

Evaluation of microbiological and physico-chemical quality of water from aquifers in the North West Province, South Africa

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ABSTRACT

Contamination of groundwater that is suitable for drinking is of growing concern as the water supply of South Africa is becoming increasingly limited. This is especially the case in the North West province, with its semi – arid climate and variable rainfall patterns. The aim of the study was to evaluate the microbiological and physico – chemical qualities of groundwater obtained from selected DWA (Department of Water Affairs) monitoring boreholes in the Mooi River and Harts River catchment areas. Physico-chemical parameters included temperature, pH, electrical conductivity (EC), salinity, total dissolved solids (TDS), sulphate and nitrate concentrations. Physical parameters were measured using a calibrated submerge-able multimeter and chemical parameters using specialised kits and a spectrophotometer. Microbiological parameters included heterotrophic plate counts and total and faecal coliform enumeration. Membrane filtration and culture based methods were followed for enumeration of bacteria. During the identification procedures multiplex PCR for *E. coli* identification and 16S rRNA gene sequencing for identification of heterotrophic plate count bacteria and amoeba resistant bacteria were used. For antibiotic resistance, the Kirby-Bauer (1996) disk diffusion method was used. During the warm and wet season high electrical conductivity and salinity were observed in the Trimpark (65.3 mS/m; 325 ppm), School (125.1 mS/m; 644 ppm), Warrenton (166.9 mS/m; 867 ppm) and Ganspan (83.3 mS/m; 421 ppm) boreholes. Warrenton borehole had a high sulphate level (450 mg/l) as well. High chemical oxygen demand was observed in the Blaauwbank (62 mg/l) and Warrenton (98.5 mg/l) boreholes. In the dry and cold season similar observations were made for the various boreholes. Electrical conductivity and salinity levels remained high for the Trimpark (70.1 mS/m; 427.5 ppm), School (127 mS/m; 645 ppm), Warrenton (173.3 mS/m; 896.5 ppm) and Ganspan (88.1 mS/m; 444.5 ppm) boreholes. Nitrate levels for the Trimpark (14.1 mg/l) and School (137 mg/l), as well as sulphate levels for the Warrenton (325 mg/l) borehole were also high. Total coliforms, faecal streptococci and HPC bacteria were enumerated from water samples from all boreholes, except Blaauwbank where no faecal streptococci were enumerated. Faecal coliforms were enumerated from 5 of the possible 7 boreholes during a warm and wet season (Trimpark – 42 cfu/100ml; School – 2 cfu/100ml; Cemetery – 175 cfu/100ml; Warrenton – 3.84×10^3 cfu/100ml; Ganspan – 1.9×10^3 cfu/100ml). Indicator bacteria (FC, TC, HPC) exceeded target water quality ranges (TWQR) for drinking water in

each case. During the cold and dry sampling season, faecal coliforms were enumerated mainly from the Trimpark (11 cfu/100ml) borehole. Total coliforms, faecal streptococci and HPC bacteria were enumerated from all the boreholes, except for Blaauwbank that contained no faecal streptococci or total coliforms. Enumerated indicator bacteria levels again exceeded TWQR for domestic use. Total coliform counts for the Pad dam borehole, however, complied with TWQR for domestic use. Identified *E. coli* were resistant to Erythromycin, Cephalothin and Amoxicillin and susceptible to Ciprofloxacin. *Escherichia coli* isolated from the Mooi River catchment shared the same antibiotic resistance phenotype. The most abundant HPC bacterial genus identified was *Pseudomonas* spp. (7 isolates). Opportunistic pathogens isolated included *Pseudomonas aeruginosa*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Flavobacterium*, *Bacillus cereus* and *Mycobacterium* spp. Varying degrees of antibiotic resistance were observed. Generally, the same pattern between the same genera were observed. All HPC isolates were resistant to Cephalothin and Amoxicillin and a lower degree Erythromycin and Streptomycin. The most abundant amoeba resistant bacteria was identified as *Pseudomonas* spp. Other isolates included *Alcaligenes faecalis* and *Ochrobactrum* sp. and *Achromobacter* sp.. All of these are opportunistic pathogens, except for *Achromobacter*. Resistance to more antibiotics (Streptomycin, Chloramphenicol, Cephalothin, and Amoxicillin) was observed in ARBs compared to HPC (Cephalothin, Amoxicillin) from bulk water from the same borehole. The water of all the aquifers sampled is of very poor physico-chemical or microbiological quality or both. Water may be used for irrigation or livestock watering only in the case where these boreholes comply with TWQR for said purposes. Results obtained indicate that the groundwater is faecally contaminated. Amongst the bacteria, opportunistic pathogens displaying various degrees of antibiotic resistance were frequently isolated. These results indicate health risks if untreated groundwater is consumed. Therefore groundwater needs to be treated before distribution especially if the water is for human consumption.

Keywords: Groundwater, rural communities, microbiological water quality, HPC, amoeba resistant bacteria, antibiotic resistance, opportunistic pathogens, 16S rRNA gene sequencing, *mdh*, *lacZ*.

Graag dra ek hierdie werkstuk op aan my Ma en Pa vir al die liefde en ondersteuning, asook my Vader in die Hemel, sonder wie se bystand hierdie droom nooit sou kon realiseer nie

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Nicolene Kleynhans for being a great pillar of support.

DECLARATION

I declare that this dissertation for the degree of Master of Science in Environmental Science (M.Sc Env.Sci) at the North West University: Potchefstroom Campus hereby submitted, has not been submitted by me for a degree at this or another university, that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

Alewyn Carstens

Date

TABLE OF CONTENTS

ABSTRACT.....	i
ACKNOWLEDGEMENTS.....	iv
DECLARATION	v
LIST OF FIGURES.....	ix
LIST OF TABLES	x
CHAPTER 1 – GENERAL INTRODUCTION AND LITERATURE REVIEW	1
1.1 INTRODUCTION	1
1.2 RESEARCH AIM AND OBJECTIVES	2
1.3 LITERATURE REVIEW.....	3
1.3.1 Water and groundwater availability	3
1.3.2 Water dependent economic activities in North West province	6
1.3.3 Factors influencing groundwater recharge (contaminant movement)	8
1.3.4 Surface and groundwater interactions	9
1.3.5 Types of pollution	10
1.3.5.1. Chemical pollution.	10
1.3.5.1.1 pH	10
1.3.5.1.2 Total Dissolved Solids.	11
1.3.5.1.3 Nitrates.....	11
1.3.5.1.4 Sulphate	13
1.3.5.2 Microbial pollution.....	14
1.3.6 Indicator organisms	16
(a) Total coliforms	17
(b) Faecal coliforms	17
(c) Faecal streptococci.....	18
(d) Heterotrophic plate count bacteria (HPC).....	18
1.3.7 Amoeba resistant bacteria (ARB)	19
1.3.8 Antibiotic resistance.....	21
1.3.9 Principles of techniques available to study the quality of groundwater	22
1.3.9.1 Sampling collection.....	22
1.3.9.2 Physico-chemical methods.....	22
1.3.9.2.1 Chemical parameters	22

(a) Nitrate	22
(b) Sulphate	22
(c) Chemical oxygen demand (COD)	23
1.3.9.3 Microbiological methods	23
1.3.9.3.1 Membrane filtration.....	23
1.3.9.3.2 Media.....	23
1.3.9.3.2.1 MFC Agar.	23
1.3.9.3.2.2 Membrane-lactose glucuronide agar (MLG agar).	24
1.3.9.3.2.3 KF-streptococcus agar.	24
1.3.9.3.2.4 Mueller-Hinton agar.	24
1.3.9.3.2.5 R2A agar.	25
1.3.9.3.3 Molecular based methods.....	25
1.3.10 Summary of literature.....	27
CHAPTER 2 - MATERIALS AND METHODS.....	29
2.1 Study site	29
2.1.1 Mooi River Catchment	29
2.1.2 Harts River Catchment	31
2.2 Mobile Research Unit	32
2.3 Measurement of physical and chemical parameters of borehole water <i>in situ</i>	33
2.4 Sample collection.....	34
2.5 Nitrate, sulphate concentration and dissolved oxygen measurements.	34
2.6 Enumeration of indicator organisms on selective media.....	34
2.7 Enumeration of heterotrophic plate count bacteria	35
2.7.1 Amoeba resistant bacteria.....	35
2.8 Identification of <i>E. coli</i> , HPC and ARB.....	35
2.8.1 DNA isolation.....	35
2.8.2 Agarose gel electrophoreses of DNA	36
2.8.3 DNA amplification.....	36
2.8.3.1 <i>E. coli</i> genome markers (<i>mdh</i> , <i>lacZ</i> genes).....	36
2.8.3.2 16S rRNA sequencing of HPC and ARBs.....	37
2.8.4 Agarose gel electrophoreses of PCR products.....	37
2.8.5 Clean-up of PCR products before sequencing PCR (HPC & Amoeba bac.).....	37
2.8.6 Sequencing PCR and clean up of HPC & ARB 16S rRNA fragments.....	37
2.8.8 Sequencing and analysis of 16S rRNA gene fragments	38

2.9 Antibiotics resistance testing of isolated <i>E. coli</i> , HPC and ARB	38
2.10 Statistical analyses	38
CHAPTER 3 – RESULTS	40
3.1 Physico-chemical data.....	40
3.1.1 Mooi River Catchment	40
3.1.2 Harts River Catchment	43
3.2 Microbiological data	45
3.2.1 Mooi River catchment	45
3.2.2 Harts River Catchment	48
3.3 Antimicrobial resistance.....	49
3.4 Molecular identification results	51
3.4 HPC identification and antimicrobial resistance results.....	52
3.4.1 Mooi River catchment	52
3.4.2 Harts River Catchment	60
3.5 Amoeba resistant bacteria identification and antibiotic resistance results	63
3.6 Redundancy analysis.....	69
3.7 Summary of results	70
CHAPTER 4 – DISCUSSION	71
4.1 Physico-chemical data.....	71
4.2 Microbial results	73
4.2.1 Compliance to target water quality ranges.....	73
4.2.2 Trends identified	73
4.3 Antibiotic resistance of isolated <i>E. coli</i>	77
4.4 Heterotrophic plate count (HPC) bacteria	78
4.5 Redundancy analyses	81
CHAPTER 5 – CONCLUSIONS AND RECOMMENDATIONS.....	83
5.1 Conclusions	83
5.2 Recommendations.....	84
REFERENCES.....	86

LIST OF FIGURES

- Figure 1.1:** Average rainfall patterns of the North West province. p 4
- Figure 1.2:** Pie chart displaying percentage of water used by the different sectors in the province. p 7
- Figure 2.1:** Google map displaying the position of the three boreholes sampled in the Potchefstroom municipal district. p 30
- Figure 2.2:** Google map displaying the position of the borehole which is situated near the Klerkskraal dam. p 30
- Figure 2.3:** Google map displaying the location of the borehole situated on the Blaauwbank farm. p 31
- Figure 2.4:** Google map of the two boreholes situated in the Harts River catchment area. p 32
- Figure 2.5:** Photo illustrating all the instruments used during sampling in the rear of the bakkie. p 33
- Figure 3.1:** A 1.5% (w/v) agarose gel illustrating the results of the multiplex PCR. p 52
- Figure 3.2:** RDA ordination triplot of environmental parameters (red vectors) and biological data (blue vectors) for the various boreholes sampled. p 79

LIST OF TABLES

Table 3.1:	Physico-chemical results for the Mooi River catchment area during a warm and wet season	p 41
Table 3.2:	Physico-chemical results for the Mooi River catchment area during a cold and dry season	p 43
Table 3.3:	Physico-chemical results for the Harts River catchment area during a warm and wet season	p 44
Table 3.4:	Physico-chemical results for the Harts River catchment area during a cold and dry season	p 45
Table 3.5:	Microbiological results for boreholes sampled in the Mooi River catchment area during a warm and wet season	p 46
Table 3.6:	Microbiological results for boreholes sampled in the Mooi River catchment area during a cold and dry season	p 47
Table 3.7:	Microbiological results for boreholes sampled in the Harts River catchment area during a warm and wet season	p 48
Table 3.8:	Microbiological results for boreholes sampled in the Harts River catchment area during a cold and dry season	p 49
Table 3.9:	Antibiotic resistance results of isolated <i>E. coli</i> from boreholes sampled in the warm and wet season	p 50
Table 3.10:	Antibiotic resistance results of isolated <i>E. coli</i> from boreholes sampled in the cold and dry season	p 51

Table 3.11:	Identification and antibiotic resistance results of HPC bacteria isolated from the Pad dam borehole	p 55
Table 3.12:	Identification and antibiotic resistance results of HPC bacteria isolated from the Blaauwbank borehole	p 56
Table 3.13:	Identification and antibiotic resistance results of HPC bacteria isolated from the Trimpark borehole	p 57
Table 3.14:	Identification and antibiotic resistance results of HPC bacteria isolated from the Cemetery borehole	p 58
Table 3.15:	Identification and antibiotic resistance results of HPC bacteria isolated from the School borehole	p 59
Table 3.16:	Identification and antibiotic resistance results of HPC bacteria isolated from the Warrenton borehole	p 61
Table 3.17:	Identification and antibiotic resistance results of HPC bacteria isolated from the Ganspan borehole	p 62
Table 3.18:	Identification and antibiotic resistance results of amoeba resistant bacteria isolated from the Pad dam borehole	p 64
Table 3.19:	Identification and antibiotic resistance results of amoeba resistant bacteria isolated from the Cemetery borehole	p 65
Table 3.20:	Identification and antibiotic resistance results of amoeba resistant bacteria isolated from the School (SK) borehole	p 66
Table 3.21:	Identification and antibiotic resistance results of amoeba resistant bacteria isolated from the School borehole	p 67

CHAPTER 1 – GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

South Africa is situated in a semi arid region of the world. The average rainfall of the country (450 mm) is far below the average rainfall per year of the world (NWRS, 2004). In relation to the country as a whole, the North West province has very little perennial surface water resources, and the little that is available is under a large threat of pollution due to anthropogenic activities (NWPG, 2002; DWAF, 2006).

People living in rural areas are largely dependent on groundwater resources to meet the demand for water used for domestic purposes. In the North West province, this amounts to 65% of 3.5 million people (DWA, 2009). As groundwater was previously considered to be of excellent quality (physico-chemically and microbiologically), this resource was usually supplied to communities without prior treatment (Momba *et al.*, 2006). Recent studies in the North West province (Ferreira, 2011) and Gauteng (Mwabi *et al.*, 2012) concluded that this perception of groundwater quality is incorrect. A high percentage of boreholes in the North West province that was tested (Ferreira, 2011) did not comply with target water quality ranges (TWQR; physico-chemical and microbiological) for drinking water (DWAF, 1996a) and none of the water tested by Mwabi *et al.* (2012) complied with the same microbiological TWQR. These examples demonstrate that the monitoring of groundwater resources needs to be performed to prevent health implications.

1.2 RESEARCH AIM AND OBJECTIVES

The aim of this study was to evaluate the microbiological and physico-chemical quality of water from aquifers in the North West province, South Africa.

The objectives of the study were to:

- i. Collect water samples from DWA monitoring boreholes
- ii. Analyse water samples on site for set physico-chemical parameters using appropriate instruments
- iii. Analyse water samples for levels of faecal indicator organisms
- iv. Isolate and identify heterotrophic plate count bacteria
- v. Identify amoeba resistant bacteria
- vi. Comment on the suitability of the water for specific applications

1.3 LITERATURE REVIEW

1.3.1 Water and groundwater availability

Water is less available on the African continent than in Asia, Europe or North America and with one of the fastest urbanisation rates in the world, this could lead to a major water shortage (Showers, 2002).

South Africa is situated in a semi-arid region of the world (NWRS, 2004). Climatic conditions associated with semi-arid regions include low average precipitation, where South Africa has an average of 450 mm/year compared to the 860 mm/year of the world (NWRS, 2004). High evaporation rates, high temperature, large variability in precipitation and low mean annual runoff contribute to stresses already placed on water resources (NWPG, 2002; NWRS, 2004).

South Africa is mainly dependent on surface water sources for irrigation, industrial and urban water supply (NWRS, 2004). For this reason, the surface water infrastructure is well developed (NWRS, 2004). The reliable local yield for the year 2000 was estimated at 13227 million m³/annum and local requirements at 12 871 million m³/annum, which gives a surplus of 186 million m³/annum (NWRS, 2004). From estimations (under a scenario of high population and economic growth) for local water requirements in the year 2025, a water deficit of 2044 million m³/annum is predicted (NWRS, 2004). Parsons (2003) estimated that ground water can supply a reliable yield of 19 250 million m³/year, from which only 2 100 million m³/annum is abstracted.

The North West province has a surface area of 116 320 km² with a geology of igneous, ancient igneous volcanic and sedimentary rocks (NWPG, 2002). From Figure 1.1, it can be observed that the rainfall averages are highly variable and decrease from the East (600 mm) to the West (less than 300 mm) (NWPG, 2002). Climatic conditions are characterised by high evaporation rates that exceed precipitation rates and a low average mean annual run-off (6% compared to 9% of South Africa) (NWPG, 2002). Perennial water sources

(Crocodile, Marico and Vaal Rivers) are concentrated to the South Eastern and North Eastern parts of the province, where these water sources are shared with bordering provinces (NWPG, 2002).

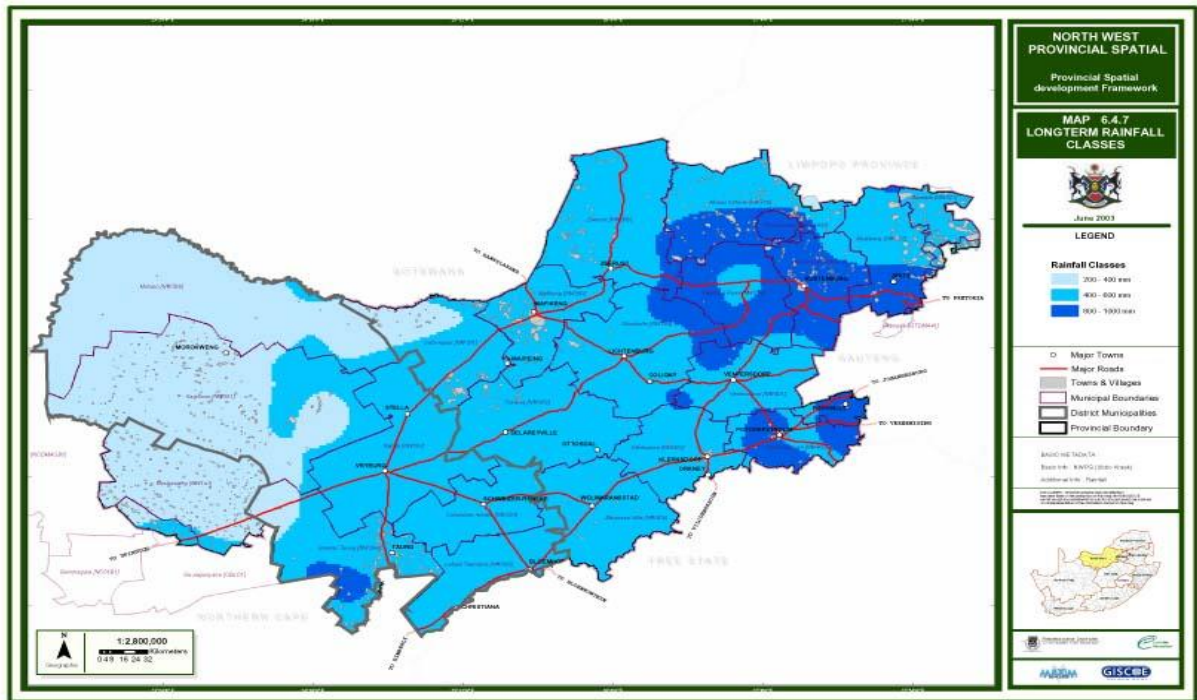


Figure 1.1: Average rainfall patterns of the North West province (DWA, 2010)

Surface water sources for the province include rivers (Crocodile, Marico, Vaal and tributaries [34]), dams, pans, wetlands and dolomite eyes (NWPG, 2002). Although surface water resources are limited in the province, a large reservoir of groundwater is available (NWPG, 2002). These reservoirs are comprised of dolomitic compartments and fractured aquifers (NWPG, 2002). Average annual recharge of groundwater resources in the province is very low (less than 10 mm) and is the lowest in the country (NWPG, 2002). Water abstraction from aquifers in the province can range from as low as 0.1 – 1 million m³/ annum (Reivilo, Stella) to as high as 27 million m³/annum in the Louwna-Ganyesa region (NWPG, 2002).

Because of the scarcity of surface water resources in the province, people are reliant on groundwater resources to fulfill the demand for water for domestic use. Sixty five percent of people from a population of 3465 million live in rural areas in the North West province, and rely solely on groundwater for domestic use (DWA, 2009; NWPG, 2002). This figure is in close correlation to the estimated 80% of the population in rural or low income communities in developing countries that rely on groundwater as sole source of drinking water (Murray *et al.*, 2004). These figures indicate the importance of groundwater as a water resource.

Groundwater was classified as private water in the previous Water Act (Act no. 54 of 1956) of South Africa. Therefore there was no need to monitor the quality of groundwater. The new Water Act (Act no. 36 of 1998) identified groundwater as a national resource and therefore the need to monitor the water quality of groundwater resources. According to the Water Act (Act no 36, 1998) all water resource must be protected. Through monitoring of the quality of the groundwater, anthropogenic impacts on water resources can be identified and remediated, and so can the water resources be protected against deterioration through pollution.

Many sources of pollution exist in the province. These include agricultural practices, the mining industry, negative impacts of population growth and urbanisation (Coetzee *et al.*, 2006; Griesel & Jagals, 2002). All of these sources could contribute to chemical and/or microbiological contamination and dewatering of aquifers (irrigation, mining) (NWPG, 2002; Coetzee *et al.*, 2006).

Groundwater is perceived as being inherently of pristine quality (Mpenyana-Monyatsi and Momba, 2012; Momba *et al.*, 2006; Ferguson *et al.*, 2012; Mackintosh & Colvin, 2003). For this reason, many of the rural areas in South Africa are supplied with groundwater for domestic use without prior treatment (Lehloesa & Muyima, 2000; Mackintosh & Colvin, 2003). This scenario would place people using the untreated groundwater at a high risk of exposure to chemical contaminants and pathogens which would lead to adverse health effects.

1.3.2 Water dependent economic activities in North West province

Because of rich ore deposits in South Africa, mining has become an important economic activity (Coetzee *et al.*, 2006). The mining sector is the largest contributor (35.5%) to the economy as well as the largest employer (22%) (NWPG, 2002). Mining plays an important part in hydraulic environments, whether active or inactive, where both states could negatively affect water resources in several different ways (DWAF, 2003b). The mining industry consumes large amounts of water for process purposes and discards polluted used water into streams, rivers or tailings dams from where toxic metals leach to groundwater resources (Winde, 2010). Mining activities cannot be safely performed under dolomite compartments, due to their large water storage capacity. Therefore the compartments (aquifers) are dewatered, leading to loss of local available groundwater yield (NWRS, 2004).

The West and East Rand basins in Gauteng contain highly polluted groundwater, due to mining activities. During dewatering, these waters are decanted into surface water resources that may infiltrate groundwater resources (Winde, 2010). It is a well known fact that ground and surface water interacts with one another (Xu *et al.*, 2002; Parsons, 2003; Le Maitre & Colvin, 2008). Contamination of one source would inevitably lead to the contamination of the other due to physical interactions. These decanting mine waters contain high concentrations of total dissolved salts (TDS), sulfates (acid mine drainage), sodium, chloride and nitrates (DWAF, 2006). Thus, return flow water from mines is usually of poor quality, which affects downstream users using the water for domestic purposes (Coetzee *et al.*, 2006). Therefore, it is imperative that mine water effluents and return flow water be of good quality.

Agriculture is recognised as the biggest consumer of groundwater resources (Fig. 1.2) for irrigation use, as well as the occupier of the largest surface area of the province (livestock farming) (Conrad *et al.*, 1999; NWPG, 2002). Although being the largest consumer of water, the agricultural sector only contributes a small percentage to the economy of the province. This is indicative of poor water use efficiency (NWRS, 2004). Over-abstraction of groundwater can lead to the depletion of localised groundwater resources and irrigation

return flows may contain a wide variety of pesticides, herbicides and leachable ions due to incorrect fertiliser applications (Harold & Bailey, 1996). Livestock farming (feedlot runoff) can contribute to antimicrobial and faecal pollution of groundwater resources (Alanis, 2006).

Urbanisation and population growth also exert pressure on available water resources (NWRS, 2004). A high influx of people to urban areas results in informal settlements expanding where sanitary infrastructure is usually lacking (DWA, 2003). On site sanitation is normally incorporated for human waste disposal in these circumstances (Mpenyana-Monyatsi & Momba, 2012). On site sanitation has been identified as a contributor to faecal pollution of groundwater resources (Godfrey *et al.*, 2005; Bonton *et al.*, 2010; Howard *et al.*, 2003).

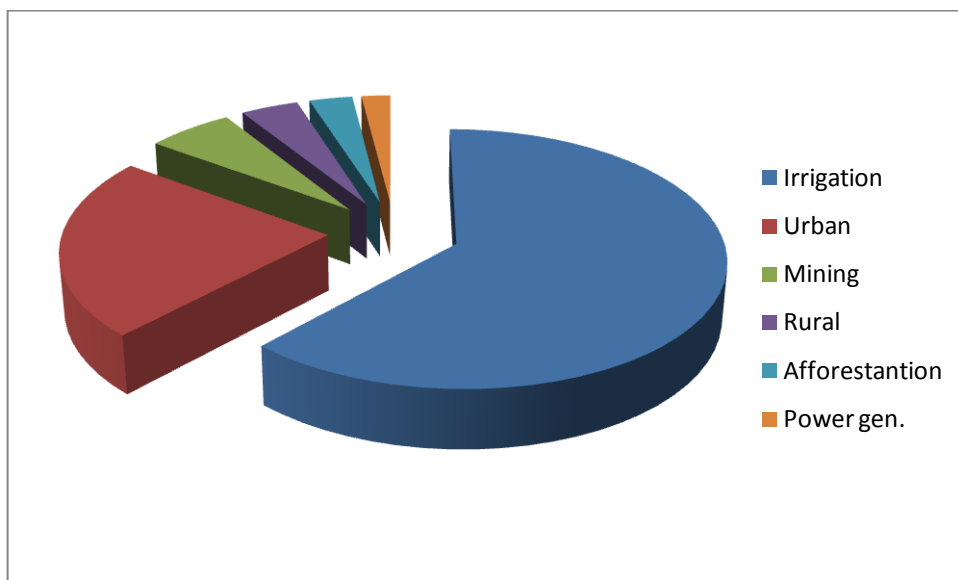


Fig 1.2: Pie chart displaying percentage of water used by the different sectors in South Africa (Data obtained from NWRS, 2004).

Where sewerage is available, many of the waste water treatment plants of the province do not adequately treat the water to remove microbes from the water. Twenty of the 32 waste water treatment plants in the province do not comply with 50% of the TWQR for microbiological removal set by the Department of Water affairs (DWA, 2012). Water

containing faecal bacteria and possibly pathogens are released into receiving water bodies exposing people downstream to these pathogens.

1.3.3 Factors influencing groundwater recharge (contaminant movement)

Topography of soils plays an important part in the absorption of water into soil. Flat surfaces would allow more time for precipitation to penetrate soil. More precipitation absorbed would allow more contaminants to be transported to groundwater sources, for example, nitrates. In contrast, steep soils would allow less precipitation to penetrate soil (Iqbal & Krothe, 1995).

The geological composition of the unsaturated and saturated zones above underground water resources also determines the quality of water entering the aquifer. Rock formations made up of coarser grains have a higher permeability and porosity as opposed to rock formations with a finer/fine grained composition (Yasmin, 2009). Coarse grained rocks would therefore allow fast and easy movement of water through the zones and less attenuation of contaminants and *vice versa* for finer grained rock formations (DWAF, 2003a). The physical properties of a specific aquifer therefore play an important role in the vulnerability to, and the rate and time of contamination (Valenzuela *et al.*, 2009; Krapac *et al.*, 2002; Gelinas *et al.*, 1996).

Borehole construction should also be considered when contamination of groundwater resources is investigated. Poor borehole construction would lead to easier groundwater contamination, as all of the inherent attenuation processes described above are bypassed. After installation of the casing of a borehole, a gravel pack needs to be installed between the actual wall of the hole in the ground and the casing. Materials used for this purpose should be of an inert nature as to not change the water quality entering the borehole. Quartzitic gravel is the most commonly used material (Barnes & Vermeulen, 2012). A sanitary seal then needs to be fitted from the ground level down to 5 meters (Barnes & Vermeulen, 2012). This is to prevent surface runoff water from entering a groundwater resource through the space between the casing and the hole in the ground (DWAF, 2003a). Bentonite is the material of choice as it forms an impermeable layer (Barnes & Vermeulen,

2012). Lastly, a concrete slab needs to be placed around the protruding casing in such a manner that water will not pool around the casing (DWAF, 2003a; Barnes & Vermeulen, 2012).

1.3.4 Surface and groundwater interactions

Considering that there are interactions between ground and surface waters, surface water can have a significant impact on groundwater (DWAF, 2003b; Xu *et al.*, 2002). This interaction is dependent on the relative level of the water referred to. For example, surface water would recharge ground water when the level of the surface water is higher than that of the groundwater (Gardner, 1999; Winter *et al.*, 1998). This interaction usually occurs at an upper catchment level (near origin of river, stream) (Xu *et al.*, 2002). The opposite applies in a lower part of the catchment, where groundwater may contribute to river flow (Parsons, 2003). These are examples of change over time in the system (Gardner, 1999).

During precipitation, and subsequent recharge of groundwater, the water level of groundwater can rise above that of a relevant surface water body. Groundwater will then move to the surface water body (Le Maitre & Colvin, 2008). This would be an example of change over time (Gardner, 1999). Ground and surface water interactions are not limited to rivers alone. Groundwater sources can contribute water to rivers in the form of springs and interact with wetlands (Parsons, 2003). The interactions of surface and groundwater sources are controlled by the geology between the separate water bodies (Le Maitre & Colvin, 2008). High transmissivity and porosity values (fast movement and large storage) would be indicative of a more pronounced interaction between water bodies (Le Maitre & Colvin, 2008). All surface and groundwater bodies are however, connected to some degree (Parsons, 2003). Because of these interactions, contamination or pollution of surface water can contribute to groundwater contamination and *vice versa* (Gardner, 1999; Winter, *et al.*, 1998).

1.3.5 Types of pollution

There are mainly two types of water pollution that should be considered, namely microbiological and chemical pollution. The former includes pollution by viruses, bacteria, protozoa and helminths. Chemical pollutants are carbon, nitrogen, phosphorous and various other minerals and metals (Fourie & van Ryneveld, 1995).

1.3.5.1. Chemical pollution.

1.3.5.1.1 pH.

Rivers and other water sources are buffered by natural buffering systems, so as to allow that the pH stays in close ranges to a neutral pH of 7 (Dallas & Day, 2004). This happens because of the action of complex acid-base equilibria of various dissolved compounds, mainly the carbon dioxide-bicarbonate-carbonate equilibrium system (DWAF, 1996a). The pH of water thus does not indicate the ability of the water to neutralise additions of bases or acids. When acids or bases are added to natural water, some elemental composition changes in water take place. For example, aluminium occurs as unavailable hydrated hydroxides in alkaline waters, and as the pH drops, it is converted to the highly toxic Al^{3+} ion (Dallas & Day, 2004). Changes in the pH of water also cause some elements to be more or less available, through altering their solubility. A decrease in pH will bring about a surface charge change of an ion or molecule, making the ion or molecule more soluble in water. This may then lead to the release of toxic substances from the sediments of water bodies (Dallas & Day, 2004). Sources of pollution that could change the pH of water include acid mine drainage and industrial processes (DWAF, 2006).

The TWQR for drinking water established by DWAF (1996a) are a pH range of between 6.0 to 9.0. Deviation from this range to more acidic levels would bring about severe danger due to toxic metal ions while deviation to more alkaline levels poses danger due to deprotonated species (DWAF, 1996a). The TWQR for irrigation purposes are between 6.5 and 8.4 (DWAF, 1996c). Using water not complying with these TWQR will result in foliar damage of crops (DWAF, 1996c).

1.3.5.1.2 Total Dissolved Solids.

Total dissolved solids (TDS) in water originate from enhanced weathering of minerals from acids in the soil, process water effluent and fissure water effluent (Atekwana *et al.*, 2004; Coetzee *et al.*, 2006). Atekwana and colleagues (2004) concluded that TDS is a likely geochemical parameter that closely links bulk electrical conductivity to microbial degradation of hydrocarbons. Electrical conductivity is directly proportional to the concentration of TDS in a water sample by a factor of 6.5 (Atekwana *et al.*, 2004; DWAF, 1996a). TDS can be measured by automated meters or the gravimetric method. In the latter case, the water sample is evaporated and the solids measured that are left behind (Atekwana *et al.*, 2004).

Various methodologies are available to remove bulk TDS from contaminated water. These are usually based on membrane separation processes and include nano filtration, reverse osmosis and electro-dialysis with bi-polar membranes (Basha *et al.*, 2008; Chandramowleeswara & Palanivelu, 2006). The TDS in water does not cause any adverse human health effects. It however affects the aesthetic value of the water (DWAF, 1996a).

Target water quality ranges for domestic water is set at less than 70 mS/m for electrical conductivity (DWAF, 1996a). Higher concentrations will lead to a disturbance in the salt balance of the body (DWAF, 1996a). Livestock can tolerate water with a TDS concentration of up to 4000 mg/l (DWAF, 1996b). Elevated levels would lead to a decrease in production, as animals would be reluctant to drink such water (DWAF, 1996b). Crops can be irrigated with water having an electrical conductivity of up to 40 mS/m, where elevated levels would lead to decreased productions in sensitive crops (DWAF, 1996c).

1.3.5.1.3 Nitrates.

Nitrates may contaminate groundwater, as well as surface water sources. This could be due to the result of incorrectly treated waste water disposal, industrial practices and agricultural practices, where too much nitrogen fertilisers are added to the soil for efficient uptake by plants (Smith *et al.*, 2005; Moreno *et al.*, 1996; Suthar *et al.*, 2009; Shroder *et al.*, 2004).

Nitrate is water soluble and therefore readily transported through sediments into groundwater (Aelion *et al.*, 1997; Tesoriero *et al.*, 2007; Andrade & Stigter, 2009). Other sources of nitrogen pollution include livestock farming (animal waste), chemical industrial effluents high in nitrogen, pit latrines and landfills (Suthar *et al.*, 2009; Al-Khatib & Arafat, 2009; Wakida & Lerner, 2005).

Accompanied with the varying types of rainfall associated with semi- and arid regions, nitrogen in the soil will leach out much faster during times of heavy precipitation when water flow through the soil is fast, causing increased nitrogen levels in groundwater resources (Tredoux, 2004; Stigter *et al.*, 2008). Other factors that also affect the movement of nitrogen include the transmissivity of soils and aquifers (Stigter *et al.*, 2008).

Problems associated with high nitrate concentrations in water (>20 mg/ml) include methaemoglobinemia, cancer and headaches (Tredoux, 2004; Almasri & Kaluarachi, 2004; Suthar *et al.*, 2009; Wakida & Lerner, 2005; Faniran *et al.*, 2001). When infants ingest large quantities of water containing high concentrations of nitrates, the nitrates would first be converted to nitrite by microbes in their digestive systems (Suthar *et al.*, 2009). The nitrites are absorbed into the blood stream where they bind to haemoglobin to form methaemoglobin. Infants do not have the metabolic pathways to detoxify the methaemoglobin (Wright *et al.*, 1999). Methemoglobin decreases the oxygen carrying capacity of blood and infants may, in the worst case scenario, die because of asphyxiation (Tredoux, 2004; Suthar *et al.*, 2009).

Cancers of the digestive tract may also be caused by nitrates. Nitrites that are formed endogenously in the digestive tract of humans undergo nitrosation reactions in the stomach with amines to form a variety of N-nitroso compounds which are carcinogens (Suthar *et al.*, 2009; Tredoux, 2004). This is, however, a controversial issue and further research and evidence is required. Nitrates also acts as vasodilators of the cardiovascular system. When nitrates are ingested through water or any other medium, the arteries in the body would dilate and a headache would develop (Suthar *et al.*, 2009; Tredoux, 2004).

Cattle are also at risk when ingesting water with high nitrate levels. Although cattle can tolerate higher nitrate concentrations than humans, negative health effects are also evident in constant exposure to high concentrations. These include increased indices of abortion, infertility and inhibition of growth. Death of cattle could also occur when there is a sudden extreme spike in the nitrate concentration of water (Tredoux, 2004; Suthar *et al.*, 2009).

Water used for domestic purposes should contain less than 10 mg/l of nitrates (DWAF, 1996a). Elevated levels would increase the occurrence of methemoglobinemia. Sensitive crops can tolerate water containing up to 5 mg/l of nitrates and most other crops a nitrate concentration of up to 30 mg/l (DWAF, 1996b). Higher levels would negatively affect yields. The TWQR for livestock water is set at 100 mg/l (DWAF, 1996c). Water containing levels of between 200 and 400 mg/l would affect monogastrics and ruminant animals alike (DWAF, 1996c).

1.3.5.1.4 Sulphate

In South Africa, gold bearing ore contains about 3% pyrite (FeS_2), which was and is still being transported to the surface due to mining activities (Tutu *et al.*, 2008). To extract gold from ore, a cyanidation process is used, because it is very selective to gold, leaving behind all other minerals contained in ore (Naicker *et al.*, 2003). Unused slurry of this process is pumped to tailings dams where it is exposed to the environment and oxygenated rain during precipitation events (Coetzee *et al.*, 2006). Pyrite and other sulphur containing minerals are oxidized in the tailings dams and sand heaps (artifact of gold extraction method used prior to cyanidation process) (Tutu *et al.*, 2008). Acidic water percolates to groundwater sources which feed streams in lower lying areas surrounding affected areas (Naicker *et al.*, 2003). Pyrite is also exposed to oxygen in abandoned mines

Sulphate is a normally occurring compound found in geologic formations and therefore in water as well with fluctuating concentrations (Naicker *et al.*, 2003). Mining activities expose pyrite to oxygen during which pyrite is oxidized into sulphate and an acid (Tutu *et al.*, 2008). Acid and sulphate containing water percolates to groundwater (tailings dams and sand heaps) or decants into surface water sources contaminating such resources (Coetzee *et al.*, 2006). Such water may contain sulphate levels of up 7500 mg/l (Tutu *et al.*,

2008). Sulphates are also contributed to surface water sources by acid rain produced by anthropogenic activities (DWAF, 1996a).

Initial exposure to water containing high levels of sulphates will cause diarrhea, but individuals are able to adapt to consuming water with elevated levels of sulphate (DWAF, 1996a). No adaptation will, however, occur when water with sulphate levels of 1000 mg/l and higher are consumed (DWAF, 1996a).

1.3.5.2 Microbial pollution.

Bacteria occur naturally in groundwater and most of these are not pathogenic to humans or animals. Only when pathogens are present in water sources will disease outbreaks occur. Conditions prevailing in groundwater favor bacterial growth and survival and could allow pathogenic organisms to survive for long periods (Murray *et al.*, 2004).

Sources of bacterial pollution include pit-latrines, incorrect sewage discharges and spills, landfill leachate and agriculture practices such as intensive cattle/sheep/dairy farming. It could also result from recharge (natural or induced) of aquifers by polluted water sources (Crowther *et al.*, 2002; Howard *et al.*, 2003; Murray *et al.*, 2004; Field & Samadpour, 2007).

Factors that influence the movement of micro-organisms through soil are grain size, soil composition (clay, loam or sandy soil), mineral content of soil, residence time of water in soil and the chemical conditions prevailing in soil (Flynn & Sinreich, 2010; Fourie & van Ryneveld, 1995).

Bacteria are usually filtered out of water by soil, and thus, the further away a water resource from a contaminant source, the less chance of contamination exist (DWAF, 2003a). Soil with small grain sizes (clayey soils) will remove (attenuate) micro-organisms more effectively than soils with larger grain sizes (sandy soil) as the retention time of water in the clayey soils is longer (DWAF, 2003a).

Larger micro-organisms, such as helminths and protozoa, would be efficiently filtered out of water if the soil has a loam or clayey-loam composition (Fourie & van Ryneveld, 1995). Smaller micro-organisms, such as bacteria and viruses, would only effectively be filtered out of water by clay/clayey soils. Micro-organisms mobility through soil is inversely proportional to their physical size. The time that it takes the micro-organisms to reach groundwater sources also plays an important role. Micro-organisms can only survive in soil for a finite period of time, and if the travel times exceed between 150 – 200 days, contamination from micro-organisms (bacteria) should not pose a health threat (DWAF, 2003b).

Strong correlations were observed between short term rainfall before sampling and the amount of indicator bacteria found in groundwater (Howard *et al.*, 2003; Fourie & van Ryneveld, 1995). Micro-organisms get trapped to the soil particles due to the difference in surface charges of the soil particles and the bacteria (Murray *et al.*, 2004; Howard *et al.*, 2003). The bacteria can, and will be, flushed out of soil during heavy precipitation events, where the micro-organisms will then end up in groundwater sources as aquifers are recharged during or after precipitation (Howard *et al.*, 2003; Fourie & van Ryneveld, 1995). Permeability of soils to bacteria also increase as the soil becomes more saturated with water, causing bacterial cells to travel along the saturated soil into groundwater resources (Howard *et al.*, 2003).

In soils, there are artificial channels that are made by decomposed roots and organisms such as earthworms. In rock formations fissures are present, and these channels present water with a pathway by which it can flow freely and where all the processes of attenuation are bypassed. This type of water movement is termed as macropore flow (Fourie & van Ryneveld, 1995). It would allow for the contamination of groundwater resources, irrespective of the depth of soil between a contamination source and groundwater resource (Fourie & van Ryneveld, 1995).

1.3.6 Indicator organisms

Most human pathogens are transported via the faecal oral route (WHO, 2011). Pathogens are then excreted in faeces from infected people. When faecal matter is not disposed of correctly, pathogens may be transported to water sources (surface or ground). When the correct physico-chemical environment presents itself, pathogens can then survive in water. Ingestion of contaminated water may lead to infection by these pathogens to cause adverse health effects, such as cholera, typhoid fever, paratyphoid fever, bacillary dysentery and diarrheal disease (Lawrence *et al.*, 2001; Fourie & van Ryneveld, 1994).

A wide range of pathogens could be present in water after contamination, and to test water for all known pathogens would not be economically viable or productive (Baghel *et al.*, 2005). Indicator organisms have therefore been identified to indicate possible faecal contamination of water sources.

Micro-organisms have to comply with certain criteria before being incorporated as an indicator organism. No one micro-organism complies with all of the set criteria, and therefore a selection of specific indicator organisms are used in conjunction with one another to obtain reliable results on the microbiological state of waters. Commonly used indicators occur together with enteric micro-organisms in the intestines of warm blooded animals and are therefore excreted together with enteric pathogens. Indicator organisms can then be used to analyse water for faecal pollution (Willey *et al.*, 2011; Atlas & Bartha, 2002; DWAF, 1996a). Micro-organisms considered to be used for indicator organisms should comply with the following criteria i) Should be present in faeces of warm blooded animals ii) Should be easily detectable using simple methods iii) Should not multiply in water sources or in any other environmental settings iv) Should be present in water together with pathogenic organisms v) Should have the same or longer die-off rate than pathogenic organisms and should not cause adverse health effects in humans (DWAF, 1996a; NHMRC, 2003; Atlas & Bartha, 2002). Using this criteria there are four generally used indicator organisms, namely total coliforms, faecal coliforms, faecal streptococci and heterotrophic plate count bacteria.

(a) Total coliforms

These micro-organisms form part of the Enterobacteriaceae family and can be isolated from a variety of environments (NHMRC, 2003), including the gastro-intestinal tract of humans. Bacteria belonging to this group include the species *Escherichia*, *Citrobacter*, *Enterobacter*, *Klebsiella* and *Serratia* (DWAF, 1996a). All of these micro-organisms can multiply in the environment and water sources (Cohen & Shival, 1972; NHMRC, 2003) and are therefore not used exclusively for detection of faecal pollution in water sources (NHMRC, 2003). This group of indicator organisms is generally used to analyse the general sanitary quality of water, as well as to indicate possible failures in distribution systems (DWAF, 1996a; NHMRC, 2003) and may indicate the potential presence of enteropathogens in water (Rompre *et al.*, 2002). The TWQR for these indicator organisms are less than 5 cfu/100 ml for domestic use (DWAF, 1996a). Levels higher than 100 cfu/100ml would lead to a significant increase in risk of infectious disease transmission (DWAF, 1996a).

(b) Faecal coliforms

Faecal coliforms are part of a subgroup of total coliforms. These bacteria are distinguished from the total coliform group in that they can grow at elevated temperatures of 44.5°C (Paruch & Maehlum, 2012; Rompre *et al.*, 2002). Incubation at this temperature inhibits growth of non faecal coliform bacteria (Rompre *et al.*, 2002). *Escherichia coli* are part of this group of indicator organisms and are the only faecal coliforms that are exclusively present in the intestinal tract of warm blooded animals (Foppen & Schijven, 2006; Paruch & Maehlum, 2012). *Escherichia coli* also outnumber the other faecal coliforms of the gastro-intestinal tract and comply with most of the criteria set for selecting an indicator organism (Foppen & Schijven, 2006). Gabutti *et al.* (2000) stated that *E. coli* detection in water samples would only indicate recent faecal pollution, as faecal coliforms (*E. coli*) are not very persistent in environmental conditions. For these reasons *E. coli* is recognised as the best indicator of faecal pollution in water sources (Paruch & Maehlum, 2012).

Target water quality ranges for faecal coliforms are set at zero for domestic use, less than 200 cfu/100ml for livestock watering purposes and up to one cfu/100ml for water used for irrigation (DWAF, 1996a; DWAF, 1996b; DWAF, 1996c). Increased levels of up to 10

cfu/100ml would have an increased risk of infectious disease transmission if water is used continuously by humans. Levels of more than 200 cfu/100ml would increase the chances of infectious disease transmission in animals. Crops irrigated with water containing up to 1000 cfu/100ml would cause transmission of infectious disease if crops like vegetables are consumed raw. Water with this level of faecal contamination can, however, be used to irrigate parks and tree plantations if human contact with this water is avoided (DWAF, 1996a; DWAF, 1996b; DWAF, 1996c).

(c) Faecal streptococci

These micro-organisms include four species of the genus *Enterococci*. They include *Enterococcus faecalis*, *E. faecium*, *E. durans* and *E. hirae* (Junco *et al.*, 2001). These indicator organisms are also present in the faeces of warm blooded animals and are more persistent in the environment than *E. coli* (Gabutti *et al.*, 2000; Willey *et al.*, 2011). Geldreich (1996) stated that faecal streptococci are more abundant in faeces from warm blooded animals than faecal coliforms. Faecal streptococci are also more persistent in environmental conditions (Gabutti *et al.*, 2000). Faecal streptococci would therefore give an indication of faecal pollution that occurred a longer time ago if no faecal coliforms are enumerated together with faecal streptococci. No TWQR are set for this indicator organism.

(d) Heterotrophic plate count bacteria (HPC)

This group of micro-organisms includes all naturally occurring bacteria that utilise organic nutrients at low concentrations for growth (Edberg *et al.*, 1997; Edberg & Allen, 2004). These bacterial counts give an indication of the general microbial quality of water and do not indicate faecal pollution (DWAF, 1996a). Counts also do not give a representation of the total number of bacteria present in water (DWAF, 1996a). Heterotrophic plate count bacteria may also decrease the sensitivity of other methods to enumerate indicator organisms (Allen *et al.*, 2004; Quiroz, 1999). Allen *et al.* (2004) stated that high numbers of HPC bacteria inhibit the growth of coliform and subsequent groups on selective media. This would obscure the presence of these important indicator organisms.

Opportunistic pathogens do make out some of the heterotrophic bacteria and include the genus *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Alcaligenes*, *Achromobacter*, *Aeromonas* and *Mycobacterium* (Quiroz, 1999; Payment *et al.*, 1991; Stelma *et al.*, 2004; Geldreich, 1996). These bacteria do not occur in enough numbers in water to cause gastrointestinal disease in healthy people, as no sufficient clinical evidence exists to suggest otherwise (Edberg *et al.*, 1997; Edberg & Allen, 2004; Allen *et al.*, 2004). Some researchers do not agree with this statement, as HPC bacteria with virulence factors were identified in water (Pavlov *et al.*, 2004; Payment *et al.*, 1991). Chances of developing disease due to ingestion of these opportunistic pathogens are higher in immune compromised individuals, the elderly and the very young (Ford, 1999; Paulse *et al.*, 2009).

Heterotrophic plate count bacteria are used as an indicator of bacterial after growth or contamination that may have occurred after treatment of water (DWAF, 1996a). Higher than normal HPC counts may therefore indicate the possible presence of pathogenic micro-organisms in water distribution systems.

The absence of a faecal indicator organism does not necessarily prove that no faecal contamination occurred in water sources (Fourie & van Ryneveld, 1994), as high HPC concentrations may obscure the identification/enumeration of faecal indicator organisms, as stated above. In conclusion, it would be advisable that all of the indicator micro-organisms should be used in conjunction with one another, as every indicator micro-organism only gives an answer to a piece of the puzzle. This would give a holistic view of the microbial quality of water. Domestically used water may contain levels of heterotrophic plate count bacteria of up to 100 cfu/100ml (DWAF, 1996a). Elevated levels would increase the possibility of infectious disease transmission if such water is consumed.

1.3.7 Amoeba resistant bacteria (ARB)

Amoebas are unicellular eukaryotes that are predators on bacteria in the environment (Moliner *et al.*, 2010). Two developmental stages can be observed from amoeba, namely the trophozoite stage and a cyst stage (Greub & Raoult, 2004; Thomas *et al.*, 2008). The trophozoite is the vegetative feeding form and the cyst is the resting form during

unfavorable environmental conditions (Greub & Raoult, 2004). Amoebas feed on bacteria through phagocytosis, where bacterial cells are engulfed and digested by enzymes (Greub & Raoult, 2004).

The diversity and abundance of amoeba in water sources are dependent on certain environmental factors. These include temperature, moisture content in non water sources, pH and nutrient availability in the form of bacterial cells (Greub & Raoult, 2004). Previously identified ARB genera include *Acinetobacter*, *Aeromonas*, *Bacillus*, *Pseudomonas*, *Alcaligenes*, *Brevundimonas*, *Chryseobacterium*, *Comamonas*, *Delftia*, *Flavobacterium* and *Ochrobactrum* (Pagnier *et al.*, 2008; Barker and Brown, 1994).

Bacteria isolated from amoeba can be divided into three groups. Some are obligate intracellular bacteria, some are facultative intracellular and other are endosymbionts (Moliner *et al.*, 2010; Thomas *et al.*, 2008; Greub & Raoult, 2004). For bacteria to be able to survive in the phagolytic environment of amoeba, they have developed certain mechanisms of resistance. Specific mechanisms include the resistance to microbicidal effectors in the phagocytes of amoeba, the ability to replicate in the intracellular environment or the secretion of toxins that kill of the amoeba before phagocytosis can be completed (Thomas *et al.*, 2006; Cosson & Soldati, 2008; Thomas *et al.*, 2008; Greub & Raoult, 2004). The mechanism of phagocytosis used by amoebas is the same as macrophages incorporated by multicellular organisms (Greub & Raoult, 2004; Thomas *et al.*, 2006; Cosson & Soldati, 2008). This would allow ARB to resist phagocytosis of the human body, which is one of the immunological responses to infection. Amoeba can also increase antimicrobial resistance of ARB (Pagnier *et al.*, 2008), either by horizontal gene transfer (genes that encode for resistance) with other ARB, the amoeba host or free DNA of digested non resistant bacteria (Moliner *et al.*, 2010). All of these mechanisms increase the pathogenicity of ARB. Amoeba resistant bacteria can therefore be considered as emerging human pathogens (Pagnier *et al.*, 2008).

When bacteria are able to resist digestion by amoeba, the amoeba can become a reservoir of these bacteria (Thomas *et al.*, 2006; Pagnier *et al.*, 2008). As amoeba can resist harsh

environments by forming a cyst, the amoeba would not be affected by disinfecting processes. These cysts forms of amoeba would then protect ARB against forms of disinfection that would normally kill bacteria. All of these factors would contribute to the spreading of inherently pathogenetic ARB bacteria.

1.3.8 Antibiotic resistance

Enteric pathogens are usually Gram negative rods (Alanis, 2006). Micro-organisms that have been routinely identified with antibiotic resistance include *Shigella* spp, *Salmonella*, *Vibrio cholera*, *E. coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* (Alanis, 2006; Jansen *et al.*, 2006). Bacteria possess the ability to adapt to their environment and therefore they can develop resistance to antimicrobials (Donskey, 2006; Alanis, 2006). Mechanisms of resistance include efflux pumps, expression of inactivation enzymes, modification of targets that antimicrobials bind to and making alterations to outer membrane proteins to inhibit antimicrobials from penetrating the intracellular environment (Donskey, 2006; Neu, 1992; Bax *et al.*, 2000). Genes in the DNA of such micro-organisms encode for all of these resistance mechanisms.

When selective pressures (antimicrobial use) are applied by an environment, micro-organisms with mutations allowing them to survive unfavorable environments are selected for (Bax *et al.*, 2000; Neu, 1992; Alanis, 2006). Micro-organisms with these mutations then transfer genes that encode for resistance to other micro-organisms (Donskey, 2006; Bax *et al.*, 2000). The intestinal tract of humans provides a favorable environment for gene transfers and is also a reservoir for antibiotic resistant conferring genes (Donskey, 2006; Newman *et al.*, 2011).

The misuse and over use of antibiotics leads to the emergence of antimicrobial resistant micro-organisms in the environment (Donskey, 2006; Alanis, 2006; Sundar *et al.*, 2005). Antimicrobials are used clinically for humans and as growth promoters in the animal husbandry industry (Alanis, 2006; Sundar *et al.*, 2005). It is possible to remediate this problem by correct and effective management strategies (Bax *et al.*, 2000; Sundar *et al.*, 2005).

1.3.9 Principles of techniques available to study the quality of groundwater

1.3.9.1 Sampling collection

Water samples obtained from boreholes should be stored immediately on ice or at 4°C and analysed within six hours of sampling (Twarakavi & Kaluarachchi, 2006; Suthar *et al.*, 2009; Griebler *et al.*, 2010; Faniran *et al.*, 2001; Howard *et al.*, 2003; Crowther *et al.*, 2002). Water should be pumped out of the borehole to ensure that a representative aquifer water sample is obtained and not stagnant water collected in the casing of the borehole (Bruce & McMahon, 1996; Suthar *et al.*, 2009; Mclay *et al.*, 2001).

1.3.9.2 Physico-chemical methods

1.3.9.2.1 Chemical parameters

Multiprobes can be used to measure the most physical parameters, including temperature, salinity, pH and electrical conductivity (EC) (Hydralab DS5). Dissolved oxygen concentration is also measured by using a handheld probe (sension156 probe, HACH, Germany). For the determination of chemical parameters, certain chemical reactions need to take place, which will be discussed accordingly.

(a) Nitrate

According to the HACH DR 2800 Spectrophotometer procedures manual (2007), nitrate concentrations in samples are measured using the cadmium reduction method. Cadmium reduces nitrate to nitrite. The nitrite then reacts with sulfanilic acid to form an intermediate diazonium salt in an acidic medium. The diazonium salt then reacts with gentisic acid to form an amber coloured solution. Absorbance of the sample is measured at 500 nm and results displayed in mg/l on the instruments screen.

(b) Sulphate

Sulphate in samples react with barium to form a barium sulphate precipitate. The resulting turbidity formed is directly proportional to the amount of sulphate present in samples. Absorbance is measured at 450 nm and results displayed as mg/l (HACH, 2007).

(c) Chemical oxygen demand (COD)

Two milliliter aliquots of water samples are added to reaction vials supplied by the manufacturer. Vials are heated to 150°C for two hours. The reaction vials contain potassium dichromate, silver and mercury ions. The potassium dichromate acts as an oxidizing agent. Oxidisable organic compounds reduce the dichromate ion to a green chromic ion. The amount of Cr⁶⁺ left in the vials is measured at 420 nm and results displayed as mg/l on the instrument's screen (HACH, 2007).

1.3.9.3 Microbiological methods

1.3.9.3.1 Membrane filtration

Membrane filtration is defined by the US EPA (2005) as a vacuum or pressure driven separation process where particulate matter larger than 1 µm is rejected by using an engineered barrier. For detection of indicator organisms in water, sterile filters of pore size 0.45 µm is used (Cohen & Shuval, 1972; Wang & Wade, 2007). A water sample of 100 ml is filtered through the membranes and then aseptically placed onto selective media and incubated at the appropriate temperatures and time (Romprè *et al.*, 2002). Nutrients and selective chemical compounds contained in the selective media diffuse through the filter to bacterial cells retained on the surface of the membrane (Wang & Wanda, 2007). Bacteria are counted and results given as colony forming units (cfu) per 100 ml.

1.3.9.3.2 Media

1.3.9.3.2.1 mFC Agar.

MFC agar is used for the detection and enumeration of faecal coliform micro-organisms by making use of the membrane filtration technique as described by Harley (2005). The agar plates are incubated at 45°C for 24 hours and the blue colonies on the agar plates are counted as faecal coliform bacteria (Grabow *et al.*, 1981; Harley, 2005). The high incubation temperature makes the test more selective for faecal coliform bacteria (Finch *et al.*, 1987).

Yeast and peptone extracts serve as nutrient sources while bile salts inhibit growth of Gram positive bacteria (Merck, 2012). Rosolic acid contained in the agar serves as a pH indicator. Lactose fermentation by faecal coliforms produces acid to bring about colour change of pH indicator (Merck, 2012).

1.3.9.3.2.2 Membrane-lactose glucuronide agar (MLG agar).

MLG agar is used for the selective detection and enumeration of total coliforms and *E. coli* (Eccles *et al.*, 2004; Fricker *et al.*, 2008). The agar contains peptone and yeast extracts as nutrient sources and laurel sulphate to inhibit growth of Gram positive bacteria (Merck, 2012). Differentiation between coliforms and *E. coli* is based on two biochemical reactions occurring. Coliforms produce acid during lactose fermentation and turn the pH indicator phenol red to yellow (Merck, 2012). *Escherichia coli* produces the enzyme glucuronidase which cleaves 5-bromo-4-chloro-3-indolyl- β -D-glucuronide to form a blue chromophore inside cells (Merck, 2012). Coliforms are identified as yellow colonies and *E. coli* as green colonies on the agar (Eccles *et al.*, 2004; Fricker *et al.*, 2008; Harley, 2005).

1.3.9.3.2.3 KF-streptococcus agar.

KF-streptococcus agar is used for the selective isolation and enumeration of faecal streptococci (Po Cataloa Dionisia & Borrego, 1995; Domig *et al.*, 2003). Faecal streptococci metabolise maltose and lactose to produce acid (Merck, 2012). During preparation of the agar, 1% of triphenyltetrazolium chloride is added to the agar, which is a stain that is assimilated by actively metabolizing cells, giving colonies a red to pink colour (Harley, 2005; Merck, 2012).

1.3.9.3.2.4 Mueller-Hinton agar.

This agar is used for antibiotic sensitivity testing to determine the susceptibility of bacteria to various antibiotics as well as the effectiveness of the different antibiotics to the bacteria. The test involves the growing of a bacterial mat on the medium on which paper disks are placed which are impregnated with an antibiotic. The antibiotic disks are standardised to contain a specific amount of antibiotic. The antibiotic will then diffuse into the agar and inhibit the growth of the bacterium (Leboffe & Pierce, 1999). Inhibition zones are then

measured, recorded and compared to standards such as those from the National Committee for Clinical Laboratory Standards (NCCLS, 1999).

1.3.9.3.2.5 R2A agar.

R2A agar was used for the enumeration of heterotrophic bacteria contained in water (Reasoner & Geldreich, 1985). This medium has a low nutrient content to simulate environmental growing conditions and is incubated at lower temperatures (22°C - 25°C) for longer periods (5 – 7 days) (Merck, 2012). This is to allow for more accurate enumerations of stressed and chlorine tolerant bacteria and other heterotrophic organisms that develop slower (Merck, 2012).

1.3.9.3.3 Molecular based methods

The polymerase chain reaction (PCR) method is used to selectively amplify specific target genes of interest from small amounts of genetic material (Muyzer *et al.*, 1993; Romprè *et al.*, 2002; Willey *et al.*, 2011). This is achieved by repeating three general steps cyclically. These include: i) Denaturing of DNA into single stranded DNA, ii) Annealing primers to single stranded DNA and, iii) Extension of the primers into new complementary DNA strands incorporating heat stable DNA polymerase and deoxyribonucleoside triphosphates (Romprè *et al.*, 2002; Willey *et al.*, 2011). These steps should be repeated for a minimum of 20 cycles up until enough product is produced. This molecular based technique is very rapid, specific and sensitive (Bej *et al.*, 1990; Pollard *et al.*, 1990; Romprè *et al.*, 2002).

Due to very specific environmental conditions and micro-organisms adaptation to these conditions, not all micro-organisms can be grown on culture media. These uncultureable micro-organisms are termed as viable but noncultureable (VBNC) (Bej *et al.*, 1990; Baggi *et al.*, 2001). Owing to this fact, environmental samples may test negative for indicators although they are present in the samples in a VBNC state. The polymerase chain reaction procedure does not discriminate between the two states of micro-organisms and indicator or pathogenic micro-organisms will be detected none the less.

Multiplex PCR refers to a PCR procedure where more than one gene target is identified in a single reaction (Romprè *et al.*, 2002; Willey *et al.*, 2011). The genes *mdh* and *lacZ* are housekeeping genes in *E. coli* (Bej *et al.*, 1990; Romprè *et al.*, 2002; Pupo *et al.*, 1997; Reid *et al.*, 2000). Multiplex PCR can therefore be used to screen for the presence of *E. coli* in samples, where both of these genes needs to be present for a sample to be positive for *E. coli*.

There are some drawbacks when using the PCR procedure. These include the fact that the procedure cannot determine that physiological state of micro-organisms, as micro-organisms may be physiologically active or in a VBNC state. Environmental samples also contain substances that are inherently inhibitory to the PCR procedure, for example humic substances. Highly skilled staff is also required as well as specialised instruments and an equipped laboratory (Romprè *et al.*, 2002).

The 16S ribosomal subunit is only found in prokaryotic organisms and archaea (Vandamme *et al.*, 1996; Mignard & Flandrois, 2006). This subunit is also very conserved, with little variable regions occurring (Stackebrandt & Goebel, 1994; Lane *et al.*, 1985). For these reasons this sub-unit has been used to identify bacteria from various sources via established databases (Mignard & Flandrois, 2006). Variations in DNA sequence results of the non conserved regions allow for the identification of bacteria and archaea (Vandamme *et al.*, 1996).

1.3.10 Summary of literature

The North West Province has an arid climate with highly variable precipitation patterns (NWPG, 2002). For this reason, perennial water sources are limited in the province and therefore people in rural areas are highly dependent on groundwater resources for water supply for domestic use (NWRS, 2004). Groundwater is perceived as being of pristine quality, and therefore communities in rural areas are usually supplied with groundwater without prior treatment (Mpenyana-Monyatsi and Momba, 2012; Ferguson *et al.*, 2012).

Water dependent economic activities, such as mining and agriculture, are present in the province. The mining sector is the biggest contributor to the economy and is also the largest employer in the province (NWPG, 2002). The agricultural sector is the largest user of both surface and groundwater resources, and both of these sectors pose the biggest threat to water resources (Coetzee *et al.*, 2006; Conrad *et al.*, 1999; Naicker *et al.*, 2003). Urbanisation and population growth also pose a threat to water resources because of inadequate sewerage and waste disposal facilities in informal settlements and rural areas (NWRS, 2004). Interactions between ground and surface waters occur (Le Maitrè & Colvin, 2008; Parsons, 2003; Tutu *et al.*, 2008). Contamination of surface water can therefore lead to groundwater contamination and *vice versa*.

Possible physico-chemical pollution indicators include pH, EC, and nitrates and sulphates levels (DWAF, 1996a). All of these parameters can have deleterious effects on human (DWAF, 1996a), livestock (DWAF, 1996b) and crop (DWAF, 1996c) health if water is consumed with levels that exceed safe limits for consumption. Microbial pollution of water sources can occur via a variety of mechanisms (Fourie & van Ryneveld, 1995; DWAF, 2003b; Howard *et al.*, 2003). Faecal pollution of water sources can lead to the possible transmission of pathogenic micro-organisms which can cause possible cholera, typhoid fever, paratyphoid fever, bacillary dysentery and diarrheal disease (Lawrence *et al.*, 2001; Fourie & van Ryneveld, 1994). Indicators of faecal pollution of water sources include faecal coliforms and streptococci (DWAF, 1996a; Romprè *et al.*, 2002; Foppen & Schijven, 2006; Paruch & Maehlum, 2012), where HPC bacteria and total coliforms are

used to assess the general microbiological quality of water (DWAF, 1996a; Gabutti *et al.*, 2000).

Adaptations by bacteria to survive the hostile intracellular environment of amoeba also allows for survival in the human body, despite immune reactions (Thomas *et al.*, 2006; Cosson & Soldati, 2008; Greub & Raoult, 2004). Bacteria can also acquire virulence genes in amoeba as well as develop antimicrobial resistance (Moliner *et al.*, 2010). For these reasons, amoeba resistant bacteria need to be considered as important emerging human pathogens (Pagnier *et al.*, 2008).

Water is a scarce and valuable resource in the North West province and needs to be protected. Many threats are present that can pollute the little water that is available. Through monitoring, parties guilty of pollution can be identified and corrective measures be implemented to rectify the problem and ensure the health of communities relying on surface and groundwater resources.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Study site

2.1.1 Mooi River Catchment

After consultation and physical inspection of all possible monitoring boreholes in the Mooi River catchment area, five boreholes were identified as suitable to be sampled. Due to restrictions imposed by the sampling equipment, other boreholes were excluded from the study. Restrictions include accessibility to boreholes and the diameter that the borehole's casing opening should be to allow the instruments to be lowered down into the borehole. Three boreholes are located in the municipal district of Potchefstroom (Figure 2.1). One of the boreholes is situated in the Trimpark, which is a public park next to the Mooi River in Potchefstroom. The other two boreholes are situated in the informal settlement called Ikageng. One of these boreholes is in a Cemetery, and the other just outside the perimeter of the Boiterilo Primary school. The other two boreholes in the Mooi River catchment are situated outside of Potchefstroom. One of these is situated near the Klerkskraal dam (Figure 2.2), which lies about 45 kilometers North of Potchefstroom. The second of these boreholes is situated on a privately owned farm called Blaauwbank (Figure 2.3). This farm is situated 40 kilometers out of Potchefstroom on the Carletonville road (R501).

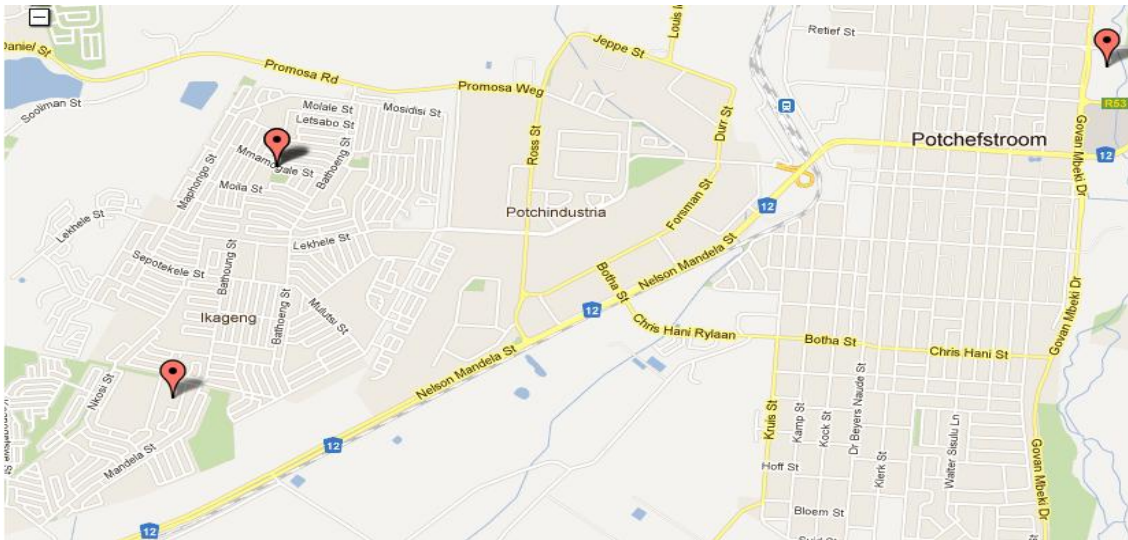


Figure 2.1: Google map displaying the positions of the three boreholes sampled in the Potchefstroom municipal district.



Figure 2.2: Google map displaying the position of the borehole which is situated near to the Klerkskraal dam (Pad dam).

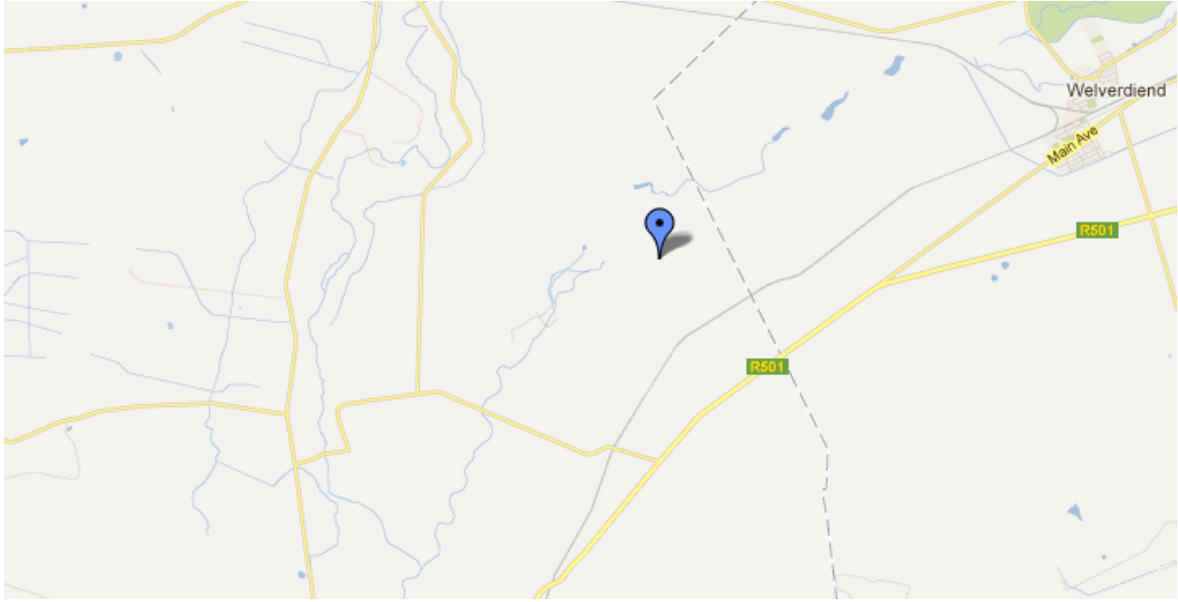


Figure 2.3: Google map displaying the locations of the boreholes situated on the farm Blaauwbank.

2.1.2 Harts River Catchment

After consultation and inspection of all possible monitoring boreholes, two boreholes were identified as suitable to be sampled. The same restrictions applied for these boreholes as described in section 2.1.1. One of the two boreholes is situated inside the town of Warrenton (Figure 2.4). The other borehole is situated on privately owned grounds in the settlement Ganspan (Figure 4). Warrenton is situated about 290 km from Potchefstroom on the N12 en route towards Kimberley. The Ganspan settlement is situated roughly 36 km away from Warrenton on the N18 moving towards Vryburg.

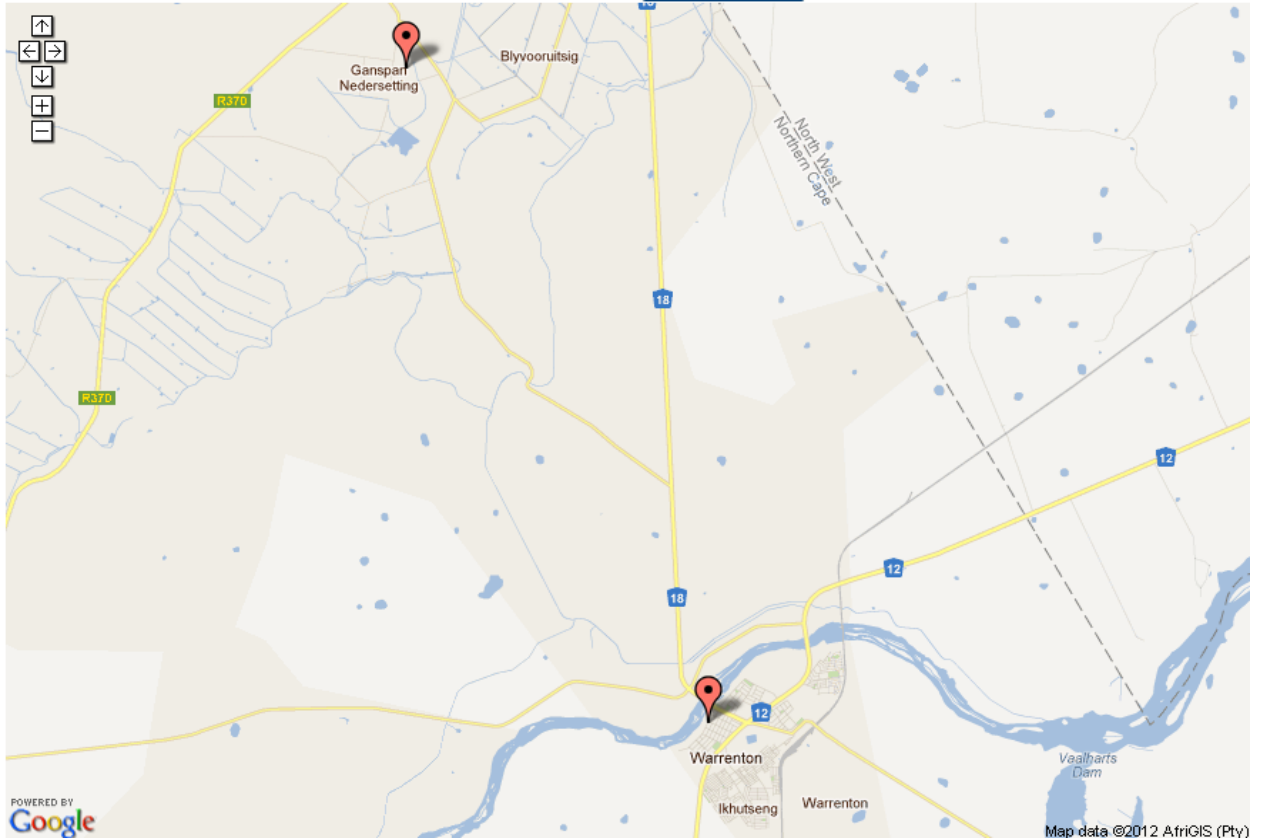


Figure 2.4: Google map displaying the locations of the two boreholes situated in the Harts River catchment area.

2.2 Mobile Research Unit

Due to the nature of this project, specialised instruments had to be used to be able to measure set parameters. For this reason, a specialised truck (Figure 2.5) was used to do all the sampling. Instruments mentioned include a Hydra multimeter (Hydralab DS5, Stevens Water Monitoring Systems, USA [Figure 2.5: D]) and an electrical water sampler (Figure 2.5: C). Each of these instruments is connected to its own spool via a unique cable for the instrument. The spools mentioned are all mounted to the back of the truck, which is covered by a canopy. The spools are operated by making use of a drill (Figure 2.5: F) which is interchangeable between gearboxes (Figure 2.5: G) on each spool. This allows for the lowering into, and abstraction of instruments from boreholes. Power is supplied to these instruments (Figure 2.5: B) via a generator which is mounted to the front bumper of

the truck. The power is first relayed through an uninterruptable power supply (UPS [Figure 2.5: E]). This device stabilizes the current to protect sensitive instruments from power fluctuations (Figure 2.5: A).

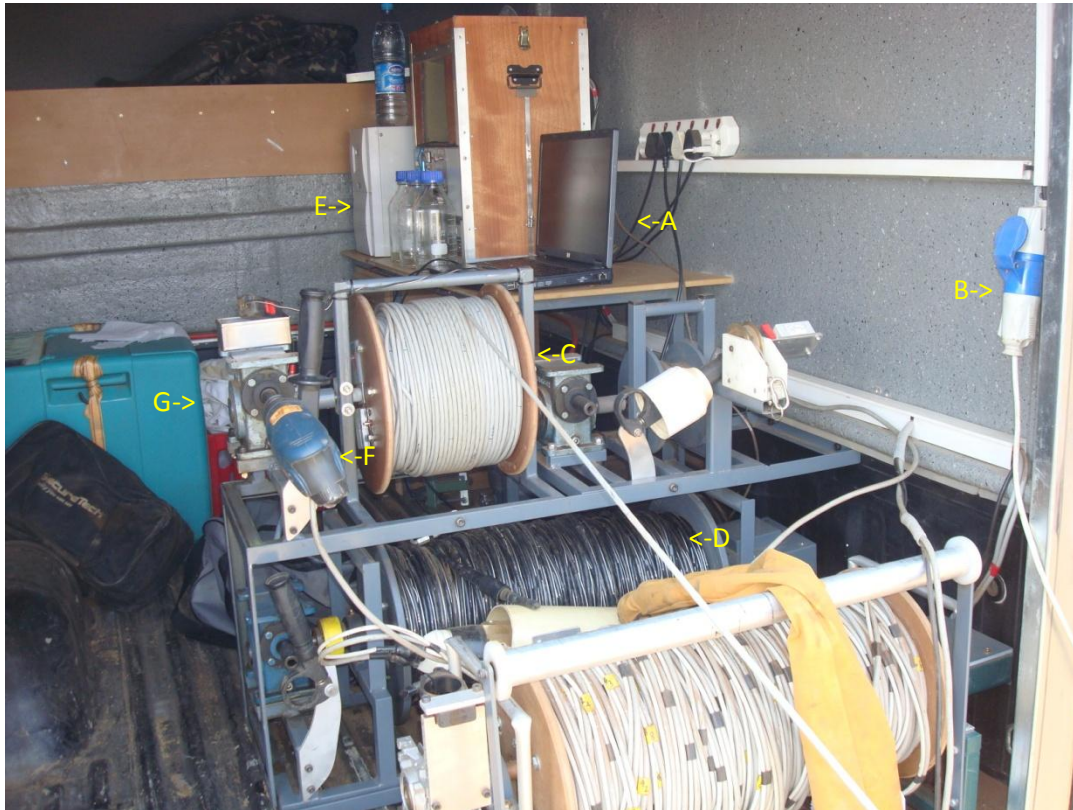


Figure 2.5: Photo illustrating all the instruments used during sampling in the rear of the bakkie. A shows the laptop needed to operate the Hydra multimeter (D). B is the cable that supplies the back of the bakkie with electricity from the generator. C is the spool onto which the water sampler is wound up. E indicates the position of the UPS.

2.3 Measurement of physical and chemical parameters of borehole water *in situ*

Before any sampling or measuring of physico-chemical parameters was done, each borehole was purged for 15 minutes using a submergible pump. This is to allow for the removal of stale water from the borehole casing and infiltration of water from the surrounding aquifer into the borehole. Before a water sample was retrieved from each borehole, a multimeter (Hydralab DS5, Stevens Water Monitoring Systems, USA) was

lowered into each borehole to measure certain physical and chemical parameters of the water *in situ*. Parameters included pH, temperature, conductivity, salinity and total dissolved salts of water.

2.4 Sample collection

Each of the seven boreholes were sampled twice during the warm and wet season (April 2012) and twice during the cold and dry season (July 2012). A time period of three weeks were given between each sampling trip. An alcohol sterilised electrical water sampler was lowered into each borehole to collect water. The water sampler is open when lowered to allow water to fill the empty cavity. The water sampler is then closed electrically from a control box on the water sampler's spool. The water sampler was then raised out of the borehole and the water poured into autoclave sterilised SHCOTT Duran one liter bottles. A sterilised funnel was used to facilitate this process. The bottles containing the water samples were put onto ice for further analyses in the laboratory within six hours of collection of samples.

2.5 Nitrate, sulphate concentration and dissolved oxygen measurements.

Nitrate and sulphate concentrations were measured by using reagents, protocols and a spectrophotometer (DR 2800) from HACH (Germany). Dissolved oxygen was measured on site using a sension156 probe (HACH, Germany).

2.6 Enumeration of indicator organisms on selective media.

Hundred milliliter aliquots of water were filtered through 0.45 µm-pore size membrane filters (Separations, USA) using the membrane filtration technique. Individually filtered membranes were placed onto various media including MLG (MERCK, Germany) agar, KF-streptococcus (Sigma-Aldrich, USA) agar and mFC (MERCK, Germany) agar. Analyses were conducted in triplicate. Preliminary investigations indicated that the water samples had to be diluted (10^{-3}) to obtain values that were measureable. MLG Agar was incubated at 35°C for 24 hours, KF-streptococcus Agar at 35°C for 48 hours and the MFC Agar at 45°C for 24 hours. Green (faecal coliforms, possible *E. coli*) and yellow (total coliforms) colonies were counted on the MLG Agar, pink colonies (faecal streptococci) on

the KF Agar and blue colonies (faecal coliforms) on the MFC agar. Results were expressed (converted where applicable) as cfu/100 ml. Putative *E. coli* isolates were purified by successive streak plating on mFC agar.

2.7 Enumeration of heterotrophic plate count bacteria

A dilution series (up to 10^{-5}) of the water samples were made up for the enumeration of heterotrophic plate count bacteria. The dilutions were spread plated onto Difco R2A (Becton, Dickinson and Company, France) agar plates and incubated at room temperature for five days. After this period the total number of cfu growing on the plates were counted and converted to cfu/ml. The various morphotypes (morphologically distinct colonies, based on shape, colony colour etc.) were identified. Representatives were also purified by successive streak plating on R2A.

2.7.1 Amoeba resistant bacteria

Water samples (500 ml) from the Mooi River catchment boreholes were collected in March 2012 and sent to the National Institute of Occupational Health and Safety (Johannesburg, SA) for the enumeration of amoeba and subsequent isolation of amoeba resistant bacteria (ARB). The latter bacteria were sent to the NWU and were identified as part of this study.

2.8 Identification of *E. coli*, HPC and ARB

Single colony isolates were streaked out at least three times to ensure that the cultures were pure. Gram staining was performed according to Bauer, *et al* (1966) and results recorded. HPC as well as the ARBs were subjected to 16S RNA gene sequencing and BLAST searches for identification. *E. coli* were subjected to PCR amplification of specific housekeeping gene fragments (*mdh* and *lacZ*).

2.8.1 DNA isolation

DNA isolation of putative *E. coli* was performed by using a Nucleospin tissue DNA isolation kit (Macherey-Nagel, Germany). The protocol given by the manufacturer was followed to obtain genomic DNA. After isolation, DNA quality and concentrations were

determined by a NanoDrop™ 1000 Spectrophotometer (Thermo Fischer Scientific, US) and the integrity as described in Section 2.8.2.

DNA was isolated from the HPC isolates and amoeba resistant bacteria using the following approach: Single colonies from streak plates were inoculated into nutrient broth and incubated at room temperature for 5 days. Twenty microliters of this broth was transferred into a 1.5 ml microfuge tube. This was centrifuged briefly (4000 rpm; for 15 seconds) to ensure that the bacterial cells collect at the bottom of the microfuge tube. Tubes containing the cells were microwaved for two minutes at the full power (700 W). Tubes were then centrifuged for one minute at 12800 rpm for cell debris to settle at the bottom. One microliter of the DNA containing supernatant was used for the PCR reactions.

2.8.2 Agarose gel electrophoreses of DNA

Integrity of DNA from putative *E. coli* was determined by electrophoreses. Two microliters of DNA was mixed with 2 µl of Orange Loading dye (Fermentas Life Science, US). The Orange loading dye contained 3 µl of Gel Red (Biotum, US). A 1.5% (w/v) agarose gel submerged in 1 x TAE buffer (40 mM Tris-HCl, 1 mM EDTA [pH 8], 20 mM Acetic Acid) was used and electrophoreses conditions were set at 80V for 50 minutes. Gels were viewed using a ChemiDoc™ TM (BioRad, US) imager.

2.8.3 DNA amplification

2.8.3.1 *E. coli* genome markers (*mdh*, *lacZ* genes)

PCR reagents for amplification consisted of: (i) ~50ng genomic template DNA, (ii) 12.5 µl double strength PCR Master Mix (0.05 U/µl Taq polymerase, 4mM MgCl₂ and 0.4mM dNTPs; Fermentas, US), (iii) 0.5 mM MgCl₂, (iv) 10.2 µl Nuclease free water (Fermentas, US) and 0.8 mM primer mix. The various primers consisted of the *mdh* (*mdhF* 5'-GGTATGGATCGTTCCGACCT-3'; *mdhR* 5'-GGCAGAATGGTAACACCAGAGT-3' [Inqaba Biotech, SA]) and *lacZ* (*lacZF* 5'-CTGGCGTAATAGCGAAGAGG-3'; *lacZR* 5'-GGATTG ACCGTAATGGGATATG-3' [Inqaba Biotech, SA]) genes. The final reaction volume was 25 µl. Primers were used in multiplex PCRs. The PCR cycling conditions consisted of an initial denaturation of 300 seconds at 95°C, followed by 40 cycles of 60

seconds at 95°C, 60 seconds at 55°C and 120 seconds at 72°C. A final extension step of 300 seconds at 72°C was included. The PCR products were resolved by agarose gel electrophoresis as described in section 2.8.2.

2.8.3.2 16S rRNA sequencing of HPC and ARBs

PCR reagents for amplification consisted of: (i) ~50ng of genomic template DNA, (ii) 12.5 µl double strength PCR Master Mix (0.05 U/µl Taq polymerase, 4mM MgCl₂ and 0.4mM dNTPs; Fermentas, US), (iii) 0.5 mM MgCl₂, (iv) Nuclease free water (Fermentas, US), and 0.3 mM primer mix (27F 5'-AGAGTTTGATCMTGGCTCAG-3'; 1492R 5'-TACGGYTACCTTGTTACGACTT-3' [Inqaba Biotech, SA]). The final reaction volume was 25 µl. The PCR cycling conditions consisted of an initial denaturation of 300 seconds at 95°C, followed by 40 cycles of 60 seconds at 95°C, 45 seconds at 51°C and 110 seconds at 72°C. A final extension step of 300 seconds at 72°C was included. The PCR products were resolved by agarose gel electrophoresis as described in section 2.8.2.

2.8.4 Agarose gel electrophoreses of PCR products

Confirmation of DNA amplification was done by electrophoresis as described in section 2.8.2. For the HPC and amoeba resistant bacteria amplicons, a 1kb ladder (O'GeneRuler™, Fermentas Life Sciences, US) was added into the first well. A 100bp ladder (O'GeneRuler™, Fermentas Life Sciences, US) was used in the case of the *E. coli* housekeeping gene amplicons.

2.8.5 Clean-up of PCR products before sequencing PCR (HPC & Amoeba bac.)

A Nucleospin Extract II PCR clean up kit (Macherey-Nagel, Germany) was used to clean up PCR following the instructions of the manufacturer. A NanoDrop™ 1000 Spectrophotometer (Thermo Fischer Scientific, USA) was used to determine DNA quantity and quality.

2.8.6 Sequencing PCR and clean up of HPC & ARB 16S rRNA fragments

A Cycle Sequencing BigDye Terminator Kit (Zymo Research, US) was used to perform the sequencing PCR. The reaction mix consisted of: (i) 4 µl Ready Reaction Premix (2x),

(ii) 2 µl BigDye Sequencing Buffer (2x), (iii) 3.2 pmol 27F primer (Inqaba Biotech, SA), (iv) 1 µl Template DNA (10-40ng) and 9.8 µl of nuclease free water (Fermentas Life Sciences, US) to make up a final volume of 20 µl. Cycling conditions (ICycler) were set at; 96°C for 60 seconds initial denaturation that was followed by 25 cycles of; 96°C for 10 seconds; 50°C for 5 seconds and 60° for 240 seconds. A ZR DNA-Sequencing Cleanup kit (Zymo Research, US), was used to clean the sequence PCR products following the manufacturers protocol.

2.8.8 Sequencing and analysis of 16S rRNA gene fragments

Amplicons were sequenced using an ABI 3130 Genetic Analyser (Applied Biosystems, UK). Chromatograms were viewed in Geospiza Finch TV (version 1.4) software and BLAST searches (<http://www.ncbi.nlm.nih.gov/BLAST>) were used to determine the identity of the amplified sequences.

2.9 Antibiotics resistance testing of isolated *E. coli*, HPC and ARB

Green colonies identified on MLG Agar were isolated and aseptically streaked out again onto MLG Agar to isolate pure *E. coli* (green) colonies. Pure colonies were transferred to Nutrient broth and incubated at 35°C for 24 hours. The nutrient broth was used to make spread plates onto Mueller-Hinton (MERCK, South Africa) agar plates. Eight different antibiotics were identified to be used. These antibiotics include Amoxicillin 10 µg, Cephalothin 30 µg, Ciprofloxacin 5 µg, Kanamycin 30 µg, Erythromycin 15 µg, Chloramphenicol 30 µg, Vancomycin 30 µg and Penicillin 10 µg (Davies Diagnostics, UK). The Mueller-Hinton plates were incubated at 35°C for 24 hours and the diameter of clear areas around the antibiotic discs were measured in millimeters and recorded. The same procedure was used to perform antibiotic resistance testing on HPC isolates, as well as the amoeba resistant bacteria.

2.10 Statistical analysis

Microsoft Windows Excel was used to calculate averages and standard deviations for all data collected. Canonical ordination redundancy analyses (RDA) was also used to

determine the correlation between environmental- (temperature, pH, EC, salinity, COD, nitrate and sulphate) and microbiological variables, as well as sampling sites. Canoco software version 4.5, developed by Ter Braak (1990), was used to perform the multivariate analysis and the results were visualised by triplots. The smaller an angle is between an environmental and microbiological vector, the more correlated factors are to each other.

CHAPTER 3 – RESULTS

Groundwater samples were collected from two different areas in the North West province. Seven boreholes were sampled directly from the aquifer. Five boreholes were sampled in the Mooi River catchment area and two boreholes in the Harts River catchment area. Data are represented in the following order: Physico-chemical data of the Mooi River catchment area boreholes for the warm and wet season followed by the results for a cold and dry season. The physico-chemical results for the Harts River catchment are presented in the same manner. Microbial results for the boreholes from the two areas are presented in the same manner as the physico-chemical results. The following section then presents the antibiotic resistance data of *E. coli* identified and isolated from sampled water. This is followed by the results of the identification and antibiotic resistance of the HPC bacteria isolated from water samples of the Mooi River catchment. The identification and antibiotic resistance results of the amoeba resistant bacteria then follows. The last section is a statistical analysis section where the microbiological and physico-chemical results are compared.

3.1 Physico-chemical data

3.1.1 Mooi River Catchment

In Table 3.1, the physico-chemical data for the Mooi River catchment area measured in the warm and wet season is shown. All of the physico-chemical parameters were below the TWQR for domestic use, as well as for livestock watering purposes. Electrical conductivity for the Trimpark and School boreholes (65.3 mS/m and 125.1 mS/m respectively) as well as nitrate concentration of the School (5.3 mg/l) was above the TWQR for irrigation purposes. The maximum value for salinity was measured at the School borehole (644 ppm) and the minimum at the Cemetery (130.5 ppm). Although there is not a TWQR for salinity, the value measured at the School and Trimpark boreholes were quite high. The maximum sulphate concentration recorded was at the Trimpark borehole (63.5 mg/l) and for nitrate at the School (5.4 mg/l).

pH measurements ranged from 6.6 to 7.7 and were generally within the TWQR for drinking, irrigation and animal watering. Blaauwbank borehole (62 mg/l) had the highest COD level. No nitrate or COD were measured in the Cemetery borehole water. It could be that the levels at these sites were out of the range of the test kits.

Table 3.1: Physico-chemical results for the Mooi River catchment area during the warm and wet season.

Borehole		Temp	pH	EC mS/m	Salinity ppm	SO ₄ mg/l	Nitrate mg/l	DO mg/l	COD mg/l
	TWQR domestic use	N/A	6 – 9	<70	N/A	<200	<10	N/A	N/A
Blaauwbank	Max	23.6	7.7	40.7	198	10	0	1.2	89
	Min	21	7.5	38.5	190	0	4	1.2	35
	Ave	22.3	7.6	39.5	194	5	2	1.2	62
	SD	±1.8	± 0.1	±1.1	±5.7	±7.1	±2.8	±0	±38.2
Pad Dam	Max	21.2	6.9	32.7	162	40	0	4.5	24
	Min	19.4	6.6	31.3	157	0	0	2.3	24
	Ave	20.3	6.7	32	159.5	20	0	3.4	24
	SD	±1.3	±0.2	±1	±3.5	±28.3	±0	±1.6	±0
Trimpark	Max	21.9	7.3	83.8	417	77	0.6	2.6	23
	Min	20.6	7.2	46.7	233	50	0	2.3	12
	Ave	21.3	7.25	65.3	325	63.5	0.3	2.5	17.5
	SD	±0.9	±0	±26.2	±130.1	±19.1	±0.4	±0.2	±7.8
School	Max	23.4	7	125.7	645	21	10.7	3.1	22
	Min	21.3	6.8	124.5	643	0	0	2.3	1
	Ave	22.4	6.9	125.1	644	10.5	5.4	2.7	11.5
	SD	±1.5	±0.1	±0.8	±1.4	±14.8	±7.6	±0.6	±14.8
Cemetery	Max	23.2	6.9	27.2	134	7	0	4.2	0
	Min	20.4	6.9	25.5	127	4	0	2.3	0
	Ave	21.8	6.85	26.4	130.5	5.5	0	3.3	0
	SD	±2.0	±0.1	±1.2	±4.9	±2.1	±0	±1.3	±0

Temp = temperature; EC = Electrical Conductivity; DO = Dissolved Oxygen; COD = Chemical Oxygen demand; mS/m = Milli-Siemens per meter; ppm = Parts per Million (mg/l); mg/l = milligrams per liter; N/A = Not Applicable

In Table 3.2, physico-chemical data is shown for the Mooi River catchment area during the cold and dry season. All of the physico-chemical parameters were below the TWQR for domestic use, except for the nitrate concentration of the Trimpark (14.1mg/l) and School (137 mg/l) borehole water samples. The School borehole also exceeded the TWQR for livestock watering regarding nitrate concentration. Electrical conductivity of the Trimpark (65.3 mS/m) and School (125.1 mS/m) boreholes exceeded the TWQR for irrigation.

Nitrate concentration of the Pad dam, School and Trim boreholes (8.15 mg/l, 14.1 mg/l and 137mg/l respectively) exceeded the TWQR for irrigation water.

The pH range for this season was between pH 7 (school) and pH 7.96 (Blaauwbank) and thus in the ideal range for water. However, the highest EC (127 mS/m) and salinity (645 ppm) values was recorded at the School borehole. The salinity of the Trimpark borehole was also high (427.5 ppm), compared to the other boreholes. Maximum sulphate concentration was recorded for the Trimpark borehole (81 mg/l) and nitrate at the School borehole (137 mg/l). The maximum COD was again recorded at the Blaauw borehole (55 mg/l).

When the results from the warm versus cold season are compared, it is evident that the temperature between the two seasons was relatively constant. This is expected since these boreholes were in the same area and protected from temperature variation. The pH, electrical conductance, salinity and nitrate concentrations for all boreholes increased from the warm to the cold season. The exceptions were that of the Blaauwbank (EC, salinity, Nitrate) and Cemetery (salinity) boreholes where concentrations declined. Sulphate and COD concentrations decreased for all boreholes, except for the Trimpark borehole. In both seasons electrical conductivity and salinity of the School borehole samples were the highest. The same trend for sulphate concentration was observed for the Trimpark borehole samples.

Table 3.2: Physico-chemical results for the Mooi River catchment area during the cold and dry season.

Borehole		Temp	pH	EC mS/m	Salinity ppm	SO ₄ mg/l	Nitrate mg/l	DO mg/l	COD mg/l
	TWQR domestic use	N/A	6 – 9	<70	N/A	<200	<10	N/A	N/A
Blaauwbank	Max	20.1	8	38.3	188	0	0	2.6	55
	Min	22	7.92	37.8	187	0	0	1.8	54
	Ave	21	7.96	38.1	188	0	0	2.2	55
	SD	±1.3	±0.1	±0.4	±0.7	±0	±0	±0.6	±0.7
Pad Dam	Max	21.3	7.18	34.7	165	2	16.3	6	27
	Min	18.6	7.1	33.4	165	0	0	5	0
	Ave	20.2	7.14	34	165	1	8.15	5.5	13.5
	SD	±1.9	±0.1	±0.9	±0	±1.4	±11.5	±0.7	±19.1
Trimpark	Max	20.5	7.6	77.3	470	103	27.1	2.7	44
	Min	18.6	7.24	62.9	385	59	0	2.6	30
	Ave	19.6	7.42	70.1	427.5	81	14.1	2.65	37
	SD	±1.3	±0.3	±10.2	±60.1	±31.1	±19.2	±0.1	±9.9
School	Max	23.4	7.05	127.2	650	0	176	2.6	0
	Min	21.6	6.95	126.8	640	0	98	2.4	0
	Ave	22.5	7	127	645	0	137	2.5	0
	SD	±1.3	±0.1	±0.3	±7.1	±0	±55.2	±0.1	±0
Cemetery	Max	22.5	7.2	28.4	137	3	9	5.3	0
	Min	20.4	7.2	24.8	122	0	0.7	2.9	0
	Ave	21.5	7.2	26.6	129.5	1.5	4.9	4.1	0
	SD	±1.5	±0	±2.5	±10.6	±2.1	±5.9	±1.7	±0

Temp = temperature; EC = Electrical Conductivity; DO = Dissolved Oxygen; COD = Chemical Oxygen Demand; mS/m = Milli-Siemens per meter; ppm = Parts per Million (mg/l); mg/l = milligrams per liter; N/A = Not Applicable

3.1.2 Harts River Catchment

In Table 3.3, the physico-chemical data are shown for the boreholes sampled in the Harts River catchment area during the warm and wet season. Some of the parameters measured were below the TWQR for water used for domestic purposes and livestock watering. The pH was around 7 which is ideal for water for all purposes. However, nitrate and sulphate concentrations and electrical conductivity were high in both cases. In the case of the Warrenton borehole, a sulphate level of 450 mg/l was measured. This exceeds the TWQR for domestic use. Electrical conductivity of both boreholes (166.9 mS/m and 83.3 mS/m) and nitrate concentration (5.65 mg/l and 5 mg/l for Warrenton and Ganspan respectively)

exceeded the TWQR for water for irrigation use. The highest values for all of the physico-chemical parameters, except nitrates, were measured in the Warrenton borehole water.

Table 3.3: Physico-chemical results for the Harts River catchment area during the warm and wet season.

Borehole		Temp	pH	EC mS/m	Salinity ppm	SO ₄ mg/l	Nitrate mg/l	DO mg/l	COD mg/l
	TWQR Domestic use	N/A	6 - 9	<70	N/A	<200	<10	N/A	N/A
Warrenton	Max	24.2	7.16	167.3	868	600	6.3	5.4	160
	Min	22.2	6.97	166.5	866	300	5	4	37
	Ave	23.2	7.07	166.9	867	450	5.65	4.7	98.5
	SD	±1.4	±0.1	±0.6	±1.4	±212.1	±0.9	±1	±87
Ganspan	Max	21.2	7.77	84.6	428	190	10	4.7	20
	Min	20.9	7.75	82	414	77	0	3	19
	Ave	21.5	7.76	83.3	421	133.5	5	3.85	19.5
	SD	±0.2	±0	±1.8	±9.9	±79.9	±7.1	±1.2	±0.7

Temp = temperature; EC = Electrical Conductivity; DO = Dissolved Oxygen; COD = Chemical Oxygen Demand; mS/m = Milli-Siemens per meter; ppm = Parts per Million (mg/l); mg/l = milligrams per liter; N/A = Not Applicable

In Table 3.4, the physico-chemical data are shown for boreholes sampled in the Harts River catchment area during a cold and dry season. Both boreholes exceeded the TWQR for electrical conductivity for irrigation water. Values were 173.3 mS/m and 88.1 mS/m for the Warrenton and Ganspan boreholes, respectively. The nitrate (7.65 mg/l) level of the Warrenton borehole also exceed the TWQR for irrigation.

From Table 3.4, it is evident that all the high values were again measured at the Warrenton borehole. The salinity at the Warrenton borehole (896.5 mg/l) was twice that of the Ganspan borehole (444.5 mg/l).

Table 3.4: Physico-chemical results for the Harts River catchment area during the cold and dry season.

Borehole		Temp	pH	EC	Salinity ppm	SO ₄ mg/l	Nitrate mg/l	DO mg/l	COD mg/l
	TWQR Domestic use	N/A	6 - 9	<70	N/A	<200	<10	N/A	N/A
Warrenton	Max	20.1	7.24	174.6	904	390	15.3	4.1	65
	Min	19.8	7.14	172.0	889	260	0	2.8	31
	Ave	19.9	7.19	173.3	896.5	325	7.65	3.45	48
	SD	±0.2	±0.1	±1.8	±10.6	±91.9	±10.8	±0.9	±24
Ganspan	Max	21.2	7.75	89.1	449	97	0	3.8	4
	Min	20.2	7.6	87.2	440	90	0	2.5	0
	Ave	20.7	7.68	88.1	444.5	93.5	0	3.15	2
	SD	±0.7	±0.1	±1.3	±6.4	±4.9	±0	±0.9	±2.8

Temp = temperature; EC = Electrical Conductivity; DO = Dissolved Oxygen; COD = Chemical Oxygen Demand; MS/m = Milli-Siemens per meter; ppm = Parts per Million (mg/l); mg/l = milligrams per liter; N/A = Not Applicable

The temperature and pH of both boreholes were similar during the two seasons. The COD level was lower in the cold and dry season, compared to the warm and wet season. Slight inter-seasonal variations were observed for the other physico-chemical parameters.

3.2 Microbiological data

3.2.1 Mooi River catchment

In Table 3.5, the microbiological data is shown for all the boreholes sampled in the Mooi River catchment area during the warm and wet season. None of the boreholes sampled complied with all the TWQR for domestic use and irrigation purposes. However, the water from all of the boreholes complied with all of the requirements of the TWQR for livestock watering. Only the Blaauwbank and Pad dam faecal coliform counts were below the TWQR for domestic water use.

Faecal coliforms were identified in three out of the five boreholes. The maximum level was obtained at the Cemetery borehole (175 cfu/100 ml) and the minimum at the School

borehole (2 cfu/100ml). Total coliforms and HPC bacteria were present in all of the boreholes. Faecal streptococci were also present in all of the boreholes, except for the Blaauwbank borehole. Total coliform counts ranged between 1.43×10^5 cfu/100ml (Blaauwbank - max) and 150 cfu/100ml (Trimpark – min). The maximum number for HPC bacteria (7.23×10^7 cfu/ml) and faecal streptococci (2.69×10^4 cfu/100ml) were obtained from the Pad dam borehole. The least number of HPC bacteria were enumerated in the Blaauwbank (1.73×10^6 cfu/ml) and faecal streptococci counts in the cemetery borehole water (92 cfu/100ml).

Table 3.5: Microbiological results for boreholes sampled in the Mooi River catchment during the warm and wet season.

Borehole		Feecal coliforms cfu/100ml	Total coliforms cfu/100ml	HPC cfu/ml	Feecal Streps cfu/100ml
	TWQR Domestic use	0	< 10	<1000	
Blaauwbank	Max	0	2.87×10^5	1.93×10^6	0
	Min	0	0	1.53×10^6	0
	Ave	0	1.43×10^5	1.73×10^6	0
	SD	±0	±2.0×10^5	±2.8×10^5	±0
Pad Dam	Max	0	300	9.43×10^7	2.92×10^4
	Min	0	0	5.03×10^7	2.46×10^4
	Ave	0	150	7.23×10^7	2.69×10^4
	SD	±0	±212	±3.1×10^7	±3.2×10^3
Trimpark	Max	79	330	1.69×10^7	220
	Min	4	0	5.5×10^4	16
	Ave	42	165	8.47×10^6	118
	SD	±53	±233	±1.1×10^7	±144
School	Max	3	1.45×10^4	2.51×10^6	1.64×10^3
	Min	0	600	1.78×10^6	370
	Ave	2	7.55×10^3	2.14×10^6	1×10^3
	SD	±2	±9.8×10^3	±5.1×10^5	±898
Cemetery	Max	350	3.2×10^3	5.36×10^7	177
	Min	0	367	2.67×10^5	6
	Ave	175	1.78×10^3	2.69×10^7	92
	SD	±247	±2.0×10^3	±3.7×10^7	±120

SD = Standard deviation

In Table 3.6, data is shown for all the boreholes sampled in the Mooi River catchment during the cold and dry season. Although all the values were lower than for the warm

season, they were still not appropriate for direct use for several purposes. The heterotrophic plate counts of all of the boreholes and total coliform counts for the Trimpark, School and Cemetery boreholes were above the TWQR for domestic use. However, all of the boreholes were suitable for irrigation as well as livestock watering. Only the Trimpark borehole water cannot be used for irrigation purposes because of the excessively high faecal coliform counts. The cemetery borehole (3.3×10^4 cfu/100ml) had the highest number of total coliforms. Heterotrophic plate count bacteria ranged from a maximum of $>1 \times 10^7$ cfu/ml (Blaauwbank) to a minimum of 4.35×10^4 cfu/ml (School). Trimpark borehole (1.6×10^2 cfu/100ml) had the highest numbers of faecal streptococci.

Table 3.6: Microbial results for boreholes sampled in the Mooi River catchment during the cold and dry season.

Borehole		Feecal coliforms cfu/100ml	Total coliforms cfu/100ml	HPC cfu/ml	Feecal Streps cfu/100ml
TWQR Drinking water		< 0	< 10	<1000	
Blaauwbank	Max	0	0	>300	0
	Min	0	0	>300	0
	Ave	0	0	>300	0
	SD	±0	±0	±0	±0
Pad Dam	Max	0	3	3×10^5	36
	Min	0	0	4.6×10^4	23
	Ave	0	1.5	3.8×10^5	30
	SD	±0	±2.1	$\pm 1.7 \times 10^5$	±9.2
Trimpark	Max	22	760	3.79×10^5	320
	Min	0	0	5.1×10^4	4
	Ave	11	380	2.1×10^5	160
	SD	±15.6	±537	$\pm 2.3 \times 10^5$	±233
School	Max	0	250	7.3×10^4	280
	Min	0	40	1.4×10^4	4
	Ave	0	145	4.35×10^4	142
	SD	±0	±148	$\pm 4.1 \times 10^4$	±195
Cemetery	Max	0	6.6×10^4	4.91×10^6	85
	Min	0	20	6.6×10^3	0
	Ave	0	3.3×10^4	2.45×10^6	43
	SD	±0	$\pm 4.6 \times 10^4$	$\pm 3.4 \times 10^6$	±60

SD = Standard deviation

3.2.2 Harts River Catchment

In Table 3.7, data is shown for all of the boreholes sampled in the Harts River catchment area during the warm and wet season. None of the two boreholes complied with the TWQR for domestic, irrigation or livestock watering. The maximum indicator bacterial levels were enumerated from the Warrenton borehole water. The numbers of enumerated bacteria decreased between the first and second sampling period.

Table 3.7: Microbiological results for boreholes sampled in the Harts River catchment during the warm and wet season.

Borehole		Feecal coliforms cfu/100ml	Total coliforms cfu/100ml	HPC cfu/ml	Feecal Streps cfu/100ml
	TWQR Drinking water	≥ 0	≥ 10	≥1000	
Warrenton	Max	7.33 x 10 ³	2.85 x 10 ⁴	3.8 x 10 ⁵	1.56 x 10 ⁴
	Min	365	1.38 x 10 ⁴	8.17 x 10 ⁴	870
	Ave	3.84 x 10³	2.11 x 10⁴	2.3 x 10⁵	8.23 x 10³
	SD	±4.9 x 10³	±1.0 x 10⁴	±2.1 x 10⁵	±1.0 x 10⁴
Ganspan	Max	3.8 x 10 ³	300	1.21 x 10 ⁶	3 x 10 ³
	Min	0	0	6.8 x 10 ³	16
	Ave	1.9 x 10³	150	6.08 x 10³	1.5 x 10³
	SD	±2.6 x 10³	±212	±8.5 x 10⁵	±2.1 x 10³

SD=Standard deviation

Table 3.8 shows the data for the boreholes sampled in the Harts River catchment during the cold and dry season. For both boreholes, the total coliform and heterotrophic plate counts were above the TWQR for domestic use. The Ganspan borehole microbial counts were within the TWQR for irrigation and livestock watering purposes. The Warrenton borehole's microbial counts comply with TWQR for livestock watering. Very little faecal coliforms were enumerated compared to the warm and wet season. In the latter season, the bacterial levels were generally higher than in the cold and dry season. Warrenton had once again the highest levels for all of the indicator bacteria.

Table 3.8: Microbial data for boreholes sampled in the Harts River catchment area during the cold and dry season.

Borehole		Feacal coliforms cfu/100ml	Total coliforms cfu/100ml	HPC cfu/ml	Feacal Streps cfu/100ml
	TWQR Drinking water	< 0	< 10	<1000	
Warrenton	Max	2	1 x 10 ⁵	1.5 x 10 ⁵	1.7 x 10 ⁴
	Min	0	3.4 x 10 ²	6.05 x 10 ⁴	105
	Ave	1	5 x 10⁴	1.07 x 10⁵	8.5 x 10³
	SD	±1.4	±7.0 x 10⁴	±6.3 x 10⁴	±1.1 x 10⁴
Ganspan	Max	0	103	5.3 x 10 ⁴	5
	Min	0	24	4.6 x 10 ³	2
	Ave	0	63.5	2.8 x 10⁴	3.5
	SD	±0	±55	±3.4 x 10⁴	±2.1

SD=Standard deviation

3.3 Antimicrobial resistance

Results for antimicrobial resistance testing performed on *E. coli* isolated from all boreholes are provided in Table 3.9. The *E. coli* included are all confirmed *E. coli*, thus only single representatives for the boreholes were tested and results presented. The table includes the isolates from the warm and wet sampling period. All of the *E. coli* were resistant to Cephalothin, Erythromycin and Amoxicillin, but were susceptible to Ciprofloxacin. Except for the Trimpark isolate, all the other isolates were resistant to Kanamycin. This isolate however, had intermediate resistance. The Warrenton and Ganspan isolates were resistant to Chloramphenicol. Isolates from the School and Cemetery were intermediately resistant to this antibiotic.

What is evident is that all the isolates tested had the same base antibiotic resistant phenotype (KF, E, A). The representative isolates from the two Harts River boreholes had the same antibiotic resistance profile (K, C, KF, E, A). These latter two boreholes are ~50 km apart. The antimicrobial resistance phenotype of the two catchments differed. The individuals from the Mooi River catchment had the same antimicrobial resistance profile and the individuals from the Harts River catchment also shared the same antimicrobial resistance profile.

Table 3.9: Antibiotic resistance results for isolated *E. coli* from boreholes sampled in the warm and wet season.

	Trimpark	Cemetery	School	Warrenton	Ganspan
Kanamycin 30µg (K)	I 14 – 17 mm	R <13 mm	R <13 mm	R <13 mm	R <13 mm
Chloramphenicol 30µg (C)	S >18 mm	I 13 – 17 mm	I 13 – 17 mm	R <12 mm	R <12 mm
Ciprofloxacin 5µg (CIP)	S >21 mm	S >21 mm	S >21 mm	S >21 mm	S >21 mm
Cephalothin 30µg (KF)	R <14 mm	R <14 mm	R <14 mm	R <14 mm	R <14 mm
Erythromycin 15µg (E)	R <13 mm	R <13 mm	R <13 mm	R <13 mm	R <13 mm
Amoxicillin 10µg (A)	R <13 mm	R <13 mm	R <13 mm	R <13 mm	R <13 mm
Antimicrobial resistance phenotype	(ii) KF, E, A	(i) K, KF, E, A	(i) K, KF, E, A	(iii) K, C, KF, E, A	(iii) K, C, KF, E, A

R = Resistant; I = Intermediate; S = Susceptible

Table 3.10 displays the results for antimicrobial resistance testing performed on the two confirmed *E. coli* isolates obtained from boreholes sampled in the cold and dry season. Both isolates were susceptible to Kanamycin, Chloramphenicol, Ciprofloxacin and Amoxicillin. The isolates were however, resistant to Erythromycin and intermediately susceptible to Cephalothin.

Table 3.10: Antimicrobial resistance results of isolated *E. coli* from all boreholes sampled in the cold and dry season.

	Trimpark	Warrenton
Kanamycin 30µg (K)	S >18 mm	S >18 mm
Chloramphenicol 30µg (C)	S >18 mm	S >18 mm
Ciprofloxacin 5µg (CIP)	S >21 mm	S >21 mm
Cephalothin 30µg (KF)	I 15 – 17 mm	I 15 – 17 mm
Erythromycin 15µg (E)	R <13 mm	R <13 mm
Amoxicillin 10µg (A)	S >17 mm	S >17 mm
Antimicrobial resistance phenotype	(iv) E	(iv) E

R = Resistant; I = Intermediate; S = Susceptible

3.4 Molecular identification results

A multiplex PCR (mPCR) protocol was followed for positive identification of presumptive *E. coli* isolated. During a mPCR, two or more gene fragments are amplified at the same time. For the positive identification of *E. coli*, fragments of the malate dehydrogenase (*mdh*) and lactose promoter (*lacZ*) were amplified (Bej *et al.*, 1990; Romprè *et al.*, 2002). Only if both gene fragments were amplified was the result accepted as positive for *E. coli*. Agarose gel electrophoresis was used for visualisation of PCR amplification products.

Figure 3.1 is an image of a 1.5 % (w/v) agarose gel that illustrates mPCR products. The first lane contains a molecular marker, followed by five lanes of *E. coli* isolated during the warm and wet sampling season and a negative control. The following four lanes consist of a molecular marker, two *E. coli* isolates isolated during the cold and dry sampling season and a negative control.

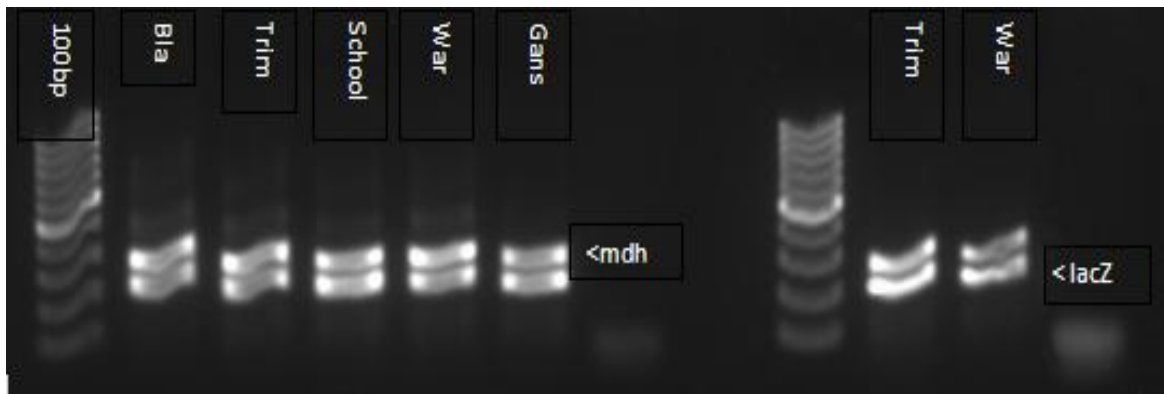


Figure 3.1: A 1.5% (w/v) agarose gel illustrating the results of the multiplex PCR. Two microliters of amplified DNA and loading dye were loaded into wells of the gel. Lane one and nine contains a 100bp molecular marker (O'GeneRuler™ 100bp DNA ladder, Fermentas Life Sciences, US). Blaauwbank, Trimpark, School, Warrenton and Ganspan in lanes 2 – 6, were presumptive *E. coli* that were isolated during the warm sampling season. Trim and War in lanes 10 and 11 were presumptive *E. coli* isolated during the cold sampling season.

Two distinct bands of the expected fragment size are visible in all of the lanes. It can therefore be ascertained that the colonies isolated from the differential media were in fact *E. coli*. These were the only samples included for antibiotic resistance testing.

3.4 HPC identification and antimicrobial resistance results

3.4.1 Mooi River catchment

In Table 3.11 – 3.15, the identification results and antimicrobial resistance patterns for HPC bacteria from the Mooi River catchment boreholes are provided. The antibiotic resistance patterns were generated in the same manner as provided in section 3.3. The initial selection of HPC isolates were targeting morphologically different colonies on the original plate and streak plated several times to purify. Finally, a total of fifteen different species were identified. The most abundant genera identified were *Pseudomonas* with

seven isolates and *Bacillus* with five isolates and *Comamonas* with three isolates. The least abundant genera identified were *Fermicutes*, *Delftia*, *Massilia*, *Siphonobacter*, *Dechlorosoma*, *Brevundimonas*, *Lysinibacillus*, *Aeromonas* and *Chitinophaga* with only one isolate each.

The borehole with the most variety of isolates was the School borehole with 7 different genera. Trimpark borehole was the one with the least variety of species (3). Amongst all isolates identified, the *Bacillus* species occurred in 4 of 5 boreholes.

In some boreholes, two or more of the same bacteria was identified by 16S rRNA sequences. These however, had distinguishing phenotypical features or antibiotic resistance phenotypes. For example, *Chryseobacterium* identified in the Pad dam borehole could be distinguished from each other by the colony colour as observed on R2A agar. One was translucent and the other a distinguishing orange.

Species that were resistant to (five out of possible eight) antibiotics tested are: *Pseudomonas putida* (Trim), *Pseudomonas fluorescens* (Trimpark + Cemetery), *Pseudomonas plecoglossicida* (Cemetery), *Comamonas* sp. (Cemetery) and *Chitinophaga* sp isolated from the School borehole. Isolates with the least resistance to antimicrobials tested include *Leifsonia xyli* from the Blaauwbank borehole with no resistance to any antibiotic and *Pseudomonas stutzeri* from the Cemetery borehole with resistance to only one (KF) antibiotic.

Chryseobacterium (iv), all *Bacillus* sp (i), *Flavobacterium cucumis* and all *Pseudomonas* species (v), isolated from different boreholes, had the same antimicrobial resistance phenotype. The exception being *Pseudomonas stutzeri* (vi) and a *Pseudomonas* sp. (i) isolated from the Cemetery borehole and *Pseudomonas anguilliseptica* (viii) isolated from the School borehole.

Isolates of different species identified displaying similar antibiotic phenotypes (i) include *Fermicutes* clone (Pad dam), *Massilia timonae* (Blaauwbank), *Siphonobacter aquaeclarae*, *Dechlorosoma suillum*, *Leifsonia xyli* (Cemetery), *Pseudomonas* sp. (Cemetery) and *Flavobacterium cucumis* (School). *Comamonas odontotermis*, *Lysinibacillus sphaericus* and *Aeromonas hydrophila* also displayed the same antibiotic resistance phenotype (iv).

Resistance to Cephalothin and Amoxicillin was a general antibiotic phenotype observed among the *E. coli* and HPC. Resistance to Erythromycin was also observed. The table also contains amoeba resistance information. This will be further explored in a section below. The Reference (Ref) column in the table indicates which previous author/s had already identified specific bacteria as ARB.

Table 3.11: Identification and antibiotic resistance results of HPC bacteria isolated from the Pad Dam borehole.

Habitat	Identity of isolate	Method of ID	Gram reaction and shape	Colony colour	Appearance on agar	Antibiotic resistance phenotype	ARB	Ref
Pad dam	2.1 Uncultured Firmicutes bacterium clone 65B	16S rRNA sequencing	+ Bacil	Transparent	Medium size colonies with flat, smooth surface and circular fringe	(i) KF, A	Unknown	
	3.1 <i>Comamonas thiooxydans</i>	16S rRNA sequencing	- Bacil	Translucent	Big colonies with granular surface	(ii) S, A	Unknown	
	4.1 <i>Delftia tsuruhatensis</i>	16S rRNA sequencing	- Bacil	White	Medium sized colonies with smooth surface	(iii) S, E, A	+	Pagnier <i>et al.</i> , 2008
	5.1 <i>Chryseobacterium</i>	16S rRNA sequencing	- Bacil	Translucent	Big colonies with non-translucent centre with translucent fringe	(iv) S, KF, A	+	Pagnier <i>et al.</i> , 2008
	6.1 <i>Chryseobacterium</i>	16S rRNA sequencing	- Bacil	Orange	Medium sized colonies with a smooth surface	(iv) S, KF, A	+	Pagnier <i>et al.</i> , 2008
	7.1 <i>B. cereus</i>	16S rRNA sequencing	+ Bacil	Translucent	Big colonies with non-translucent centre and ripples fanning out from centre	(i) KF, A	+	Pagnier <i>et al.</i> , 2008

Table 3.12: Identification and antibiotic resistance results of HPC bacteria isolated from the Blaauw borehole.

Habitat	Identity of Isolate	Method of ID	Gram reaction and shape	Colony colour	Appearance on agar	Antibiotic resistance phenotype	ARB	Ref
Blaauwbank	1.1 <i>Massilia timonae</i>	16S rRNA sequencing	- Bacil	Light green	Medium sized colonies with granular surface and circular fringe	(i) KF, A	Unknown	
	2.1 <i>Leifsonia xyli</i>	16S rRNA sequencing	+ Bacil	Yellow	Medium sized colonies with smooth surface and circular fringe	-	Unknown	
	3.1 <i>B. cereus</i>	16S rRNA sequencing	+ Bacil	Transparent	Medium sized colonies with granular surface and irregular fringe	(i) KF, A	+	Pagnier <i>et al.</i> , 2008
	4.1 <i>Siphonobacter aquaeclarae</i>	16S rRNA sequencing	- Bacil	Light green	Small colonies with smooth surface and circular fringe	(i) KF, A	Unknown	
	6.1 <i>Comamonas odontotermitis</i>	16S rRNA sequencing	- Bacil	Translucent centre, transparent fringe	Medium sized colonies with granular surface and circular fringe	(iv) S, KF, A	Unknown	
	9.1 <i>Dechlorosoma suillum</i>	16S rRNA sequencing	- Bacil	Transparent	Very small colonies, smooth surface and circular fringe	(i) KF, A	Unknown	

Table 3.13: Identification and antibiotic resistance results of HPC bacteria isolated from the Trimpark borehole.

Habitat	Identity of isolate	Method of ID	Gram reaction and shape	Colony colour	Appearance on agar	Antibiotic Resistance phenotype	ARB	Ref
Trimpark	1.1 <i>Chryseobacterium</i> sp.	16S rRNA sequencing	- Bacil	Cream	Medium sized colonies with smooth surface and circular fringe	(iv) S, KF, A	+	Pagnier <i>et al.</i> , 2008
	2.1 <i>Pseudomonas putida</i>	16S rRNA sequencing	- Bacil	Yellow	Medium sized colonies with smooth surface and circular fringe	(v) S, C, KF, E, A	+	Pagnier <i>et al.</i> , 2008
	3.1 <i>Pseudomonas fluorescens</i>	16S rRNA sequencing	- Bacil	Translucent	Medium sized colonies with smooth surface and circular fringe	(v) S, C, KF, E, A	+	Pagnier <i>et al.</i> , 2008

Table 3.14: Identification and antibiotic resistance results of HPC bacteria isolated from the Cemetery borehole.

Habitat	Isolate	Method of ID	Gram reaction and shape	Colony colour	Appearance on agar	Antibiotic resistance Phenotype	ARB	Ref
Cemetery	1.1 <i>Pseudomonas plecoglossicida</i>	16S rRNA sequencing	- Bacil	Cream	Medium sized colonies, smooth surface, circular fringe	(v) S, C, KF, E, A	Unknown	
	2.1 <i>Leifsonia xyli</i>	16S rRNA sequencing	+ Bacil	Neon Yellow	Small colonies, smooth surface, circular fringe	(i) KF, A	Unknown	
	2.2 <i>Pseudomonas</i> sp.	16S rRNA sequencing	- Bacil	Neon Yellow	Medium sized colonies, smooth surface, circular fringe	(i) KF, A	+	Pagnier <i>et al.</i> , 2008
	3.1 <i>Pseudomonas fluorescens</i>	16S rRNA sequencing	- Bacil	Translucent white	Big colonies, umbonate elevation, irregular fringe	(v) S, C, KF, E, A	+	Pagnier <i>et al.</i> , 2008
	4.1 <i>Pseudomonas stutzeri</i>	16S rRNA sequencing	- Bacil	Translucent	Medium sized colonies, darker centre, translucent fringe with ripples fanning out towards fringe	(vi) KF	Unknown	
	6.1 <i>Comamonas</i> sp.	16S rRNA sequencing	- Bacil	Transparent	Pinhead sized colonies, smooth surface, circular fringe	(v) S, C, KF, E, A	+	Walchonik <i>et al.</i> , 1999
	7.2 <i>Brevundimonas diminuta</i>	16S rRNA sequencing	- Bacil	White	Small sized colonies, smooth surface, circular fringe	(vii) CIP, A	Unknown	

Table 3.15: Identification and antibiotic resistance results of HPC bacteria isolated from the School borehole.

Habitat	Identification of Isolate	Method of ID	Gram reaction and shape	Colony colour	Appearance on agar	Antibiotic resistance Phenotype	ARB	Ref
School	1.1 <i>B. cereus</i>	16S rRNA sequencing	+ Bacil	Pink	Medium sized colonies, smooth surface, circular fringe	(i) KF, A	+	Pagnier <i>et al.</i> , 2008
	2 <i>Pseudomonas anguilliseptica</i>	16S rRNA sequencing	- Bacil	Light Yellow	Small colonies, smooth surface, circular fringe	(viii) KF, E, A	Unknown	
	3.3 <i>Flavobacterium cucumis</i>	16S rRNA sequencing	- Bacil	Yellow	Medium sized colonies, smooth surface, circular fringe	(i) KF, A	+	Pagnier <i>et al.</i> , 2008
	4.1 <i>Lysinibacillus sphaericus</i>	16S rRNA sequencing	+ Bacil	White	Big colonies, filamentous surface	(iv) S, KF, A	Unknown	
	5.1 <i>Bacillus</i> sp. NT4	16S rRNA sequencing	+ Bacil	Neon Yellow	Small colonies, smooth surface, circular fringe	(i)KF , A	Unknown	
	7.2 <i>Comamonas</i> sp	16S rRNA sequencing	- Bacil	Transparent	Very small colonies, smooth surface, circular fringe	(viii) KF, E, A	Unknown	
	8.2 <i>B. cerues</i>	16S rRNA sequencing	+ Bacil	White	Medium sized colonies, smooth surface, circular fringe	(i) KF, A	+	Pagnier <i>et al.</i> , 2008
	8.3 <i>Aeromonas hydrophila</i>	16S rRNA sequencing	- Bacil	White	Medium sized colonies, smooth surface, circular fringe	(iv) S, KF, A	Unknown	
	9.1 <i>Flavobacterium cucumis</i>	16S rRNA sequencing	- Bacil	Opaque Yellow	Small colonies, smooth surface, circular fringe	(i) KF, A	+	Pagnier <i>et al.</i> , 2008
	10.1 <i>Chitinophaga</i> sp.	16S rRNA sequencing	- Bacil	Translucent Yellow	Medium sized colonies, smooth surface, circular fringe	(v)S, C, KF, E, A	Unknown	

3.4.2 Harts River Catchment

In Table 3.16 – 3.17, the identification and antimicrobial resistance results are given for all of the boreholes sampled in the Harts River catchment area. Seven different genera were identified. The most abundant include *Comamonas* and *Acinetobacter*. The least abundant species are *Lysinibacillus* and *Aeromonas*, both with only one isolate. Warrenton had six different species and Ganspan four. *Comamonas*, *Acinetobacter* and *Pseudomonas* occurred in both boreholes and *Lysinibacillus* and *Aeromonas* in only one of the two boreholes.

Isolates displaying resistance to five antibiotics include an uncultured *Acinetobacter* (Ganspan) and *Pseudomonas plecoglossicida* from Warrenton and Ganspan. Isolates that were the least resistant to antibiotics include an uncultured *Acinetobacter* (from both boreholes), *Acinetobacter johnsonii* (Warrenton) and *Lysinibacillus sphaericus* (Warrenton). These were resistant to only two different antibiotics that they were tested against. *Comamonas* (iv), *Lysinibacillus* (ii), *Acinetobacter* (i), *Aeromonas* (iv) and *Pseudomonas* (v) isolated from both boreholes had the same antimicrobial resistance phenotype respectively, except for an uncultured *Acinetobacter* (xiii) isolated from the Ganspan borehole.

Table 3.16: Identification and antimicrobial resistance results of HPC bacteria isolated from the Warrenton borehole.

Habitat	Identity of Isolate	Method of ID	Gram reaction and shape	Colony colour	Appearance on agar	Antibiotic Resistance phenotype	ARB
Warrenton	2.1 <i>Comamonas testosteroni</i>	16S rRNA sequencing	- Bacil	White	Medium sized colonies, smooth surface, circular fringe	(iv) S, KF, A	Unknown
	2.3 <i>Lysinibacillus sphaericus</i>	16S rRNA sequencing	+ Bacil	White	Big colonies, filamentous surface, irregular fringe	(ii) S, A	Unknown
	2.6 <i>Acinetobacter</i> sp.	16S rRNA sequencing	- Bacil	White	Small colonies, smooth surface and circular fringe	(i) KF, A	Unknown
	3.1 <i>Comamonas testosteroni</i>	16S rRNA sequencing	- Bacil	Transparent	Very big colonies, smooth surface, somewhat irregular fringe	(iv) S, KF, A	Unknown
	4.1 <i>Uncultured bacterium clone 31</i>	16S rRNA sequencing	+ Bacil	Orange	Medium sized colonies, smooth surface	(iv) S, KF, A	Unknown
	4.3 <i>Aeromonas hydrophila</i>	16S rRNA sequencing	- Bacil	Orange	Medium sized colonies, smooth surface	(iv) S, KF, A	Unknown
	5.1 <i>Comamonas testosteroni</i>	16S rRNA sequencing	- Bacil	Translucent	Medium sized colonies, translucent centre, transparent irregular ,fringe,	(iv) S, KF, A	Unknown
	6.1 <i>Pseudomonas plecoglossicida</i>	16S rRNA sequencing	- Bacil	Beige	Small colonies, irregular fringe	(v) S, C, KF, E, A	Unknown
	7.2 <i>Acinetobacter johnsonii</i>	16S rRNA sequencing	- Bacil	Beige	Darker centre moving towards irregular fringe	(i) KF, A	Unknown

Table 3.17: Identification and antimicrobial resistance results of HPC bacteria isolated from the Ganspan.

Habitat	Identification of Isolate	Method of ID	Gram reaction and shape	Colony colour	Appearance on agar	Antibiotic resistance Phenotype	ARB
Ganspan	1.1 <i>Uncultured bacterium clone</i>	16S rRNA sequencing	+ Bacil	Translucent	Darker centre, transparent fringe, smooth surface, circular fringe	(i) KF, A	Unknown
	2.1 <i>Pseudomonas plecoglossicida</i>	16S rRNA sequencing	- Bacil	Cream	Medium sized colonies, radially striated, lobed surface	(v) S, C, KF, E, A	Unknown
	3.1 <i>Uncultured Comamonas</i>	16S rRNA sequencing	- Bacil	Yellow	Small colonies, smooth surface, circular fringe	(iv) S, KF, A	Unknown
	4.1 <i>Uncultured Acinetobacter</i>	16S rRNA sequencing	- Bacil	Whitish yellow	Small colonies, smooth surface, circular fringe	(i) KF, A	Unknown
	5.1 <i>Comamonas testosteroni</i>	16S rRNA sequencing	- Bacil	White	Medium sized colonies, smooth surface, circular fringe	(iv) S, KF, A	Unknown
	5.2 <i>Uncultured Acinetobacter</i>	16S rRNA sequencing	- Bacil	Translucent white	Small colonies, smooth surface, circular fringe	(xiii) C, CIP, KF, E, A	Unknown

3.5 Amoeba resistant bacteria identification and antibiotic resistance results

In Table 3.18 – 3.20, the identification and antimicrobial resistance results are given for all the amoeba resistant bacteria isolated. Five different bacterial genera were isolated with *Pseudomonas* (7) being the most abundant followed by *Ochrobactrum* with three isolates. The least abundant were *Alcaligenes* and *Achromobacter* with only two isolates. These isolates were all isolated from amoebas and are thus resistant to amoebas. Six different bacterial species were isolated and identified from the School borehole. Only two different species were identified from the Cemetery borehole. *Pseudomonas aeruginosa* was identified in the School and Pad dam boreholes

All bacteria identified had resistance to three or more antibiotics with the exception of *Achromobacter piechaudii* (School) with resistance to only one antibiotic. *Pseudomonas* and *Ochrobactrum* (identified) were resistant to most of the antibiotics (five) tested against, except for *Pseudomonas* sp. (Pad dam), all pseudomonads from Cemetery amoeba and *P. nitroreductans* from the School amoeba. *Achromobacter* from the School amoeba was resistant to only one antibiotic. Eight of the isolates were resistant to antibiotics. Again, the antibiotic to which most bacterial individuals were resistant to is Cephalothin and Amoxicillin. Erythromycin and Chloramphenicol resistance was also common.

Table 3.18: Identification and antibiotic resistance results of Amoeba resistant bacteria isolated from the Pad dam borehole

Habitat	Identity of isolate	Method of ID	Gram reaction and shape	Colony colour	Appearance on agar	Antibiotic resistance phenotype	ARB	Ref
Pad dam	<i>Pseudomonas aeruginosa</i>	16S rRNA sequencing	- Bacil	Light yellow	Medium sized, finely granular, round fringe	(v) S, C, KF, E, A	+	Pagnier <i>et al.</i> , 2008
	<i>Alcaligenes faecalis</i>	16S rRNA sequencing	- Bacil	Translucent cream	Small colonies, granular surface, round fringe	(ix) S, C, KF, A	+	Barker and Brown 1994
	Uncultured bacterium clone	16S rRNA sequencing	- Bacil	Cream yellow	Medium colonies, granular, round fringe	(x) C, KF, E, A	Unknown	
	<i>Pseudomonas</i> sp.	16S rRNA sequencing	- Bacil	Translucent	Small colonies, smooth, round fringe	(x) C, KF, E, A	+	Pagnier <i>et al.</i> , 2008

Table 3.19: Identification and antibiotic resistance results of Amoeba resistant bacteria isolated from the Cemetery borehole

Habitat	Identity of isolate	Method of ID	Gram reaction and shape	Colony colour	Appearance on agar	Antibiotic resistance phenotype	ARB	Ref
Cemetery	<i>P. putida</i>	16S rRNA sequencing	- Bacil	Translucent green	Small colonies, smooth surface, round fringe	(x) C, KF, E, A	+	Pagnier <i>et al.</i> , 2008
	<i>Ochrobactrum</i>	16S rRNA sequencing	- Bacil	Translucent white	Medium colonies, smooth surface, round fringe	(ix) S, C, KF, A	Unknown	
	<i>P. plecoglossicida</i>	16S rRNA sequencing	- Bacil	Translucent green	Small colonies, smooth surface, round fringe	(x) C, KF, E, A	Unknown	

Table 3.20: Identification and antibiotic resistance results of Amoeba resistant bacteria isolated from the School (Sk) borehole

Habitat	Identity of isolate	Method of ID	Gram reaction and shape	Colony colour	Appearance on agar	Antibiotic resistance phenotype	ARB
School-SK	<i>Ochrobactrum</i>	16S rRNA sequencing	- Bacil	Light white	Medium colonies, smooth surface, round fringe,	(v) S, C, KF, E, A	Unknown
	<i>Ochrobactrum</i>	16S rRNA sequencing	- Bacil	Light white	Medium colonies, smooth surface, round fringe	(x) S, C, KF, A	Unknown
	Uncultured bacterium clone	16S rRNA sequencing	- Bacil	Translucent	Medium colonies, granular surface, round fringe	(x) S, C, KF, A	Unknown

Table 3.21: Identification and antibiotic resistance results of Amoeba resistant bacteria isolated from the School borehole

Habitat	Identity of isolate	Method of ID	Gram reaction and shape	Colony colour	Appearance on agar	Antibiotic resistance phenotype	ARB	Ref
School	<i>P. fluorescens</i>	16S rRNA sequencing	- Bacil	White	Small colonies, smooth surface, round fringe	(v) S, C, KF, E, A	+	Pagnier <i>et al.</i> , 2008
	<i>Achromobacter piechaudii</i>	16S rRNA sequencing	- Bacil	Translucent white	Small colonies, smooth surface, round fringe	(xi) E	Unknown	
	<i>Alcaligenes faecalis</i>	16S rRNA sequencing	- Bacil	Cream	Small colonies, smooth surface, round fringe	(xii) S, C, A	+	Barker and Brown 1994
	<i>P. nitroreductans</i>	16S rRNA sequencing	- Bacil	Cream	Small colonies, smooth surface, round fringe	(x) C, KF, E, A	Unknown	
	<i>P. aeruginosa</i>	16S rRNA sequencing	- Bacil	Light green	Big colonies, granular surface, oval fringe	(v) S, C, KF, E, A	+	Pagnier <i>et al.</i> , 2008,
	<i>Achromobacter</i> sp.	16S rRNA sequencing	- Bacil	White	Small colonies, smooth surface, round fringe	(x) C, KF, E, A	Unknown	

ARBs are Gram negative bacteria, while the HPC bacteria isolated directly from the bulk water were either Gram negative or positive. The ARBs displayed resistance to a greater number of antibiotics compared to the bulk water isolated HPC. The ARBs from the Pad dam borehole were all resistant Streptomycin, Cephalothin and Amoxicillin. The Gram negative HPCs from the bulk water were also all resistant to Streptomycin and Amoxicillin. Two of these were also resistant to Cephalothin.

The ARBs from the Cemetery borehole all had the same antibiotic resistance pattern. These have not previously been reported as ARBs. Two of the *Pseudomonas* sp. and the *Comamonas* sp. from bulk water had the same antibiotic resistance phenotype (S, C, KF, E, A) as the ARBs from the borehole. The only bacterium isolated from both bulk water and amoeba was *P. plecoglossicida* from the Cemetery borehole. Both these isolates were resistant to Chloramphenicol, Cephalothin, Erythromycin and Amoxicillin.

Amongst the bacteria that were isolated and identified from the amoebas, 5 belonging to 4 different Gram negative genera had the same antibiotic resistance profile (S, C, KF, E, A). This pattern was also observed in one of the species from the bulk water (*Chitinophaga* sp.). Two of the ARB isolates (*P. nitroreductans* and *Achromobacter* sp.) were resistant to all the antibiotics mentioned above, except Streptomycin. The other isolates from the bulk water were all resistant to Cephalothin and Amoxicillin and in two cases to either Streptomycin or Erythromycin.

There was thus considerable similarity and differences between the isolates from various boreholes. These similarities were observed for isolates from the same boreholes and different compartments, the bulk water on the one and amoebas on the other.

Many of the amoeba resistant bacteria were previously reported. A number of the species identified in this study is reported to be ARBs for the first time. Furthermore, no reports could be found for the presence of amoeba resistant bacteria in borehole water in the North West Province. This is cause for concern since the amoeba resistant genotype is associated with pathogenicity and virulence.

3.6 Redundancy analysis

A redundancy analysis (RDA) was performed to determine if there were any correlations between the environmental and the microbiological parameters, as well as the sampling sites. The results of the multi-variate analysis are displayed as a triplot (Figure 3.2). Angles between the vectors are used to determine the correlation between the environmental and microbial parameters and the sampling sites.

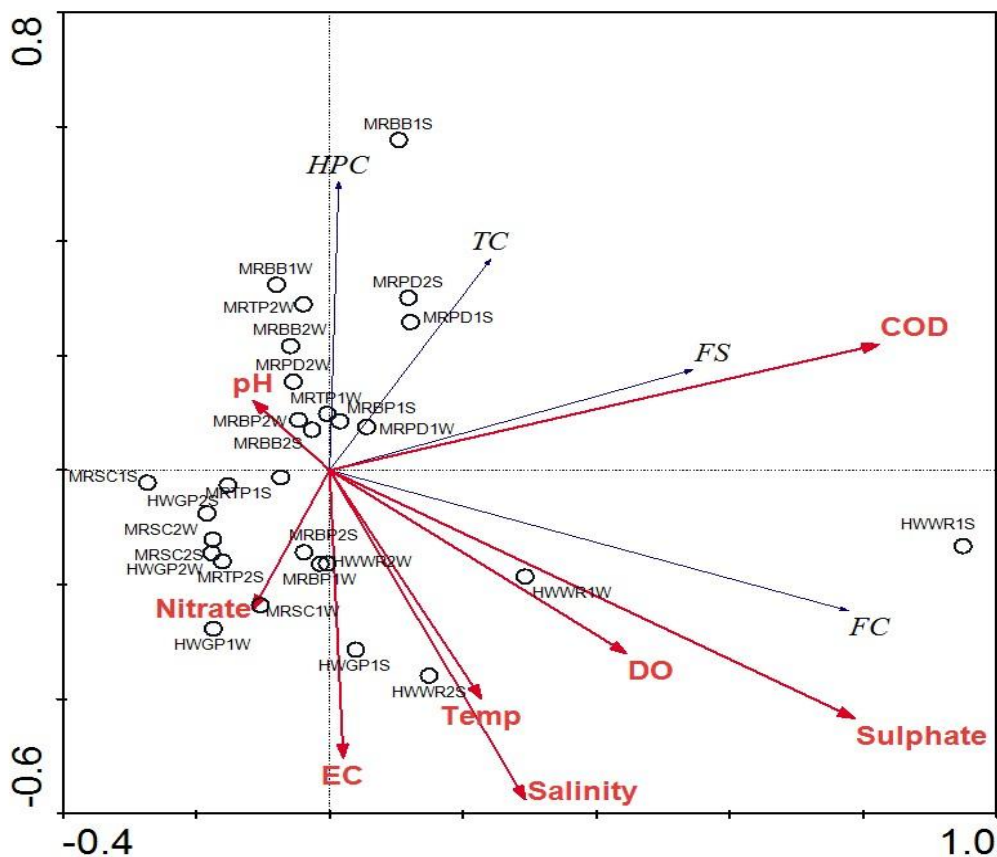


Figure 3.2: RDA ordination triplot of environmental parameters (red vectors) and biological data (red vectors) for the various boreholes sampled. Eigenvalues for the first two axes were 0.24 and 0.12 respectively ($p=0.0485$).

From Figure 3.2, it can be observed that there is a strong correlation between faecal streptococci (FS) and chemical oxygen demand (COD). Total coliforms (TC) also correlated with COD as well as (to a lesser degree) FS. Chemical oxygen demand had a greater effect on FS and TC than any of the other environmental parameters. Total coliforms and HPC bacteria also correlated with each other. Faecal coliform levels correlated with sulphate and dissolved

oxygen. HPC correlated with pH and nitrates do not have any impact on any of the microbial levels measured.

This analysis demonstrates that some of the physico-chemical parameters had impacts on the bacterial levels observed in the boreholes. The relationships demonstrated here could be further explored.

3.7 Summary of results

The results presented here indicate that the aquifers in the Mooi and Harts River catchments are chemically as well as microbiologically polluted. Chemical oxygen demand correlated the most with numbers of indicator organisms enumerated with varying degrees between the different groups. Nitrate and EC did not correlate to any of the bacterial indicators.

Temperature and pH levels in all of the boreholes over both sampling seasons were within the TWQR for drinking water. The Blaauwbank borehole had the best physico-chemical quality water during both sampling seasons while the Trimpark and School boreholes had the worst quality during both sampling seasons. These two boreholes had constantly high levels of pollution over both seasons, indicative of constant pollution input.

Microbial indicator organisms were identified in all of the boreholes in both seasons and did not comply with the TWQR for drinking water. Levels of indicator organisms generally declined during the cold and dry sampling season. During both seasons, the Trimpark and Warrenton boreholes had the worst microbiological water quality.

Varying levels of antibiotic resistance were observed for isolated *E. coli* from both catchments. *Escherichia coli* from the Mooi River catchment shared the same antibiotic resistance profile, as well as *E. coli* from the Harts River catchment. Several different genera of HPC were isolated and identified. Generally, the same antibiotic resistance profiles were observed for bacteria belonging to the same genus. Resistance was observed to specific antibiotics by all bacteria originating from the same borehole. The same scenario was identified with ARBs. Comparing antibiotic resistance profiles of the HPC bacteria with those of the ARB, similarities were identified between bacteria from the same borehole.

CHAPTER 4 – DISCUSSION

Seven boreholes were sampled during this study. Five boreholes were situated in the Mooi River catchment area and two in the Harts River catchment area. All boreholes were sampled two times during a wet season and two times during a dry season in 2012, with a period of three weeks between each sampling session for every season. Set physico-chemical parameters were measured and microbiological parameters analysed. Physico-chemical results will be discussed first followed by microbial results.

4.1 Physico-chemical data

The EC, salinity and nitrate concentrations of all the boreholes in both catchments increased from the warm to the cold season. Exceptions are the nitrate concentrations of the Blaauwbank (2mg/l – 0mg/l) and Ganspan boreholes (3.85 mg/l – 0 mg/l), EC of the Pad dam borehole (65.3 mS/m – 34 mS/m), salinity of Blaauwbank (194 ppm – 188 ppm) and Cemetery (130.5 ppm -129.5 ppm) boreholes and nitrate of the Blaauwbank borehole (2 mg/l – 0 mg/l) which decreased. Temperature and pH remained relatively constant between the seasons. Sulphate and COD concentrations decreased for all of the boreholes, except for the sulphate concentration of the Trimpark borehole (63.5 mg/l – 81 mg/l). For boreholes in the Mooi River catchment, the DO concentration increased from the warm to the cold season where the DO concentration for the Harts River catchment boreholes decreased.

Rozemeijer *et al.* (2009) conducted a study in the Netherlands on groundwater quality to compare groundwater quality and meteorological events. The authors (Rozemeijer *et al.*, 2009) concluded that precipitation had an effect on the concentration of chemical constituents in groundwater. Concentrations increased due to the diluting effect of infiltrating water that is lost during periods with less or no precipitation. The predominant rainfall season for the North West province is during the summer months (NWPG, 2002). Concentrations of EC, salinity and nitrate increased during the cold and dry sampling season. Less recharge of groundwater in the province may explain the increase in EC, salinity and nitrate levels during winter months. The EC, salinity and sulphate levels in the Trimpark, School, Warrenton and Ganspan remained high over the two sampling seasons. This is indicative of a constant source of pollution on these boreholes.

A study done by Harold and Bailey (1996) on the long term salt balance of the Vaalharts irrigation scheme concluded that fertilisers contribute to salinisation of groundwater sources. A large phospho gypsym heap is situated in the area of Ikageng (informal settlement) in Potchefstroom. This was a waste heap of fertiliser byproducts for a chemical fertiliser manufacturing company in Potchefstroom. The School borehole is situated roughly two kilometers away down gradient from the waste heap. The area between the School borehole and the phospho gypsym heap is underlain by dolomites. Due to the inherent qualities of dolomites (high transmissivity, rapid recharge), these types of aquifers are more vulnerable to contamination (DWAF, 2006). As the phospho gypsym heap was a disposal area for fertilizer byproducts, the leaching of specific ions into the soil may be the reason for the high EC and salinity concentrations measured in this borehole. The Ganspan borehole is situated in the Vaalharts irrigation scheme next to a field where a variety of crops are grown throughout the year. Leaching of excess fertilisers may explain the high EC and salinity values for this borehole.

According to DWAF (2006), the Boskop dam is the receiving water body for most of the mining influenced water that drains from the West and far West Rand. These waters have high concentrations of salts and sulphates. The Mooi River, which flows through the town of Potchefstroom, is fed by water from the Boskop dam. Comparing sulphate and salt concentrations of this study with results obtained through personal communication with K. Jordaan on the Mooi river (physico-chemical and microbiological quality), a clear correlation can be seen between the results. The sulphate (81 mg/l-borehole and 86 mg/l-surface water) and electrical conductivity (70.1 mS/m-borehole and 68.2 mS/m-surface water) concentrations are near similar. The Trimpark borehole is situated roughly 20 meters away from the banks of the Mooi River. In dolomitic terrain, groundwater and surface water displays similar characteristics, which is indicative of a close relationship between these two water bodies (DWAF, 2006). As the Trimpark borehole is situated so close to the river, it can be hypothesised that the Mooi River does have an influence on the water quality of the Trimpark borehole and therefore explain the high EC and sulphate levels throughout the two seasons.

Borehole construction sets the Warrenton borehole apart from the Trimpark and School boreholes. Where there is a cement collar around the Trimpark and School borehole, there is none around the Warrenton borehole. According to Howard *et al.* (2003), poor sanitary

completion of boreholes is one of the many contributing factors to pollution of groundwater. As there is no collar around the borehole, urban run-off contaminated with pollutants can easily drain into the ground by means of the spaces between the casing of the borehole and the soil (Howard *et al.*, 2003).

High EC and nitrate values for groundwater were recorded in other studies (Mpenyana-Monyatsi and Momba, 2012; Ferreira, 2011). Values and occurrence in groundwater were greater in these studies, but may be attributed to the fact that more samples were collected from a wider selection of boreholes across the whole North West province.

4.2 Microbial results

4.2.1 Compliance to target water quality ranges

During the warm and wet season, the water quality in all boreholes was very poor. None of the boreholes' indicator organism counts were within the TWQR for domestic use, except for faecal coliform counts from the Blaauwbank and Pad dam boreholes. The water from all of the boreholes may however be used for livestock watering purposes, except for Warrenton and Ganspan. Only the water from Blaauwbank and Pad dam boreholes may be used for irrigational purposes, considering faecal coliform counts.

Microbial water quality did improve to some degree during the cold and dry sampling season for both catchments. None of the water from all of the boreholes may, however, be used for drinking. Blaauwbank and Pad dam boreholes had the best microbiological water quality. During the cold and dry sampling season, faecal coliforms were only enumerated from the Trimpark and Warrenton boreholes. Water from all of the boreholes may be used for livestock and irrigational purposes, except for water from the Trimpark borehole, which may only be used for livestock watering.

4.2.2 Trends identified

In the warm season, five boreholes (Trim, School, Cemetery, Warrenton and Ganspan) tested positive for faecal coliform bacteria. The maximum was detected at the Warrenton borehole (3.84×10^3 cfu/100 ml). Total coliforms, HPC and faecal streptococci were present in all of the water samples for both catchments, except for the Blaauwbank borehole, where no faecal streptococci were enumerated.

During the cold and dry sampling season, only the Trimpark (11 cfu/100 ml) and the Warrenton (1 cfu/100 ml) boreholes tested positive for faecal coliforms. Total coliforms were also enumerated from all of the boreholes in both catchments, except for the Blaauwbank borehole. Heterotrophic plate count bacteria were present in all of the boreholes and faecal streptococci in only six out of the possible seven boreholes. No faecal streptococci were enumerated from the Blaauwbank borehole.

In boreholes of both the Mooi River and Harts River catchments, faecal and total coliforms, faecal streptococci as well as HPC levels were lower in the cold and dry season compared to the warm season. Exceptions were total coliforms from the Trimpark, Cemetery and Warrenton boreholes, HPC from the Blaauwbank borehole and faecal streptococci counts for the Trimpark and Warrenton boreholes. A two log increase was observed from the HPC of the Blaauwbank borehole. Heterotrophic plate count bacteria levels increased from 1.73×10^5 cfu/ml to 1×10^7 cfu/ml.

From the results, it is indicative that groundwater sources in the North West Province are potentially contaminated by faecal matter. These results are in accordance with a study done by Mpenyana-Monyatsi & Momba (2012). The authors sampled 100 boreholes across the province between September and November in 2008. Water samples were taken from rotary hand pumps or a standpipe connected to a groundwater source. Total and faecal coliforms were enumerated from all of the sampled water, indicating possible faecal pollution of groundwater sources.

Ferreira (2011) also conducted a study on the microbiological quality of groundwater in the North West province. The author sampled an array of boreholes over a two year period (2009 and 2010). Standard cultivation and molecular techniques were used to identify specific indicator organisms. The author enumerated faecal and total coliforms as well as faecal streptococci and HPC from most of the sampled water. In some instances, pathogenetic *P. aeruginosa* and *S. aureus* were identified as well.

It is not groundwater of the North West Province only in which faecal coliforms were detected in the recent past. Mwabi *et al.* (2012) stated that borehole water sources in the Gauteng province, South Africa, tested positive for *E. coli*, indicating possible faecal

pollution. In the Eastern and Western Cape provinces, Mackintosh and Colvin (2003) found that water samples contained HPC bacteria, total and faecal coliforms levels that did not comply with TWQR for domestic use.

Momba *et al.* (2006) conducted a study in the Nkonkobe district in the Eastern Cape province from November 2001 to March 2002. Molecular techniques were used to test for *E. coli* and toxigenic *V. cholerae*. The authors found that *E. coli* was present in 75% of the boreholes and *V. cholera* in 25%.

The occurrence of faecally polluted groundwater sources is not limited to South Africa alone. Dzwauro *et al.* (2006) analysed water from wells for total and faecal coliforms in the Kamangita village in Zimbabwe between February and May in 2005. Twenty two percent of the wells analysed exceeded the South African TWQR for domestic use (DWAF, 1996a). Wright (1986) analysed water from four springs and wells, as well as a river and a stream in Sierra Leone. Sampling occurred between August 1979 and July 1980. Faecal coliforms and faecal streptococci were identified in all of the well and spring water samples. Gèlinas *et al.* (1996) studied water from fountains, modernised as well as traditional wells in the Conakry district of the Republic of Guinea. Faecal coliforms and faecal streptococci were enumerated from 100% of the water samples from the modernised and traditional wells. A study was also performed on 42 private wells in the Bio-Bio region in Chile over a 10 month period (Valenzuela *et al.*, 2009). Total and faecal coliforms and faecal streptococci were enumerated from the water samples.

As water moves through the unsaturated zone, natural processes remove harmful chemical and organic compounds (Fourie & van Ryneveld, 1995). For this reason, it is perceived that groundwater is naturally of good drinking quality (Mackintosh & Colvin, 2003). Many rural communities of South Africa are therefore supplied with untreated groundwater for domestic use (Mwabi *et al.*, 2012; Lehloesa & Muyima, 2000; Mackintosh & Colvin, 2003). The results presented in this study, and similar other studies, provide evidence that groundwater can become contaminated (chemically or microbiologically). As the useable surface water of the country is becoming limited, groundwater would be more relied on to provide water for domestic use. For this reason, groundwater resources need to be monitored so that it can be protected.

From the results, it is observed that the microbial water quality is worst during the warm and wet season. Annual rainfall for the province accounts to approximately 540 mm, with the most precipitation occurring in the summer months (DWA, 2009). Howard *et al.* (2003) conducted a study on the risk factors contributing to microbial contamination of water from wells in Kampala city, Uganda. A sanitary inspection was completed which included identifying solid waste dumps and scattered waste (faecal origin) around each well. The authors concluded that faecal matter of wild (rodents, birds etc.) and domesticated animals (goats, chickens, dogs etc.) were some of the main sources of faecal pollution of shallow groundwater sources after precipitation events (urban runoff). Other studies also displayed strong correlations between precipitation events and faecal contamination of ground water sources (Kravitz, *et al.*, 1999; Howard, *et al.*, 2003; Douagui, *et al.*, 2012; An & Breidenbach, 2005; Lawrence *et al.*, 2001)

The depth to groundwater level was the least at the Warrenton and Trimpark (~3 m) boreholes and the deepest at the Blaauwbank (~52 m) borehole. The microbial quality of the Warrenton and Trimpark boreholes was generally poorer compared to the Blaauwbank quality. Krapac and his colleagues (2002) conducted a study on the possible impacts of swine manure pits on groundwater quality. Microbial composition of raw swine manure was analysed and compared to indicator organisms enumerated from groundwater samples collected at different depths. Shallow groundwater contained more faecal indicator organisms than deeper groundwater samples (Krapac *et al.*, 2002). Geldreich (1996) also stated that shallow wells are more frequently highly polluted and Wakida and Lerner (2005) concluded that depth to the water table can prevent contamination. Thus, previous studies have made similar observations as was made in the present study with respect to the greater chance of pollution of shallow boreholes, compared to deeper ones.

When boreholes are drilled, a casing should be installed. A sanitary seal should be installed in the upper part of the casing and a concrete collar placed around the casing (Geldreich, 1996; Howard *et al.*, 2003). This is to prevent polluted surface runoff water from seeping directly into the borehole (DWA, 2003). The Warrenton borehole had no concrete collar around the casing and dog excrement was observed around the casing of the borehole during each sampling session. A concrete collar was present around the Trimpark borehole casing, but it was eroded at the fringes. This allows for polluted water to collect in close proximity to the borehole casing and probable seeping of polluted water directly into the borehole.

In comparison to the Trimpark and Warrenton boreholes, the Blaauwbank borehole had a larger concrete collar around the casing, as well as a cement column which extends from the collar to about a height of 50 cm above the surface. Although cattle faeces was scattered around the borehole at every sampling session, no faecal coliforms or streptococci were enumerated from this borehole, indicating the importance of such collars. Preferential flow paths are present in the subsurface (section 1.3.5.2). Deeper boreholes can therefore be vulnerable to contamination under certain conditions and it should not be accepted that deeper groundwater sources are always of good quality.

From Tables 4.5 – 4.8, it can be observed that on average, more faecal streptococci were enumerated from sample water than faecal coliforms. Exceptions are the Cemetery borehole in the warm season (FC – 175; FS – 92) and the Ganspan borehole (FC – 1.9×10^3 ; FS – 1.5×10^3) in the same season. It was suggested that there are more faecal streptococci present in faecal matter from warm blooded animals than faecal coliforms (Geldreich, 1996). Faecal streptococci also have a higher persistence in non-enteric environments (Geldreich, 1996; Howard *et al.*, 2003). Faecal coliforms do, however, possess the ability to multiply in the environment under favorable environmental conditions (Howard *et al.*, 2003), and therefore may account for the results observed in the cemetery and Ganspan boreholes.

4.3 Antibiotic resistance of isolated *E. coli*

All the *E. coli* isolates were resistant to Cephalothin, Erythromycin and Amoxicillin and susceptible to Ciprofloxacin. These results are in accordance to another study, where antibiotic resistance testing formed part of the battery of tests on groundwater samples from the North West province, South Africa (Ferreira, 2011). Faecal coliforms isolated by the author had varying percentages of resistance to Amoxicillin and Cephalothin and susceptibility to Ciprofloxacin and Chloramphenicol. In the present study, *E. coli* isolates from the Harts River catchment were resistant to Chloramphenicol. *Escherichia coli* isolated from the Mooi River catchment (Trimpark, Cemetery, School) shared the same antibiotic resistance phenotype while those from the Harts River catchment also shared a similar phenotype.

Antibiotics are used sub-therapeutically as growth promoters and as prophylaxis in animal husbandry environments (Bester & Essack, 2008; Alanis, 2006). Application regimes administered to animals and antibiotics prescribed to patients can vary between regions

(Thompson, 1999). Exposure of bacteria to antibiotics exerts selective pressure which leads to bacteria acquiring resistance to those antibiotics (Donskey, 2006; Alanis, 2006; Bax *et al.*, 2000). This may explain the different antibiotic resistance patterns observed for the *E. coli* isolates from the two catchments.

4.4 Heterotrophic plate count (HPC) bacteria

Heterotrophic plate count bacteria are an indicator of the general microbial water quality and should not exceed 1000 cfu/ml for water used for domestic purposes (DWAf, 1996a). In the present study, HPC bacteria were isolated from all of the boreholes during both sampling seasons. The highest count was obtained from the Blaauwbank borehole (1×10^8 cfu/ml – summer) and the lowest from the Ganspan borehole (2.8×10^4 cfu/ml – winter). None of the water samples complied with the TWQR for domestic use in South Africa (DWAf, 1996a). Behardien and colleagues (2011) conducted a study on a spring water distribution system in the Western Cape Province, South Africa. Water samples were taken from different points in the system over a period of 9 months in 2004. HPC counts enumerated from water samples taken at a borehole were 1.34×10^8 cfu/ml for week one, 4.50×10^6 cfu/ml for week four and 2.7×10^8 cfu/ml for week eight. These results are in accordance with the results obtained in this study.

Heterotrophic plate count bacteria were grown on R2A agar and incubated at $\sim 25^\circ\text{C}$ for 5 days. After incubation, colonies were selected based on colony morphology and identified as described in section 2.8. These were further identified using Gram staining and 16S rRNA gene fragments. The most abundant genus identified was *Pseudomonas*. Others included *Flavobacterium*, *Acinetobacter*, *Alcaligenes* and *Aeromonas*. Individual representatives from the genera *Fermicutes*, *Delftia*, *Massilia*, *Siphonobacter*, *Dechlorosoma*, *Brevundimonas*, *Lysinibacillus* and *Chitinophaga* were also isolated and identified.

Opportunistic pathogens that were enumerated as HPC bacteria in previous studies include *Pseudomonas aeruginosa*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Acinetobacter*, *Flavobacterium*, *Bacillus cerues* and *Mycobacterium* (Thompson, 1999; Donskey, 2006; Stelma *et al.*, 2004; Payment *et al.*, 1991; Ford, 1999; Quiroz, 1999). All of these species have been identified as HPC bacteria in the present study.

Controversy exists in the literature over the importance of HPC bacteria as a microbial water quality indicator of human health significance (Thompson, 1999; Donskey, 2006; Stelma *et*

al., 2004; Payment *et al.*, 1991; Ford, 1999; Quiroz, 1999). Although certain bacterial species are opportunistic pathogens, and form part of the HPC bacteria, clinical evidence of these species causing gastro intestinal tract infections are lacking (Edberg *et al.*, 1997; Edberg & Allen, 2004; Allen *et al.*, 2004). The listed studies state that not enough evidence is present to decisively conclude if ingestion of water with opportunistic HPC bacteria will lead to gastro intestinal disease.

These opportunistic pathogens also proliferate when they are deposited onto food sources (Allen *et al.*, 2004). Ingestion of such contaminated food would then most probably cause gastroenteritis. This may constitute other infection pathways that indicate the importance of these opportunistic pathogen HPC bacteria. Thus, not only the direct ingestion of these bacteria is of concern, also the preparation of food using this water may be of concern.

Opportunistic pathogens are of a greater threat to the elderly, the very young, and people that are immuno-compromised (Ford, 1999; Quiroz, 1999; Paulse *et al.*, 2009). All of the studies by the authors were conducted on healthy population groups, which may explain the lack of evidence. Sixty five percent of the total population of the North West province resides in rural areas (NWPG, 2002). As stated before, many rural communities are reliant on groundwater sources for drinking water. HIV/AIDS has been identified as the cause of death of 30% of fatalities in the province (Bradshaw *et al.*, 2004). This is indicative of a high incidence of HIV infection. According to the State of the Environment report (NWPG, 2002), the largest age group in the province is younger than five years old. Supplying these rural communities with water containing high levels of HPC bacteria may lead to detrimental health effects because of the increased risk of infection by HPC opportunistic pathogens.

Antibiotic resistance of the HPC bacteria varied, but similarities in resistance to some of the antibiotics were observed. This also applied for the amoeba resistant bacteria. Resistance to Cephalothin and Amoxicillin was observed amongst most of the isolates, as well as resistance to Erythromycin and Streptomycin, to a lower degree.

Knapp and his colleagues (2010) conducted a study on antibiotic resistance conferring genes of soil bacteria. Soil samples were collected from 1970 to 2008. Extracted DNA was screened for genes that confer antibiotic resistance. The authors (Knapp *et al.*, 2010) then concluded that a general increase of antibiotic resistant genes occurred over time in accordance to land use practices and anthropogenic activities. It can be concluded that the micro-organisms were exposed to different chemicals over a period of 38 years, which applied selective pressure to

develop resistance against these chemicals. This would explain why the different HPC species identified in the present study had similar resistance to the same antibiotics. These bacteria from the same borehole potentially had the same chemical exposure history. The same could apply for the amoeba resistant bacteria.

4.5 Amoeba resistant bacteria identification

Amoebae were isolated from the water collected from the boreholes in the Mooi River catchment by the National Institute of Occupational Health. Bacteria resistant to these amoebas were isolated. The bacterial cultures were identified as described in section 2.8.

In this case, *Pseudomonas* sp. was also the most abundant species identified. The other were identified as *Alcaligenes* sp. (2 isolates), *Ochrobactrum* (3 isolates) and *Achromobacter* (2 isolates). *Pseudomonas* sp., *Alcaligenes faecalis* and *Ochrobactrum* sp. are known ARB opportunistic pathogens.

Amoebae resistant bacteria have developed certain mechanisms for surviving the intracellular environment of amoeba. These mechanisms allow ARBs to survive macrophagic attacks by the immune system of humans (Cosson & Soldati, 2008; Pagnier *et al.*, 2008). Amoebae can form very resistant cysts if undesirable conditions preside in an environment (Greub & Raoult, 2004; Thomas *et al.*, 2006). The gastrointestinal tract of humans would be an example of such an environment. These cysts would protect ARB from the first immune defense mechanisms of the human body, increasing the chance of infection. Studies also stated that amoeba can contribute to antibiotic resistance of ARB (Greub & Raoult, 2004; Thomas *et al.*, 2006; Walchonik *et al.*, 1999). Horizontal gene transfer has been identified as processes that are favoured in the intracellular environment of amoebae (Pagnier *et al.*, 2008; Moliner *et al.*, 2010). Genes are selected and incorporated into the DNA of ARB that would help ARB to better survive the macrophagic lifestyle (Moliner *et al.*, 2010). All of these factors can contribute to the pathogenicity of ARB to humans, and it can therefore be concluded that ARB has the potential to become emerging opportunistic human pathogens.

ARB were resistant to more antibiotics than the HPC that were isolated from bulk water. For example, all of the ARB was resistant to Streptomycin, Chloramphenicol, Cephalothin and Amoxicillin, whereas all of the HPC bacteria from the same borehole were only resistant to Cephalothin and Amoxicillin.

Studies proposed that amoebae's internal environment may contribute to increased antibiotic resistance of ARB (Greub & Raoult, 2004; Thomas *et al.*, 2006; Walchonik *et al.*, 1999). Mechanisms of acquisition are not understood in detail, but it is proposed that horizontal gene transfer may play an integral role (Moliner *et al.*, 2010). Gene transfers can be between two different bacteria in the intracellular environment of the amoeba. It can also be between the genes of the amoeba and a bacterium. Free DNA of a digested amoeba susceptible bacterium can be incorporated into the DNA of ARB (Moliner *et al.*, 2010). Such genes would only be acquired and incorporated if it can provide a metabolic advantage, such as encoding for antibiotic resistance. Greub and Raoult (2004) also proposed that amoebae may have a decreased rate of uptake of antibiotics, thus allowing ARB to adapt to antibiotics. The authors (Greub and Raoult, 2004) also stated that amoebae may be able to inactivate antibiotics in their intracellular environment. This metabolic feature may then be obtained by ARB through horizontal gene transfer. All of these may explain why ARB isolated from amoebae in this study was resistant to more antibiotics than the HPC bacteria from bulk water of the same borehole.

4.6 Redundancy analyses

Chemical oxygen demand indirectly determines the amount of organic matter present in samples (Willey *et al.*, 2011). Organic carbon is utilised by bacteria as an energy source and to synthesise new cellular material (LeChevallier *et al.*, 1991; Willey *et al.*, 2011). Organic carbon can then be considered to be a limiting growth nutrient. LeChevallier *et al.* (1991) conducted a study on a water distribution system plant in New Jersey. The plant experienced bacterial regrowth in the distribution system. Physico-chemical parameters were compared to the microbial quality of water. The authors (LeChevallier *et al.*, 1991) concluded that an increase in the organic carbon concentration leads to an increase in the HPC and coliform bacteria levels. Strong correlations were observed between COD concentration and faecal streptococci, faecal coliforms and total coliform counts. A strong correlation between the COD concentration and indicator bacteria could mean that the COD concentration is influencing the growth of these bacteria the most.

A large variance can be observed between the two sampling sessions during a warm and wet season of the Warrenton borehole. This would imply that the borehole is greatly susceptible to environmental impacts. This statistical observation supports the hypothesis that shallow

groundwater is prone to pollution as suggested by Geldreich (1996), Krapac *et al.* (2002) and Wakida & Lerner (2005).

CHAPTER 5 – CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The aim of the study was to evaluate the physico-chemical and microbiological quality of water from aquifers in the North West province of South Africa. To reach this aim, six objectives were formulated in guidance to achieve this aim.

i) Collect water samples from DWA monitoring boreholes

Aquifer representative water samples were collected by means of the specialised truck. After liaising with the Department for permission, access to aquifers were obtained using Department of Water Affairs monitoring boreholes. Selected monitoring boreholes did not have any instruments in the casing, allowing for instruments to be lowered down into the casing of the boreholes.

ii) Analyse water samples on site for set physico-chemical parameters using appropriate instruments

Specialised instruments incorporated into the truck (Section 2.4 - Figure 2.4) allowed for the *in situ* measurement of physico-chemical parameters. High levels of electrical conductivity, sulphate and nitrates were observed in selected boreholes from both catchments. Water from these boreholes may not be used for drinking purposes. The Trimpark and School boreholes in the Mooi River and Warrenton borehole in the Harts River catchment had the worst physico-chemical water quality during both seasons.

iii) Analyse water samples for levels of faecal indicator organisms

Faecal indicator organisms were enumerated from all boreholes during both sampling seasons. Aquifers in the North West Province are therefore faecally contaminated and opportunistic pathogens may be transmitted to healthy individuals if water from these aquifers is consumed. Borehole construction techniques (sanitary completion) and physical borehole attributes (depth of borehole) have been identified that may impact on pollution to boreholes. Identities of potential *E. coli* isolates were confirmed using PCR amplification of *mdh* and *lacZ* genes. Antibiotic resistance patterns of these *E. coli* were determined and it was demonstrated that all were resistant to Cephalothin and Amoxicillin.

iv) Isolate and identify heterotrophic plate count bacteria

The levels of HPC in the bulk water were very high. Bacteria were isolated, purified and identified using 16S rRNA gene sequencing. Several different genera have been identified and *Pseudomonas* spp. was the most abundant. Identified bacteria also included several species that had previously been reported as opportunistic pathogens. Immuno-compromised, the elderly and very young individuals may be more prone to infections by these microorganisms if contaminated water is consumed. The HPC from the bulk water were all resistant to Cephalothin, Amoxicillin and to Erythromycin and Streptomycin to a lower degree.

v) Identify amoeba resistant bacteria

Several different genera of ARBs were identified. These were resistant to several antibiotics including Cephalothin and Amoxicillin, and Erythromycin and Chloramphenicol to a lesser extent. Some of the bacterial species were previously reported as ARBs and others were identified in this study for the first time. *Pseudomonas plecoglossicida* was the only species identified in both the bulk water and as an ARB. Inherent factors of ABRs contribute to their pathogenicity in multicellular organisms and should therefore be considered as an indicator of water quality.

vi) Comment on the suitability of the water for specific applications

Based on physico-chemical and microbiological parameters, not one of the boreholes water can be used for consumption for humans as drinking water. However, water from these boreholes may be used for irrigation or livestock watering in certain circumstances if water quality complies with the TWQR for these uses.

5.2 Recommendations

i) Water treatment

Results from this study indicate that groundwater of the North West province is vulnerable to physico-chemical as well as microbiological pollution. Therefore groundwater may not always be of pristine quality as is perceived. For this reason, it is recommended that groundwater for human consumption are treated in the same manner as surface water sources before distribution to users.

ii) Borehole installation regulations

Poor sanitary completion of boreholes may lead to contamination of groundwater. Implementation of standardised regulations ensuring good sanitary installation of boreholes can prevent this from happening.

iii) Education

Infrastructure and implementation takes time and funds may not always be available. Communities supplied with groundwater should be educated of the possible risks when groundwater is used for drinking. Typical symptoms of infection by water transmitted pathogens can be communicated. Early and correct diagnosis could decrease morbidity and mortality rates. Education should also include possible means of treatment of water (boiling, chlorination pills) as to prevent possible adverse health effects.

iv) Monitoring regime

A more frequent and inclusive monitoring regime of groundwater sources needs to be practiced. Early detection of possible contamination can lead to faster implementation of corrective measures, preventing a possible disease outbreak.

v) Molecular techniques

Molecular techniques, such as pyro-sequencing, should be considered for further studies of the diversity of bacteria in groundwater. Information generated would lead to better comprehension of the possible interactions between groundwater bacteria and amoeba.

vi) Amoebae

Amoeba can increase the pathogenicity of bacteria. More work needs to be performed on this subject in terms of possible health impacts on humans. Pending results of such work, it is recommended that amoeba be included into the battery of microbial tests for water quality.

REFERENCES

Acts: See SOUTH AFRICA.

Aelion, C.M., Shavo, J.N., & Wahl, M. 1997. Impact of suburbanisation on ground water quality and denitrification in coastal aquifer sediments. *Journal of Experimental Marine Biology and Ecology*, 213: 31-51.

Alanis, A. 2006. Resistance to antibiotics: Are we in the post-antibiotic era? *Archives of Medical Research*, 36: 697-705.

Al-Khatib, I.A. & Arafat, H.A. 2009. Chemical and microbial quality of desalinated water, groundwater and rain-fed cisterns in the Gaza strip, Palestine. *Desalination*, 249: 1165-1170.

Allen, M.J., Edberg, S.C. & Reasoner, J. 2004. Heterotrophic plate count bacteria – what is their significance in drinking water? *International Journal of Food Microbiology*, 92: 265-274.

Almasri, M.N. & Kaluarachchi, J.J. 2004. Assessment and management of long term nitrate pollution of groundwater in agriculture-dominated watersheds. *Journal of Hydrology*, 295: 225-245.

An, Y. & Breidenbach, G.P. 2005. Monitoring *E. coli* and total coliforms in natural spring water as related to recreational mountain areas. *Environmental Monitoring and Assessment*, 102: 131-137.

Andrade, A.I.A.S.S. & Stigter, T.Y. 2009. Multi-method assessment of nitrate and pesticide contamination in shallow alluvial groundwater as a function of hydrogeological setting and land use. *Agricultural Water Management*, 96: 1751-1765.

Atekwana, E.A., Rowe, R.S., Werkema, P.D. & Legall, F. 2004. The relationship of total dissolved solids measurements to bulk electrical conductivity in an aquifer contaminated with hydrocarbon. *Journal of Applied Geophysics*, 56: 281-294.

Atlas, R.M. & Bartha, R. 2002. *Microbial Ecology: Fundamentals and Application*. 4th ED. America: Addison Wesley Longman Inc.

Baggi, F., Demarta, A. & Peduzzi, R. 2001. Persistence of viral pathogens and bacteriophages during sewage treatment: lack of correlation with indicator bacteria. *Research in Microbiology*, 152: 743-751.

Baghel, V.S., Gopal, K., Dwivedi, S. & Tripathi, R.D. 2005. Bacterial indicators of faecal contamination of the Gangetic river system right at its source. *Ecological Indicators*, 5: 49-56.

Barker, J. & Brown, M.R.W. 1994. Trojan horses of the microbial world: Protozoa and the survival of bacterial pathogens in the environment. *Microbiology*, 140: 1253-1259.

Barnes, M.R. & Vermeulen, P.D. 2012. Guide to groundwater monitoring for the coal industry. *Water SA*, 38(5): 831-836.

Basha, C.A., Ghosh, P.K. & Gajalakshmi, G. 2008. Total dissolved solids removal by electrochemical ion exchange (EIX) process. *Electrochimica Acta*, 54: 474-483.

Bauer, A., Kirby, W.M.M., Sherris, J.C. & Turck, M. 1966. Antibiotic susceptibility testing by single disc method. *American Journal of Clinical Pathology*, 45: 493.

Bax, R., Mullan, N. & Verhoef, J. 2000. The millennium bugs – the need for and development of new antibacterials. *International Journal of Antimicrobial Agents*, 16: 51-59.

Behardien, L., Paulse, A., Jackson, V., Khan, S. & Khan, W. 2011. Investigation into the microbial contamination in a spring water distribution system, Western Cape, South Africa. *African Journal of Microbiology Research*, 5(20): 3200-3214.

Bej, A.K., Steffan, R.J., DiCesare, J., Haff, L. & Atlas, R.M. 1990. Detection of coliform bacteria in water by polymerase chain reaction and gene probes. *Applied and Environmental Microbiology*, 56(2): 307-314.

- Bester, L.A. & Essack, S.Y.** 2008. Prevalence of antibiotic resistance in *Campylobacter* isolates from commercial poultry suppliers in Kwazulu-Natal, South Africa. *Journal of Antimicrobial Chemotherapy*, 62: 1298-1300.
- Bonton, A., Rouleau, A., Bouchard, C. & Rodriguez, M.J.** 2010. Assessment of groundwater quality and its variations in the capture zone of a pumping well in an agricultural area. *Agricultural Water Management*, 97: 824-834.
- Bradshaw, D., Nannan, N., Laubscher, R., Groenewals, P., Joubert, J., Nojilana, B., Norman, R., Pieterse, D. & Schneider, M.** 2004. South African national burden of disease study, 2000. Estimates of provincial mortality. Medical Research council report.
- Bruce, B.W. & McMahon, P.B.** 1996. Shallow groundwater quality beneath a major urban centre: Denver, Colorado, USA. *Journal of Hydrology*, 186: 129-151.
- Chandramowleeswara, M. & Palanivelu, K.** 2006. Treatability studies on textile effluent for total dissolved solids reduction using electro dialysis. *Desalination*, 201: 164-174.
- Coetzee, H., Winde, F. & Wade, P.W.** 2006. An assessment of sources, pathways, mechanisms and risks of current and potential future pollution of water and sediments in gold – mining areas of the Wonderfonteinspruit catchment. Water Research Commission Report. Report No 1214/1/06.
- Cohen, J. & Shuval, H.** 1972. Coliforms, faecal coliforms, and faecal streptococci as indicators of water pollution. *Water, Air and Soil Pollution*, 2: 85-95.
- Conrad, J.E., Colvin, C., Sililo, O., Gorgens, A., Weaver, J. & Reinhardt, C.** 1999. Assessment of the impact of agricultural practices on the quality of groundwater resources in South Africa. Water Research Commission Report. Report No. 641/1/99.
- Cosson, P. & Soldati, T.** 2008. Eat, kill or die: when amoeba meets bacteria. *Current Opinions in Microbiology*, 11: 271-276.

Crowther, J., Kay, D. & Wyer, M.D. 2002. Faecal-indicator concentrations in waters draining lowland pastoral catchments in the UK: relationships with land use and farming practices. *Water Research*, 36: 1725-1734.

Dallas, H.F. & Day, J.A. 2004. The effect of water quality variables on aquatic ecosystems: A Review. Water Research Commission Report. Report No. TT 224/04.

Domig, K.J., Mayer, H.K. & Kneifel, W. 2003. Methods used for the isolation, enumeration, characterisation and identification of *Enterococcus* spp. 1. Media for isolation and enumeration. *International Journal of Food Microbiology*, 88: 147-164.

Donskey, C.J. 2006. Antibiotic regimes and intestinal colonization with antibiotic-resistant Gram-negative bacilli. *Clinical Infectious Diseases*, 43: S62-S69.

Douagui, A.G., Kouame, I., Koffi, K., Goula, A.T., Dibi, B., Gone, D.L., Coulibaly, K., Seka, A.M., Kouassi, A.K., Mangoua, J.M. & Savane, I. 2012. Assessment of the bacteriological quality and nitrate pollution risk of quaternary groundwater in the southern part of Abidjan district (Côte d'Ivoire). *Journal of Hydro-Environment Research*, 6: 227-238.

DWA: Department of Water Affairs. 2009. Executive summary: Municipal wastewater treatment base information for targeted risk-based regulation. North West province. Pretoria, South Africa: Government Printer.

DWA: Department of Water Affairs. 2010. Map of average rainfall of the North West province. (www.dwa.gov.za). Date accessed: 11 October.

DWA: Department of Water Affairs. 2012. Green drop progress report. Pretoria, South Africa: Government Printer.

DWAF: Department of Water Affairs and Forestry. 2004. National Water Resource Strategy 1sted. Pretoria, South Africa: Government Printer.

DWAF: Department of Water Affairs and Forestry, 1996b. South African Water Quality Guidelines (second edition). Volume 5: Agricultural use: Livestock watering. Pretoria, South Africa: Government Printer.

DWAF: Department of Water Affairs and Forestry, 1996c. South African Water Quality Guidelines (second edition). Volume 4: Agricultural use: Irrigation. Pretoria, South Africa: Government Printer.

DWAF: Department of Water Affairs and Forestry. 1996a. South African Water Quality Guidelines (second edition). Volume 1: Domestic Use. Pretoria, South Africa: Government Printer.

DWAF: Department of Water Affairs and Forestry. 2003a. A protocol to manage the potential of groundwater contamination from on site sanitation (second edition). Pretoria, South Africa: Government Printer.

DWAF: Department of Water Affairs and Forestry. 2003b. Development of internal strategic perspectives. Groundwater overview for middle Vaal catchment management area. 1st ed. Pretoria, South Africa: Government Printer.

DWAF: Department of Water Affairs and Forestry. 2006. A guideline for the assessment, planning and management of groundwater resources within dolomitic areas in South Africa. 1st ed. Pretoria, South Africa: Government Printer.

Dzwairo, B., Hoko, Z., Love, D. & Guzha, E. 2006. Assessment of the impacts of pit latrines on groundwater quality in rural areas: A case study from Marondera district, Zimbabwe. *Physics and Chemistry of the Earth*, 31: 779-788.

Eccles, J.P., Searle, R., Holt, D. & Dennis, P.J. 2004. A comparison of methods used to enumerate *Escherichia coli* in conventionally treated sewage sludge. *Letters in Applied Microbiology*, 96: 375-383.

Edberg, S.C. & Allen, M.J. 2004. Virulence and risk from drinking water of heterotrophic plate count bacteria in human population groups. *International journal of food microbiology*, 92: 255-263.

Edberg, S.C., Kops, S., Kontnick, C. & Escarzaga, M. 1997. Analysis of cytotoxicity and invasiveness of heterotrophic plate count bacteria (HPC) isolated from drinking water on blood media. *Journal of applied microbiology*, 82: 455-461.

Faniran, J.A., Ngceba, F.S., Bhat, R.B. & Oche, C.Y. 2001. An assessment of the water quality of the Isinuka springs in the Transkei region of the Eastern Cape, Republic of South Africa. *Water SA*, 27: 241-249.

Ferguson, A.S., Layton, A.C., Mailloux, B.J., Culligan, P.J., Williams, D.E., Smartt, A.E., Sayler, G.S., Feighery, J., McKay, L.D., Knappett, P.S.K., Alexandrova, E., Arbit, T., Emch, M., Escamilla, V., Ahmed, K.M., Alam, J., Streatfield, K., Yunus, M. & van Geen, A. 2012. Comparison of faecal indicators with pathogenic bacteria and rotavirus in groundwater. *Science of the Total Environment*, 431: 314-322.

Ferreira, S.L. 2011. Microbial and physico-chemical quality of groundwater in the North West province, South Africa. Potchefstroom: NWU. (Dissertation – M. Sc.)

Field, K.G. & Samadpour, M. 2007. Fecal source tracking, the indicator paradigm, and managing water quality. *Water Research*, 41: 3517-3538.

Finch, G. R., Stiles, M. E. & Smith, D. W. 1987. Recovery of a marker strain of *Escherichia coli* from ozonated water by membrane filtration. *Applied and Environmental Microbiology* 53. p 2894-2896.

Flynn, R.M. & Sinreich, M. 2010. Characterisation of virus transport and attenuation in epikarts using short pulse and prolonged injection multi-tracer testing. *Water Research*, 44: 1138-1149.

Foppen, J.W.A. & Schijven J, F. 2006. Evaluation of data from the literature on the transport and survival of *Escherichia coli* and thermotolerant coliforms in aquifers under saturated conditions. *Water Research*, 40: 401-426.

Ford, T.E. 1999. Microbiological safety of drinking water: United States and global perspectives. *Environmental Health Perspectives*, 107(1): 191-206.

Fourie, A.B. & van Ryneveld, M.B. 1994. Environmental impact of on-site sanitation. A literature review with particular application to South Africa. Water Research Commission Report. Report. No. KV 57/94.

Fourie, A.B. & van Ryneveld, M.B. 1995. The fate in subsurface of contaminants associated with on-site sanitation: A review. *Water SA*, 21: 101-111.

Fricker, C.R., DeSarno, M., Warden, P.S. & Eldred, B.J. 2008. False negative β -D-glucuronidase reactions in membrane lactose glucuronide agar medium used for simultaneous detection of coliforms and *Escherichia coli* from water. *Letters in Applied Microbiology*, 47: 539-542.

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

Gardner, K.M. 1999. The importance of surface water/groundwater interactions. Issue paper. Environmental Protection Agency Report. Report No. EPA 910-R-99-013.

Geldreich, E.E. 1996. Microbial quality of water supply in distribution systems. Boca Ranton, FL: CRC Press.

Gelinas, Y., Randall, H., Robidoux, L. & Schmit, J. 1996. Well water survey in two districts of Conakry (Republic of Guinea), and comparison with the piped city water. *Water Research*, 30(9): 2017-2026.

Godfrey, S., Timo, F. & Smith, M. 2005. Relationship between rainfall and microbiological contamination of shallow groundwater in Northern Mozambique. *Water SA*, 31(4): 609-614.

- Grabow, W.O.K., Hilner, C.A. & Coubrough, P.** 1981. Evaluation of standard and modified MFC, McConkey and Teepol media for membrane filtration counting of fecal coliforms in water. *Applied and Environmental Microbiology*, 42: 192-199.
- Greub, G. & Raoult, D.** 2004. Micro-organisms resistant to free-living amoeba. *Clinical Microbiology Reviews*, 17(2): 413-433.
- Griebler, C., Stein, H., Kellerman, C., Berkhoff, S., Brielmann, H., Schmidt, S., Selesi, D., Steube, C., Fuchs, A. & Hahn, H.J.** 2010. Ecological assessment of groundwater ecosystems – Vision or illusion? *Ecological Engineering*, 1618: 1-17.
- Griesel, M. & Jagals, P.** 2002. Faecal indicator organisms in the Renoster Spruit system of the Modder – Riet River catchment and implications for human users of the water. *Water SA*, 28(2): 227-234.
- HACH.** 2007. DR 2800 Spectrophotometer. Procedures manual. 2nd ed. Germany.
- Harley, J.P.** 2005. Laboratory exercises in Microbiology. 6th ED. USA: McGraw-Hill.
- Herold, C.E. & Bailey, A.K.** 1996. Long term salt balance of the Vaalharts irrigation scheme. Water Research Commission Report. Report No. 420/1/96.
- Howard, G., Pedley, S., Barrett, M., Nalubega, M. & Johal, K.** 2003. Risk factors contributing to microbiological contamination of shallow groundwater in Kampala, Uganda. *Water Research*, 37: 3421-3429.
- Iqbal, M. & Krothe, N.C.** 1995. Infiltration mechanisms related to agricultural waste transport through the soil mantle to karst aquifers of southern Indiana, USA. *Journal of Hydrology*, 164: 171-192.
- Jansen, W.T.M., van der Bruggen, J.T., Verhoef, J. & Fluit, A.C.** 2006. Bacterial resistance: A sensitive issue. Complexity of the challenge and containment strategy in Europe. *Drug Resistance Updates*, 9: 123-133.

Junco, M.T.T., Martin, M.G., Toledo, M.L.P., Gomez, P.L. & Barrasa, J.L.M. 2001. Identification and antibiotic resistance of faecal enterococci isolated from water samples. *International Journal of Hygiene and Environmental Health*, 203: 363-368.

Karen Jordaan, personal communication, 21 September, 2012.

Knapp, C.W., Dolfing, J., Ehlert, P.A.I. & Graham, D.W. 2010. Evidence of increasing resistance gene abundances in archived soils since 1940. *Environmental Science and Technology*, 44: 580-587.

Krapac, I.G., Dey, W.S., Roy, W.R., Smyth, C.A., Storum, E., Sargent, S.L. & Steele, J.D. 2002. Impacts of swine manure pits on groundwater quality. *Environmental Pollution*, 120: 475-492.

Kravitz, J.D., Nyaphisi, M., Mandel, R. & Petersen, E. 1999. Quantitative bacterial examination of domestic water supplies in the Lesotho Highlands: Water quality, sanitation, and village health. *Bulletin of the World Health Organisation*, 77(10): 829-836.

Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogen, M.I. & Pace, R. 1985. Rapid detection of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences*, 82: 6955-6959.

Lawrence, A.R., Macdonald, D.M.J., Howard, A.G., Barrett, M.H., Pedley, S., Ahmed, K. M. & Nalubega, M. 2001. Guidelines for assessing the risk to groundwater from on-site sanitation. British geological survey report. CR/01/142.

Le Maitre, D.C. & Colvin, C.A. 2008. Assessment of the contribution of groundwater discharges to rivers using monthly flow statistics and flow seasonality. *Water SA*, 34(5): 549-564.

Leboffe, M.J., & Pierce, B.E. 1999. A photographic atlas for the Microbiology Laboratory. USA: Morton Publishing Company.

LeChevallier, M.W., Schulz, W. & Lee, R.G. 1991. Bacterial nutrients in drinking water. *Applied and Environmental Microbiology*, 57(3): 857-862.

Lehloesa, L.J. & Muyima, N.Y.O. 2000. Evaluation of the impacts of household treatment procedures on the quality of groundwater supplies in the rural community of the Victoria district, Eastern Cape. *Water SA*, 26(2): 285-290.

Mackintosh, G. & Colvin, C. 2003. Failure of rural schemes in South Africa to provide potable water. *Environmental Geology*, 44: 101-105.

McLay, C.D.A., Dragten, R., Sparling, G. & Selvarajah, N. 2001. Predicting groundwater nitrate concentrations in a region of mixed agriculture land use: a comparison of three approaches. *Environmental Pollution*, 115: 191-204.

MERCK. 2012. Microbiology Manual. 12th ed. Web: <http://www.scribd.com/doc/94454909/Merck-Microbiology-Manual-12th> [Date accessed: 12 November 2012].

Mignard, S. & Flandrois, J.P. 2006. 16S rRNA sequencing in routine bacterial identification: A 30-month experiment. *Journal of Microbiological Methods*, 67: 574-581.

Moliner, C., Fournier, P. & Raoult, D. 2010. Genome analysis of microorganisms living in amoebae reveals a melting pot of evolution. *Federation of European Microbiological Societies Microbiology Reviews*, 34: 281-294.

Momba, M.N.B., Tyafa, Z., Makala, N., Brouckaert, B.M. & Obi, C.L. 2006. Safe drinking water still a dream in rural areas of South Africa. Case study: The Eastern Cape province. *Water SA*, 32(5): 715-720.

Moreno, F., Gayuela, J.A., Fernandez, J.E., Fernandez-Boy, E., Murillo, J.M. & Cabrera, F. 1996. Water balance and nitrate leaching in an irrigated maize crop in SW Spain. *Agricultural Water Management*, 32: 71-83.

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

Murray, K., du Preez, M., Taylor, M.B., Meyer, R., Parsons, R., van Wyk, E., Kuhn, A., van Niekerk, H. & Ehlers, M.M. 2004. National microbial monitoring program for ground water. Water Research Commission Report. Report No. 1277/1/04.

Muyzer, G., De Waal, E. & Uitterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, 59(3): 695-700.

Mwabi, J.K., Mamba, B.B. & Momba, M.N.B. 2012. Removal of *Escherichia coli* and faecal coliforms from surface water and groundwater by household water treatment devices/systems: A sustainable solution for improving water quality in rural communities of the Southern African Development Community Region. *International Journal of Environmental Research and Public Health*, 9: 139-170.

Naicker, K., Cukrowska, E. & McCarthy, T.S. 2003. Acid mine drainage arising from gold mining activity in Johannesburg, South Africa and environs. *Environmental Pollution*, 122: 29-40.

NCCLS (National Committee for Clinical Laboratory Standards). 1999. Performance standards for antimicrobial susceptibility testing. Ninth international supplement, Vol.19 (1). NCCLS document M100-S9.

Neu, H.C. 1992. The crisis in antibiotic resistance. *Science*, 257: 1064-1073.

Newman, M.J., Frimpong, E., Donkor, E.S., Opintan, J.A. & Asamoah-Adu, A. 2011. Resistance to antimicrobial drugs in Ghana. *Infection and Drug Resistance*, 4: 215-220.

NHMRC: National Health and Medical Research Council. 2003. Recommendations to change the use of coliforms as microbial indicators of drinking water quality. Canberra, Australia: Biotext.

NWPG (North West Provincial Government). 2002. State of the Environment Report, South Africa. Web: <http://www.nwpg.gov.za/soer/fullreport/html> [Date accessed: 12 June 2010].

NWRS: See DWAF 2004.

Pagnier, I., Raoult, D. & La Scola, B. 2008. Isolation and identification of amoeba-resisting bacteria from water in human environment by using *Acanthamoeba polyphaga* co-culture procedure. *Environmental Microbiology*, 10(5): 1135-1144.

Parsons, R. 2003. Surface water – groundwater interaction in a Southern Africa context. Water Research Commission Report. Report no TT 218/03.

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

Paulse, A.N., Jackson, V.A. & Khan, W. 2009. Comparison of microbial contamination at various sites along the Plakenburg- and Diep Rivers, Western Cape, South Africa. *Water SA*, 34(4): 469-478.

Pavlov, D., de Wet, C.M.E., Grabow, W.O.K. & Ehlers, M.M. 2004. Potentially pathogenic features of heterotrophic plate count bacteria isolated from treated and untreated drinking water. *International Journal of Food Microbiology*, 92: 275-287.

Payment, P., Franco, E., Richardson, L. & Siemiatycki, J. 1991. Gastrointestinal health effects associates with the consumption of drinking water produced by point-of-use domestic reverse-osmosis filtration units. *Applied and Environmental Microbiology*, 57(4): 945-948.

Po Cataloa Dionisia, L. & Borrego, J.J. 1995. Evaluation of media for the enumeration of faecal streptococci from natural water samples. *Journal of Microbiological Methods*, 23: 183-203.

Pollard, D.R., Johson, W.M., Lior, H., Tyler, S.D. & Rozee, K.R. 1990. Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction. *Journal of Clinical Microbiology*, 28(3): 540-545.

Pupo, G.M., Karaolis, K.R., Lan, R. & Reeves, P.R. 1997. Evolutionary relationships among pathogenic and nonpathogenic *Escherichia coli* strains inferred from multilocus enzyme electrophoresis and *mdh* sequence studies. *Infection and Immunity*, 65(7): 2685-2692.

Quiroz, C.C. 1999. Risk assessment of selected opportunistic pathogens in drinking water. Arizona: University of Arizona. (Thesis – PhD).

Reasoner, D.J. & Geldreich, E.E. 1985. A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology*, 49: 1-7.

Reid, S.D., Herbelin, C.J., Bumbaugh, R.K.S. & Whittam, T.S. 2000. Parallel evolution of virulence in pathogenic *Escherichia coli*. *Nature*, 406: 64-67.

Romprè, A., Servais, P., Baudart, J., de-Roubin, M. & Laurent, P. 2002. Detection and enumeration of coliforms in drinking water: Current methods and emerging approaches. *Journal of Microbiological Methods*, 49: 31-54.

Rozemeijer, J.C., Broers, H.P., van Geer, F.C. & Bierkens, M.F.P. 2009. Weather induced temporal variations in nitrate concentrations in shallow groundwater. *Journal of Hydrology*, 378: 119-127.

Showers, K.B. 2002. Water Scarcity and urban Africa: An overview of urban-rural water linkages. *World Development*, 30: 621-648.

Shroder, J.J., Scholefield, D., Cabral, F. & Hofman, G. 2004. The effects of nutrient losses from agriculture on ground and surface water quality: the position of science in developing indicators for regulation. *Environmental Science & Policy*, 7: 15-23.

Smith, R.L., Buckwater, S.P., Repert, D.A. & Miller, D.N. 2005. Small scale, hydrogen-oxidising-denitrifying bioreactor for treatment of nitrate contaminated drinking water. *Water Research*, 39: 2014-2023.

SOUTH AFRICA. 1956. Act No. 54 of 1956. Pretoria: Government Printer

SOUTH AFRICA. 1996. Act No. 36 of 1998. Pretoria: Government Printer

Stackebrandt, E. & Goebel, B.M. 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology*, 44(4): 846-849.

Stelma, G.N., Lye, D.J., Smith, B.G., Messer, J.W. & Payment, P. 2004. Rare occurrence of heterotrophic bacteria with pathogenic potential in potable water. *International Journal of Food Microbiology*, 92: 249-254.

Stigter, T.Y., Ribeiro, L. & Carvalho Dill, A.M.M. 2008. Building factorial regression models to explain and predict nitrate concentrations in groundwater under agricultural land. *Journal of Hydrology*, 357: 42-56.

Sundar, S., Rai, M., Charavarty, J. & Agrawal, S. 2005. Prevention of antibiotic resistance/antibiotic misuse – abuse. *Medicine Update*, : 695-699.

Suthar, S., Bishnoi, P., Singh, S., Mutiyar, P.K., Nema, A.K. & Patil, N.S. 2009. Nitrate contamination in groundwater of some rural areas of Rajasthan, India. *Journal of Hazardous Materials*, 171: 189-199.

- Ter Braak, C.J.F.** 1990. Interpreting canonical correlation analysis through biplots of structure correlations and weights. *Psychometrika*, **55**: 519–531.
- Tesoriero, A.J., Saad, D.A., Burow, K.R., Frick, E.A., Puckett, L.J. & Barbash, J.C.** 2007. Linking groundwater age and chemistry data along flow paths: Implications for trends and transformations of nitrate and pesticides. *Journal of Contaminant Hydrology*, **94**: 139-155.
- Thomas, V., Herrera-Rimann, K., Blanc, D.S. & Greub, G.** 2006. Diversity of amoeba and amoeba-resisting bacteria in hospital water network. *Applied and Environmental Microbiology*, **72**(4): 2428-2438.
- Thomas, V., Loret, J., Jousset, M. & Greub, G.** 2008. Biodiversity of amoebae and amoebae-resisting bacteria in a drinking water treatment plant. *Environmental Microbiology*, **10**(10): 2728-2745.
- Thompson, C.** 1999. The global epidemiology of resistance to ciprofloxacin and the changing nature of antibiotic resistance: a 10 year perspective. *Journal of Antimicrobial chemotherapy*, **43**: 31-40.
- Tredoux, G.** 2004. Nitrate and associated hazard quantification and strategies for protecting rural water supplies. Water Research Commission Report. Report No. 1058/1/04.
- Tutu, H., McCarthy, T.S. & Cukrowska, E.** 2008. The chemical characteristics of acid mine drainage with particular reference to sources, distribution and remediation: The Witwatersrand basin, South Africa as a case study. *Applied Geochemistry*, **23**: 3666-3684.
- Twarakari, N.K.C. & Kaluarachchi, J.J.** 2006. Sustainability of groundwater quality considering land use changes and public health risks. *Journal of Environmental Management*, **81**: 405-419.
- US EPA** (United States Environmental Protection Agency). 2005. Membrane filtration guidance manual. EPA 815-R-06-009, Office of Water, US Environmental Protection Agency, Cincinnati, OH.

- Valenzuela, M., Lagos, B., Claret, M., Mandaca, M.A., Perez, C. & Parra, O.** 2009. Fecal contamination of groundwater in a small rural dryland watershed in central Chile. *Chilean Journal of Agricultural Research*, 69(2): 235-243.
- Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K. & Swings, J.** 1996. Polyphasic taxonomy, a consensus approach to bacterial systematic. *Microbiological Reviews*, 60(2): 407-438.
- Wakida, F.T. & Lerner, D.N.** 2005. Non-agricultural sources of groundwater nitrate: A review and case study. *Water Research*, 39: 3-16.
- Walchonik, J., Picher, O., Aspöck, C., Ullmann, M., Sommer, R. & Aspöck, H.** 1999. Interactions of “limax amoebae” and Gram-negative bacteria: Experimental studies and review of current problems. *Tokai Journal of Experimental and Clinical Medicine*, 23(6): 273-278.
- Wang, D. & Wanda, F.** 2007. Evaluation of media for the simultaneous enumeration of total coliform and *Escherichia coli* in drinking water supplies by membrane filtration techniques. *Journal of Environmental Sciences*, 20: 273-277.
- WHO:** World Health Organisation. 2011. Guideline for drinking-water quality. 4th ed. Geneva.
- Willey, J.M., Sherwood, L.M. & Woolverton, C.J.** 2011. Prescott, Harley and Klein’s Microbiology. 8th ed. NY: McGraw-Hill.
- Winde, F.** 2010. Uranium pollution of the Wonderfonteinspruit, 1997-2008. Part 1: Uranium toxicity, regional background and mining related sources of uranium pollution. *Water SA*, 36(3): 239-256.
- Winter, T.C., Harvey, J.W., Franke, O.L. & Alley, W.M.** 1998. Groundwater and surface water. A single resource. U.S Geological survey circular 1139.
- Wright, R.C.** 1986. The seasonality of bacterial quality of water in a tropical developing country (Sierra Leone). *Journal of hygiene. Cambridge* 96: 75-82.

Wright, R.O., Lewander, W.J. & Woolf, A.D. 1999. Methemoglobinemia: etiology, pharmacology and clinical management. *Annals of Emergency Medicine*, 34(5): 646-656.

Xu, Y., Titus, R., Holness, S.D., Zhang, J. & van Tonder G.J. 2002. A hydrogeomorphological approach to quantification of groundwater discharge to streams in South Africa. *Water SA*, 28(4): 375-380.

Yasmin, R. 2009. The fate of microbial contaminants in the subsurface with a South African case study. Cape Town: UWC. (Dissertation – M. Sc.)