauteng) significantly impact the quality of water (DWAF, 2004). There are two major water quality problems within the North West Province, notably eutrophication and salinization. Both of these arise because of excessive loads of chemicals from industrial and domestic sources (DWAF, 2004).

1.3 Drinking water production practices and processes

In order to provide the community with safe drinking water, appropriate drinking water production practices must be implemented. Water purification is essential for all surface water in South Africa and is critical in removing waterborne pathogens, thus controls disease transmission and render water fit for human consumption (Momba et al., 2009). Water suppliers use a variety of treatment processes to remove contaminants from drinking water (EPA, 2004). The process involves various steps depending on the type of raw water. Firstly when water is abstracted it is passed through screens to keep out of weeds, algae and floating debris (DWAF, 2002). This water could undergo coagulation and flocculation steps. Coagulation is the process of adding chemicals (coagulants) to water to destabilise the naturally occurring particles to aggregate and form flocs and can be removed by flocculation (DWAF, 2002). Different chemicals such as aluminium sulphate (alum), ferric chloride, lime, aluminium polymers and polyelectrolytes can be used as coagulants. The general trend is the use of polyelectrolyte as a substitute for alum and ferric chloride as coagulants. However, few treatment plants in North West province use ferric chloride (Momba et al., 2009). Flocculation is considered to be part of the coagulation process and can take place in different types of equipment in which the individual destabilised colloidal particles are allowed to collide with one another to form larger floc particles (DWAF, 2002). Sedimentation is the process in which the water is passed through a sedimentation tank (clarifier) where large solid particles (flocs) that have been formed during coagulation and flocculation are allowed to settle out (DWAF, 2002). Partially clarified water is channelled to flotation tank where water is mixed with air dissolved in a small amount of water under high pressure, for the removal of light types of flocs. When water is aerated in this manner the dissolved air comes out of solution in the form of fine bubbles and attach to the floc causing them to rise to the surface (dissolved air flotation) that is skimmed off. During these process microorganisms, organic matter, toxic contaminants and
suspended fine particles are removed (DWAF, 2002). The resultant water is further purified by passing it through rapid gravity filtration system or sand filters which traps fine flocs or non-flocculated colloidal material to achieve low turbidity. A sufficiently low turbidity level is required for the effective disinfection of the water (DWAF, 2002). Most of the remaining bacteria are removed here. However, *Gardia*, *Cryptosporidium* and viruses cannot be effectively removed. Removal of these organisms can be achieved by allowing water to pass through slow sand filters. This treatment involves the slow passage of water through a bed of sand in which a microbial layer covers the surface of each sand grain. Waterborne microorganisms are removed by adhesion to the gelatinous surface microbial layer. Water is then softened by removing calcium and magnesium. Taste and odour is removed by aeration, chemical oxidation and adsorption (EPA, 2004). Finally the disinfection process, which entails the addition of the required amount of a disinfectant for the destruction of harmful micro-organisms found in water, to make it fit for domestic use (DWAF, 2002). The processes involve chlorination, irradiation, ozonation, reverse osmosis, electro-dialysis, advanced coagulation and oxidation methods (EPA, 2004). However, some of the disinfectants can cause the production of disinfectant by-products, potentially carcinogenic. The predominant types of disinfectants employed in North West Province were chlorine gas followed by sodium and calcium hypochlorite (Momba et al., 2009). Chlorine gas is used for disinfection in Mafikeng and Mmabatho Water Treatment plants (Mr. Maboka, Operation manager, Mmabatho Water Treatment plant). Free chlorine residual concentration of at least 0.2 mg/l - 0.5 mg/l in the final water leaving the plant is necessary to protect the drinking water against the pathogenic microorganisms (WHO, 2004). However, South African Assessment Guide for the Quality of Domestic Water Supply recommends 0.3 to 0.6 mg/l as the ideal residual chlorine at the consumer’s tap water in order to combat any possible contamination in the network and to protect public health (DWAF, 1998).

1.4 Drinking water provision in the North West Province (NWP)

Every water services authority has a duty to all customers in its area of jurisdiction to progressively ensure efficient, affordable, economical and sustainable access to water services (National Water Act, 1997). Drinking water services providers do the work of providing water to customers according to its contract with the Water
Services Authority who can contract a community based organisation, a Water Board, a private company, an NGO or an adjoining local authority to be the Water Services Provider. Raw water is supplied in bulk quantity by Bulk Water Service Provider to a Water Services Provider in an area. This Bulk Water Service Provider may be a Water Board, NGO, private company or Local government (www.eWISA.co.za, 2012).

The North West Province is classified as a water scarce province and the available water is not equally distributed. The North West's surface water comprises of rivers, dams, pans, wetlands and dolomitic eyes fed by underground water sources. Therefore, water quality and quantity issues affecting groundwater also have implications for surface waters. In the North West, ground and surface water are integrated and interdependent as dolomitic eyes or springs are the sources of several major rivers which rise within the boundaries of the Province, such as Groot Marico, Mooi and Molopo Rivers (SER, 2004). There are four main driving forces affecting surface water resources in the North West Province, namely climatic conditions, increased population growth, industrial demand, and policy and legislation.

Water services delivery is performed by eleven (11) Water Services Authorities in North West via 43 drinking water supply systems a total design capacity of 170.9 Ml/day (DWAF, Blue Drop Report, 2011). Operational data is not available for all systems; however the existing data indicates operating capacities between 55% and 87% (DWAF, Blue Drop Report, 2011). This result in an average output volume (final water) of 122 Ml/day (DWAF, 2011). In the urban areas water is supplied by a combination of surface water and ground water sources which is purified at Water Treatment Works. A few of the treatment plants draw water from unprotected springs also (Momba et al., 2009).

1.5 Drinking water provision in Mafikeng
Water provision in Mafikeng is based on a two source approach. The groundwater is only chlorinated and presented for distribution. Surface water is sourced from a eutrophic dam that received treated sewage upstream from the abstraction point. Mafikeng Local Municipality, water services authority, which falls under Ngaka Modiri
Molema District Municipality, supplies water to Mafikeng and Mmabatho residents (DWAF, Blue Drop Report, 2009). Botshelo water is the water services provider (Water Resource Profile, Version 2004). Drinking water is produced from a mixture of surface water, boreholes and treated effluents from the sewage treatment plants in Mafikeng and Mmabatho. Grootfontein eye was the only water source in the early times of Mafikeng (Eddie van der Heiden, Director of Operations, Botshelo Water). Water from this source was used for domestic and irrigational purposes. Eventually the level of water dropped drastically. Hence Grootfontein boreholes were drilled to address the water shortage. Water from the boreholes is dolomitic water with high calcium level. Later, in 1988, water from Molopo eye was sourced. At that stage Molopo eye and ground water from Grootfontein boreholes were the only raw water sources for Mafikeng. With the economic growth and exponential increase in population, the demand for water increased and the absence of alternative water sources the municipality struggled to meet the demand for water. To address this problem the Modimola dam (Setumo dam) and the associated Mmabatho water treatment plant was commissioned in 1994 and constructed in 1996. This was done as part of Molopo eye augmentation plan (Eddie van der Heiden, Director of Operations, Botshelo Water). The dam which is one of the water sources is a eutrophic dam with lush algal growth which is indication of organic and inorganic pollution. Treated waste water from the Mmabatho sewage treatment plant which is located upstream from the Mmabatho water treatment plant, is discharged in to Modimola dam where the effluent is diluted as it reticulates in the dam (Figure 1.1)
Figure 1.1: Schematic representation of water provision of Mafikeng
Treated effluent from Mafikeng sewage treatment plant is released into the Molopo River and flows through Cookes Lake in order to aerate the water and to remove the nutrients from the water by the water reeds (Eddie van der Heiden, Director of Operations, Botshelo Water). Water cascades to Lotlamoreng dam which cascades to Molopo River which joins Modimola dam. The dam is also a receptacle of storm water runoff. Therefore water in the dam is a mixture of environmental water and recycled water and is sufficient enough to dilute the waste water discharged.

Drinking water for the Mafikeng is thus supplied to the consumers from two different sources (Figure 1.1). Raw water from these sources is treated differently. Water from Molopo eye weir gravitates and borehole water is pumped to the treatment plant in Mafikeng where chlorine is added for disinfection (Momba et al., 2009). This water is distributed to Mafikeng residents and pumped to the Signal Hill reservoirs. At the Mmabatho water treatment plant raw water is abstracted from the dam and undergoes various treatment processes such as sedimentation, dissolved air flotation, filtration and disinfection using chlorine (Mr. Maboka, Operation manager, Mmabatho water treatment plant). Powdered activated carbon is used to remove odour and taste caused by algal cell lyses. Softening and absorption processes are not done in the plant (Mmabatho water treatment plant). Treated water is pumped to Lokaleng pump station from where water is distributed to a few locations. The bulk of the water goes to Signal Hill reservoirs. Treated water from both plants is blended at these reservoirs at Signal hill, before distribution to the major part of Mmabatho residents (Source of information: Eddie van der Heiden, Director of Operations, Botshelo Water). The mixing is done in order to mitigate the effect of sewage water released into Modimola dam.

Innovative water certification systems were introduced by the Department of Water Affairs to the water sector in September 2008. The Blue Drop certificate is awarded to municipalities for excellence in drinking water management and water quality. The Green Drop certification on the other hand is for waste water management and the quality of effluent (DWAF, Green Drop Report 2009). According to the Green Drop Certification Programme (2009) most of the municipalities in the North West Province and their waste water treatment works (WWTW) had relatively low scores. Average score for Ngaka Modiri Molema District Municipality is 5. For Mafikeng and
Mmabatho the waste water quality compliance score is G and Green Drop score is 10. These municipalities fall under Ngaka Modiri Molema District Municipality and the waste water quality management is poor and substantial effort is needed in all areas to improve the quality of the effluent released. The Green Drop results for 2010-2011 indicated that municipal wastewater management in North West was not in a satisfactory state. The majority of wastewater systems still resided in high risk state, compared to 2009. Furthermore, the average Green Drop score for North West province decreased from 33% (2009) to 29% (2010/2011). The Provincial Green Drop score of 50%. Mafikeng and Mmabatho waste water quality compliance was 0 and Green Drop score 29.1 and 35.2 respectively. According to Green Drop report Mafikeng and Mmabatho waste water treatment plants are positioned under high risk category and needs urgent intervention. According to 2012 report, the waste water risk rating is 77.3% and 74.1% for Mafikeng and Mmabatho, respectively. The highest risk area is the poor effluent compliance for Mafikeng and Mmabatho waste water treatment plants, hence rated in high and critical risk positions (DWAF, Green Drop Report, 2012). There was no information about the chemical, physical and microbiological compliance. Waste water performance is substandard and as a result water resources and public health will suffer and significant effort is needed to ensure acceptable effluent quality. This scenario is unsatisfactory when one considers that the effluents from these sewage treatment systems are diluted and sourced as source water for drinking water production within a few kilometres from where they are released. Urgent intervention is required as such practices put the drinking water production systems under tremendous pressure and consumers of the drinking water may be at risk.

A previous study in Mafikeng has demonstrated that this municipal waste water treatment plant acts as a source of antimicrobial resistant bacteria and their genes (Mulamattathil et al., 2000). Similar observations were made in another recent study (Siri et al., 2011). Contamination of sewage water with Cryptosporidium spp. and Giardia is a major problem experienced at the Mmabatho water treatment (Mr. Maboka, Operation manager, Mmabatho water treatment plant, personal communication). These are protozoa transmitted through water or food causing mild to severe diarrhoeal diseases in humans (DWAF, 1996; Moulin et al., 2010). These
are some of the challenges faced by the Mmabatho water treatment works and may affect the participation of the city in the Blue Drop certification programme.

The Blue Drop score for Mafikeng drinking water in 2011 was an 8.85%, a decline in performance from the 2010 score of 31.88%. However, the score for 2012 has improved to 46%. This is still unsatisfactory. This score is an indication of inadequate monitoring, treatment of drinking water as well as several management aspects that are being neglected. The situation demands more stringent application of rules to provide water of acceptable quality. Several studies conducted in Mafikeng reported the isolation of coliforms and pathogenic bacteria from surface and drinking water (Wose kinge and Mbewe, 2010; Ateba and Mbewe, 2011). Botshelo water regularly receives verbal complaints, from the consumers regarding water quality problem in Mafikeng area of taste and smell in the finished water (Botshelo water report, 2012). Department of Water Affairs and Forestry also issued warning to public not to consume tap water without proper care (DWAF, My Water, 2012). According to records, the treated sewage is compromising the water provision and may have serious implications for continued drinking water supply to a rapidly growing city.

1.6 Water quality parameters

Water quality is a significant problem in most countries and pollution-induced quality deterioration leads to harmful environmental and health hazards (DWAF, 2012, National Water Resource Strategy 2). Anthropogenic activities, surface runoff and discharge of waste water can cause contamination of fresh water bodies and becomes a threat to public water supplies. Treated drinking water should be of acceptable quality for human consumption. With the increased concern for drinking water quality, information regarding the physico-chemical and bacteriological parameters is of paramount importance to assess the threat to human health. Microbial and chemical parameters contribute to the deterioration of water quality (Lehtola et al., 2004; Chidya et al., 2011).

Total coliforms, faecal coliforms, heterotrophic bacteria, are indicators commonly used to assess the microbiological safety and quality of drinking water (Nevodo and Cloete, 1999; Obi et al., 2002; Whitlock et al., 2002; Pavlov et al., 2004). Water
quality is often related to the degree of bacterial contamination (da Silva et al., 2008). Although many indicator organisms are not pathogenic, their presence in water signals the presence of potentially pathogenic organisms (Rompré et al., 2002). Heterotrophic bacteria are generally considered as harmless organisms and pose no health risks. However, several organisms associated with opportunistic infections have been reported to be present among heterotrophic bacteria (Pavlov et al., 2004). These organisms include Aeromonas, Acinetobacter, Aureobacterium, Bacillus, Chryseobacterium, Klebsiella, Moraxella, Pseudomonas, Staphylococcus and Vibrio (Pavlov et al., 2004; Messi et al., 2005). Aeromonas and Pseudomonas opportunistic pathogens and limited attention has been given to the presence of these species in drinking water. Aeromonas are human pathogens commonly found in aquatic ecosystem and are the leading causes of enteric and non-enteric diseases (Fontes et al., 2010; Alcaide et al., 2010; Pablos et al., 2011; Parker and Shaw, 2011). Pseudomonas sp. are opportunistic pathogens known to cause several infections, particularly in immunocompromised patients, those with catheters, open wounds or cystic fibrosis and ICU related infections (Trautmann et al., 2005; da Silva et al., 2008; Waszczuk et al., 2010; Fricks-Lima et al., 2011). While water is known to be the common vehicle for the transmission of Aeromonas and Pseudomonas, several authors have proposed that Pseudomonas sp. and Aeromonas sp. be included as bacterial indicator of water quality (Pavlov et al., 2004; da Silva et al., 2008; Pablos et al., 2011; Parker and Shaw, 2011).

1.6.1 Physico-chemical parameters
The physico-chemical factors that affect the quality of water include amongst others colour, odour, taste, turbidity, temperature, pH, electric conductivity (EC), total dissolved salts (TDS), dissolved organic carbon, total trihalomethanes, phenols, macro and micro-nutrients (SANS 241: 2011). Water temperature influences microbial growth, biofilm formation and other chemical reactions (Pritchard et al., 2007). The taste of water, its corrosiveness, solubility and speciation of metal ions are all influenced by pH. At low pH water may taste sour while at high pH water taste bitter or soapy (DWAF, 2006). The main significance of pH in domestic water supplies relates to its effects on water treatment process. There is no health consequences attributed to pH of water, except at extreme values. Total heavy metal content in water could increase at low pH which is a matter of public concern
Very low pH makes water corrosive and creates strain on equipments (Schäfer et al., 2009).

Electric conductivity is an indication of water salinity and mineral content and is directly proportional to total dissolved solids (TDS) (Virkutyle and Sillanpää, 2006). TDS is calculated by adding the ions measured in solution. Consumers find water distasteful when a TDS value is above 1200 Mg/L (Schäfer et al., 2009). According to South African Water Quality Guidelines (DWAF, 1996) the accepted value of TDS ranges from 0-450 Mg/l and that of EC is 0-70 mS/m. TDS in water could be attributed to the release of deposits from the pipes in to the water but also as reminance from the raw water. High EC, as TDS, is attributed to high salinity and high mineral content. In raw water elevated EC is attributed to pollution of water by soil through surface run off (Chidya et al., 2011) and by climate (Delpa et al., 2009). Mineral ions naturally occur in water and they are essential for various processes in the body. However, high concentration of these may make the water unfit for living organisms and detrimental to human health (Azizullah et al., 2011). It can adversely affect kidney functions, as well as cardiac and hypertension sufferers (DWAF, 2006). Excessive levels of minerals in distribution systems may cause corrosion of plumbing and appliances. In stream activities of people and livestock affected the chemical quality of water (Yilla et al., 2008).

1.6.2 Bacteriological quality

Access to safe drinking water is considered as a human right (WHO, 2004). Deterioration of water quality is a major problem experienced worldwide and with no exception, South Africa (Nevondo and Cloete, 1999; Lehtola et al., 2004). Many drinking water sources are not of good quality and several disease causing microorganisms are linked to water and therefore many disease outbreaks and deaths occur as a result of consuming contaminated water (Schäfer et al., 2009). Raw water sources can be contaminated by immense amount of microbes and hazardous toxic chemicals harmful to human health, entering the system through surface run-off, agricultural inputs, and disposal of industrial, municipal and domestic wastes, mixing sewage effluent and from wild life (Vega et al., 1998; Azizullah et al., 2011). Recent studies showed an increasing interest on the role of surface water as natural reservoir and in the transmission of enteric pathogens (Schriewer et al., 2011).
Contaminated water might contribute to the dissemination of human pathogens through direct ingestion or indirect contamination of food (Elhariry, 2011). Rural populations use unprotected water from rivers, dams and streams for domestic purposes. As a result these populations are exposed to contaminated and polluted water which may cause serious water related health problems (Obi et al., 2002; Lehtola et al., 2004). Hence the health risks associated with consumption of contaminated water are of great interest (Eckner, 1998). Water intended for human consumption and for all other domestic purposes, including personal hygiene and recreational activities should be free of harmful microorganisms (Nevondo and Cloete, 1999). Contamination of drinking waters with pathogenic and toxigenic microbes is a growing public health and environmental problem (Patel et al., 2011) and the consequent effects is an issue of great concern. The impact of water borne pathogens in human health is significant. To protect consumers from waterborne diseases, the distributed water must be completely free of pathogenic microorganisms (Pereira et al., 2009). There are several reports on the prevalence of microorganisms in surface water, drinking water and water used for irrigation (Müller et al., 2001; Germs et al., 2004; Mukherjee and Chakraborty, 2006; Yáñez et al., 2006; Revetta et al., 2010) which reflect the concern relating to the quality of drinking water. A wide variety of pathogenic viruses, protozoa, fungi and bacteria may be transmitted by water (Regli et al., 1991; Pereira et al., 2009; Lee et al., 2010; Moulin et al., 2010). These micro-organisms cause diseases such as gastroenteritis, giardiasis, hepatitis, typhoid fever, cholera, salmonellosis, dysentery and eye, ear, nose and skin infections, which have been associated with polluted water (Grabow, 1996; Genthe and Seager, 1996; Momba et al., 2009). Infections are generally contracted by drinking polluted water, recreational exposure to contaminated water, inhaling contaminated aerosols or the consumption of raw food (that is, irrigated vegetables and shellfish) exposed to polluted water (DWAF, 1996, South African Water Quality Guidelines).

Surface water appeared to have a direct influence on the bacteriological quality of drinking water. Source water is treated with various procedures depending on its quality (Brettar and Höfle, 2008) and high bacterial count in treated drinking water is an indication of ineffective disinfection processes at water treatment plants. Fluctuating temperatures, stagnation (residence time), pipe material and decreasing
pipe diameters can all promote bacterial growth in the distribution system which will, in turn, affect the aesthetic quality of water. Moreover, it bears the potential risk of pathogenic proliferation (Lautenschlager et al., 2010). Water pollution by microorganisms of faecal origin is a current world wide public health concern (Okeke et al., 2011). Total coliforms, faecal coliforms, enterococci and heterotrophic bacteria are indicators commonly used to assess the microbiological quality of water resources (Yañez et al., 2006; Okeke et al., 2011), in order to obtain the most reliable indication of potential risks of infection. Total coliform bacteria comprise faecal and non-faecal origin. Their presence in water is a general indication of the hygienic quality of water (Zamxaka et al., 2004). Total coliforms produce metallic sheen colonies when incubated at 35°C on mEndo agar and will give an indication of the general sanitary quality of water (DWAF, 1996, South African Water Quality Guidelines). Presence of faecal coliforms in water indicates recent faecal or other contamination, inadequate treatment or post-treatment deficiencies (Zamxaka et al., 2004). These organisms produce a typical blue colour on mFC agar when incubated at 44.5°C and indicates probable faecal pollution of water (DWAF, 1996, South African Water Quality Guidelines). However, Heterotrophic bacterial counts are used to indicate the general microbial quality of water. They are used to assess the efficiency of water treatment and disinfection processes, to test the integrity of distribution systems for after growth and to determine the quality of water used in industrial processes.

South African Bureau of Standards specifies 100 cfu/1 ml as the accepted limit of heterotrophic bacteria. Between 100-1000 cfu/1 ml is an indication of inadequate treatment, post-treatment contamination or after growth in the water distribution system and pose slight risk of microbial infection. The acceptable limit of total coliforms is 0-5 cfu/100 ml and that of faecal coliforms is 0 cfu/100 ml. High levels of these two indicating poor sanitary quality of water and poses risk of infectious disease transmission (DWAF, 1996, South African Water Quality Guidelines). It is therefore critical to understand the relevance of surface and drinking water contribution to the transmission of pathogenic microorganisms to humans.
1.7 Heterotrophic bacteria, particularly *Aeromonas* and *Pseudomonas* in drinking water

The wide range of microorganisms recovered from water that requires organic carbon for growth is collectively known as heterotrophic bacteria (Roslev *et al*., 2004; Chu *et al*., 2005) and are generally used to assess the effectiveness of water treatment and disinfection processes (WHO, 2002; Pavlov *et al*., 2004). High levels heterotrophic bacteria in treated water indicate inadequate treatment of the water, post-treatment contamination or bacterial after growth in the distribution system and may harbour opportunistic pathogens with virulence factors (Lye and Dufour, 1991). These bacteria may have a negative impact on human health especially immunocompromised individuals are at risk (Pavlov *et al*., 2004). All bacterial pathogens and opportunistic pathogens are heterotrophic bacteria (Allen *et al*., 2004). Heterotrophic bacteria harbouring opportunistic pathogens such as *Aeromonas*, *Pseudomonas*, *Serratia*, *Salmonella*, *Acinetobacter*, *Klebsiella* and *Flavobacterium* have been reported (Messi *et al*., 2005).

*Aeromonas* spp. and *Pseudomonas* spp. are ubiquitous opportunistic pathogens responsible for various infections (Kim and Wei, 2007; Alcaide *et al*., 2010; Fontes *et al*., 2010; Moritz *et al*., 2010; Figueira *et al*., 2011; Parker and Shaw 2011). Their presence in drinking water and food is a cause of concern as water and food act as vehicles for the dissemination of these pathogens (Chang *et al*., 2007; Emekdas *et al*., 2009; Ottaviani *et al*., 2011).

*Aeromonas* species are Gram-negative facultative anaerobic rods. They are common inhabitants of natural habitats such as soil, fresh and brackish water, sewage and waste water. Members of this genus had been implicated in a number of intestinal and extra intestinal infections in humans as well as other animals (Janda and Abbott, 2010; Pablos *et al*., 2011; Parker and Shaw, 2011). Among the leading pathogenic species are *A. hydrophila*, *A. bestiarum*, *A. sobria*, *A. caviae* (synonym of *A. punctata*) and *A. veronii* (Lamy *et al*., 2009; Beaz-Hidalgo *et al*., 2010). The environmental ubiquity associated with the potential pathogenicity of these bacteria has been illustrated also in recent natural disasters (Chang *et al*., 2007; Pablos *et al*., 2011). Some species, mainly the *A. salmonicida* and *A. hydrophila* and *A. veronii* are recognized causative agents of fish disease (Beaz-Hidalgo *et al*., 2010; Janda...
and Abbott, 2010). Antibiotic resistant *Aeromonas* species have also been isolated from drinking water, food and patients with diarrhoea (Chang et al., 2007; Alcaide et al., 2010; Pablos et al., 2011) and from the diarrhoeal water samples of HIV patients suffering from gastroenteritis and their household drinking water in Limpopo Province, South Africa (Obi et al., 2004; Ramalivhana et al., 2010).

*Pseudomonas* species are non-spore forming Gram-negative facultative anaerobic rods. They are commonly found in soil and water with great adaptability and metabolic versatility (Kim and Wei, 2007). Infectious species include *P. aeruginosa, P. oryzihabitans* and *P. plecoglossicida*. These organisms are associated with wound and pulmonary infections, urinary tract infections and septicaemia (National Nosocomial Infection Surveillance (NNIS), 2004). The potentially pathogenic bacterium *P. aeruginosa* is regarded as a contaminant of drinking water environments, where it can present a hazard to human health. The main water related routes of transmission are exposure of damaged skin, ears and eyes to contaminated water and inhalation of *P. aeruginosa* containing aerosols. The risk of gastrointestinal infection via water ingestion is, however, low (Mena and Gerba, 2009). It is an important human opportunistic pathogen causing intensive care unit nosocomial infections and immunocompromised patients (Trautmann et al., 2005; Durojaiye et al., 2011; Kowada et al., 2011). *P. aeruginosa* has the pronounced capacity to flourish in hospital environments and possesses a wide range of protein secretion mechanisms which is responsible for its pathogenicity (Dwidjosiswojo et al., 2012). The widespread distribution of *Pseudomonas* spp. may pose some public health concerns.

The WHO, (2004) expert group on heterotrophic plate count (HPC) in drinking water argues that *Aeromonas* may not be a risk factor for the general community but agreed that the immune compromised individuals of the communities are at risk. For this they recommended that *Aeromonas* not be included in water quality standards. With an increasing number of HIV positive individuals in the population of Sub-Saharan Africa it may be necessary to consider including testing for opportunistic pathogenic microorganism such as *Aeromonas*. 
Several studies have demonstrated that many *Aeromonas* spp. and *Pseudomonas* spp. isolated from drinking water may exhibit a vast array of virulence factors. Although the pathogenesis of *Aeromonas* infections remains poorly understood, mesophilic *Aeromonas* spp. can express a range of virulence factors including attachment mechanisms and production of a number of haemolysins including aerolysin, proteases, adhesins, invasions, enterotoxins, phospholipase and lipase (Gosling, 1996; Yogananth *et al*., 2009; Parker and Shaw, 2011). Isolates from food and surface water frequently had toxin gene patterns similar to those of clinical strains and expressed virulence properties at human body temperature. This implies that they have the potential to cause human illness (Ottaviani *et al*., 2011). *Aeromonas* strains isolated from untreated water displayed virulence related phenotypes such as extracellular lipolytic and proteolytic activities as well as enterotoxins and haemolysins such as aerolysin related genes (Carvalho *et al*., 2012) linked to diarrhoea (Galindo *et al*., 2006). Virulence factors enable them to colonise, invade, establish in and infect different hosts (Galindo *et al*., 2006).

Health concerns regarding *Pseudomonas* spp. depends on the presence of virulence factors. *Pseudomonas* spp. is able to secrete a large number virulence associated factors that have great influence on pathogenesis (Van Delden, 2004; Lin *et al*., 2006). Virulence factors include the secretion of proteins with toxic effects (Winstanley and Fothergill, 2008) directly in to the cytoplasm of host cells (Ajayi *et al*., 2003). Type 11 (T2SS) and Type 111 (TTSS) secretion systems are important in the secretion of these proteins which can be ADP-ribosylating enzymes, cytotoxins or adenyl-cyclases among others (Sato and Frank, 2004). Virulent strains of *Pseudomonas* spp. carry virulent related genes (*exoA*, *exoU*, *exoT*, *exoS* and *exoY*) encoding toxic proteins (Kaszab *et al*., 2011). Another virulence feature is the ability to adhere to the human extracellular matrix protein, fibronectin (Pimenta *et al*., 2003), to A549 pneumocyte cells causing respiratory infections (Di Martino *et al*., 2002) and to human nerve cells (Picot *et al*., 2001). Various types of drug resistance are also common among *Pseudomonas* spp. (Drenkard, 2003; Kim and Wei, 2007; Fricks-Lima *et al*., 2011). Depending on the virulence and antibiotic resistance properties, these opportunistic pathogens may be a cause of concern to susceptible individuals.
1.8 Regrowth of organisms in the distribution system and biofilm formation

Prior to distribution, drinking water is purified, treated with disinfectants and the bacteriological quality of water is carefully monitored. Yet a few bacteria survive the treatment procedures; enter a viable but non-culturable (VBNC) state (WHO, 2002). The bacteria remain unnoticed and if the conditions become favourable they regrow and multiply thus may serve as an environmental reservoir for pathogenic microorganisms. The principal determinants of regrowth are temperature, availability of nutrients and lack of residual disinfectant (WHO, 2002). If left unnoticed they may result in a potential health risk for humans (Moritz et al., 2010; Wingender and Flemming, 2011). A characteristic of the VBNC condition is the ability of bacteria to become culturable again upon resuscitation (Oliver, 2005). Dwidjosiswojo et al., (2012) demonstrated the ability of copper ions to induce VBNC state in *P. aeruginosa* accompanied by the loss of culturability and cytotoxicity. Copper pipes are commonly used in distribution system in South Africa (Lehtola et al., 2004).

Aquatic microorganisms have the ability to attach to a surface and form biofilms (Muñoz-Berbel et al., 2006). Organisms such as faecal indicator bacteria, obligate bacterial pathogens of faecal origin, opportunistic pathogens, enteric viruses and parasitic protozoa are found to colonize drinking water biofilms (Wingender and Flemming, 2011).

Heterotrophic bacteria, including *Pseudomonas* and *Aeromonas* have the potential to grow on surfaces in contact with water as biofilms (da Silva et al., 2008; Moritz et al., 2010). The capacity to produce biofilm is related to virulence in bacteria (Pimenta et al., 2003). Bacterial biofilms are complex microbial depositions enclosed in an exopolysaccharide matrix (Sun et al., 2011, Wingender and Flemming, 2011) and express properties distinct from planktonic cells. One of these is an increased resistance to antimicrobial agents (Muhammad and Eberl, 2011; Drenkard, 2003; Wunder et al., 2011). Biofilm communities can develop in spatially highly irregular morphological structures, in which individual colonies are separated by voids and channels (Muhammad and Eberl, 2011). From time to time biofilm material becomes detached from the pipes, and thus enters the water supply system. In many clinical and industrial settings, biofilm represents a hazardous and costly problem. Many human infections are caused by bacteria that form biofilms (Waszczuk et al., 2010).
Infection episodes caused by biofilm associated *Pseudomonas aeruginosa* transmitted from intensive care unit tap water to patients (Trautmann *et al.*, 2005).

In biofilms, pathogens are protected from disinfection and cause a public health risk (Berry *et al.*, 2006). As a result of resistance to antimicrobial compounds, infections caused by bacterial biofilms that are persistent are very difficult to eradicate (Drenkard, 2003). Elhariry, (2011) demonstrated the biofilm forming ability of aquatic bacterial strains on leafy vegetables. These bacteria were isolated from a drinking water distribution network.

Studies found that the application of two common disinfectants, chlorine and monochloramine (Berry *et al.*, 2006) as well as an ultraviolet light/ hydrogen peroxide advanced oxidation treatment (Metz *et al.*, 2011) did not eliminate bacteria from accumulating in biofilms. This is attributed to the decrease in the efficiency of disinfectants within the biofilms because of the limited diffusion into the biofilm (Bridier *et al.*, 2011). In such cases regrowth of hygienically relevant microorganisms could occur and affect drinking water quality (Roeder *et al.*, 2010). The disinfection methods induce selection pressure on the biofilm population and cause considerable population shifts (Roeder *et al.*, 2010). Biofilms in drinking water distribution networks can become transient or long term habitats for hygienically relevant microorganisms including pathogens such as protozoa, bacterial pathogens and enteric viruses (Lehtola *et al.*, 2004; Moritz *et al.*, 2010). These undesired microorganisms can be dislodged into the bulk water causing the deterioration of the microbiological quality of drinking water (Le Chevallier, 1999). Growth of biofilms in systems for storage and distribution of purified water is caused by a combination of temperature, total organic compounds, increased stagnation periods and low residual chlorine (Goudier *et al.*, 2009; Velten *et al.*, 2011). Florjanič and Kristl, (2011) observed that HPCs in biofilm were proportional to the number of detached bacteria in effluent water that is responsible for the deterioration of water quality. Drinking water biofilms could pose serious health implications to humans, more especially to immuno-compromised individuals when they harbour pathogens (Bressler *et al.*, 2009). Bacterial biofilms can also have detrimental effects in *in situ* operational conditions such as corrosion, in particular in drinking water supply, the food industry and medicine (Pavanello *et al.*, 2011; Muhammad and Eberl, 2011).
1.9 Water reuse practice and the associated health implications

As urbanisation increases, so does the pressure to provide adequate clean drinking water. Many countries experience severe water scarcity and in an effort to combat this problem authorities have focussed their attention on the utilization of treated waste water for irrigation and surface or ground water replenishment purposes (Fatta-Kassinos et al., 2011). Many communities in South Africa struggle to access reliable and adequate quantities of potable water, in particular poor people who do not have access to reliable water supply (DWAF, 2012, National Water Resource Strategy 2). The water resources of South Africa are well utilised and in many areas show signs of stress because of high demand. Hence reuse of treated wastewater is a viable alternative to overcome water shortage and encompass numerous reuse options (Adewumi et al., 2010). However, if not treated effectively, waste water reuse can be harmful and pauses potential health risks to public (Adewumi et al., 2010). In the North West province water is scarce and the answer to our looming water crisis is to recycle water.

Waste water reclamation and reuse can cause pharmaceutical end products and illicit drugs to reach the environment leading to the flourishing of antibiotic resistant organisms in water. Reuse of treated waste water introduces the antibiotics into the drinking water, thus increases the opportunity of human exposure to such antibiotics (Bartlet-Hunt et al., 2009; Khalaf et al., 2009; Kümmerer, 2009). Emerging concerns about chemicals such as antibiotics, antidepressants, hormones and personal care products has spawned a new generation of water quality issues (Watkinson et al., 2009). Because these compounds are biologically active, ecotoxicological and human health impacts are of serious concern (Bartelt-Hunt et al., 2009). Antibiotics are extensively used in human and veterinary medicine as well as in aquaculture, for prophylaxis or treating infectious diseases, as growth promoters of animals, to improve the quality of the products, in fruit growing and bee-keeping (Kümmerer et al., 2009; Jiang et al., 2011). Antibiotic consumption has received a lot of attention due to the increasing numbers of diseases and infections becoming resistant to traditional treatments (Turkdogan and Yetilmezsoy, 2009). However, antibiotics used in human therapy or their metabolites are excreted in urine and faeces and reach the sewage treatment plants and are only partly eliminated in the sewage treatment
plant (Murata et al., 2011). They pass through the sewage system and may end up in the environment, mainly in the water compartment with the potential of adversely affecting aquatic and terrestrial organisms (Kümmerer, 2009; Murata et al., 2011) and are able to be stable for up to one year in the environment (Khalaf et al., 2009).

The presence of antibiotics in water resources has been disturbing news for the stakeholders who are responsible for public health and drinking water supply (Alighardashi et al., 2009). Antibiotics persist in the environment or surface run-off is a significant source of contaminants and reaches humans through drinking contaminated water. Many researchers have documented the occurrence of antibiotics used in humans and veterinary practices in surface waters, ground waters, hospital waste waters, biosolids, sediments and sewage effluents (Castiglioni et al., 2008; Bartelt-Hunt et al., 2009; Khalaf et al., 2009; Kümmerer, 2009; Munir et al., 2011; Thevenon et al., 2011). Sediment contaminants can accumulate or be remobilized from the sediments into the water column, and affect water quality causing potential irreversible adverse effects to human health (Thevenon et al., 2011). Waste water treatment plants are the major source for loading antibiotics into the water body and are regularly detected in surface and ground waters (Loganathan et al., 2009; Li and Zhang, 2011; Bartelt-Hunt et al., 2009; Börjesson et al., 2009), dominated by the β-lactam, quinolone and sulphonamide group (Watkinson et al., 2009). Risks associated with the occurrence of antibiotics and the impact on human health is a global concern (Deblonde et al., 2011; Wunder et al., 2011). Low levels of antibiotic residues in the environment impose selective pressure on bacterial population and can cause selection of antibiotic resistance in bacteria and the resistance occurring in pathogenic organisms that compromise the use of antimicrobial therapy (Hoa et al., 2011; Kümmerer, 2009; Castiglioni et al., 2008). Identification of antibiotic resistant bacteria has been observed in wastewater due to elevated levels of antibiotics released in to the environment and their occurrence in waters receiving wastewater effluents is of great concern (Schwartz et al., 2003).

Human and animal pathogenic and potentially pathogenic bacteria are constantly released with wastewater into the water environment and many of these organisms harbour antibiotic resistant genes (Huang et al., 2011; Baquero et al., 2008). Activated sludge system of the waste water treatment plants act as suitable habitat
where bacteria from many different individuals have the opportunity to interact and are able to spread their resistant genes to water-indigenous bacteria. These bacteria thus become a reservoir of resistant genes (Fatta-Kassinos et al., 2011). The emergence of resistance to all classes of antibiotics in previously susceptible bacterial pathogens is a major challenge to infectious disease medicine (Wright, 2010). Plasmid mediated dissemination of antibiotic resistance genes takes place in many environmental compartments (Merlin et al., 2011). Wastewater treatment processes reduce the number of bacteria. However, some wastewater treatment processes may sustain multiple antibiotic resistant microbial communities (Merlin et al., 2011) because chlorination of drinking water and wastewater affects the selection of antibiotic resistant strains (Huang et al., 2011). Antibiotic resistant bacteria are emerging as important waterborne contaminants (Li et al., 2009) and therefore water acts as a vehicle in the dissemination of the antibiotic resistant organisms among human and animal populations (Baquero et al., 2008). It has been speculated that the waste water treatment process may not effectively remove resistant organisms.

1.10 Emergence of resistant bacteria in drinking water

The wide use and abuse of antibiotics in human therapy has contributed to the emergence of antibiotic resistant bacteria, especially in aquatic environments. This can potentially increase the number of infections each year and is associated with the emergence of more virulent bacterial pathogens (Kim and Wei, 2007). Occurrence of resistant bacteria in the distribution system or in biofilms in water may transfer resistant genes to non-resistant pathogens (Wright, 2010) and introgression to gut micro flora when ingested. Most resistance genes found in pathogens are acquired through horizontal gene transfer via mobile genetic elements such as plasmids (Wright, 2010), conjugation, transduction and transformation (Zhang et al., 2009).

Moreover, recent and emerging antibiotic resistance to various classes and combinations of antibiotics seems to be common in different species of Pseudomonas (Kim and Wei, 2007; Wright, 2010) and Aeromonas (Figueira et al., 2011). Different studies have shown that antibiotic resistant bacteria widely occur in different environments (Licht et al., 2003; Yoo et al., 2003; Mukherjee and
Chakraborty, 2006; Volkmann et al., 2007). Water borne bacterial infections are of concern because they are becoming increasingly common. Moreover, their antibiotic resistance is particularly alarming (Huang et al., 2011). Untreated drinking water is frequently used in the rural parts of many countries for drinking, cooking, bathing and irrigation and this water is frequently overlooked as a source of antibiotic resistance in developed countries (Macedo and Freitas, 2011). Hence untreated drinking water may be reservoirs of or vehicles for antibiotic resistant bacteria and genes and can be disseminated to humans through water ingestion.

1.11 Principles of methods used to study bacteriological quality of water
In order to assess water quality, indicator and pathogenic microorganisms should be monitored periodically. The latter group is diverse and expensive to monitor regularly. Total coliforms, faecal coliforms and heterotrophic bacteria are indicator organisms and are generally recommended for assessment of the microbiological safety and the potential occurrence of pathogens in potable water (DWAF, 1996, South African Water Quality Guidelines; Pavlov et al., 2004; Pereira et al., 2009; SANS 241: 2011). Level of indicator bacteria in water is an indication of faecal pollution and possible presence of human pathogens. Survivals of these organisms depend on the physico-chemical parameters (DWAF, 1996, South African Water Quality Guidelines).

1.11.1 Conventional methods
Presence of coliforms and faecal coliforms could be detected in water systems using multiple tube fermentation technique to estimate the most probable number (MPN) (Cochran, 1950), pour plate, spread plate (Hoa et al., 2011), membrane filtration, presence-absence test (Standard methods, 9610; Lye and Dufour, 1991), ColilertTM defined substrate test etc. (Pereira et al., 2009). Faecal coliforms using mFC agar and total coliforms are identified using mEndo agar (APHA et al., 2005; Lye and Dufour, 1991; Schraft and Watterworth, 2005). In order to isolate faecal coliforms and total coliforms, 100 ml of the samples can be filtered through membrane filters with 0.45 μm pore size filters and place the filters on mFC agar and mEndo agar. Identification can be done after incubation at 44.5°C for mFC and 35°C for mEndo agar for 24 h (da Silva et al., 2007; da Silva et al., 2008). Faecal coliforms will form
blue colonies on mFC agar and metallic sheen colonies of total coliforms will be observed on mEndo agar.

*Aeromonas* and *Pseudomonas* species are natural inhabitants of aquatic environments worldwide and have been isolated from a variety of sources including wild and domestic animals, diarrhetic and asymptomatic humans as well as drinking water and various foods (Jeppesen, 1995). Several selective media are proposed for the detection of *Aeromonas* species and *Pseudomonas* species from environmental samples. These include Starch ampicillin agar (SSA), Rippey Cabelli agar (mA), ampicillin bile salts inositol xylose (MIX) agar, ampicillin dextrin agar (ADA), dextrinfuchsin sulphite agar (DFS), bile salts irgasan brilliant green agar (BIBG) ampicillin sheep blood agar (ASBA) and starch glutamate ampicillin penicillin C-glucose agar (SGAP-10C) (Jeppesen, 1995; Gobat and Jemmi, 1995). Ampicillin prevents growth of most of the competing faecal flora while allowing efficient recovery of *Aeromonas* species.

M-Aeromonas selective agar which contains ampicillin, sodium deoxycholate and ethanol as selective agents and trehalose as a differential agent is recommended for the detection of *Aeromonas* species in water sample by the membrane filter technique. Suspected colonies on mA agar are yellow (Rippey and Cabelli, 1979). In this study also Aeromonas selective agar is used for the selection of *Aeromonas* and *Pseudomonas*. Membrane filters through which water samples have been passed are aseptically placed on M-Aeromonas selective agar base plates. After incubation at 35-37°C for 24 h presumptive *Aeromonas* species appear as large, yellow colonies and green colonies were selected as presumptive *Pseudomonas* species.

Membrane filtration uses different selective media for the isolation of targeted organisms. The membrane filtration procedure is advantageous as large volume of samples can be processed to increase assay sensitivity, filters can be transferred between different media, good reproducibility and single step result is possible. However, it has its own drawbacks also. High turbidity of water limits volumes sampled, high populations of background bacteria causes overgrowth and metals and phenols can absorb to filters and inhibit growth (Schaft and Watterworth, 2005). Another limitation is the length of time required to complete the identification of the
organism (Yáñez et al., 2006). The MPN technique requires at least four days for the completion of the whole process. Presence absence test uses broth containing lactose broth, lauryl tryptose broth and bromcresol purple indicator. Positive test result produces yellow colour which indicates the production of acid.

Colilert™ defined substrate test uses a specialized medium which contain two nutrients, o-nitrophenyl-β-D-galactopyranoside (ONPG) and 4-methylumbelliferyl- β-D-glucuronide (MUG) (Chao, 2006). The development of the Defined Substrate Technology using 4-methyl-umbellfieryl-β-D-glucuronide (MUG) as the substrate enabled the rapid, specific and simple identification of β-glucuronidase. This enzyme is present in >95% of all the E. coli isolates tested (Rice et al., 1990). Hence, E. coli can now be effectively used as a bio-safety indicator for drinking water. In presence of coliforms the medium will turn yellow with in 24 hours at 35°C due to the hydrolysis of ONPG by β-galactosidase, which releases o-nitrophenol. When E. coli is present, the MUG is metabolized by β-glucuronidase to yield a fluorescent product (Graves et al., 2007). If the test is negative the water is considered acceptable for human consumption (Geissler et al., 2000). This method offers several advantages such as shortened analysis and response time and no interference of particular matter with the reading results (Pereira et al., 2009). Inspite of the advantages, the major disadvantage is the cost involved (Yáñez et al., 2006).

1.11.2 Polymerase chain reaction (PCR) and virulence determination

The evaluation of the diversity, community structure, virulence factor and antibiotic resistance in environmental samples with conventional methods are laborious and not always sufficient (Sanz and Köchling, 2007). To circumvent some of the problems associated with conventional techniques, molecular tools have been used to provide information regarding the identification and detection of specific genes (Rousselon et al., 2004). PCR based assays are specific, reliable and results can be obtained rapidly (Dang et al., 2006; Fontes et al., 2010; Nawaz et al., 1997; Sartory and Watkins, 1999; Tan, 2002) and based on these observations, this study employs PCR based assays since they are specific and reliable.

PCR has also been used in clinical diagnosis of infection, identification of a certain known disease molecular marker, or amplification of a specific gene and
identification of mutation or polymorphism. Detection and quantification of non-culturable bacteria can be achieved by the application of DNA extraction and PCR (Khan and Yadav 2004). Several PCR protocols have been used for the detection of Aeromonas and Pseudomonas species from clinical and environmental samples (Lavenir et al., 2007; Anuj et al., 2009; Yogananth et al., 2009; Revetta et al., 2010; Kaszab et al., 2011). Some of these protocols employ the use of primer sets that amplify the different virulent molecular markers (aerA, hylA, exoA, exoS, exoT, ecfX) that are carried on the bacterial chromosome and are used as a means of identification (Lavenir et al., 2007; Anuj et al., 2009; Yogananth et al., 2009; Kaszab et al., 2011). These PCR assays are specific for the identification of Aeromonas and Pseudomonas species and hence can be effectively employed in the genotypic detection of these pathogens. Moreover, duplex real time PCR assays have been developed to facilitate the detection of Pseudomonas species (Anuj et al., 2009) and multiplex PCR to determine the distribution of different types of multidrug resistance (Yoo et al., 2003).

1.12 Problem statement

In developing countries sustained access to safe and clean drinking water is a challenge. Quality of drinking water is often related to the physico-chemical parameters and the degree of bacterial contamination. As pressures mount on limited water resources water reuse practice is an alternative to address the problem of water scarcity. Releasing of inadequately treated waste water from sewage treatment plant to surface water is a major source of bacterial pathogens and various chemical pollutants including antibiotics and illicit drugs. This in turn increases exposure of environment and humans to pathogenic microorganisms may have profound consequences on human health. It is well known that water constitutes an important contamination route for microorganisms and outbreaks of infections result from consumption of water contaminated with bacterial pathogens. Constant exposure of bacterial population to antibiotics can increase resistance to antibiotics of clinical interest and compromise drug therapy. Furthermore, infections caused by antibiotic resistant pathogenic organisms harbouring virulent genes can be fatal.

Potable water quality can deteriorate immensely along the distribution system due to numerous factors. One of the reasons is the development of bacterial biofilms inside
the pipes in the reticulation system. Microorganisms survive the treatment procedures and enter into drinking water bulk distribution system, sustained by organic and inorganic nutrients present within the pipe and develop into biofilm. Sloughing off of organisms can cause significant drop in water quality at the point of usage.

Modimola dam in Mmabatho is one of the water sources which supplies drinking water to the community receives treated sewage effluent from Mmabatho sewage treatment plant and is therefore under constant threat. Although majority of the pathogenic organisms can be eliminated by sewage treatment, many end up in the effluent which is then discharged into receiving waters. Therefore one of the major sources of pollution in the dam is the disposal of treated sewage effluent into the water. The pressure of water quality is also increased by the anthropogenic activities in and around both Molopo eye. Incidences of possible faecal contamination can occur from the domestic animals grazing in the vicinity of water sources. Therefore there is a great need to investigate the occurrence faecal coliforms, total coliforms and heterotrophic bacteria to assess the contamination levels and indicates the efficiency of water treatment processes. Improper treatment of raw water causes the survival of organisms and persistence in drinking water.

*Aeromonas* and *Pseudomonas* species are ubiquitous and are most often associated with fresh water. These organisms have been isolated from a variety of sources, including wild and domestic animals, humans as well as drinking water and various foods. These species have been implicated in human intestinal infections. Of considerable concern are the increasing levels of resistance displayed by both clinical and environmental isolates. There is no report available on the prevalence of *Aeromonas* and *Pseudomonas* species in drinking water in the North West Province in particular, Mafikeng is available. Eventhough there is no incidences of infections caused by these organisms have been reported in Mafikeng, North West Province, the possibilities of their occurrence and possible infections cannot be underestimated.

According to the Blue Drop report the water in Mmabatho area is not of acceptable quality and there were periods when the water did not comply with standard. The
municipality even failed to monitor and confirm the actual quality of water. Therefore
the purpose of this study is to assess the bacteriological quality of raw and treated
water from different sources and the impact of reused water on the finished water.
The identification of opportunistic pathogens in water is highly important to assist in
the control and prevention of waterborne infections and may be used to assess the
degree of risk associated with the transmission of pathogens to humans through
drinking water.

1.13 Aim of the study
The general aim of the study was to analyse the impact of physico-chemical and
bacterial parameters of source water on drinking water quality, biofilm formation and
antibiotic resistant bacteria in the drinking water distribution system in Mafikeng,
North West Province, South Africa.

1.14 Objectives of the study
The objectives of the study were:
i) To analyse the physico-chemical parameters and bacteriological quality of
source and drinking water.
ii) To isolate and characterise *Pseudomonas* and *Aeromonas* species from
drinking water distribution system.
iii) To investigate the development of biofilm in raw source and drinking water
compartments in Mafikeng.
iv) To determine the antibiotic resistance profiles of the bacterial isolates from
drinking water and biofilm.
v) To determine if virulence gene determinants are present in the isolates.
vi) To determine the impact of two raw water sources on the quality of mixed
drinking water of Mafikeng.
CHAPTER 2

Analysis of physico-chemical and bacteriological quality of drinking water in Mafikeng, South Africa

2.1 Introduction
Water is generally a scarce resource in the North West province of South Africa. With the increasing demand on water for drinking, irrigation and industrial purposes this scarce resource should be well protected and managed (Brettar and Höfle, 2008). Successful management depends on the regular monitoring of the physico-chemical and bacteriological quality of water. Potable water should be clear, not saline, and free from compounds that can cause colour, taste and odour (Pritchard et al., 2007). Bacteria, inorganic, organic and water soluble radioactive substances are considered as the major water pollutants contributing to the deterioration of water quality and responsible for various public health problems (Azizullah et al., 2011; Butiuc-Keul et al., 2012). To protect consumers from waterborne diseases, drinking water utilities should ensure that the distributed water is completely free of pathogenic or potential pathogenic microorganisms as well as harmful chemicals (Pereira et al., 2009). Potable water of poor quality can cause social and economic problems through water related epidemics (Pritchard et al., 2007).

The analysis of microbiological quality of water aims to ensure that the consumer is protected from pathogenic organisms such as bacteria, viruses and protozoa (Figuera & Borrego, 2010). Sampling and analysis of microbiological parameters must be done more frequently than physico-chemical parameters, because microbial contamination can have acute health effects on consumers (DWA, 2005). Bacteria can be used either as indicators of faecal pollution or to indicate the effectiveness of a water treatment system (Wingender & Flemming, 2011). Indicator organisms are generally used for the surveillance of the potential presence of pathogens in water.

However, absolute water quality is impossible to achieve, due to the intrusion of hazardous pollutants into the water from various sources. In drinking water quality management, faecal coliforms are used as indicators of faecal contamination and
Heterotrophic plate count (HPC) levels as a measure to indicate the effectiveness of the water purification processes (Hurst et al., 1997).

Coliform bacteria are present in the environment and faeces of all warm-blooded animals and humans. Coliform bacteria are unlikely to cause illness. However, their presence in drinking water indicates that disease-causing organisms (pathogens) that can cause diseases such as intestinal infections, dysentery, hepatitis, typhoid fever, cholera and other illnesses could be in the water system (Emmanuel et al., 2009). Their presence in drinking water can thus be seen as an indication of faecal pollution and possible deteriorating water quality (Rompré et al., 2002). There are three groups of coliform bacteria. Each is an indicator of drinking water quality and each has a different level of risk. Total coliform is a large collection of different kinds of bacteria. Faecal coliform are types of total coliform that exist in faeces. E. coli is a subgroup of faecal coliform (United State Environmental Protection Agency (EPA), 2013.

Total coliforms are a group of closely related bacteria that are (with few exceptions) not harmful to humans. Because total coliforms are common inhabitants of ambient water and may be injured by environmental stresses (e.g., lack of nutrients) and water treatment (e.g., chlorine disinfection) in a manner similar to many pathogens, EPA (2013) considers them a useful indicator of these pathogens. Health problems associated with these pathogens include diarrhea, cramps, nausea and vomiting. Together these symptoms comprise a general category known as gastroenteritis. Gastroenteritis is not usually serious for a healthy person, but it can lead to more serious problems for people with weakened immune systems, such as the very young, elderly, or immuno-compromised (EPA, 2011). Total coliforms do not necessarily indicate recent water contamination by faecal waste. However, the presence or absence of these bacteria in treated water is often used to determine whether water disinfection is working properly and also serve as a parameter to provide basic information on surface water quality (WHO, 2006).
Heterotrophic bacteria includes all bacteria that use organic nutrients for growth and Heterotrophic plate count (HPC) bacteria represent those microbes isolated by a particular method, whose variables include media composition, time of incubation, temperature of incubation and means of medium inoculation. All Heterotrophic plate count methods enumerate only a fraction or subpopulation of heterotrophic bacteria (Allen et al., 2004). A high density of heterotrophic bacteria found in treated drinking water is a cause of concerns. This is due to the fact that a wide range of water associated opportunistic pathogens which may cause health problems in humans are represented by heterotrophic bacteria (Lye and Dufour, 1991; Chowdhury, 2012). Some heterotrophic bacteria are opportunistic pathogens such as *Pseudomonas aeruginosa* (WHO, 2011), *Klebisella* and *Aeromonas* (Allen et al., 2004).

*Pseudomonas aeruginosa* is a waterborne opportunistic pathogen which may have impacts on human health, especially in immunocompromised populations (Wang et al., 2012). There is no SANS 241: 2011 standard for *Pseudomonas* in drinking water. High levels of this bacterium in water may cause taste, odour and turbidity problems (WHO, 2011). *Pseudomonas* spp. can survive extreme physical conditions (Völker et al., 2010) and is an opportunistic pathogen implicated to cystic fibrosis infections, septicemia, pneumonia, endocarditis, otitis and keratitis (Lavenir et al., 2007) infections in high risk populations (da Silva et al., 2008).

Worldwide water borne diseases are the cause of death and suffering of millions of people, especially children in developing countries (Schäfer et al., 2009). Many countries in Africa are faced with a serious shortage of drinking water. South Africa is also faced with a critical shortage of water. Therefore in some areas of the country reused water is released in to the source water which may affect the quality of the receiving water. In South Africa, bacteriological contamination is the major threat to the quality of water. Source water associated microorganisms sporadically cause infections especially to immunocompromised patients.

Mafikeng is the capital of the North West Province and has a population of about 260 000 people (Statistics South Africa, Census 2011). The potable water is obtained from two sources. The Molopo-eye is a natural spring that is situated 30 km from town. Its water is clear and the total dissolved salts (TDS) are generally very low. For
this reason no sedimentation and filtration is required. Water is thus directly chlorinated and supplied to the community. The other source is the Modimola dam which lies on the banks of Molopo River. The water works is down-stream from the waste water treatment plant. This water source is thus impacted on by the sewage works, human settlements, farming and other anthropogenic activities are prevalent in this area. However, the water samples are treated at the Mmabatho water works where chemical dosing, sedimentation, sand filtration and chlorine sanitation are the processes followed (Mmabatho water works, Modimola, Mmabatho). This water is then stored in reservoirs before mixed and supplied to the Mmabatho community.

Recent studies in Mafikeng revealed that ground water and surface water had bacteriological contamination (Wose-Kinge and Mbewe, 2010; Siri et al., 2011; Ateba and Maribeng, 2011). *E. coli* O157, a virulent strain, implicated in waterborne infections have been reportedly isolated from South African water sources intended for direct and indirect human consumption (Müller et al., 2001; Ateba and Mbewe, 2011). According to the Blue Drop report of 2010, a certification programme that was introduced by the South African Department of Water Affairs to encourage drinking water provision by municipalities, a score of only 30% was achieved. This certification process evaluates drinking water quality, as well as several management aspects including water safety plans. The blue drop score of for Mafikeng in 2011 was reduced to a dismal 8.85% (DWAF, Blue Drop Report, 2011). The Blue Drop certification process evaluates drinking water quality as well as several management aspects including water safety plans. The scores for both Mmabatho water works and Mafikeng indicate is inadequate monitoring, treatment of drinking water as well as several management aspects that are being neglected. The drinking water standard did not comply with the drinking water quality proposed by WHO (2008), SANS 241: (2011) and DWAF (2012) and. Noncompliance to national legislation SANS 241, DWAF and WHO standards pose a significant risk of infection. According to Blue Drop report there is no information about the microbiological and chemical compliance of drinking water in Mmabatho/Mafikeng drinking water. For this reason a study on the chemical and microbiological quality of drinking water in this area is necessary.
This study was conducted with the aim of assessing the physico-chemical and microbial quality of drinking water that were obtained from Modimola dam, Molopo eye. A secondary aim was to determine whether the water produced from these sources had a negative impact on the quality of the mixed water.

2.2 Materials and methods

2.2.1 Sampling sites
Several sampling points were selected located along the distribution system. Source water is obtained from two different sites. One of them is Molopo eye which is a natural spring where human settlements and other anthropogenic activities are prevalent. Modimola dam is the other catchment area which receives effluent from Mmabatho sewage treatment plant. Treated water from both sources are mixed in Signal hill reservoir and distributed to some areas in the city.

2.2.2 Physico-chemical parameters
The physico-chemical parameters of the water were analysed three times a week for four months on water samples collected from three sites which received treated water from Modimola dam, Molopo eye (Mafikeng) and mixed water (Mmabatho) from these two sites. Temperature was measured at the sites and water samples for other parameters were collected in sterile Schott bottles, immediately stored on ice in a cooler box and transported to the laboratory. Physico-chemical parameters analyzed were pH, TDS and temperature using Crison pH 25 multimeter (Corison Instruments, South Africa).

2.2.3 Sample collection and isolation of bacterial consortia
For the bacteriological analysis water samples were collected from the Modimola dam, Molopo eye before and after treatment as well as the mixed water (Fig. 2.1). Treated water was collected from taps in the city. At each of the three different sites two taps were used as sampling points. One tap had a point of use carbon filter connected. Each of the sample points was supplied either by water from one of the sources or mixed water.

Collection of samples was from August to November 2010. Water samples were collected in sterile Schott bottles, immediately stored on ice in a cooler box,
transported to the laboratory and analysed within 3-6 hours. For all the samples, 100 ml were filtered through 0.45 µm pore sized filter (cellulose nitrate membranes, 45 µm diameter, Whatman Laboratory Division, Maidstone, England) using a membrane filtration unit and vacuum pump (model Sartorius 16824). These membranes were aseptically placed on petridishes containing appropriate selective media, such as mFC agar, mEndo agar and Aeromonas selective media (Biolab, Merck, South Africa). All the media was prepared according to the manufacturer’s instruction (Biolab, Merck, South Africa). The membranes were placed on the agars ensuring that no air bubbles were trapped. The selective media used are as follows; mFC agar used as a selective medium for faecal coliforms, mEndo for total coliforms, nutrient agar for heterotrophic bacteria and Aeromonas selective medium for *Aeromonas* and *Pseudomonas*. In order to isolate heterotrophic bacteria 100 µl of the treated water samples were spread on to the nutrient agar plates. Water samples from the dam and Molopo eye were serially diluted and 100 µl of the 5-fold serial dilutions was spread on to the nutrient agar plates.

The plates were incubated at 35°C except for mFC agar which was incubated at 45°C for 24 hours. The results were expressed as the number of faecal coliforms, total coliforms, *Pseudomonas* and *Aeromonas* in 100 ml of water and heterotrophic bacteria in 1 ml of water. Blue colonies from mFC agar (presumptive coliforms), metallic-sheen colonies from mEndo agar (presumptive total coliforms) and yellow (presumptive *Aeromonas*) and green colonies (presumptive *Pseudomonas*) from Aeromonas selective media were enumerated. The results for faecal coliforms, total coliforms, and *Pseudomonas* were expressed as number of colony forming units per 100 ml of water and the results for Heterotrophic bacteria were expressed in number of colony forming units per 1 ml of water.
2.3 Statistical analysis
The data obtained were subjected to statistical analysis using Excel 2007 (Microsoft) for mean average and standard deviation and SPSS (version 14.0) programme for Pearson’s correlation. Pearson’s correlation product of the moment was used to determine the correlation between EC, TDS, pH and temperature. The two tailed test of significance (p<0.05) was used in order to determine the significance of the result.

2.4 Results and Discussion
The physico-chemical parameters and microbiological quality of drinking water in Mafikeng in the North West province in South Africa were analysed over a period of four months. Results were compared to WHO (2008), SANS 241: (2011) and DWAF (2012) drinking water quality guidelines (Table 2.2) to ascertain if the quality of the drinking water is in accordance with the appropriate drinking water standards. In South Africa (SA), the quality of the domestic supply that is considered safe for human consumption is assured by monitoring for compliance with the South African National Standard (SANS 241: 2011).
2.4.1 Physico-chemical parameters

Table 2.1 represents the minimum, maximum and standard deviation of pH, TDS and temperature of the water samples tested. Chlorine level ranged from 0.2 – 0.7 mg/l.

**Table 2.1: Maximum, minimum and standard deviation of physico-chemical parameters of treated water in each site tested over the study period between August to November 2010**

<table>
<thead>
<tr>
<th>Site</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>TDS (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max</td>
<td>Min</td>
<td>Ave ±SD</td>
</tr>
<tr>
<td>Molopo eye derived water</td>
<td>8.1</td>
<td>5.7</td>
<td>7.7 ±0.18</td>
</tr>
<tr>
<td>Modimola dam derived water</td>
<td>8.7</td>
<td>8.0</td>
<td>8.3 ±0.15</td>
</tr>
<tr>
<td>Mixed water</td>
<td>8.6</td>
<td>5.6</td>
<td>7.9 ±0.14</td>
</tr>
</tbody>
</table>

SD = Standard deviation, Min = Minimum, Max = Maximum, Ave=average, TDS=Total Dissolved Salts

**Table 2.2: Recommended limits for no risk**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>pH</th>
<th>TDS (mg/l)</th>
<th>TC cfu/100ml</th>
<th>FC cfu/100ml</th>
<th>HPC cfu/1ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO (2008)</td>
<td>6.5-8.5</td>
<td>1000</td>
<td>0</td>
<td>0</td>
<td>≤1000</td>
</tr>
<tr>
<td>DWAF(2012)</td>
<td>6.0-9.0</td>
<td>≤1000</td>
<td>0</td>
<td>0</td>
<td>≤1000</td>
</tr>
<tr>
<td>SANS 241 (2011)</td>
<td>6.0-9.0</td>
<td>≤1200</td>
<td>0</td>
<td>≤10</td>
<td>≤1000</td>
</tr>
</tbody>
</table>

TC=Total coliform, FC=Faecal coliform, HPC=Heterotrophic plate count bacteria

All the values were within the accepted range for no health risk as proposed by WHO, DWAF and SANS 241 (Table 2.2). Water temperature ranged from 18.3 to 25.3°C which would pose no adverse effects on living organisms. Temperature is the
main factor which affects almost all physico-chemical equilibriums and biological reactions (Delpla et al., 2009). Water temperature can have a direct or indirect effect on physical parameters (LeChevallier et al., 1996). It can influence the pH, dissolved oxygen, redox potentials and microbial activity (Park et al., 2010). Temperatures in this range and higher enhance microbial activity and other chemical reactions (Pritchard et al., 2007). This would encourage biofilm formation and regrowth potential in the distribution system which could serve as an environmental reservoir for pathogenic microorganisms (Wingender and Flemming, 2011).

It was observed that the pH and TDS concentration was higher in the Modimola dam derived (pH 8.0 to 8.7; TDS 226.3 to 364.4 mg/L) than in the Molopo eye derived water (pH 5.7 to 8.1; TDS 159.9 to 238.6 mg/L). Biological and anthropogenic activities can give rise to pH and TDS fluctuations. The elevated pH and TDS in the Modimola dam derived water could be attributed to the wastewater effluent released into the dam from the sewage treatment plant where lime is used for treatment. The low values of water derived from the Molopo eye impacted positively the quality of mixed water.

The pH of water is a reflection of the degree of acidity (pH lower than 7) or alkalinity (pH greater than 7). The pH of most unpolluted water lies between 6.5-8.5 and pH is an important operational water quality parameter (WHO, 2011). The taste of water, its corrosiveness and solubility and speciation of metal ions are all influenced by pH. At low pH water may taste sour while at high pH water taste bitter or soapy (DWAF, 2006). Water with a low pH level may cause corrosion in galvanised or copper pipes (DWAF 1998). The main significance of pH in domestic water supplies relates to its effects on water treatment process. There is no health consequences attributed to pH of water, except at extreme values. The direct health effects of low and high pH levels include acid and alkali burns, respectively. These extreme pH levels may also cause irritation of the mucous membranes (DWAF, 1998). The main significance of pH in domestic water supplies relates to its effects on water treatment process. To ensure effective disinfection, the pH levels must be controlled when disinfection products are added (WHO, 2011). Total heavy metal content in water could increase at low pH which is a matter of public concern (Virkutyle and Sillanpää, 2006).
Parameter variations were not always statistically significant as a result of occasional peaks that occurred. Pearson’s correlation analysis showed a significant (p<0.05) positive correlation (r = 0.99) between TDS and temperature in water from the Molopo eye and the mixed water (r = 0.928, p = 0.000). The same was not true for the water derived from the Modimola dam. Furthermore, TDS values for the mixed water was lower than the Modimola dam derived water but higher than the Molopo-eye derived water (Table 2.1). It thus appears as if the Molopo-eye derived water diluted the Modimola dam derived water. A strong positive correlation (r = 0.931, p = 0.000) between pH from Molopo eye water and mixed water was observed, but a weak negative correlation (r = 0.360, p = 0.170) observed between TDS from both sites. Modimola dam pH and TDS showed a weak negative correlation to pH and TDS of mixed water. For pH and temperature, significant (p<0.05) positive correlations (r = 0.78 to 0.99) for treated water from all three water types were calculated. There was also a general significant (p<0.05) correlation between pH and TDS measurements for water from the various samples.

2.4.2 Bacteriological quality
Total coliforms (TC), faecal coliforms (FC) and Heterotrophic plate count (HPC) bacteria are indicator organisms and 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). TC bacteria and Heterotrophic plate count (HPC) are primarily used to determine the general hygienic quality of water and to evaluate the efficiency of treatment procedures. FC bacteria are used to detect faecal pollution and pathogens are associated with faecal pollution (Lin et al., 2004; Rousselon et al., 2004). Pseudomonas spp. is common inhabitants of aquatic environments, including drinking water (Vaz-Moreira et al., 2012). This ubiquitous genus includes species considered opportunistic pathogens that can colonize animals and humans (Mena and Gerba, 2009). Periodical enumeration of Pseudomonas is vital to assess the drinking water quality (da Silva et al., 2008). If contaminated water is consumed these organisms may cause diseases such as gastroenteritis, dysentery, cholera and typhoid fever (Azizullah et al., 2011). Results of the microbiological tests conducted during the study period are given in Table 2.3.
<table>
<thead>
<tr>
<th>Site</th>
<th>H (cfu/1ml)</th>
<th>TC (cfu/100ml)</th>
<th>FC (cfu/100ml)</th>
<th>P (cfu/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max</td>
<td>Min</td>
<td>Ave±SD</td>
<td>Max</td>
</tr>
<tr>
<td>Molopo eye</td>
<td>&gt;100</td>
<td>0</td>
<td>&gt;73±44.7</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Raw water</td>
<td>&gt;100</td>
<td>0</td>
<td>&gt;12±30.3</td>
<td>0</td>
</tr>
<tr>
<td>Unfiltered water</td>
<td>&gt;100</td>
<td>0</td>
<td>&gt;12±30.3</td>
<td>0</td>
</tr>
<tr>
<td>Filtered water</td>
<td>50</td>
<td>0</td>
<td>3.4±12.9</td>
<td>0</td>
</tr>
<tr>
<td>Modimola dam</td>
<td>&gt;100</td>
<td>1</td>
<td>85±34.4</td>
<td>100</td>
</tr>
<tr>
<td>Raw water</td>
<td>&gt;100</td>
<td>0</td>
<td>33.3±46.8</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Unfiltered water</td>
<td>&gt;100</td>
<td>0</td>
<td>33.4±48.8</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Filtered water</td>
<td>&gt;100</td>
<td>0</td>
<td>33.4±48.8</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Mixed water</td>
<td>60</td>
<td>0</td>
<td>4.13±15.5</td>
<td>0</td>
</tr>
<tr>
<td>Unfiltered water</td>
<td>9</td>
<td>0</td>
<td>0.6±2.3</td>
<td>0</td>
</tr>
<tr>
<td>Filtered water</td>
<td>9</td>
<td>0</td>
<td>0.6±2.3</td>
<td>0</td>
</tr>
</tbody>
</table>

(H=Heterotrophic bacteria, TC=Total coliform, FC=Faecal coliform and P=Pseudomonas)
The WHO (2008) and DWAF (2012) standards for drinking water quality limit is 0 cfu/100ml for TC and FC. Raw water in both sites is heavily contaminated with all the organisms tested for. Similar results were observed by Chidya et al., (2011), Moulin et al., (2010) and Schraft and Watterworth, (2005). This result may imply that TC and FC bacteria are present in water subjected to faecal pollution. Pollution may have been caused by sewage effluent released into the dam and herds of cattle and sheep grazing around the dam and also pollutants being washed to the dam by rain water. Input of untreated or partly treated waste water cause fresh water pollution (Thevenon et al., 2011). We have noticed an increase in the TDS and bacteria in Modimola dam, the most extensive increase observed was the Pseudomonas counts. Occurrence of nutrients enhances bacterial growth in the distribution system (Lehtola et al., 2004).

In this study we have observed that both unfiltered and filtered treated water samples from all sites possessed high Pseudomonas and heterotrophic bacteria (>100 cfu/mL). This observation was in accordance with the findings of Völker et al., (2010), Ghizellaoui, (2008) and da Silva et al., (2008). There were no significant changes in the number of Pseudomonas in filtered and unfiltered water samples and during the sampling period. The observation demonstrated that carbon filters did not effectively removed Pseudomonas. This result is comparable with the ineffectiveness of filters in the removal of bacteria demonstrated by Fengyi et al., (2009). In Modimola dam water we have observed elevated levels of heterotrophic bacteria, TC and Pseudomonas but significantly low levels of FC. There was no reduction in the number of any of these organisms when the levels in the filtered and unfiltered water were considered. However, TC and FC were not observed in the treated water from the Molopo eye and mixed water.

Lower levels of heterotrophic bacteria were observed in mixed treated water compared to the Modimola dam treated water. The low levels of these organisms in the mixed water could potentially be attributed to the impact of Molopo eye derived water. However, elevated levels of heterotrophic bacteria and Pseudomonas were observed in treated water samples from all sites. In a similar study conducted by
Feretti et al., (2007) and IFOWAHB (2009), drinking water samples were contaminated with HPC could be attributed to the presence of nutrients in the water.

The trends of *Pseudomonas* from all sites were similar at all sampling periods and the exceptionally high level of *Pseudomonas* is a cause of concern. *Pseudomonas* spp. can survive extreme physical conditions (Völker et al., 2010) and is an opportunistic pathogen implicated to cystic fibrosis infections, septicemia, pneumonia, endocarditis, otitis and keratitis (Lavenir et al., 2007) infections in high risk populations (da Silva et al., 2008). The number of *Pseudomonas* in the filtered water was still very high. This could be attributed to the ability of *Pseudomonas* to form biofilm (Moritz et al., 2010; Waszczuk et al., 2010). Fengyi et al., (2009) demonstrated the formation of biofilms inside filters affecting the bacterial quality of the effluent water. Filter could retain bacteria inside and the trapped organic matter within the filter support the growth of biofilm. Periodical enumeration of *Pseudomonas* is vital to assess the drinking water quality (da Silva et al., 2008).

The results suggest that the counts of faecal coliforms, total coliforms and heterotrophic bacteria of Modimola dam and Molopo eye during this study period were significantly lower than those reported by Germs et al., (2004). High levels of heterotrophic bacteria were observed in raw water and treated water from Modimola dam. The Modimola dam receives sewage effluent and occurrence of high levels of heterotrophic bacteria entails that treatment process at the sewage treatment plant failed to eliminate bacteria successfully before discharge. Water associated opportunistic pathogens have raised concerns about high densities of heterotrophic bacteria found in both potable source waters and treated drinking waters (Lye and Dufour 1991). Opportunistic pathogens express activities which by themselves are not sufficient to cause disease, unless interacting with a host that is debilitated or whose body defence system has been compromised in some way (Lye and Dufour 1991). Pereira et al., (2009) and (1995) detected high levels of total coliforms and *E. coli* spring water and in all surface water. Occurrence of *E. coli* 0157:H7 was reported in South African waters (Müller et al., 2001; Ateba and Mbewe, 2011). This bacterial strain is responsible for bloody and non-bloody diarrhoea, haemorrhagic colitis and haemolytic uremic syndrome (Tozzi et al., 2003; Olsen et al., 2002). The level of contamination can also be compared to those reported by Lin et al., (2004).
Drinking water that failed to meet the standards set by WHO (2008), SANS 241: (2011) and DWAF (2012) guidelines and failure to comply with bacteriological standards demonstrate that this water is not safe for human consumption. The supply of clean drinking water is a major public health milestone (Berry et al., 2006). One of the most important requirements for domestic water is that the water should be safe to drink (Schutte, 2006; Momba et al., 2009). Studies have shown that water purification plants in South Africa may not always produce the quality and quantity of drinking water they are designed for (Momba et al., 2006). To determine if the water provided to communities is safe to drink the physico-chemical and microbiological analysis of water must be performed.

2.5 Conclusion

Increased TDS and microbial numbers can cause drinking water quality deterioration and it is a major problem faced. The quality of drinking water distributed to the consumers must the standard for physico-chemical and microbial quality. The result presented in this study is not unique as similar results were demonstrated by other researchers. The result of this study revealed that the physico-chemical parameters of drinking water were constant over time and within the limits set by WHO and DWAF and this would pause no significant threat to domestic use. However, bacteriological quality of the water was unacceptable as the study demonstrated the wide spread occurrence of *Pseudomonas* and heterotrophic bacteria. A comparison of the levels of organisms in the drinking water from each site demonstrated an increased level in the treated water from the Modimola dam and low in mixed water, attributed to the impact of Molopo eye water on mixed water. Modimola dam is the main supply for drinking water to Mmabatho community and it receives treated waste water which accounts for the increased levels of bacteria. Waste water treatment plant can have a significant impact on the microbiological quality of source water. Moreover, *Pseudomonas* and heterotrophic bacterial counts were high in all the drinking water samples tested. These organisms can harbour pathogenic bacteria and opportunistic pathogens. This episode can cause adverse health effects in humans. To protect consumers from waterborne pathogens, drinking water supplied to the consumers should be free of pathogens. Therefore it is recommended to evaluate the levels of *Pseudomonas* periodically in addition to the routine faecal indicator check. Based on the results presented herein, we suggest that the
prevalence of bacteriological pollutants in the drinking water ratify ineffectiveness of chlorine to eradicate microorganisms from the drinking water. Therefore it is recommended that periodical monitoring of drinking water should be carried out to assess the levels of microorganisms in drinking water.
CHAPTER 3

Isolation of environmental bacteria from surface and drinking water in Mafikeng, South Africa and characterization using their antibiotic resistance profiles

3.1 Introduction

Water is considered as a vehicle for the propagation and dissemination of human associated bacteria (Faria et al., 2009). Safe drinking water is a fundamental human right and if contaminated with opportunistic pathogenic environmental bacteria it may have health implications for consumers (Fawell and Nieuwenhuijsen, 2003; WHO, 2004). Human health should therefore be protected by preventing microbial contamination of water that is intended for consumption (Volker et al., 2010). In rural communities untreated surface water from rivers, dams and streams is directly used for drinking and other domestic purposes (Biyela et al., 2004). These unprotected water sources can be contaminated with microbes through rainfall run-offs, agricultural inputs, mixing with sewage effluents and faeces from wildlife (Obi et al., 2002; Sharma et al., 2005) which renders them unacceptable for human consumption. Faecal coliforms, Aeromonas and Pseudomonas are used as indicators of faecal contamination in water (Webster et al., 2004). These pathogens may have severe health implications on consumers especially those that are immuno-compromised (Dumotent et al., 2000; Biyela et al., 2004; Pavlov et al., 2004).

South Africa is a semi-arid country with very low rainfall and high evaporation (Eberhard and Robinson, 2003). There is scarcity for fresh water systems in the country due to the highly variable and spatial distribution of rainfall (Adewumi et al., 2010). Moreover, safe drinking water is used for non-drinking applications such as irrigation, toilet and urinary flushing (Adewumi et al., 2010). This practice is usually not sustainable. To manage the existing water resources and to address some of the challenges associated with water shortages in South Africa and the world at large, waste water reuse can form an important component of water demand management (International Water Association (IWA), 2008). Despite this waste water reuse may
affect the quality of drinking water if proper treatment procedures are not implemented. Waste water reuse has been extensively implemented in some European and African countries (IWA, 2008). However, in South Africa, only a few waste water reuse schemes have been documented (Mckenzie et al., 2003; Dimitriadis, 2005) and there is limited implementation of this alternative in communities.

There is an alarming increase in the consumption of antibiotics through human therapy and agricultural process (Vaseeharan et al., 2005; Martinez, 2009). This extensive usage of antibiotics in both human and animal medicine has resulted in the development of antibiotic resistant bacteria and affects treatment of infections (Akram et al., 2007; Luczkiewicz et al., 2010). Antibiotic resistance has therefore become a major public health issue (Moore et al., 2010) and their presence in waste water, surface water and drinking water is well documented (Schwartz et al., 2003; Lobova et al., 2008; Pathak and Gopal, 2008; Luczkiewicz et al., 2010; Moore et al., 2010). The hazard associated with the pathogenicity of microbes is aggravated by its ability to resist destruction by antibiotics. Biological treatment process in a waste water treatment plants may result in a selective increase of antibiotic resistant bacteria and therefore increase occurrence of multidrug resistant organisms (Zhang et al., 2009). Although microorganisms in drinking water are reduced by chlorination, they may survive the treatment process and enter the distribution system (Faria et al., 2009). Moreover, the presence of antibiotic resistance in microorganisms has been previously reported. (Mulamattathil et al., 2000; Lin and Biyela, 2005; Wose Kinge and Mbewe, 2010). Considering the fact that the public health of a community may be related to the quality of treated waste water supplied and that public health can be protected by reducing the pathogenic microorganisms in drinking water, the present study was designed to isolate environmental bacteria from surface and drinking water in Mafikeng. A further objective was to characterise the isolates using their antibiotic resistance profiles. Determining the antibiotic resistant phenotype of bacteria present in water will provide suggestions that could help in the control of antibiotic resistance.
3.2 Materials and methods

3.2.1 Study area

Water samples were collected around Mafikeng and which includes five sampling points. Raw water from Molopo eye, treated water from Molopo eye, raw water from Modimola dam, treated water from Modimola dam and mixed water. These sampling points were chosen for the study because water from Molopo eye and Modimola dam, after purification, is used for human consumption, recreational, agricultural and industrial purposes. Around Molopo eye and Modimola dam, few small scale farmers live there and the dam receives water after treatment from the sewage plant. Modimola dam receives treated sewage effluent from the sewage treatment plant and sewage effluent is the major source of pathogens. It is therefore important to investigate the microbiological quality of water at these points.

3.2.2 Sampling

Raw and treated water samples were collected during a one year period, in February, April, July and October, to cover the four different seasons. Water samples from the Molopo eye and Modimola dam were collected aseptically in sterile 500 ml Duran Schott glass bottles from different sampling points by directly dipping the bottles into the surface of the water. Purified water samples are collected directly in to the sterile bottles from the tap, after letting the tap to run for a minute. The samples were labelled properly and transported on ice to the laboratory for analysis. Aliquots of the samples were used for selective isolation of faecal coliforms, total coliforms, Aeromonas, Pseudomonas and heterotrophic bacteria based on standard microbiological procedures (SANS 241: 2011).

3.2.3 Isolation of planktonic bacteria by membrane filtration

For all the samples, three volumes of 100 ml were filtered through 0.45 µm pore sized filter (cellulose nitrate membranes, 45 µm diameter. Whatman Laboratory Division, Maidstone, England) using a water pump (model Sartorius 16824). These membranes were aseptically placed upon plates with appropriate selective media, such as mFC agar, mEndo agar and Aeromonas selective media. Each sample was analysed in triplicate. In order to isolate heterotrophic bacteria 1 ml of the treated water samples were spread on to the nutrient agar plates. Water samples from the dam and Molopo eye were serially diluted and 1 ml of the 5-fold serial dilutions was
spread on to the nutrient agar plates. All the media were prepared according to the manufacture’s instruction. The membranes were placed on the agars ensuring that no air bubbles were trapped. The selective media used are as follows. mFC agar used as a selective medium for faecal coliforms, mEndo for total coliforms, nutrient agar for heterotrophic bacteria and Aeromonas selective medium for Aeromonas and Pseudomonas.

The plates were incubated at 37°C except for mFC agar which were incubated at 45°C for 24 hours. The colonies were enumerated, characterized and recorded. The results were expressed as the number of faecal coliforms, total coliforms, Pseudomonas and Aeromonas in 100 ml of water and heterotrophic bacteria in 1 ml of water. Blue colonies from mFC agar (presumptive coliforms), metallic-sheen colonies from mEndo agar (presumptive total coliforms) and yellow (presumptive Aeromonas) and green colonies (presumptive Pseudomonas) from Aeromonas selective media were picked for further work (Biolab Catalogue).

3.2.4 Purification of colonies
Colonies were purified by sub-culturing twice using streaking plate method. Young cultures were used for Gram staining and all isolates were identified as Gram negative bacilli. All the Gram negative isolates were subjected to primary and secondary biochemical identification tests. The bacteria that were picked to create antibiograms were streaked on to nutrient agar slants to make sample cultures and for PCR purpose.

3.2.5 Antimicrobial susceptibility testing
An antibiotic susceptibility test was performed using Kirby-Bauer disk diffusion method (Kirby et al., 1966). The following antibiotic discs (Mast Diagnostics-United Kingdom) at the final concentrations that are indicated were used; ampicillin (AP) 10µg, cephalothin (KF) 5 µg, streptomycin (S) 10 µg, erythromycin (E) 15 µg, chloramphenicol (C) 30 µg, neomycin (NE) 30 µg, amoxyccillin (A) 10 µg, ciprofloxacin (CIP) 5 µg, trimethoprim (TM) 25 µg, kanamycin (K) 30 µg, oxytetracycline (OT) 30 µg. These antibiotics were chosen because they are either used in both human and animals or they have been reported to be resistant in previous studies (Ateba and Bezuidenhout, 2008).
Three colonies were picked from each sample and each colony was transferred in to 3 ml sterile distilled water to prepare bacterial suspension. Aliquots of 1000μl from each suspension were spread-plated on Mueller-Hinton agar plates. Antibiotic discs were applied on to the plates using sterile needles and the plates were incubated at 37°C for 24 hours (National Committee for Clinical Laboratory Standards (NCCLS), 1999). After incubation the antibiotic inhibition zone diameters (IZD) were measured. Results obtained were used to classify isolates as being resistant, intermediate resistant or susceptible to a particular antibiotic using standard reference values according to National Committee for Clinical Laboratory Standards (NCCLS, 1999). MAR phenotypes were generated for isolates that showed resistance to 3 or more antibiotics.

3.2.6 Primary identification tests

3.2.6.1 Triple sugar iron (TSI) test

Triple sugar iron (TSI) agar obtained from Biolab, Merck (South Africa) was used to assay *E. coli* content, with the substrates glucose, sucrose and lactose at sample concentrations of 0.1, 1.0 and 1.0% respectively.

3.2.6.2 Oxidase test

This test was performed using the Test Oxidase™ Reagent (PL.390) from Mast Diagnostics (Nesto, Wirral, UK) in accordance with the manufacturer’s published protocol. A well isolated pure colony was placed on a filter paper using a sterile wire loop. A drop of Test Oxidase™ Reagent was added on to it and mixed. After 30 seconds the filter was observed for the colour change. Oxidase positive colonies produced dark purple colour and were taken as presumptive *Aeromonas* and *Pseudomonas* isolates.

3.2.7 Secondary identification tests

3.2.7.1 Analytical profile index (API) 20E test

The API 20E test was performed in accordance with the manufacturer’s protocol (Bio-Mérieux, 69280, Marcy l’Etoile, France) and the organisms were identified at species level.
3.2.8 Haemolysis on blood agar
Haemolysis on blood agar (Biolab, Merck, SA) supplemented with 5% (v/v) sheep blood was determined. After incubation at 37°C for 24 hours, plates were examined for haemolysis.

3.2.9 Extraction of genomic DNA and Polymerase chain reaction (PCR) for the identification of culture species
DNA from the isolates was extracted using the peqGOLD (PEQLAB Biotechnologie GmbH 12-3450) bacterial DNA extraction kit according to the manufacturer’s protocol. The concentration of the extracted DNA in solution was determined spectrophotometrically (NanoDrop ND 1000) at a wavelength of 260 nm and the purity was measured at 280 nm. The integrity of the purified template DNA was assessed by conventional 1% (w/v) agarose gel.

3.2.10 Identification of the isolates by PCR assays
The identities of the presumptive Pseudomonas and Aeromonas were confirmed through amplicons of gyrB 222 (Qin et al., 2003), toxA 367 (Khan and Cerniglia., 1994) and ecfX, 528 (Lavenir et al., (2007) gene fragments respectively. PCRs were performed using oligonucleotide primer combinations and cycling conditions that appear in tables 3.1 and 3.2. Amplifications were performed using a Peltier Thermal Cycler (model-PTC-220DYAD™ DNA ENGINE, MJ Research Inc. USA). The reactions were prepared in 25 µl volumes that constituted 1 µg/µl of the template DNA, 50 pmol of each oligonucleotide primer set, 1X PCR master mix and RNase free water. All PCR reagents used were Fermentas, USA products supplied by Inqaba Biotechnological Industries Pty Ltd, Pretoria, South Africa. All products were stored at 4°C.

3.2.11 Electrophoresis of PCR products
PCR products were separated by electrophoresis on 2% (w/v) agarose gel. Electrophoresis was conducted in a horizontal Pharmacia biotech equipment system (model Hoefer HE 99X; Amersham Pharmacia biotech, Sweden) for 2 h at 60 V using 1X TAE buffer (40mM Tris, 1mM EDTA and 20 mM glacial acetic acid, pH 8.0). Each gel contained a 100 bp DNA molecular weight marker (Fermentas, USA). The gel was stained in ethidium bromide (0.1 µg/ml) for 15 min and amplicons were
visualised under UV light (Sambrook et al., 1989). A Gene Genius Bio-imaging system (Syngene, Synoptics; UK) was used to capture the image using GeneSnap (version 3.07.01) software (Syngene, Synoptics; UK) to determine the relative size of amplicons.

**Table 3.1:** Oligonucleotide primers that were used for specific detection of *Aeromonas* and *Pseudomonas* species

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Target and size (bp)</th>
<th>PCR cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECF1</td>
<td>ATGGATGAGCGCTTCCGTG</td>
<td><em>ectX</em>, (528)</td>
<td>35X 94°C for 45s</td>
</tr>
<tr>
<td>ECF2</td>
<td>TCATCCTTGCCCTCCCTG</td>
<td></td>
<td>58°C for 45s 72°C for 1m</td>
</tr>
<tr>
<td>GyrPA-398</td>
<td>CCTGACCATCCGTCGCCACAAC</td>
<td><em>gyrB</em>, (222)</td>
<td>35X 94°C for 45s</td>
</tr>
<tr>
<td>GyrPA-620</td>
<td>CGCAGCAGGATGCCGACGCC</td>
<td></td>
<td>66°C for 45s 72°C for 1m</td>
</tr>
<tr>
<td>ETA1</td>
<td>GACAACGCCCTCAGCATCACCAGC</td>
<td><em>toxA</em>, (367)</td>
<td>35X 94°C for 45s</td>
</tr>
<tr>
<td>ETA2</td>
<td>CGCTGGCCCATTCCGCTCCAGCCT</td>
<td></td>
<td>66°C for 45s 72°C for 1m</td>
</tr>
</tbody>
</table>

Initial denaturing step of 95°C for 5 min and final strand extension of 72°C for 5 min

**Table 3.2:** Oligonucleotide primers that were used to detect virulence genes in *Aeromonas* species

<table>
<thead>
<tr>
<th>Genes</th>
<th>Oligonucleotide sequence (5’ – 3’)</th>
<th>Target gene and size (bp)</th>
<th>PCR cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>aer A</em></td>
<td>Aer 2F: AGCGGCAGAGCCCGTCTATCCA</td>
<td><em>aerA</em> (416)</td>
<td>30X 95°C for 2m</td>
</tr>
<tr>
<td></td>
<td>Aer 2R: AGTTGGTGCCCGTCTCGTAGCG</td>
<td></td>
<td>55°C for 1m 72°C for 1m</td>
</tr>
<tr>
<td><em>hyl H</em></td>
<td>Hyl 2F: GGCCCGTGCCCGGAAGATGCAGG</td>
<td><em>hylH</em> (597)</td>
<td>30X 95°C for 2m</td>
</tr>
<tr>
<td></td>
<td>Hyl 2R: CAGTCCACCCACTTC</td>
<td></td>
<td>55°C for 1m 72°C for 1m</td>
</tr>
</tbody>
</table>

Initial denaturing step of 95°C for 5 min and final strand extension of 72°C for 7 min
3.3 Statistical analysis
Cluster analysis based on the antibiotic inhibition zone diameter data of different organisms isolated from different sites was determined using Wards algorithm and Euclidean distance of Statistica version 7.

3.4 Results
3.4.1 Occurrence of coliform bacteria, *Aeromonas* and *Pseudomonas* species in water
The primary aim of this study was to isolate and identify environmental bacteria from drinking water from Mafikeng. Raw water and treated water from five different sites were analysed for the presence of total coliforms, faecal coliforms, heterotrophic bacteria, *Aeromonas* and *Pseudomonas* species. Table 3.3 shows the average number of different organisms isolated from different sites for summer (October) and winter (July). Heterotrophic bacteria were isolated from all the sampling sites in both seasons and their occurrence was high. Moreover, *Pseudomonas* species, faecal and total coliforms were the most prevalent in all the seasons in both raw and treated water from Modimola dam. However, *Aeromonas* species were isolated only from the raw water samples, not from treated water from all the sites. The numbers of the different organisms isolated were higher in summer than in winter.
Table 3.3: Average number of organisms isolated

<table>
<thead>
<tr>
<th>Site</th>
<th>Seasons</th>
<th>FC</th>
<th>TC</th>
<th>H</th>
<th>A</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molopo eye (ME)</td>
<td>Summer (October)</td>
<td>30</td>
<td>40</td>
<td>&gt;100</td>
<td>42</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>Winter (July)</td>
<td>50</td>
<td>40</td>
<td>5</td>
<td>28</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Modimola dam (MD)</td>
<td>Summer</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>35</td>
<td>60</td>
<td>20</td>
<td>6</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Treated water ME</td>
<td>Summer</td>
<td>15</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Treated water MD</td>
<td>Summer</td>
<td>15</td>
<td>6</td>
<td>20</td>
<td>0</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Mixed water</td>
<td>Summer</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

FC= faecal coliforms, TC= total coliforms, H= heterotrophic bacteria, A= *Aeromonas*, P= *Pseudomonas*

Total coliforms were not observed in treated water from Molopo eye. *Pseudomonas* species were isolated in large numbers from Modimola dam and treated water from this site. The results obtained from the present study indicated that the organisms in treated water may have survived the treatment process. The large proportion of bacteria species isolated from the Modimola dam may be due to the fact that this dam receives sewage effluent from the sewage treatment plant.

3.4.2 Biochemical tests used to identify the isolates

Metallic sheen colonies on EMBA were considered as presumptive *E. coli* isolates. All the isolates were subjected to oxidase test and the oxidase positive colonies were taken as presumptive *Pseudomonas* and *Aeromonas* species. Further identification is done using TSI and API tests and the results are listed in table 3.4. Haemolysis on blood agar demonstrated that *Aeromonas* and *Pseudomonas* species were positive for β-haemolytic activity.
Table 3.4: Identification of the drinking water isolates using biochemical tests

<table>
<thead>
<tr>
<th>Site</th>
<th>TSI</th>
<th>API 20E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modimola dam (MD)</td>
<td><em>Pseudomonas</em></td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td><em>Aeromonas</em></td>
<td><em>Pseudomonas luteola</em></td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td><em>Aeromonas hydrophila</em></td>
</tr>
<tr>
<td></td>
<td><em>Serretia</em></td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td><em>Shigella</em></td>
<td><em>Serratia odorifera</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Serratia liquefaciens</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Proteus vulgaris</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Providentia rettgeri</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Chryseobacterium meningosa</em></td>
</tr>
<tr>
<td>MD-Treated water</td>
<td><em>Pseudomonas</em></td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td><em>Aeromonas</em></td>
<td><em>Pseudomonas luteola</em></td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td><em>Serratia odorifera</em></td>
</tr>
<tr>
<td></td>
<td><em>Serretia</em></td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td><em>Shigella</em></td>
<td></td>
</tr>
<tr>
<td>Molopo eye (ME)</td>
<td><em>Pseudomonas</em></td>
<td><em>Serratia odorifera</em></td>
</tr>
<tr>
<td></td>
<td><em>Aeromonas</em></td>
<td><em>Serratia liquefaciens</em></td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td><em>Aeromonas salmonicida spp</em></td>
</tr>
<tr>
<td></td>
<td><em>Serretia</em></td>
<td><em>Pseudomonas oluore</em></td>
</tr>
<tr>
<td></td>
<td><em>Shigella</em></td>
<td><em>Chryseobacterium meningosepticum</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Salmonella choleraesais</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Enterobacter asburiae</em></td>
</tr>
<tr>
<td>ME-Treated water</td>
<td><em>Pseudomonas</em></td>
<td><em>Serratia liquefaciens</em></td>
</tr>
<tr>
<td></td>
<td><em>Aeromonas</em></td>
<td><em>Myroides spp</em></td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Serretia</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Shigella</em></td>
<td></td>
</tr>
<tr>
<td>Mixed water</td>
<td><em>Pseudomonas</em></td>
<td><em>Serratia liquefaciens</em></td>
</tr>
<tr>
<td></td>
<td><em>Aeromonas</em></td>
<td><em>Serratia odorifera</em></td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td><em>Aeromonas salmonicida spp</em></td>
</tr>
<tr>
<td></td>
<td><em>Serretia</em></td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td><em>Shigella</em></td>
<td><em>Myroides spp</em></td>
</tr>
</tbody>
</table>
3.4.3 Antibiotic resistant data of different isolates from drinking water

All the isolates were subjected to antibiotic susceptibility test using eleven (11) different antibiotics and their antibiotic resistance profile. Multiple antibiotic resistance phenotypes were compiled. The results obtained are depicted in tables 3.5–3.8. These individuals are only those were identified. The results revealed that a large proportion of the environmental isolates were resistant to erythromycin followed by trimethoprim and amoxicillin. None of the isolates were resistant to ciprofloxacin and only very few isolates from Modimola dam were resistant to streptomycin and neomycin respectively. *Pseudomonas* spp. isolated from Modimola dam, Molopo eye and mixed water were resistant to most of the antibiotics except K, S, Ne and CIP. The number of resistant bacteria recovered from the dam was higher than other sources. Resistance could be attributed to heavy contamination from sewage effluent, surface runoff or from the birds that inhabit in the dam. Modimola dam received treated sewage effluent and also small scale farming communities dwell around the dam. The livestock graze around the dam and their faeces is washed off into the dam during the rainy season. Therefore the dam could serve as a reservoir of antibiotic resistant bacteria and sewage contamination contribute to the dissemination of antibiotic resistant bacteria in the environment. This demonstrated a link between the resistant isolates from sewage treatment plant, Modimola dam and treated water. Molopo eye is a protected spring where there is no sewage effluent discharged. Hence occurrence of antibiotic resistant bacteria reflected contamination from human activity, pets and wild birds.
### Table 3.5: Percentage antibiotic resistance of total coliforms

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>KF</th>
<th>AP</th>
<th>C</th>
<th>E</th>
<th>OT</th>
<th>K</th>
<th>TM</th>
<th>S</th>
<th>A</th>
<th>NE</th>
<th>CIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molopo eye N= 5</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>100</td>
<td>80</td>
<td>40</td>
<td>80</td>
<td>0</td>
<td>60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Modimola dam N= 5</td>
<td>40</td>
<td>60</td>
<td>20</td>
<td>100</td>
<td>40</td>
<td>20</td>
<td>80</td>
<td>0</td>
<td>80</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Treated Molopo eye N= 1</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Treated dam N= 2</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>100</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mixed water N=2</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>50</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

KF= cephalothin, AP=ampicillin, C= chloramphenicol, E= erythromycin, OT= oxytetracycline, K= kanamycin, TM= trimethoprim, S= streptomycin, A= amoxicillin, NE= neomycin, CIP= ciprofloxacin

### Table 3.6: Percentage antibiotic resistance of faecal coliforms

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>KF</th>
<th>AP</th>
<th>C</th>
<th>E</th>
<th>OT</th>
<th>K</th>
<th>TM</th>
<th>S</th>
<th>A</th>
<th>NE</th>
<th>CIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molopo eye N= 5</td>
<td>40</td>
<td>60</td>
<td>0</td>
<td>100</td>
<td>40</td>
<td>20</td>
<td>80</td>
<td>0</td>
<td>60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Modimola dam N= 4</td>
<td>50</td>
<td>50</td>
<td>25</td>
<td>50</td>
<td>50</td>
<td>25</td>
<td>50</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Treated Molopo eye N= 1</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Treated dam N= 1</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mixed water N1</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
### Table 3.7: Percentage antibiotic resistance of heterotrophic bacteria

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>KF</th>
<th>AP</th>
<th>C</th>
<th>E</th>
<th>OT</th>
<th>K</th>
<th>TM</th>
<th>S</th>
<th>A</th>
<th>NE</th>
<th>CIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molopo eye N= 5</td>
<td>80</td>
<td>60</td>
<td>40</td>
<td>80</td>
<td>20</td>
<td>40</td>
<td>80</td>
<td>0</td>
<td>80</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Modimola dam N= 5</td>
<td>60</td>
<td>80</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>80</td>
<td>0</td>
<td>60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Treated Molopo eye N= 4</td>
<td>50</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>25</td>
<td>50</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Treated dam N= 3</td>
<td>66.7</td>
<td>66.7</td>
<td>66.7</td>
<td>33.3</td>
<td>66.7</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>66.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mixed water N=2</td>
<td>50</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 3.8: Percentage antibiotic resistance of Aeromonas spp**

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>KF</th>
<th>AP</th>
<th>C</th>
<th>E</th>
<th>OT</th>
<th>K</th>
<th>TM</th>
<th>S</th>
<th>A</th>
<th>NE</th>
<th>CIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molopo eye N= 4</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>100</td>
<td>50</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>75</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Modimola dam N= 2</td>
<td>50</td>
<td>100</td>
<td>50</td>
<td>100</td>
<td>50</td>
<td>100</td>
<td>50</td>
<td>100</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 3.9: Different sites showing resistance to different antibiotics**

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>KF</th>
<th>AP</th>
<th>C</th>
<th>E</th>
<th>OT</th>
<th>K</th>
<th>TM</th>
<th>S</th>
<th>A</th>
<th>NE</th>
<th>CIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molopo eye</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Modimola dam</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Treated Molopo eye</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Treated dam</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mixed water</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = resistant, - = sensitive
3.4.4 Predominant multiple antibiotic resistant (MAR) phenotypes of isolates isolated from different sites

Different types of multiple antibiotic resistance patterns were observed in all the sites in all the organisms tested for (Table 3.9). Resistance towards nine different antibiotics were observed. The predominant antibiotic resistant phenotypes that were obtained for the isolates from different sites were KF-AP-C-E-OT-K-TM-A, KF-AP-C-E-OT-K-TM-A-NE, and KF-AP-E-OT-K-TM-A respectively. Similar types of MAR phenotypes were observed in isolates from different sites.

3.4.5 Cluster Analysis of the isolates for multiple antibiotic resistance (MAR) relationship on a dendogram

All the Pseudomonas, Aeromonas and heterotrophic bacterial isolates were subjected to cluster analysis based on their Inhibition Zone Diameter (IZD) data and a dendogram was generated using Ward’s method. This approach was used as a tool in determining the commonness and in resolving differences between the MAR phenotypes of different isolates.

3.4.6 Pseudomonas, Aeromonas and Heterotrophic bacteria

Pseudomonas, Aeromonas and heterotrophic bacteria isolated from all sites were subjected to cluster analysis and two main clusters were observed (Fig. 3.1) and the number of isolates from different sites within the clusters is depicted in Table 3.10. Both clusters have sub-clusters. Cluster 1 is a large cluster contained a total of 21 Aeromonas and heterotrophic bacterial isolates. Cluster 2 is a mixed cluster with Pseudomonas, Aeromonas and heterotrophic bacteria. Pseudomonas is only found in cluster 2. Cluster analysis indicated that isolates from all sites potentially had similar antibiotic resistance profiles and thus could be related to phenotypic typing.
Table 3.10: Number of *Pseudomonas*, *Aeromonas* and heterotrophic bacteria isolated from different sites within the various clusters

<table>
<thead>
<tr>
<th>Site</th>
<th>Cluster 1 (N=21)</th>
<th>Cluster 2 (N= 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>A</td>
</tr>
<tr>
<td>Dam</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Eye</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Treated water dam</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Treated water eye</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Mixed water</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

H= Heterotrophic bacteria, A= *Aeromonas*, P= *Pseudomonas*

Figure 3.1: Cluster analysis data for *Pseudomonas*, *Aeromonas* and Heterotrophic bacteria isolated from different sites within the various clusters (PS= *Pseudomonas*; AE= *Aeromonas*, H=Heterotrophic bacteria)
3.4.7 Molecular identification of *Pseudomonas* and *Aeromonas* species

3.4.7.1 Chromosomal DNA

Chromosomal DNA was extracted from the isolates and the quality of the DNA was determined by gel electrophoresis. Figure 3.2 indicates an image of a 1% (w/v) agarose gel depicting genomic DNA extracted from *Pseudomonas* and *Aeromonas* species.

![Image of a composite agarose (1% w/v) gel depicting genomic DNA extracted from *Pseudomonas* and *Aeromonas* species. Lane M (1 kb DNA Ladder); Lane 1-10 DNA extracted from *Pseudomonas* and *Aeromonas* species isolated from different sites.]

**Figure 3.2:** Image of a composite agarose (1% w/v) gel depicting genomic DNA extracted from *Pseudomonas* and *Aeromonas* species. Lane M (1 kb DNA Ladder); Lane 1-10 DNA extracted from *Pseudomonas* and *Aeromonas* species isolated from different sites.

3.4.7.2 PCR analysis for identification of *Pseudomonas* and *Aeromonas* species through *GyrB, toxA and ecfX* PCR amplification

Specific PCR was used to determine the identities of presumptive *Pseudomonas* species through amplification of the *gyrB, toxA* and the *ecfX* gene fragments. Figures 3.3 and 3.4 show agarose (1% w/v) gels indicating *gyrB* and *ecfX* gene fragments generated by PCR using genomic DNA extracted from *Pseudomonas* species isolated from different sites. Gel electrophoresis of PCR products revealed the
desired 222 bp and 528 bp fragments for the gyrB and ecfX gene fragments, respectively. A total of 61 isolates were screened and 17 were positively identified.

Figure 3.3: Image of a composite agarose (1% w/v) gel depicting DNA extracted from Pseudomonas species. Lane 1 (1 kb DNA Ladder); Lane 2-10 (gyrB gene fragments (222 bp) from Pseudomonas species isolated from different sites

Figure 3.4: Image of a composite agarose (1% w/v) gel depicting genomic DNA extracted from Pseudomonas species. Lane 1 (1 kb DNA Ladder); Lane 2-8 (ecfX gene fragments from Pseudomonas species isolated from different sites)
The identity of *Aeromonas* species were determined by screening them for the presence of *gyrB* genes (Yáñez *et al*., 2003) and specific virulent genes aerolysin (*aerA*) (Kaszab *et al*., 2011) and haemolysin (*hylH*) (Yousr *et al*., 2007). Figure 3.5 show agarose (1% w/v) gels indicating *hylH* gene fragments generated by PCR using genomic DNA extracted from *Aeromonas* species isolated from different sites.

![Image of agarose gel](image)

**Figure 3.5:** Image of a composite agarose (1% w/v) gel depicting the *hylH* gene from *Aeromonas* species. Lane M (1 kb DNA Ladder); Lanes 1-4 (*hylH* gene fragments from *Aeromonas* species isolated from different sites.

### 3.5 Discussion

One of the objectives of this study was to isolate environmental bacteria from surface and drinking water in Mafikeng. A motivation for this study was the numerous reports about the occurrence of pathogenic microrganisms in drinking water and the associated diseases (Schäfer *et al*., 2009; Yilla *et al*., 2008; Schraft and Watterwoth, 2005; Germs *et al*; 2004; Obi *et al*., 2002; Carter *et al*., 2000). Moreover, the resistance of microorganisms to antibiotics of clinicals interest has previously been reported in the area (Mulamattathil *et al*., 2000; Atebe *et al*., 2008; Wose Kinge and Mbewe, 2010). The study demonstrated the occurrence of total coliforms, faecal
coliforms, heterotrophic bacteria, *Aeromonas* and *Pseudomonas* in water samples analyzed which indicated incidence of water contamination as they are indicators of faecal contamination (Webster *et al.*, 2004). These organisms may harbour potential pathogens and presence of pathogenic organisms that can pose severe health risks to consumers in general and immuno-compromised individuals in particular (Biyela *et al.*, 2004). The water after-treatment possessed potential pathogens and the health risk caused by these pathogens should be taken into consideration. Molopo eye is a protected spring where there is no sewage effluent discharged. Occurrence of antibiotic resistant bacteria may be due to contamination from human activity, pets and wild birds.

A further objective of this study was to characterise the isolates using their antibiotic resistance profiles. All the isolates from the different sampling sites were assayed for resistance to 11 antibiotics. The results revealed that 80 to 100% of the environmental isolates were resistant to erythromycin followed by trimethoprim and amoxycillin. The trend was in accordance with earlier studies that showed resistance towards β-lactam, macrolides and Phenicols (Obi *et al.*, 2004; Messi *et al.*, 2005). Lin *et al.*, (2004) also observed high level of resistance among isolates from Mhlathuze River, Kwazulu Natal, South Africa.

Total coliforms and faecal coliforms were isolated from raw and treated water. All the isolates from all the sites were resistant to erythromycin which was in accordance with the study conducted by Moore *et al.* (2010) where highest resistance towards erythromycin and tetracycline (92.9%) was observed. Large percentage resistance was observed for trimethoprim and ampicillin also (60%-100%). All isolates were found to be susceptible to streptomycin and ciprofloxacin in line with the observation made by Lin *et al.*, (2004). This is not in agreement with the observation made by Oudhuis *et al.*, (2008). They observed increased resistance against ciprofloxacin in *E. coli* and *Pseudomonas*. Despite the fact that all isolates from Molopo eye, treated water from Molopo eye and Modimola dam and mixed water were susceptible to neomycin, only a small proportion (20%) from Modimola dam was resistant to neomycin. *Coliforms* expressed MAR to 3 or more classes of antibiotics and were detected in all samples as observed by Luczkiewicz *et al.*, (2010) and Moore *et al.*, (2010). Sorlozano *et al.*, (2007) observed an elevated resistance to quinolones
(>70%), in clinical isolates of *E. coli*. Faecal coliforms from all sites were susceptible to streptomycin neomycin and ciprofloxacin, respectively. Highest resistance was towards erythromycin. However, two isolates from Modimola dam were susceptible towards erythromycin. MAR observed towards 3 to 8 antibiotics (Tables 3.5-3.9). These results accords with the findings of others who observed MAR resistance in *E. coli* and *Pseudomonas* spp. isolated from environmental samples and hospitalized patients (Siewick *et al.*, 2007; Sorlozano *et al.*, 2007; Scott *et al.*, 2003., Tumeo *et al.*, 2008; Bredenstein *et al.*, 2011). Antibiotic resistance patterns were generally similar in all different sites. However, Modimola dam had highest level of resistance against the antibiotics tested.

Heterotrophic bacteria were isolated from all sites and exhibited antibiotic resistance. Isolates from all sites were susceptible to streptomycin, neomycin and ciprofloxacin and agrees with the susceptibility to ciprofloxacin observed by Biyela *et al.*, (2004). Highest percentage resistance was towards trimethoprim followed by β-lactam and tetracycline as observed by Biyela *et al.*, (2004). All isolates from treated water from Molopo eye and mixed water were sensitive to erythromycin. In a previously published data by Lobova *et al.*, (2008) multiple resistences to ampicillin and kanamycin predominated in all sites in lake Shira. High loads of heterotrophic bacteria and opportunistic pathogens that harbour multiple drug resistance determinants pose significant health hazards to consumers, especially those whose immune system are compromised (Jeena *et al.*, 2006). Pavlov *et al.*, (2004) detected heterotrophic bacteria with associated virulence factors that give them the ability to act as opportunistic pathogens. Heterotrophic organisms are generally harmless, but may harbour pathogenic organisms which may cause potential health risks to humans.

*Aeromonas* species isolated exhibited 100% resistance towards erythromycin. All of them were susceptible to ciprofloxacin as observed by Emekdas *et al.*, (2009). However, isolates from Molopo eye susceptible to kanamycin, neomycin and streptomycin also. Isolates from the dam were resistant to most antibiotics. Resistance could be attributed to the release of sewage effluent, faecal contamination from farm animals or birds. 100% resistance towards ampicillin, oxytetracycline, trimethoprim and amoxycillin were observed in isolates from the
dam. In a study conducted by Sharma et al., (2005) all Aeromonas species isolated from the fresh water were resistant to Ampicillin and Emekdas et al., (2009) observed resistance towards tetracycline, trimethoprim and cephalothin in the isolates from municipally treated tap water samples. Dumontent et al., (2000) reported resistance towards cephalothin, ampicillin and amoxicillin in Aeromonas species in coastal waters.

Pseudomonas samples from all the sites exhibited 100% resistance to up to eight antibiotics and susceptible to streptomycin, neomycin and ciprofloxacin. Pseudomonas isolate recovered from a river exhibited MAR and were sensitive to ciprofloxacin as observed in this study (Anguzu and Olila, 2007). Wide distribution of antibiotic resistant bacteria in surface and ground waters has been reported in many studies (Harakeh et al., 2005). There is tremendous increase in the incidence of MAR in organisms from various sources (Kümmerer, 2009). Considering the high incidence of HIV/AIDS in South Africa, the importance of such findings cannot be over-emphasized. Moreover, the results of this study on bacterial resistance profiles are consistent with previous studies in other surface and drinking water systems (Shrivastva et al., 2004; Pavlov et al., 2004; Moore et al., 2010).

Another objective of this was to identify the Pseudomonas and Aeromonas species using PCR. Isolates were identified using gyrB, toxA, ecfX, aerA and hylH gene fragments using PCR. The gyrB, ecfX and hylH fragments were amplified. The ecfX gene encodes an extra cytoplasmic function sigma factor, which may be involved in haem uptake or virulence (Anuj et al., 2009), whereas the gyrB gene encodes the DNA gyrase subunit B, a protein which plays a crucial role in the DNA replication process (Yáñez et al., 2003) and toxA encoding the exotoxin A precursor (Lavenir et al., 2007). PCR assay targeting the ecfX and gyrB genes is highly suitable for the identification of P. aeruginosa (Anuj et al., 2009). Application of PCR technique to target gyrB, aerA and hylH genes are excellent molecular chronometers for screening potentially virulent Aeromonas species in food and the environment (Yáñez et al., 2003., Yousr et al., 2007). The detection of Aeromonas species that harbour putative virulence factors and resistance to β-lactam antibiotics indicates that drinking water that is supplied to the community could serve as a source for the transmission of pathogens to humans (Emekdas et al., 2006; Li et al., 2009). Isolates
were also found to be potentially pathogenic based on their haemolytic activity. Further studies will be conducted to determine the prevalence of antibiotic resistant genes among the pathogenic isolates and the dissemination of resistant genes among the pathogens. This would provide information about the health risks associated with the consumption of contaminated water.

3.6 Conclusions

An evaluation of the bacteriological quality of drinking water confirmed the presence of faecal indicator organisms as well as opportunistic pathogens such as *Aeromonas* and *Pseudomonas*. Moreover, these organisms were resistant to several classes of antibiotics. Undesirable properties of water quality caused by the presence of drug resistant bacteria can pose a negative impact on human health.

The data on multiple antibiotic resistance (MAR) profiles of bacterial isolates from water and the resistance patterns of organisms in drinking water in Mafikeng suggested that there has been an indiscriminate use of the antibiotic tested. The high prevalence of multiple antibiotic resistant organisms in the drinking water distribution system could pose a potential threat to humans consuming this water. The presence of MAR organisms in the drinking water of Mafikeng, South Africa is an important health concern due to the risk of developing waterborne diseases and the health risks associated with immuno-compromised patients living in the area. It is therefore imperative to monitor the quality of water and strict quality control measures should be put in place to ensure the effective treatment of waste water and drinking water. This would decrease the load of microorganisms in the drinking water and thereby prevent the outbreaks and spread of water borne diseases. Antibiotic resistance surveillance can be used as tool to control the problem of antibiotic resistance and educate the public the consequences of the misuse of antibiotics and to regulate the usage of drugs in both human and veterinary medicine. It is also helpful to formulate guidelines for the optimal use of antibiotics. Moreover, these findings should be disseminated to the community. Further studies should be conducted to assess the level of antibiotics in water and the potential risks associated with human consumption of polluted water.
CHAPTER 4

Biofilm formation in the surface and drinking water distribution systems in Mafikeng, North West Province, South Africa

4.1 Introduction
Water is a very important resource for life and access to safe drinking water is basic right of every individual (WHO, 2004). South Africa is semi-arid country with very little rainfall resulting in high water stress and as such individuals in many communities struggle to access potable water (Adewumi et al., 2010). Water scarcity problems can be addressed through the recycling of municipal waste water for reuse in households and this practice is increasingly being utilized worldwide (Revit et al., 2011). However, reclaimed water may be a major source of pathogenic and opportunistic microorganisms as well as pharmaceutical waste products (Yi et al., 2011). The presence of microorganisms in treated water sources is usually due to the fact that they are able to withstand and survive treatment process. Factors such as availability of nutrients favour the regrowth of the organisms in the water distribution system (Le Chevallier et al., 1996; Chu et al., 2005). Moreover, in most developing countries water treatment plants are usually faced with maintenance problems and lack of qualified personnel.

Water is an environmental reservoir for microorganisms (Park et al., 2001). In aquatic environments, microorganisms have the ability to adhere to solid surfaces and form biofilms (Castonguay et al., 2006). Biofilms are bacterial communities embedded in a polysaccharide matrix and this gives them the opportunity to resist destruction by antibiotics, environmental stress, biocides and detergents, therefore increases the chances of them causing infections in humans when they become dislodged (Simpson, 2008). Generally most water distribution systems are characterised by the presence biofilms, regardless of its purity, type of pipe material used for distribution or the presence of a disinfectant (Lehtola et al., 2006). Moreover, the development of biofilms inside water distribution pipes facilitates the
propagation of mixed microbial populations and is considered the main source of planktonic bacteria in water supply systems (Momba et al., 2006). This is further aggravated by the presence of opportunistic pathogens such as *Pseudomonas, Aeromonas, Klebsiella, Mycobacter, E. coli, Helicobacter, Salmonella* and *Legionella* spp. that may increase the health risks associated with the consumption of water (Critchley et al., 2003; Tozzi et al., 2003). Biofilms consisting of *Pseudomonas aeruginosa* and different faecal bacteria species have been detected in water distribution systems even in countries that have more advanced water treatment facilities (Kilb et al., 2003; Werner et al., 2004). Understanding the mechanisms of pathogen attachment to biofilms developed in drinking water distribution system is of crucial interest to ensure the quality of the drinking water (Janjaroen et al., 2013).

Different types of materials such as cast iron galvanized steel, stainless steel, copper and polyethylene have been used to manufacture water distribution pipes and these favour biofilm formation in the water distribution systems (Skjervak et al., 2005; Van der Kooij et al., 2005; Lehtola et al., 2006; Teng et al., 2008). Differences in the pipe materials greatly favour the survival of different bacterial species (Kalmbach et al., 2000) and affect the effectiveness of disinfectants (Lehtola et al., 2005). The development of biofilms in copper pipes facilitates cuprosolvency and this increases the release of copper into the distribution system (Roslev et al., 2004). Detachment of bacteria from the biofilm may affect the quality of the water (Elenter et al., 2007). Therefore, the deterioration of the quality of drinking water due to biofilm formation is a major concern for most municipal supply agencies and communities.

The presence of biofilms in drinking water distribution systems may lead to a number of undesirable effects on the quality of water that is supplied to consumers (Tien et al., 2009). In spite of the different treatment processes, the occurrence of biofilm in drinking water is attributed to the bacterial resistance to disinfectants and species association which increases the proportion of viable cells (Simões et al., 2009). Different disinfectant methods have a long term effect on the biofilm community (Roeder et al., 2010). Biofilms consisting of *Pseudomonas aeruginosa* and different faecal bacteria species resistant to antimicrobials have been detected in water distribution systems even in countries that have more advanced water treatment facilities (Kilb et al., 2003; Werner et al., 2004; Toté et al., 2009).
Mafikeng is the capital of the North West Province of South Africa. This city uses both ground water as well as dam water for drinking water production. Some areas receive a mixture of the two water types and other only one or the other. The water purification plant of the surface water is at the Modimola dam that receives treated wastewater. The water purification plant is down-stream from sewage treatment plant. This is thus a semi-closed water conservation system.

The study was designed to investigate the biofilm forming ability and virulence gene determinants of biofilm bacteria especially *Pseudomonas* and *Aeromonas* species in the water distribution systems in Mafikeng, North-West Province, South Africa.

### 4.2 Materials and methods

#### 4.2.1 Biofilm formation device

To study biofilm growth, a flow system technique that utilizes a biofilm developing device was used (Fig 4.1). The biofilm developing device pipe system was made from clear plastic pipe with a diameter of 16 mm, installed with copper and galvanized coupons to serve as solid surfaces for bacteria to adhere and form biofilms. The coupons were held in place by screws. The device was mounted horizontally to the main pipe of a building of the North-West University in Mafikeng which receives mixed water and also in the treatment plant exposed to raw water. The coupons were held at the different sampling points for four months. Mini tap filters; a point-of-use (POU) treatment device (Fig 4.2) which can be used at a single faucet, under constant flow was also used to form biofilms during the second collection. The filters were placed on cold water taps in participating households that received treated ground water (Molopo eye water), only Modimola dam water or mixed water (North-West University as well).
Figure 4.1: Biofilm device

Figure 4.2: Mini tap filter
4.2.2 Sampling of biofilm
Biofilm samples were analysed twice during the study period. To achieve this, the biofilm developing device pipe was closed with valves before disconnecting them and the coupons were removed using sterile forceps. The filters in the Mini tap filter devices were also removed from the cartridge aseptically. Coupons were placed immediately into sterile 100 ml Schott bottles that contained water from the particular sampling site. Samples were transported on ice to the laboratory for analysis. Upon arrival in the laboratory, the coupons were removed from the bottles. Those for scanning electron microscopy were stored in 100% alcohol. The rest of the coupons were analysed for bacterial growth.

4.2.3 Scanning electron microscopy (SEM)
Investigation of the biofilm structure was achieved using SEM. Biofilm samples were fixed, dehydrated sequentially in increasing ethanol concentration (70%, 90% and 100%) for 15 minutes and critically dried in liquid carbon-dioxide. The samples were mounted on SEM stub using double sided carbon tape. These were then carbon coated. Finally, the samples were super coated with gold/palladium and viewed using a Philips XL30 Scanning Electron Microscope (Philips, Germany). Enlargements ranged from 63X to 20 000X.

4.2.4 Isolation of bacteria from the biofilm
Isolation of bacteria was done using standard methods. Biofilm bacteria on the coupons were removed by swiping the surfaces with sterile cotton swabs and immediately streaking it onto selective media for the isolation of targeted organisms. The media used were as follows: mFC agar for the isolation of faecal coliforms, mEndo for total coliforms and Aeromonas selective agar for Aeromonas and Pseudomonas species. The plates were incubated aerobically at 35°C for 24 hours except for mFC agar which were incubated at 45°C for 24 hours. Blue colonies from mFC agar, metallic-sheen colonies from mEndo agar were considered as presumptive faecal coliforms and total coliforms respectively. Moreover, yellow and green colonies on Aeromonas selective agar represented Aeromonas and Pseudomonas species, respectively. These isolates were sub-cultured on the respective selective media and plates were incubated aerobically at 35°C and 45°C, respectively for 24 hours. Presumptive pure colonies were subjected to specific
preliminary and confirmatory biochemical tests. All pure isolates were Gram stained using standard methods.

4.2.5 Preliminary biochemical tests
Triple Sugar Iron (TSI) agar test (Biolab, Merck, South Africa), Oxidase test using the Test Oxidase™ Reagent (PL.390) from Mast Diagnostics (Nesto, Wirral, U.K) and API 20E test (Bio-Mérieux, 69280, Marcy l'Etoile, France) were performed in accordance with the manufacturer’s published protocol.

4.2.5.1 Triple sugar iron agar (TSI) test
Triple sugar iron (TSI) agar obtained from Biolab, Merck (South Africa) was used to determine the ability of isolated organism to utilize the substrates glucose, sucrose and lactose at sample concentrations of 0.1, 1.0 and 1.0% respectively. Isolates were inoculated as required and bottles that contained the cultures were incubated at 37°C for 24 hours. Isolates were evaluated based on the formation of gas, hydrogen sulphide, and fermentation of carbohydrates to produce acids.

4.2.5.2 Oxidase test
This test was performed using the Test Oxidase™ Reagent (PL.390) from Mast Diagnostics (Nesto, Wirral, UK) in accordance with the manufacturer’s published protocol. A well isolated pure colony was placed on a filter paper using a sterile wire loop. A drop of Test Oxidase™ Reagent was added on to it and mixed. After 30 seconds the filter was observed for colour change. Bergey's manual (Garrity et al., 2001) of systematic bacteriology was used as an identification aid. Isolates that produced a purple colour were presumptively considered to be E. coli. Oxidase positive colonies were taken as presumptive Aeromonas and Pseudomonas species.

4.2.6 Analytical profile index (API) 20E test
The API 20E test was performed in accordance with the manufacturer’s protocol (Bio-Mérieux, 69280, Marcy l'Etoile, France) and the organisms were identified at species level.
4.2.7 Antibiogram

Antibiotic resistance was assayed using Kirby- Bauer disk diffusion (Kirby et al., 1966) method using Mueller-Hinton agar plates. The following antibiotic discs were used ampicillin (10 µg), cephalothin (5 µg), streptomycin (10 µg), erythromycin (15 µg), chloramphenicol (30 µg), neomycin (30 µg), amoxycillin (10 µg), ciprofloxacin (5 µg), trimethoprim (2 5µg), kanamycin (30 µg) and oxytetracycline (30 µg). The antibiotics were chosen because they are frequently in human therapy. Three colonies were picked and transferred in to 3ml sterile distilled water to prepare bacteria suspensions. Aliquots of 1 ml from these suspensions were spread-plated onto Mueller-Hinton agar (Biolab, Merck, South Africa) plates. The antibiotic discs were placed at equiTable distances on the inoculated plates using a sterile needle. The plates were then incubated aerobically at 37°C for 24 hours. After incubation the antibiotic inhibition zone diameter (IZD) were measured. Results obtained were used to classify isolates as being resistant, intermediate resistant or susceptible to a particular antibiotic using standard reference values according to National Committee for Clinical Laboratory Standards (NCCLS, 1999). MAR phenotypes were generated for isolates that showed resistance to 3 or more antibiotics.

4.2.8 Confirmatory DNA test

4.2.8.1 Genomic DNA extraction

Genomic DNA was extracted from all the presumptive *Pseudomonas* and *Aeromonas* isolates using the alkaline lysis method. The concentration, quality of the extracted DNA in solution was determined using a spectrophotometer (NanoDrop ND 1000) and 1% (w/v) agarose gel electrophoresis. The latter was also used for determining the integrity of the genomic DNA.

4.2.8.2 PCR assays for the identification and detection of virulence gene markers in *Pseudomonas* and *Aeromonas* species

The identities of the presumptive *Pseudomonas* and *Aeromonas* species were confirmed through amplification of the *gyrB* (222 bp) (Qin et al., 2003), *toa* (397 bp) (Khan and Cerniglia., 1994) and *ecfX* (528 bp) (Lavenir et al., 2007) gene fragments respectively. PCRs were performed using oligonucleotide primer combinations and cycling conditions that appear in Table 4.1. *Pseudomonas* species were screened for the presence of the *exoA* (396 bp) (encoding for exotoxin A), *exoS* (118 bp)
(encoding for exotoxin S) and exoT (152 bp) (encoding for exotoxin T) virulence gene determinants (Kaszab et al., 2011) while specific PCR for the detection of aerolysin (aerA) (416 bp) and haemolysis (hylH) (597 bp) genes was performed on all positively identified Aeromonas species (Yogananth et al., 2009) (Table 4.2).

**Table 4.1:** Oligonucleotide primers that were used for specific detection of *Aeromonas* and *Pseudomonas* species

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Target and size (bp)</th>
<th>PCR cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECF1</td>
<td>ATGGATGAGCGCTTCCGTG</td>
<td>ecfX, (528)</td>
<td>35X</td>
</tr>
<tr>
<td>ECF2</td>
<td>TCATCCTTGCCCTCCCTG</td>
<td></td>
<td>94°C for 45s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>58°C for 45s</td>
</tr>
<tr>
<td>GyrPA-398</td>
<td>CCTGACCATCCGTCCCGACCAAC</td>
<td>gyrB, (222)</td>
<td>35X</td>
</tr>
<tr>
<td>GyrPA-620</td>
<td>CGCAGCAGGATGCCGACGCC</td>
<td></td>
<td>94°C for 45s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>66°C for 45s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72°C for 1m</td>
</tr>
<tr>
<td>ETA1</td>
<td>GACAACGCCCTCAGCATCACCAGC</td>
<td>toxA, (367)</td>
<td>35X</td>
</tr>
<tr>
<td>ETA2</td>
<td>CGCTGGCCCATTCGCTCCAGCGCT</td>
<td></td>
<td>94°C for 45s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>66°C for 45s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72°C for 1m</td>
</tr>
</tbody>
</table>

Initial denaturing step of 95°C for 5 min and final strand extension of 72°C for 5 min
Table 4.2: Oligonucleotide primers that were used to detect virulence genes in *Pseudomonas* and *Aeromonas* species

<table>
<thead>
<tr>
<th>Genes</th>
<th>Oligonucleotide sequence</th>
<th>Target gene and size (bp)</th>
<th>PCR cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>exoA</td>
<td>F: 5’ AACCAGCTCAGCCACATGTC 3’&lt;br&gt;R: 5’ CGCTGGCCCATTCCGCTCCAGCGCT 3’</td>
<td>exoA 396</td>
<td>30X 94°C for 1m 68°C for 1m 72°C for 1m</td>
</tr>
<tr>
<td>exo S</td>
<td>F: 5’ GCGAGGTACGAGATGTATCG 3’&lt;br&gt;R: 5’ TTTCCGCTCAGTGATGC 3’</td>
<td>exoS 118</td>
<td>36X 94°C for 30s 58°C for 30s 68°C for 1m</td>
</tr>
<tr>
<td>exo T</td>
<td>F: 5’ AATCGCCGTCCAATGCATGCG 3’&lt;br&gt;R: 5’ TGTTCGCCGGAGTACTGCTC 3’</td>
<td>exoT 152</td>
<td>36X 94°C for 30s 58°C for 30s 68°C for 1m</td>
</tr>
<tr>
<td>aer A</td>
<td>Aer 2F: 5’ AGCCGCCAGCCGTCTATCCA3’&lt;br&gt;Aer 2R: 5’ AGTTTGTTGGCGGTGCTAGCG3’</td>
<td>aerA 416</td>
<td>30X 95°C for 2m 55°C for 1m 72°C for 1m</td>
</tr>
<tr>
<td>hyl H</td>
<td>Hyl 2F: 5’ GGCCCGTGGCCAGGCCGAAGATGCAGG3’&lt;br&gt;Hyl 2R: 5’ CAGTCCCCACCCCACCTTC3’</td>
<td>hylH 597</td>
<td>30X 95°C for 2m 55°C for 1m 72°C for 1m</td>
</tr>
</tbody>
</table>

*exoA*- initial denaturing step of 95°C for 2 min and final strand extension of 72°C for 7 min

*exo S* and *exo T*- initial denaturing step of 94°C for 2 min and final strand extension of 68°C for 7 min

*aer A* and *hyl H*- initial denaturing step of 95°C for 5 min and final strand extension of 72°C for 7 min

Standard 25 µl reactions that consisted of 1 µg/µl of the template DNA, 50 pmol of each oligonucleotide primer set, 1X PCR master mix and RNase free water were prepared. Amplifications were performed using a Peltier Thermal Cycler (model-PTC-220 DYAD™ DNA ENGINE). All PCR reagents used were Fermentas, USA products supplied by Inqaba Biotec Pty Ltd, Pretoria, South Africa. PCR products were subjected to 1% (w/v) agarose gel electrophoresis. The gels were stained in ethidium bromide (0.1 µg/ml) for 15 minutes and amplicons were visualised under UV light. A Gene Genius Bio imaging system (Syngene, Synoptics; UK) was used to capture the image using GeneSnap (version 3.07.01) software (Syngene, Synoptics; UK) to determine the relative size of amplicons.
4.3 Results

4.3.1 Occurrence and diversity of microorganism in the biofilms

Table 4.3 indicates the different organisms isolated from the biofilm. The results only indicate the numbers of isolates that were positive for the various categories when subjected to preliminary tests (TSI, oxidase and in the case of *Pseudomonas* sp. and *Aeromonas* sp. also API 20E). It is evident from Table 4.3 that total coliforms and faecal coliforms were present in the biofilms from the raw Modimola dam water but were not detected in the biofilms of the treated water. Furthermore, the POU biofilm of the treated Modimola dam drinking water and mixed water contained both *Aeromonas* sp. as well *Pseudomonas* sp. Only *Pseudomonas* sp. was isolated from the biofilm of the Modimola dam raw water.

Table 4.3: Bacteria isolated from biofilms, cultivated on different growth media

<table>
<thead>
<tr>
<th>Biofilm development device site</th>
<th>Number of organisms isolated from different sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TC</td>
</tr>
<tr>
<td>Raw water Modimola dam</td>
<td>39</td>
</tr>
<tr>
<td>Mixed water</td>
<td>0</td>
</tr>
<tr>
<td>Filtered : treated dam water</td>
<td>0</td>
</tr>
<tr>
<td>Filtered: mixed water</td>
<td>0</td>
</tr>
</tbody>
</table>

TC= Total coliforms, FC= Faecal coliforms

4.3.2 Antimicrobial susceptibility test

All the organisms were subjected to antibiotic sensitivity test using 11 antibiotics of clinical importance. Multiple antibiotic resistance phenotypes were generated for isolates resistant to three or more drugs and results are shown in Table 4.4.
Table 4.4: Prevalent antibiotic resistance phenotype of biofilm

<table>
<thead>
<tr>
<th>Site</th>
<th>Isolate</th>
<th>Antibiotic resistance phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dam FC TC P</td>
<td>FC</td>
<td>KF-AP-C-E-OT-TM-S-A-NE</td>
</tr>
<tr>
<td>Treated A P</td>
<td>A</td>
<td>KF-AP-C-E-OT-TM-A</td>
</tr>
<tr>
<td>Dam water P</td>
<td>P</td>
<td>KF-AP-C-E-OT-TM-A</td>
</tr>
<tr>
<td>Mixed water P A</td>
<td>P A</td>
<td>KF-AP-C-E-TM-A KF-AP-C-E-OT-TM-A</td>
</tr>
</tbody>
</table>

Fc=faecal coliforms, TC= total coliforms. P= Pseudomonas, A= Aeromonas

Ampicillin (AP), cephalothin (KF), streptomycin (S), erythromycin (E), chloramphenicol (C), neomycin (NE), amoxycillin (A), ciprofloxacin (CIP), trimethoprim (TM), kanamycin (K) and oxytetracycline (OT)

4.3.3 Antibiotic Resistance Phenotype

All the isolates tested were resistant to Ampicillin, amoxicillin, cephalothin, erythromycin, chloramphenicol and trimethoprim. All the organisms tested were susceptible to ciprofloxacin. Four different multiple antibiotic resistance patterns were observed all the isolates were resistant to three or more different classes of antibiotics. The highest level of resistance was observed for Isolates from biofilms and the phenotype KF-AP-C-E-OT-K-TM-A indicating resistance to eight drugs was observed. From these results, it is evident that ciprofloxacin (CIP) and streptomycin (S) were the most effective, since all or a large proportion of the isolates were susceptible to both. These results indicate that biofilm grown organisms may serve as a reservoir for antibiotic resistance organisms, and therefore may have the potential to cause infections. This is a cause for concern, particularly to infants, old and immuno-compromised individuals in the Mafikeng community. Despite this, all the isolates from Molopo eye were susceptible to most of the antibiotics tested and none was resistant to more than two drugs.
4.3.4 Scanning electron micrograph (SEM) analysis of biofilm structure
The scanning electron micrograph (SEM) revealed the existence of bacterial cells within the biofilm matrix. Figures 4.3-4.5 depict the surfaces of coupons and filters exposed to raw water and drinking water distribution systems. Different organisms were isolated from the metal surfaces as well as planktonic bulk water samples. The scanning electron micrograph (SEM) revealed the existence of bacterial cells within the biofilm matrix, with a greater amount of bacterial cells on the galvanized coupons than on the copper coupons (Figures 4.3a, 4.3b, 4.4a and 4.4b). From the micrographs it was also evident that high bacterial densities were found in biofilm from the raw water of the Modimola dam as well as in drinking water distribution system in Mafikeng. This was a cause for concern since water from the dam goes to the treatment plant where it is purified and supplied to homes for consumption.

Figure 4.3a: Electron micrograph of biofilm from Modimola dam using galvanised coupons
Figure 4.3b: Electron micrograph of biofilm from Modimola dam using copper coupons
Figure 4.4a: Electron micrograph of biofilm from mixed water using galvanised coupons

Figure 4.4b: Electron micrograph of biofilm from mixed water using copper coupons
When the coupons were removed from the biofilm development device, a green colour was noticed on the copper coupons and it could be attributed to the corrosion products that are visible like crystals in the scanning electron micrograph. Attached cells in association with exopolysaccharide (EPS) were visible in galvanised, copper and POU filter surfaces in the SEM. It is therefore suggested that the suspended cells have the potential to attach to the surface and participate during biofilm formation. Figures 4.5a, 4.5b and 4.5c (POU filters) show evidence of biofilm formation on the surface of filters from mixed water, dam and Molopo eye, respectively. Rod-shaped bacteria are the dominating organisms in the biofilm and the aggregation of rod-shaped bacteria entangled in the EPS as seen in the micrographs usually reflect a mature biofilm. It was found that galvanized coupons from raw and treated water contain thicker biofilms than the copper coupons. In the present study coliforms were not detected but that does not mean that they were absent.

Figure 4.5a: Electron micrograph of biofilm from mixed water using carbon filter
Figure 4.5b: Electron micrograph of biofilm from dam water using carbon filter
4.3.5 PCR analysis for the detection of virulence genes in *Pseudomonas* and *Aeromonas* species

Specific PCR assays for the detection of virulence genes (*aerA* and *hylH* in *Aeromonas* and *exoA*, *exoS* and *exoT* in *Pseudomonas*) produced DNA fragments of the expected size of some of the markers. The isolates were screened for the presence of virulence genes and the combination of genes detected in the isolates from the different areas is shown in Table 4.5. Out of the 12 *Aeromonas* sp. that were isolated from Modimola dam treated water biofilm 83% and out of the 10 isolates from mixed water, 10% harboured the *hylH* gene. These were more prevalent in isolates from the dam. However, *aerA* genes were not detected in the
isolates from both sites. The exoA gene was detected in *Pseudomonas* sp. from the raw water biofilm and biofilm isolates from the treated dam water. Isolates from the biofilm from all sites harboured exoT genes. Of the 27 isolates tested from raw dam water, 6 isolates (18%), 14 out of 15 (85%) from treated dam water and 6 out of 8 (73%) harboured virulence gene determinants. However, none of the *Pseudomonas* sp. isolates possessed exoS gene.

**Table 4.5:** Virulence gene determinants detected in isolates from the different areas

<table>
<thead>
<tr>
<th>Site</th>
<th>Isolate</th>
<th><em>Aeromonas</em> species</th>
<th><em>Pseudomonas</em> species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>aerA</td>
<td>hylH</td>
</tr>
<tr>
<td>Treated dam water</td>
<td>P</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Mixed water</td>
<td>P</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Raw water-dam</td>
<td>P</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

A= *Aeromonas*, P= *Pseudomonas*, NT=Not tested

**Figure 4.6:** Image of a composite agarose (1% w/v) gel depicting the hlyH gene from *Aeromonas* species. Lane M (1 kb DNA Ladder); Lanes 1-4 (*hlyH* gene fragments from *Aeromonas* species isolated from different sites.)
Figure 4.7: Image of a composite agarose (1% w/v) gel depicting the \textit{exoT} gene from \textit{Pseudomonas} species. Lane M (1 kb DNA Ladder); Lanes 1-6 (\textit{exoT} gene fragments from \textit{Pseudomonas} species isolated from different sites)
4.4 Discussion

Water is a vital component of life but can serve as an important vehicle of dissemination of potential pathogens to humans (Kilb et al., 2003). Organisms that survived the treatment process may be able to grow in the aquatic environment. A further concern is that at times the treatment processes fail and the potential exist that pathogenic and opportunistic pathogens such as *Aeromonas hydrophila* and *Pseudomonas aeruginosa*. Both these species have a tendency to form biofilms. This study thus aimed at determining whether *Aeromonas* and *Pseudomonas* spp. occur in the drinking water biofilms of Mafikeng.

Organisms isolated in this study included total coliforms, faecal coliforms, *Aeromonas* and *Pseudomonas*. *Aeromonas* species are implicated to gastroenteritis and are generally considered water-borne pathogens (Pablos et al., 2011) while *Pseudomonas* species are opportunistic pathogens that causes nosocomial infections susceptible patients (Fricks-Lima et al., 2011). *Aeromonas* species has the
potential to grow in water distribution systems especially in biofilms, where it is resistant to chlorination and produces many different putative virulence factors (Pablos et al., 2009). Isolates that are resistant to chlorine may be present in tap water that is intended for human consumption and therefore cause diseases to humans.

The regrowth and formation of biofilms in drinking water distribution pipes has been detected even in countries with advanced water treatment and health care facilities (Kilb et al., 2003). This has been found to significantly cause corrosion of pipe materials and subsequently the addition of inorganic and organic matter resulting in poor aesthetic quality of water (Teng et al., 2008). Skejevrak et al., (2005) demonstrated that free chlorine reacts with compounds present in biofilm inside water pipes, producing unpleasant taste and odour. Adherent bacteria are more resistant to antimicrobial agents and could contribute to the planktonic cells present in the bulk water (Romaní and Sabater, 2013). Prevalence of planktonic bacteria in the drinking water may be due to sloughing of biofilm. This assumption is supported by the observation of planktonic bacterial episodes in treated drinking water by Castonguy et al., (2006). If biofilms contain any pathogenic bacteria, detachment of biofilms could release these bacteria to drinking water and affect risk levels of consumers (Lehtola et al., 2006). Biofilm formation is facilitated by many factors (Le Chevallier et al., 1996; Manuel et al., 2007) including available nutrients, characteristics of pipe material, disinfectants used, physico-chemical parameters and ability of the microorganisms to resist destruction by antimicrobial agents. Lethola et al., (2004) suggested significant release of nutrients from the surface material to water promotes growth of bacteria.

Cementitious, metallic and plastic materials are the three types of plumbing materials commonly used. Plumbing materials chosen for this study were copper and galvanized steel that is commonly used in domestic plumbing systems in South Africa. The results have demonstrated biofilm formation on the both plumbing materials that were used in the present study. Clote, (2003) observed higher density of bacteria on polyethylene and polyvinylchloride when compared to galvanized steels. In the present study thicker biofilms were observed on the micrographs of galvanized steel compared to copper. However, the present study did not compare
the galvanised steel coupons to polyethylene and polyvinylchloride. Moritz et al., (2010) observed that biofilm on copper had low concentrations of culturable bacteria. It is thus not uncommon to find low levels of culturable bacteria in biofilms on copper coupons. Moreover, it had been reported that the formation of biofilm was slower on copper pipes than in polyethylene pipes. However, after 200 days there was no difference in microbial numbers in biofilms within the two pipe materials (Lehtola et al., 2004). This implies that during long term use similar biofilm levels will develop on metal surfaces as those on polyethylene and polyvinylchloride surfaces. However, due to their nature biofilms on metal surfaces will contribute to microbial induced corrosion which can increase the metal concentration in water distributed by copper pipes (Roslev et al., 2004). This has the potential to health problems.

The present study did not focus on corrosion potential of the biofilms but rather on whether or not Pseudomonas and Aeromonas spp. colonise the biofilm. Moritz et al., (2010) previously demonstrated that Pseudomonas spp. is opportunistic pathogens that can integrate in to drinking water biofilms on materials which are relevant for domestic plumbing systems. Biofilms have been implicated in human infections and are particularly recalcitrant to antibiotic compounds (Fricks-Lima et al., 2011).

**Pseudomonas** and **Aeromonas** spp. isolated in this study carried some gene sequences encoding toxic proteins, indicting the possibility of these organisms to cause diseases in humans. The ability of *Pseudomonas* to express these virulence determinants also enhances their capabilities to produce biofilms (Pimenta et al., 2003). *Pseudomonas aeruginosa* is able of synthesizing a large number of virulence proteins that have great influence on pathogenesis (Kaszab et al., 2011). *Pseudomonas* species produces extracellular compounds which promotes adhesion and the ability of the isolates to attach surfaces. This therefore, increases the virulence properties of *Pseudomonas* strains. The pathogenicity of *Aeromonas* spp. has been linked to exotoxins such as cytolytic enterotoxin, haemolysin/aerolysin, lipase and protease (Yogananth et al., 2009). Detection of these genes amongst the *Pseudomonas* and *Aeromonas* spp. isolated from drinking water sources of Mafikeng is cause for concern as these organisms were resistant to many antibiotics tested and should be further investigated.
4.5 Conclusion

The potential for raw and potable water to produce biofilms was demonstrated in this study. Biofilms on pipe walls in water distribution systems can affect chlorine demand, pipe corrosion and hygienic and aesthetic quality of drinking water, therefore, responsible for the deterioration of the drinking water quality during the distribution from waterworks to the consumer. Opportunistic pathogens such as *Pseudomonas* and *Aeromonas* species were isolated from the Modimola dam raw water and drinking water as well mixed drinking water biofilms, which is a cause of concern. These isolates were found to harbour virulence gene determinants indicating that they have the potential to cause diseases in humans. This result has important implications for the understanding of the spread and control of disease resulting from bacterial infections in humans. Therefore, it is important to constantly determine the occurrence of these species in water bodies and the drinking water distribution systems in particular and whether conditions prevail that may allow these opportunistic species to survive water purification processes. Such a strategy will be particularly of importance in scenarios where treated wastewater is reused for drinking water purposes. A clear understanding of the different mechanisms by which biofilm bacteria harbour and distribute virulence factors as well as protect themselves from the action of disinfectants and antibiotics is vital to formulate control and management strategies.
CHAPTER 5

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The primary objective of this research was to analyse the physico-chemical parameters, biofilm formation and determine antibiotic resistance profiles of bacterial communities isolated from the drinking water. An assessment of physico-chemical characteristics of water was carried out in order to ascertain the quality of water for domestic purposes. Water quality assessment can be defined as the evaluation of physical, chemical and biological nature of water in relation to natural quality, human effects and intended uses (Wanda et al., 2012). Opportunistic pathogens such as Aeromonas and Pseudomonas species were isolated with the aim of detection of virulence genes. Part of the reason this work was undertaken was the fact that there has been increasing reports on the occurrence of opportunistic pathogens, biofilm formation in drinking water distribution system and isolation and identification of antibiotic resistant organisms from drinking water (Momba et al., 2006; Drenkard, 2003; Henriques et al., 2006; Chen et al., 2010; Figueira et al., 2011; Pablos et al., 2011; Hoa et al., 2011). This study was undertaken with a purpose of establishing the possible presence of faecal indicators and opportunistic pathogens such as Aeromonas and Pseudomonas species in the drinking water systems, whether the overall quality of water poses a potential threat to various uses of water, such as drinking water supplies. Environmental factors may influence the survival of various microorganisms. Thus, it is important to carry out water quality monitoring in order to detect the changes of the water quality. Overall water quality analyzed were pH, temperature, electric conductivity (EC) and total dissolved salts (TDS) of the tap water from Modimola dam, Molopo eye and mixed water were measured thrice a week over a period of four months. The values were well within the limits as laid down by WHO and no substantial variation was observed during the assessed period.

Among all the physico-chemical parameters considered, pH is an important parameter which determines the suitability of water for various purposes (Wanda et
pH value of water is significant indication of its quality and it is dependent on carbon-dioxide carbonate-bicarbonate equilibrium (Rosli et al., 2012). The pH of water from Molopo eye and mixed water was considered as neutral. Neutral pH of mixed water could be attributed to the dilution effect of Molopo eye derived water on mixed water. However the pH of Modimola dam water was slightly alkaline. Similar results were reported by other researchers. Wanda et al., (2012) observed a pH for water samples ranging between 6.40 and 6.90 indicating weak acidic character of the waters and registered a good water quality rating for both raw and treated water. Rosli et al., (2012) reported neutral and slightly alkaline water, (pH ranging from 6.98 to 7.51) and pH of these ranges is classified as Class I. Within this pH range excessive corrosion of piping materials or other unpleasant aesthetic or health related effects could not be expected. Water temperature observed in this study would pose no adverse effects on living organisms. High temperature enhances microbial activity and other chemical reactions (Pritchard et al., 2007). The higher counts of Pseudomonas species observed in higher temperature are attributed to the temperature which would support their survival in summer. Higher concentration of bacteria in water may affect the aesthetic quality of drinking water (Hoehn, 1988).

Electric conductivity and TDS values fall within the recommended limits and these values are expected from adequately treated water. The EC values did not vary significantly in different sites except in Modimola dam where the value was high. It was observed that in Modimola dam both EC and TDS were high compared to the other two sites. High TDS is more of an aesthetic rather than a health hazard. EC is one of the important parameter in water quality as it indicates the amount of dissolved inorganic ion in water (Singh et al., 2013). However, the results by Ramdani et al., (2012) showed that the physical and chemical quality of the waters of Southern Algeria is poor and rich in mineral salts and the conductivity is between a minimum of 1374 $\mu$S/cm and and a maximum of 4070 $\mu$S/cm. The amount of EC in Salak River range from 36000 $\mu$S/cm to 41000 $\mu$S/cm and was found to be higher at points which are nearer to the seawater and TDS ranged between 31.7 to 398.8 mg/L,where they can be classified as non-saline (<1000 mg/L) (Rosli et al., 2012). The increase in conductivity is due to a high concentration of minerals (Ramdani et al., 2012; Das et al., 2013), they pose a health risk. High EC is attributed to high salinity and high mineral content.
Anthropogenic activities and livestock could affect the chemical quality of water (Yilla et al., 2008) and elevated amount of TDS may cause corrosion of plumbing materials and appliances as well as scale build up, can cause eye and skin irritation and can permit an algae bloom (WHO, Guidelines for drinking-water quality, 1996). The physico-chemical properties of the water (ionic strength, hardness) govern the biofilm adhesion rate on pipe materials (Janjaroen et al., 2013). In comparison with treated water, raw water registered a lower water quality indicating its unsuitability for direct consumption without treatment.

The danger posed by drinking water contamination with faecal indicators and pathogenic organisms cannot be overemphasized. Total coliforms, faecal coliforms, heterotrophic bacteria, Aeromonas and Pseudomonas species were successfully isolated from raw water, treated water and biofilms. Aeromonas and Pseudomonas species are resistant to environmental stress and therefore survive in the aquatic environment. High concentrations of faecal indicator organisms in raw water suggest contamination by human activities and can come from a variety of other sources including inputs from farm animals, wild life and sewage effluent. Faecal indicator organisms from human and animal origin are major contributors to decrease water quality (Van der Kooij, 2000; Okeke et al., 2011). Moreover, higher concentrations of organisms observed during summer indicate that rain-fall surface runoff which plays a major role. Presence of these organisms may cause sporadic outbreak of water borne diseases if used as drinking water. Pseudomonas was the most prevalent organism in the source water and treated Modimola dam water, in both summer and winter. Modimola dam receives treated sewage effluent from the sewage treatment plant and this may account for the occurrence of heavy loads of microorganisms. Water that is contaminated with these organisms, act as potential source for the dissemination of pathogens to humans. Based on the results, presented herein, we reinforce the importance of effective treatment procedures. Occurrence of microorganisms in treated water is an indication of bacterial resistance to chlorine or insufficient chlorine application and could cause possible regrowth within the distribution system. Similar studies have also been conducted to determine the prevalence of these organisms in drinking water (Lee et al., 2010; Okeke et al., 2011; Pablos et al., 2011; Patel et al., 2011). Pathogenic bacteria in drinking water originate from the gastrointestinal tract of warm blooded animals (Okeke et al., 2011).
and are a cause of numerous waterborne disease outbreaks in the developing world (Tao et al., 2010). Public health can be protected by reducing pathogenic microorganisms and controlling the quantities of different chemicals within the treated waste water (Adewumi et al., 2010).

Water is a scarce resource and there has been a significant increase in the use of reclaimed water to address the problem of water scarcity (Meneses et al., 2010), augmenting the limited water resource to meet the growing demands (Li et al., 2011., Wang et al., 2012). Most municipalities treat human sewage to reduce the bacterial load before releasing the water into surrounding lakes, rivers, and oceans or spreading it on land. However, studies indicate that treated sewage and the water into which it is circulated remain heavily contaminated with antimicrobial resistant bacteria (Iwane et al., 2001; Edge and Hill, 2005; Koczura et al., 2012). The two main sources of resistant bacteria and resistance genes are human sewage and animal.

Due to more advanced waste water treatment technologies reuse potential for reclaimed water is expanding. Subsequently water reuse forms an integral part of water and waste water management (Li et al., 2011). However, health risks are associated with reuse of reclaimed water. It is the major source of nutrients, salts, pathogenic organisms, toxic substances and pharmaceuticals (Dominguez-Chicas and Scrimshaw, 2010) including antibiotics (Köck-Schulmeyer et al., 2011). High concentration of pollutants in the sewage effluent may negatively impact the quality of source water and may affect the health of consumers, change the colour and odour of drinking water (Rodriguez et al., 2012). Transfer of pathogenic organisms from drinking water which receives reclaimed water to consumers is a pressing issue (Revit et al., 2011). In addition to this, contaminated water used for irrigation has been implicated to a number of incidences of gastrointestinal illness caused by contaminated produce (Adewumi et al., 2010; Shelton et al., 2013).

Another objective of the study was to assess antibiotic resistance profiles of the isolates from the raw and drinking water. Large proportions of these organisms were resistant to three or more antibiotics and hence were termed multiple antibiotic resistant (MAR) isolates. Resistance observed was towards erythromycin,
tetracycline, amoxicillin, trimethoprim and ampicillin. Erythromycin resistant strains had a high percentage share of total bacterial counts in all the sites, which confirm patterns observed in the previous findings of Guo et al., (2013). They have detected heterotrophic bacteria resistant to erythromycin and tetracycline in the wastewater samples. The prevalence of erythromycin-resistant bacteria in their study was significantly higher. This can be attributed to the over use of these antibiotics. However, resistance to erythromycin was a cause for concern because this antibiotic is not used in animals in Mafikeng area (Ateba and Bezuidenhout, 2008). High levels of resistance to other antibiotics were also detected. The results obtained on the prevalence of antibiotic resistance organisms in this study coincided with other data published (Kim and Wei, 2007; Tao et al., 2010). The prevalence of antibiotic resistant bacteria in China was confirmed by Shi et al., (2013) who reported the occurrence of antibiotic resistant bacteria in drinking water even after chlorination.

Large quantities of antibiotics are used in human therapy, prophylaxis and added as growth promoters in animal feeds (Murata et al., 2011; Deblonde et al., 2011; Jiang et al., 2011; Bouki et al., 2013). Tetracycline and penicillin are the most commonly used antibiotics in humans for the treatment of bacterial infections and also as growth promoters in veterinary medicine (Tao et al., 2010; Mathers et al., 2011). Long term use of these antibiotics in food producing animals may result in selection and potentially increase the prevalence of antibiotic resistant bacteria in animals (Mathers et al., 2011). The residues of these antibiotics reach the surface water through the discharge of waste water receiving hospital waste water, agricultural runoff and from the use of antibiotics in aquaculture (Bartelt-Hunt et al., 2009; Watkinson et al., 2009; Wright, 2010; Murata et al., 2011) can cause environmental and health hazard problems (Turkdogan and Yetilmeszoy, 2009). Waste water treatment plants are potential reservoirs contributing to the evolution and spread of antibiotic resistance (Gao et al., 2012; Guo et al., 2013). Most commonly detected antibiotics belong to different classes such as macrolides, β-lactams, aminoglycosides and tetracycline, which are commonly prescribed antibiotics (Loganathan et al., 2009; Hijosa-Valsero et al., 2010; Jiang et al., 2011; Li and Zhang, 2011). Waste water effluents from hospitals and intensive farming facilities is the major contributor of antibiotics contamination of the surface water (Baquero et al., 2008; Watkinson et al., 2009) carrying human and animal pathogenic bacteria,
harbouring antibiotic resistance genes (Hoa et al., 2011). In many countries several hospitals do not treat their wastewater before disposing it into the general sewage collecting system (Picão et al., 2013). The hospital sewage may constitute the perfect scenario for exchange of resistance genes between clinical pathogens and environmental bacteria due to the presence of broad-spectrum antimicrobials along with such bacteria (Brown et al., 2006; Martinez, 2009; Taylor et al., 2011).

Large amounts of antibiotics are released into municipal waste water due to incomplete metabolism in humans (Bouki et al., 2013). Reuse of treated waste water increases the introduction of antibiotics into the drinking water distribution system and the concentration of antibiotics in waste waters depends on various factors (Hijosa-Valsero et al., 2011). Elimination of these compounds is incomplete as they are not completely degraded or absorbed during sewage treatment process, hence found in finished drinking water (Maycock and Watta, 2011). The occurrence of antibiotics in the aquatic environment can cause selection and spread of antibiotic resistance in bacteria (Kümmerer, 2009; Fatta-Kassinos et al., 2011) and probably the spread of antibiotic resistance determinants between aquatic bacteria and human pathogens (Baquero et al., 2008; Jiang et al., 2013; Rizzo et al., 2013; Shi et al., 2013). The problem of resistance is worsened if resistance is associated with pathogenic bacteria. Waste water treatment involves primary secondary and tertiary treatment processes and in large urban areas often applies the activated sludge process. Briefly, after a primary treatment consisting of grading to remove bulky materials, wastewater is aerated. In this step, microorganisms metabolize the suspended and soluble organic matter, a flocculent sludge is formed, and, after settling, the biomass is removed from the liquid stream (Chasick, 1996; Kim and Aga, 2007). The secondarily treated sewage is then discharged into rivers, estuaries, or oceans. Although secondary treatment seems to decrease the amount of bacteria in sewage, compared to the incoming sample a significant number of bacteria still remain, especially those that are resistant to antimicrobials (Kim and Aga, 2007; Galvin et al., 2010). The biological treatment process creates an environment potentially suitable for resistance development and spread because bacteria are continuously mixed with antibiotics at sub-inhibitory concentrations (Davies et al., 2006; da Silva et al., 2006; Auerbach et al., 2007). These multidrug resistant isolates are continually disposed in the urban river. The discharge of multidrug-resistant
bacteria into an urban river is worrisome, since these isolates could persist in the environment and act as opportunistic pathogens and/or resistance reservoirs that could accelerate the evolution of antimicrobial resistance in the community (Kim and Aga, 2007; Baquero et al., 2008; Martinez, 2009). During all these processes considerable changes occur in the distribution of bacterial population ([Bouki et al., 2013; Rizzo et al., 2013]. However, treatment plant designs and operating conditions influence the proliferation of antibiotic resistant bacteria, which in turn may transfer resistance genes to non-resistant bacteria (Bouki et al., 2013; Jiang et al., 2013). Many studies have demonstrated the presence of clinically relevant antibiotic genes along with the water cycle, with potential hotspots of waste water discharge into other aquatic environments (Volkmann et al., 2004; Schwartz et al., 2006; Zhang et al., 2009). Antibiotic resistance in bacteria is a worldwide problem and there is an apparent increase in developing countries. Clinical infections, diseases and death caused by resistant bacteria are increasingly being reported (Kim et al., 2007).

The resistance profiles of the E. coli isolates in a study conducted by Coleman et al., (2013) support the findings that livestock are a likely source of contamination. Tetracycline was the most common resistance in the drinking water samples, followed by sulphas and aminoglycosides. In a research conducted by Graves et al., (2007) the major source of pollution of antibiotic resistant organisms in over 60% of the water samples tested were by cattle. However, Bartelt-Hunt et al., (2009) detected more human antibiotics in water than veterinary antibiotics. Contamination of water is of concern, not just because of the spread of resistant bacteria to humans and other animals, birds, and aquatic life, but also due to the horizontal transfer of resistance mechanisms between bacteria in the water and its environ (Walia et al., 2004; Ozgumus et al., 2007).

In the present study, we found potable water contaminated by faecal indicator bacteria and opportunistic pathogens such as Aeromonas and Pseudomonas species resistant to several classes of antibiotics. This is in accordance with the observation by Picão et al., (2013). These workers isolated resistant Aeromonas and Pseudomonas species from a waste water treatment plant. Aeromonas and Pseudomonas species are considered as important human opportunistic pathogens, with the ability to cause various types of diseases and are common inhabitants of
aquatic ecosystems (Sharma et al., 2005; Emekads et al., 2006; Oudhuis et al., 2008). *Pseudomonas aeruginosa* is also regarded as one of the most important Gram-negative pathogens, inhabiting aquatic environment, and is responsible for a number of opportunistic nosocomial infections due to its multidrug-resistance (Vaz-Moreira et al., 2012; Keating et al., 2013; Suzuki et al., 2013). Very little information is available on the ecology of *Pseudomonas aeruginosa* in water environment and its association with antimicrobial agents (Suzuki et al., 2013). Their increasing resistance to antibiotics and associated pathogenicity predict poor clinical outcome as well as impose economic burden for the health system (Trautmann et al., 2005). *Aeromonas* are widespread in untreated and treated waters. Their real incidence of virulence determinants/antibiotic resistance in environmental isolates is still poorly described. Therefore the implications of human/animal contact with non-monitored waters are unknown (Carvalho et al., 2012). *Aeromonas* spp. have been frequently implicated in diarrhoeal episodes (Janda and Abbott, 2010; Ahmed et al., 2012) Resistance to diverse groups of antibiotics is a concerning characteristic of *Aeromonas* species (Janda and Abbott, 2010; Figueira et al., 2011). Drug resistant *Aeromonas* are relevant agents for antimicrobial resistance spreading in the environment (Figueira et al., 2011). Blasco et al., (2008) reported an increase in antibiotic resistance strains that belong to pathogenic bacteria such as *Aeromonas* and *Pseudomonas* spp. Taking into account the array of infections in which aeromonads are implicated, it is mandatory to conduct the surveillance of water of public use and to explore aeromonads’ risk factors (Carvalho et al., 2012).

Occurrence of high densities of microorganisms in potable water is an area of concern and this problem is worsened by the presence of antibiotic resistant organisms which may have a negative impact on human health. Antibiotic resistant bacteria can be found in all natural environments (Harnisz et al., 2011; Coleman et al., 2013) and poses a daunting problem in hospital acquired infections (Delcour, 2009). Different strategies that bacteria have deployed to resist antibiotics have been well documented by different researchers (Malléa et al., 1998; Zhanel et al., 2004; Ghisalberti et al., 2005; Ghisalberti et al., 2006; Kim and Wei, 2007; Pagès and Amaral, 2009; Martins et al., 2010) who pointed out that efflux pump and limited permeability of outer membrane contribute to multidrug resistance in bacteria. Animal and human pollution in freshwater sources pose a risk by introducing genetic
elements responsible for bacterial resistance that may be perpetuated in environmental species (Farkas et al., 2013). In an era when antimicrobial resistance evolution and dissemination are not accompanied by the development of new antimicrobials, controlling the dissemination of antimicrobial-resistant bacteria is absolutely necessary (Picão et al., 2013). Hence, constant monitoring of the antibiotic resistance profiles of bacterial isolates, from drinking water, is essential in order improve treatment options for bacterial infections in humans.

Although the present study suggests that a large proportion of the isolates were resistant to most of the antibiotics tested, all isolates were susceptible to ciprofloxacin (CIP) and streptomycin (S) as opposed to the resistance observed by Figueira et al., (2011). From the result it is evident that ciprofloxacin (CIP) and streptomycin (S) were the most effective antibiotics, since all the isolates were susceptible to both. Resistance to these antibiotics was a cause of concern, since they are used to treat human infections. Similar observations were made by other authors (Alighardashi et al., 2009; Wunder et al., 2011). However, Picão et al., (2013) isolated ciprofloxacin resistant Aeromonas and Pseudomonas spp. from the WWTP effluent.

Resistance is inevitable and understanding the origins, evolution and dissemination of antibiotic resistance elements provides vital information for antibiotic drug discoverers (Wright, 2010). The reduction of antibiotic contamination requires the implementation of methods capable of removing these compounds during wastewater treatment (Fatta-Kassinos et al., 2011), thereby minimizing the risk of antibiotic resistance in the environment (Schwartz, 2012). Resistance to antibiotics of clinical importance is a bit alarming since it could compromise treatment. Organisms with antibiotic resistance coupled with virulence genes have the highest potential for causing diseases.

Another objective of this study was to investigate the biofilm formation in drinking water. In fact, it has been estimated that the majority of bacteria in natural aquatic ecosystems are organized in biofilms (Donlan and Costerton, 2002). Drinking water biofilms represent complex organomineral deposits with a diversified microbial community (bacteria, fungi, free-living protozoa, etc.) (Wingender and Flemming,
Bacteria in drinking water systems can grow in bulk water and as biofilms attached to pipe walls, both causing regrowth problems in the distribution system (Srinivasan et al., 2008). In a biofilm, a microbial community is attached to a surface and embedded in a self-produced matrix composed of extracellular polymeric substances which provides the bacteria with several advantages compared to those living as planktonic cells (Le Chevallier et al., 1987; Kiristis et al., 2005). First, the bacteria are maintained in the selected microenvironment where population survival does not depend on rapid multiplication (Jefferson, 2004). This is especially advantageous in environments where the bacteria are exposed to constant liquid movements, as, for example, in aquatic environments. Additionally, the bacterial cells present in a biofilm have an increased resistance to desiccation, grazing, and antimicrobial agents compared to their planktonic counterparts (Mah and O'Toole, 2001; Sutherland, 2001; Jefferson, 2004; Fux et al., 2005; Matz and Kjelleberg, 2005). The findings of Srinivasan et al., (2008) suggest that bulk water bacteria may dominate in portions of a distribution system that have low chlorine residual.

An investigation conducted in river biofilms by Proia et al., (2013) observed higher levels of antibiotics induced changes in the bacterial community structure of biofilms by favouring the antibiotic resistant bacteria. Biofilms are generally recognised as the primary source of microorganisms in drinking water distribution systems (Liu et al., 2013) and can lead to various health issues such as protecting and supporting pathogenic microorganisms, harbouring antibiotic resistant organisms and exchange of genetic materials between the attached and planktonic populations (Farkas et al., 2013). In addition, many outbreaks of pathogens have been found to be associated with biofilms (Flemming et al., 2002). Scanning electron microscopy revealed the presence of biofilms on copper and galvanised steel harbouring faecal indicators and opportunistic pathogens which is in agreement with the observation made by Farkas et al., (2012). This observation suggests that faecal indicator organisms and opportunistic pathogens occur in a culturable state in drinking water and form biofilms. Growth of biofilms in the distribution system initiates metal corrosion and eventually affects the quality of drinking water (Lee et al., 1980). The ability to produce biofilm is attributed to virulence (Pimenta et al., 2003). Episodes of water related disease outbreaks can be attributed to the consumption of drinking water that passed through biofilm contaminated drinking water distribution system (Farkas et
This is because it is possible to detach biofilm due to the hydrodynamic shear stress commonly encountered in drinking water distribution system (Abe et al., 2012). Biofilm bacteria are important in water distribution system because drinking water is considered as oligotrophic environment (Lee et al., 2010). The similarity of antibiotic resistance patterns of the isolates from biofilms and planktonic bacteria suggests that they may originate from a common source of contamination. Resistance was observed against erythromycin, followed by trimethoprim and amoxycillin in planktonic bacteria, and similar patterns were observed in biofilms also. Six different antibiotic resistance patterns were observed in biofilms.

A further objective of this study was to use PCR technique to investigate the presence of virulence gene determinants in *Aeromonas* and *Pseudomonas* and thereby demonstrate the potential pathogenicity of these organisms. The presence of *hylH*, and *aerA* were detected in *Aeromonas* and *exoA* and *exoT* were detected in *Pseudomonas* species using specific oligonucleotide primers. The prevalence of these genes in these isolates was a cause for concern. The pathogenicity of *Aeromonas* has been linked to exotoxins such as cytolytic enterotoxin, hemolysin/aerolysin, lipases and proteases (Yogananth et al., 2009). Haemolysins include aerolysin, proteases, adhesins, invasins, enterotoxins phospholipase and lipase (Parker and Shaw, 2011). The pathogenicity of *Pseudomonas* is linked to its ability to secrete different exotoxins as they possess gene sequence encoding toxic proteins (Kaszab et al., 2011). These virulent factors significantly increase the potential to infect a wide range of host organisms more especially cause diseases in humans. Therefore, presence of virulent organisms in drinking water poses a threat to humans when ingested. Organisms with antibiotic resistance coupled with virulence genes have the highest potential for causing diseases (Kaszab et al., 2011). Similar observations have been reported by Alcaide et al., (2010) and Figueira et al., (2011). This indicates the need to detect not only faecal indicators but also opportunistic pathogens in drinking water and to implement proper treatment procedures.

World Health Organization estimated in the 2000 assessment that there are four billion cases of diarrhoea each year, in addition to millions of other cases of illness associated with the lack of access to clean water. More than 5 million people die
each year from diseases caused by unsafe drinking water, lack of sanitation, and insufficient water for hygiene. In fact, over 2 million deaths occur each year from water-related diarrhoea alone. At any given time, almost half of the people in developing countries suffer from water-related diseases (Johannesburg Summit, 2002). Millions of deaths will continue to occur every year from water-related diseases unless far more aggressive actions are taken to meet basic human needs for safe water and sanitation.

Gastrointestinal infections are one of the principal causes of morbidity and mortality among children. Emerging pathogens in drinking-water have become increasingly important since the late 1980s (WHO, 2010). Diarrhoea caused by these pathogens is the second leading contributor to global burden of disease, ahead of heart disease and (HIV/AIDS). Water-borne diseases represent a major burden on human health worldwide. Every year, 1.8 million people die from diarrheal diseases, of which 1.5 million are children under the age of 5 (WHO, 2007). The consequences of these diseases include lost work days, missed educational opportunities, official and unofficial health care costs, and the draining of family resources (WHO, 2002). If no action is taken to address the basic human needs for water, as many as many as 76 million people will die by 2020 of preventable water-related diseases. This problem is one of the most serious public health crisis facing us, and deserves far more attention and resources than it has received so far (Gleick, 2002).

5.2 Conclusion
In conclusion, the level of contamination of drinking water samples in Mafikeng, coupled with antibiotic resistant bacteria and their ability to form biofilms, is of high concern. Results indicated the occurrence of antibiotic resistant faecal indicator bacteria and opportunistic pathogens such as Aeromonas and Pseudomonas species in the drinking water destined for human consumption. Faecal indicator bacteria are the major contributors of poor drinking water quality and may harbour opportunistic pathogens. Their presence in water highlighted survival of organisms against treatment procedures and the possible regrowth as biofilms in plumbing materials. Further in the present study multiple antibiotic resistance (MAR) was observed in all the organisms isolated. These isolates may have negative clinical implications and hence hamper the treatment of infections caused by these
organisms. The detection of large proportion of MAR *Aeromonas* and *Pseudomonas* species which possessed virulent genes was a cause for further concern as these could pose health risks to humans. These isolates are opportunistic pathogens and are often implicated to infectious diseases. The pathogenicity of *Aeromonas* and *Pseudomonas* species depend essentially on their virulence genes. Resistance determinants from these isolates could also be transferred to enteric bacteria of clinical importance. It highlights the necessity to have surveillance programmes and risk assessment pertaining to antimicrobial resistance to alleviate the antibiotic resistance problem. These findings have general implications for public health especially in terms of antibiotic resistance and virulent genes observed in the tested organisms. Although there is immediate observation of water borne disease through the ingestion of antimicrobial resistant strains of pathogenic bacteria, there are longer-term threats as well. The problem of MAR strains dissemination is further aggravated by the misuse and self-prescription of antibiotics. Therefore, judicious use of antibiotics in agriculture, veterinary and human medicine is needed to reduce the emergence, transmission, and persistence of antimicrobial resistance.

Drinking water quality is a global issue and the protection of both surface and ground water from pollutants is paramount for the prevention of waterborne diseases. Waterborne diseases include those where transmission occurs by drinking contaminated water, particularly contamination by pathogens transmitted from human excreta. These include most of the enteric and diarrheal diseases caused by bacteria and viruses. These diseases have always been a threat to human health and many outbreaks have been found to be associated with biofilm formation. Biofilm forming bacteria play a key role in many infections as they are more resistant to antimicrobials. Bacteria in natural habitats commonly exist in biofilm consortia. These microorganisms can also be released into the bulk water causing a decrease in the microbiological quality of water. Furthermore, it could lead to decrease in the efficiency of disinfectants and corrosion of pipes used in the distribution system affecting the aesthetic quality of water. Therefore proper biofilm control strategies are required to control drinking water biofilm formation.

Water is a limited resource and the demand gets higher as population is rapidly growing. Reclamation and reuse of waste water is one of the most effective ways to
alleviate water resource scarcity. If the final waste water effluent does not meet the regulatory standards, before being released in to the environment, it may cause adverse effect. Results from this study provide insight into the quality of treated water distributed from Mmabatho water treatment works. The poor quality of the water could be the result of release of treated waste water from Mmabatho sewage treatment plant into Modimola dam. In order to rectify the problem, periodically monitoring the quality of waste water effluent released from Mmabatho sewage treatment works could go a long way. This could contribute to reducing the occurrence of Aeromonas and Pseudomonas infections in humans and ensure proper public health. Results obtained from this study will add to the existing knowledge of concurrent emergence of multidrug resistance in bacterial communities. In addition, the findings will offer opportunities to address the problems associated with resistance which compromise drug therapy because bacteria will continue to develop resistance during waste water treatment by mutation or gene transfer. This study would create an awareness of the presence of pathogenic Aeromonas and Pseudomonas species in drinking water and will minimise the risk of contracting water-borne diseases in the community.

The provision of microbial safe drinking water is a major problem in South Africa and is one of the main requirements of drinking water supply. Water borne epidemics are linked to consumption of contaminated drinking water. Therefore, the monitoring of drinking water quality from source to tap is an essential step towards hygiene safety. This work did not look into statistics of disease incidence in surrounding hospitals and clinics. Further, other microorganisms/ pathognes such as rotaviruses have not been investigated. This could make a good future study.

5.3 Recommendations

Aeromonas and Pseudomonas species are opportunistic pathogens and have the ability to form biofilms as a survival strategy. The implication is that these organisms can be dislodged from the pipe walls and enter in to the water circulated for human consumption. Some of these are commonly implicated in water borne diseases in many countries including South Africa. These infections more severely affect children, aged and immuno-compromised individuals due to the HIV/AIDS burden. It is thus imperative that the effectiveness of treatment technologies and disinfectants used be
strengthened. The prevalence of these organisms harbouring antibiotic resistant genes coupled with virulent determinants cannot be overemphasised. Based on the findings of this study, it is therefore recommended that measures be implemented at the treatment plants for the complete inactivation or elimination of opportunistic pathogens. The fact that these opportunistic pathogens are antibiotic resistant organisms makes the urgency of elimination measures imperative. Initiatives should be taken to increase the efficiency of waste water treatment facilities to ensure that these pathogens cannot slip through the purification system. The treatment plant is only focusing on the application of minimum standard procedures. By comparing the effectiveness of different treatment technologies more elaborate treatment process such as disinfection with chloramine or ozone/UV treatment technologies can be adopted. Unlike chlorine, chloramine has a longer half-life in the distribution system and still maintains effective protection against pathogens. Helicobacter pylori, Escherichia coli and other organisms resistant to chlorine are susceptible to the disinfecting effect of chloramine. The use of membrane filtration (microfiltration, ultrafiltration and nanofiltration) has increased over the past decade mainly due the high level removal of bacteria, viruses and protozoa cysts such as Giardia and Cryptosporidium. These advanced processes may be implemented individually or as a package, depending on the need of the water treatment plant. Transmission of these organisms to humans through drinking water is something that needs to be avoided at all cost in all municipalities due to the vulnerability that exist in the South African society. It is also recommended that innovative control strategies such as reduction of biodegradable organic matter (BOM) concentration and new antimicrobials which promote high biofilm removal and inactivation activities should be implemented to combat the problem of biofilms.

Further research is needed to establish the source of development of antibiotic resistance observed among opportunistic pathogens encountered in this work or the diversity of resistance genes and mode of transfer. Extensive research is also required to evaluate how pipe material may affect the potential of biofilms and the factors that contribute to the rate and extend of biofilm development.
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