Antimicrobial resistant bacteria and genes in selected surface water bodies of the North West Province

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ABSTRACT

It has recently been established that apart from the over or misuse of antibiotics, metal pollution in the natural environment may also contribute to antibiotic resistance even when antibiotics are absent. The Wonderfonteinspruit (WFS) is highly impacted by a century of gold mining activities taking place in South Africa. Therefore, this system was identified as a possible driver for metal and antibiotic resistance. The system is also impacted on by agricultural activities and urbanization. The overall aim of this study was to investigate antimicrobial (metals and antibiotics) resistant heterotrophic plate count (HPC) bacterial levels in the mining impacted WFS and receiving Mooi River (MR) system and to identify and characterize multiple antibiotic resistant (MAR) bacteria based on their resistance levels and detection antibiotic resistance genes (ARGs) they may host. Surface water sampling took place at six sites in close vicinity to the confluence of the WFS and MR on three sampling occasions in 2015. R2A agar and R2A agar supplemented with antimicrobials (ampicillin, copper (Cu), iron (Fe), lead (Pb) and zinc (Zn)) individually were used to isolate HPC bacteria. Various physico-chemical properties were measured using standard methods and brought into context with antimicrobial HPC levels. Morphologically distinct antimicrobial resistant isolates were purified and screened for antibiotic susceptibility to seven antibiotics (ampicillin, amoxicillin, tetracycline, erythromycin, streptomycin, trimethoprim and chloramphenicol) from six antibiotic classes by a disc diffusion method. Selected MAR isolates were identified by 16S rRNA amplification, sequencing and comparison to the BLASTn database. The MIC ranges for the identified isolates towards the original antimicrobial they were isolated from and other (amoxicillin, tetracycline, erythromycin and streptomycin) antibiotics were determined by agar dilution. Finally, the presence of five ARGs (*bla*TEM*, amp*C*, tet*A*, tet*L*, tet*K) were screened for by PCR amplification of the gene and sequencing verification. Physico-chemical parameters generally exceeded the recommended water quality objectives for the catchment. From the statistical analysis of physico-chemical and HPC results it was evident that most of the HPC results related to the mining impacted site WFS 1. Co-resistance was observed as 82% of the isolates isolated from metal containing media were resistant to at least one antibiotic and over 30% of all the antimicrobial resistant isolates were MAR at all of the sites. A large proportion of isolates were resistant to all 7 antibiotics tested. Phyla detected among the 72 MAR isolates were Proteobacteria, Firmicutes, Bacteriodetes and Actinobacteria in descending order. *Pseudomonas* and *Acinetobacter* genera from the Gammaproteobacteria class were most frequently identified among the isolates. High minimum inhibitory concentration (MIC) levels for metals and antibiotics were detected amongst all the genera. In general, it was observed that the bacterial community was most susceptible to Cu and most resistant to Pb. Resistance to the β-lactam antibiotics were most prevalent and most of the identified MAR

isolates had high levels of resistance (MIC >100 mg/L) to the antibiotics of this class. *bla*TEM was most prevalent among the ARGs and found in 78% of the MAR bacteria. The *amp*C and *tet*A genes were detected in four isolates each, whereas *tet*L and *tet*K were not detected among the MAR isolates of the current study. The study could successfully conclude that metal and antibiotic resistance co-occurred in isolates from all of the sites. β-lactamase resistance was widespread as was found in previous studies. However, antimicrobial resistance was more prevalent in the mining impacted WFS sites compared to the MR and it is therefore concluded that there is a form of co-selection taking place for metal and antibiotic resistance.

Key words

Antibiotic resistance genes, Antimicrobial resistance, co-occurrence of metal and antibiotic resistance, metal pollution, multi-antibiotic resistance, minimum inhibitory concentration, surface water.

It always seems impossible, until it's done (Nelson Mandela).

Let us not be discouraged to aim for sufficient, sustainable and good quality water to all South Africans.

This work is dedicated to my parents Dianne and Michael, sister Marishka and beloved Quinton. Your continued support, love and encouragement motivated me throughout this study. May God bless each of you as He has blessed me.

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DECLARATION

I, Janita Bosch, declare that this dissertation is my own work in design and execution. It is being submitted for the degree Magister Scientiae in Microbiology at the North West University, Potchefstroom Campus. It has not been submitted before for any degree or examination at this or any other university. All material contained herein has been duly acknowledged.

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Janita Bosch **Date**

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ABBREVIATIONS

AAC	Aminoglycoside acetyltransferase
AC	After confluence
AHC	Agglomerative hierarchical clustering
Am.cons.	Antimicrobial concentration
Ap	Ampicillin
ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistance genes
ARP	Antibiotic resistant phenotype
Avg.	Average
Ax	Amoxicillin
BC	Before confluence
Bp	Base pair
BRG	Biocide resistance genes
CC	Cluster centroid
CFU	Colony forming units
COD	Chemical oxygen demand
Cp	Chloramphenicol
Cu	Copper
DEA	Department of Environmental Affairs
DO	Dissolved oxygen
DPW	Department of Public Works
DWA	Department of Water Affairs
DWAF	Department of Water Affairs and Forestry
e.g.	Example
Er	Erythromycin
ESBL	Extended spectrum β-lactamases
Fe	Iron
HGT	Horizontal gene transfer
HPC	Heterotrophic plate count
Isol.am.	Isolation antimicrobial
kb	Kilobase
MAR	Multiple antibiotic resistant
MCC	Minimum co-selective concentration
MFS	Major facilitator superfamily
MGE	Mobile genetic elements
MIC	Minimum inhibitory concentration
MR	Mooi River
MRG	Metal resistance genes
n	Sub sample size
NWA	National Water Act
NWDACE	North West Department of Agriculture, Conservations and Environment
NWP	North West Province
NWPG	North West Provincial Government
Pb	Lead
PCR	Polymerase chain reaction
RDA	Redundancy analysis
RWQO	Resource water quality objectives
St	Streptomycin
TB	Tuberculosis
TDS	Total dissolved solids
Tm	Trimethoprim
Tt	Tetracycline
WMA	Water management area
WFS	Wonderfonteinspruit
Zn	Zinc

CHAPTER 1

Literature study

1.1 Water availability in South Africa and the North West Province

The South African National Water Act (1998) gives every person in this country the even right to potable water. The government is responsible to evenly distribute the source in such a manner that human needs are met while protecting the ecosystem and increasing the availability thereof (NWA, 1998). A census done in 2011 by StatsSA estimated that the population of South Africa in that year was 51.77 million people (DWA, 2013). An increase in population requires an increase in resources such as clean or renewable water (DWA, 2013; Oberholster and Ashton, 2008; NWPG, 2002). As stated by the Department of Environmental Affairs (DEA, 2012), environmental sustainability can only be reached by maintaining environmental systems at healthy levels. In order to manage unexpected or long-term water quality problems, records with data on trends and history are required. This type of data which aids in prediction of disasters, implementation of remedial strategies and uphold of good quality water is still lacking in South Africa and places the available water sources in great danger (Fatoki *et al.*, 2001). The water resources of South Africa is made up of rivers, dams, lakes, wetlands and subsurface aquifers (DWAF, 2004).

The average rainfall of 450 mm per annum in 2010 is far below the world average of 860 mm per annum and the country is seen as the 30th driest country in the world (DWA, 2013). The rainy season of 2015 was seen as one of South Africa's driest yet. This is because of the effects of the El Nino weather phenomenon. The North West Province (NWP) was declared as a drought disaster area in 2015 by the Cooperative Governance and Traditional Affairs minister (http://www.news24.com/SouthAfrica, 2015). There is a variation in rainfall throughout the year in this Province with the rainy season taking place in the months from October to March. The NWP consists of four water management areas (WMAs) (Crocodile-(West) Marico, Upper Vaal, Middle-Vaal and Lower-Vaal) (DWAF, 2010). It shares these WMAs with an independent country (Botswana) as well as neighboring Provinces. Water resources of this Province are derived from rivers, dams, pans, wetlands and dolomite eyes fed by aquifers (DWA, 2013; NWPG, 2002). However, many rivers in the Province are dry for most of the year. The lack of adequate water limits economic growth and and development in the Province (NWDACE, 2008).

1.2 Economic and social relevance of the Wonderfonteinspruit and Mooi River

The area selected for this study is the upper Mooi River (MR) and Wonderfonteinspruit (WFS) catchments. Six sample sites surrounding the confluence of the upper MR and lower WFS were

selected. Both these systems support important economic activities such as the mining, agriculture and urban areas (Kalule-Sabiti and Heath, 2008).

The WFS, originates south of Krugersdorp in Gauteng and passes through an area known to have the richest gold deposits in the world (Coetzee *et al*., 2006). Its name literally means "Wonderful-Fountain- Stream" and dolomite rich groundwater feeds the stream through karst springs (Hamman, 2012, Winde, 2010). The catchment area is about 1600 km^2 and the stream about 90 km long (Winde, 2010). The upper WFS passes Kagiso, Azaadville and Randfontein into Donaldson Dam in the West Rand goldfield (Coetzee *et al*., 2006). This area was first mined for gold in 1887, only one year after gold was first discovered in the Witwatersrand (McCarthy, 2006). Mining in the area has been ceased and the area is left with abandoned and un-rehabilitated slime dams and rock and sand dumps (Coetzee *et al.,* 2006). The Donaldson Dam deposits its water into a 1-m pipeline (Winde, 2010). The pipeline stretches over 32 km and discharges in Carltonville which is known as the lower WFS (Coetzee *et al*., 2006). The WFS in this area supported 10 major mines which produced a total of 7300 tons of gold (Winde, 2010; Coetzee *et al*., 2006). After passing Carletonville and Welverdiend the WFS finally joins the MR upstream from Potchefstroom (Coetzee *et al*., 2006). In addition to the mining industry, the WFS is used for irrigation and livestock watering (Hamman, 2012).

The MR catchment area forms part of the Upper Vaal WMA (Van der Walt *et al*., 2002). This river has its origin in Derby in the North West Province (NWP) from where the river flows through agricultural land to the Klerkskraal Dam (Barnard *et al*., 2013). Several kilometers after Klerkskraal Dam the MR is joined by the WFS. The river then flows into Boskop Dam and Potchefstroom Dam which regulates the flow of the river, before it reaches the town of Potchefstroom. The Boskop and Potchefstroom dams are both used for certain recreational activities and informal settlements are increasingly developing on the banks of the MR (Jordaan and Bezuidenhout, 2016; Barnard *et a*l., 2013; Van der Walt *et al*., 2002). The approximately 124,000 inhabitants of Potchefstroom use the water of the MR as the sole source of domestic and drinking water (Jordaan and Bezuidenhout, 2016; Barnard *et al*., 2013; Coetzee *et al*., 2006). The MR has been an important source of irrigation for many generations of farms surrounding the river (Van der Walt *et al*., 2002).

Water management of this catchment has become more and more complex as the level of pollution and water demand increased. Awareness has been on the contribution of mining industries to pollution of ground- and surface water of the WFS (Durand, 2012; Coetzee *et al*, 2006; Van der Walt *et al.,* 2002). Though the major pollutant of the WFS is mining activity (point source) it is not the sole perpetrator. Other influences include 21 discharge points, a number of

non-point mine discharges, sewage works, formal and informal settlements, industries and agriculture (Coetzee *et al*., 2006).

Applications associated with agriculture such as run-off from nutritional additives, fertilizers, pesticides and fungicides are released and spread into the environment via manure (Seiler and Berendonk, 2012; Zhang *et al*., 2012; Dallas and Day, 2004). These sources of pollution may contribute to chemical as well as microbiological contamination of surface- and ground waters (Kalule-Sabiti and Heath, 2008, Coetzee *et al*., 2006; Griesel and Jagals, 2002). Polluted water have the potential to host pathogenic or potentially pathogenic organisms that may contribute to water borne diseases (Cho *et al*., 2010; Pereira, 2009; Darakas *et al*., 2009).

1.3. Water Quality

The biological, physical and chemical properties of water describes the quality of the water (DWAF, 1996). Water quality is influence by many factors including human and natural factors. Bezuidenhout (2012) outlined the importance of regular monitoring of the physico-chemical and microbiological quality of surface water. Each application of water (aquatic ecosystem health, domestic, agricultural, industrial and recreational) has its own specific standard for water quality parameters known as the Target Water Quality Range (TWQR) (DWAF, 1996). As the climate, geomorphology, geology and biotic composition vary in different regions, the water quality standards also vary for each region (Dallas and Day, 2004). The DWA has released unique Resource Water Quality Objectives (RWQOs) (summarized in Table 2.1) for the MR catchment (DWAF, 2009). Jordaan and Bezuidenhout (2016) detected that most of the water quality parameters were outside the RWQOs set out for the MR.

1.3.1 Bacteriological quality of water with a focus on HPC

Human activities cause undesirable and potentially irreversible changes in the environment. These impacts include adverse effects on ecosystem structures and biodiversity (Jordaan and Bezuidenhout, 2016). Biomonitoring is the process in which living organisms sensitive to toxic agents are monitored in the environment (Al-Bahry *et al*., 2012). Analysis of the bacterial community is a general practice in monitoring water quality as they are ubiquitous in aquatic environments and can be used to indicate sources of pollution (Molale and Bezuidenhout, 2016; Dunn *et al*., 2014).

HPC bacteria include any bacteria that grow on low concentrations of organic nutrients (Edberg and Allen, 2004). This covers a broad spectrum of bacteria including pathogens or potential pathogens and coliforms (Allen *et al*., 2004). Isolation of heterotrophic bacteria on organic nutrient media only yield a small percentage of the total number of heterotrophic bacteria from the

environment (Allen *et al*., 2004). There is no standard for the allowable number of HPC bacteria in surface waters. Some common pathogens found among heterotrophic bacteria include members of the genera: *Pseudomonas*, *Aeromonas*, *Acinetobacter*, *Flavobacterium*, *Alcaligens*, *Achromobacter* and *Mycobacterium* (Stelma *et al*., 2004). These bacteria may not occur in the natural environment in enough numbers to have adverse effects on healthy humans, however, their presence pose a risk to immunocompromised individuals and children (Jordaan and Bezuidenhout, 2016; Paulse *et al*., 2009).

A study of the MR that used a metagenomics approach and linking this data to physico-chemical parameters to bacterial dynamics (Jordaan and Bezuidenhout, 2016) found 10 phyla, 16 classes and 75 genera from all of the sites studied. Of the 10 phyla detected by this approach the most abundant in descending order were Proteobacteria, Bacteriodetes and Actinobacter. Thus, there is a diverse bacterial community present in the MR.

These phyla that were detected in the MR, have previous been found to be resistant to both metals and antibiotics. In recent studies it was demonstrated that Proteobacteria were of the most frequently detected antimicrobial resistant bacteria (Henriques *et al*., 2016; Pal *et al*., 2015; Moller *et al*., 2014; Vaz Moreira *et al*., 2014). Vaz Moreira and co-workers (2014) also detected antimicrobial resistant Bacteriodetes and Firmitcutes in water of their study that investigated the link between bacteria in water and the human biome. The co-selection of resistance to antibiotics and metals was also recently found in arctic bacteria from the Proteobacteria, Bacteriodetes, Firmicutes and Actinobacter phyla (Moller *et al*., 2014). *Pseudomonas* is the most predominant genus detected with this trait and this is attributed to the fact that species in this genus host a number of intrinsic resistance determinants (Henriques *et al*., 2016; Hwang *et al*., 2005). Also the plasticity of their genome should allow members of this genus to acquire all known antibiotic resistance mechanisms (Luczkiewics *et al*., 2015).

1.3.2 Temperature of surface waters

Temperature directly or indirectly influences the equilibrium of each of the other physico-chemical parameters (Delpla *et al*., 2009). The geographical properties of the region, seasonal changes, time of day, water flow, depth, air circulation, altitude and latitude and anthropogenic activities all alter the temperatures measured in the environment (Makhlough, 2008; Ahipathy and Puttaiah, 2006; DWAF, 1996). Thermal pollution may alter surface water temperatures and involves heated discharges into the environment usually from metal foundries, returned irrigation water, sewage treatment and power plants (Dallas and Day, 2004). Temperature deviations can have a lethal effect on organisms found in water resources and a distinct difference in the bacterial community is often found between warmer and colder temperatures (Henne *et al*., 2013; Dallas and Day,

2004). A similar result was found by Jordaan and Bezuidenhout (2013) in the Vaal River, South Africa, in their study on the seasonal changes of physico-chemical parameters in this water source.

1.3.3 pH of surface waters

pH is determined by bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), hydroxyl (OH-) and hydrogen ion concentrations in a sample, and is an indication of how acidic (0-7) or alkaline (8-14) a source is at a given temperature (DWAF, 1996). A lower pH leads to the release of elements from the sediment which may be toxic to organisms found in the water (Venkatesharaju *et al.*, 2010). Natural factors that may lower the pH of surface waters include: (i) leaching of water from the water table with a lower pH (Winde, 2010), (ii) temperature changes (Ideriah *et al*., 2010), and (iii) acid rain (Sutcliff, 1998). Point source pollution such as wastewater dumping or industrial pollution (e.g. metals or acid mine drainage from mining) also changes the chemical character of the source and alters the pH (Barnard *et al*., 2013; Coetzee *et al.*, 2006). Certain buffering systems present in water strive to maintain the pH at a neutral range of 7 (Dallas and Day, 2004). The main acid-base equilibria system is that of dioxide-bicarbonate-carbonate (DWAF, 1996). This buffering system has been observed in the surface water bodies of the MR catchment area (Barnard *et al*., 2013).

1.3.4 Total dissolved solids (TDS)

TDS is an indication of the level of inorganic salts and traces of organic materials present in water sources and is influenced by the solubility of minerals found in the environment such as soil, rock- and plant material (Dallas and Day, 2004; DWAF, 1996). TDS levels are usually impacted on by sources such as industrial effluent, sewage waste water, urban- and agricultural run-off (WHO, 2003). Natural causes of high TDS can be attributed to weathering of minerals in the environment or temperature changes (Delpla *et al*., 2009; Coetzee *et al.*, 2006; Atekwana *et al*., 2004). TDS has an influence on the aesthetic value of water and may indicate the presence of harmful chemical contaminants (Dallas and Day, 2004; DWAF, 1996). TDS levels exceeding the acceptable standards for various uses have been measured in parts of the MR (Monapathi, 2014; Barnard *et al*., 2013). This was attributed to agricultural- and wastewater run-off and the geology of the environment as described by Van Wyk *et al*. (2012) and Moniruzzaman *et al*. (2009).

1.3.5 Nutrients (Nitrates and Phosphates)

Commonly released nutrients into water sources include nitrogen and phosphorous which become available for up-take by plants, algae and cyanobacteria, this could result in eutrophication (Dallas and Day, 2004; Walmsley, 2000). Nutrients settle to sediments and may re-suspend into the water column if the sediment is disturbed for example by cattle that walk into the river as observed by

Line and co-workers (1998). Nitrogen is most commonly present in water sources in three ionic reactive forms namely ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-). Natural sources of nitrate to surface water include leaching nitrogen from natural soils or bedrock nitrogen (Holloway and Dahlgren, 2002). Farmers apply fertilizers to crops to enhance growth, which contain these nutrients which ultimately reach rivers in the form of agricultural run-off (Suthar *et al*., 2009; Yang *et al*., 2007). The products from the nitrification process pose great dangers for both human and animal health as it possess carcinogenic effects (Sutton *et al*., 2011; Gatseva and Argiova, 2008; Yang *et al*., 2007). Phosphates are usually seen as a limiting factor for eutrophication (Oberholster and Ashton, 2008). Dallas and Day (2004) attribute the presence of phosphate to poor managed sewage water, animal waste, crop residues and anthropogenic run-off. Both nitrates and phosphates have been measured to exceed the RWQOs in the MR, with the highest levels generally detected in Potchefstroom Dam (Barnard *et al*., 2013).

1.3.6 Sulphates in surface waters (SO_4^{2-})

Sulphates (SO_4^{2-}) occur naturally in the environment in forms such as barite ($BaSO_4$), gypsum ($CaSO_4.2H_2O$) and epsomite ($MgSO_4.7H_2O$) (Swamy *et al*., 2013). Chemical processes that release sulphates are commonly associated with mining effluent (DWAF, 2009). Limestone is often incorporated to raise the pH of mining effluent to please downstream consumers. However, a reaction between sulphuric acid with alkaline dolomite and limestone forms sulphate salts in the environment (Durand, 2012). Furthermore, sulphuric acid is a by-product of fertilizers and many agrochemicals applied in agricultural activities (Kume *et al*., 2010; Koh *et al*., 2007). Past studies in the MR measured sulphate levels to be higher after the WFS confluence and attributed these levels to mining pollution from the WFS (Barnard *et al*., 2013; Durand, 2012; Van der Walt *et al*., 2002).

1.3.7 Chemical oxygen demand (COD)

COD is a measurement of the level of oxygen as a strong oxidizing agent consumed during the oxidization of organic matter (Noguerol-Arias *et al*., 2012). It indicates the presence of organic matter which also contributes to the total amount of suspended solids in the water (Hur *et al*., 2010; DWAF, 1996). COD loads in surface water are usually attributed to agricultural, industrial or domestic pollution (DWAF, 1996). The RWQOs do not stipulate acceptable levels of COD for the MR, however, usually <75 mg/L COD is acceptable for surface water (DPW, 2012).

1.4 Antimicrobials

An antimicrobial is any compound that exerts a deadly effect on microbes or which inhibits the growth of microorganisms (Leekha *et al*., 2011). In this document antimicrobials referred to are metals and antibiotics.

1.4.1 Metals in the environment and their antimicrobial mode of action

Metals incorporated as antimicrobials have been popular since antiquity (Lemire *et al*., 2013). Heavy metals are defined as metals with a density above 5 g/cm^3 (Amalesh *et al*., 2012). There are 53 naturally occurring heavy metals (Nies, 1999). Metals in the environment are usually found within rock in the form of insoluble silicates and sulphates. Atmospheric pressures cause these compounds to decompose and metals are released into water bodies (Spitz and Trudinger, 2009).

Leaching of metals is strongly depended on the solubility of the metal and this is depended on pH of the environment (Spitz and Trudinger, 2009). Anthropogenic activities that lead to the accumulation of heavy metals in the environment include mining activities, agriculture and domestic chemical applications. The act of mining itself, which involves the removal and processing of ores, does not contaminate the environment. However, mine wastes are transferred to the environment by leaching into ground- and surface waters, dust emission and tailing solutions (DEAT, 2008). Furthermore, the diverse and abundant use of metals in feed additives, fertilizers and pesticides also contribute to the release of heavy metals such as Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn into the environment (Seiler and Berendonk, 2012; Burridge *et al*., 2010). The sediment often acts as a sinkhole for metals as it contains oxides, hydroxides and oxyhydroxides.

Most heavy metals are essential trace elements, thus cells have natural uptake mechanisms for some metals (Amalesh *et al*., 2012; Silver and Pung, 2009; Nies, 1999). Their cellular functions are critical especially with regards to their role in structure of DNA, proteins and cell membranes (Lemire *et al*., 2013). However, they have the ability to form complex compounds that accumulate within the cell and become toxic (Lemire *et al*., 2013; Nies, 1999). Certain physical parameters of the water such as salinity, acidity and hardness determines the rates of accumulation and toxicity of the metals in organisms (Durand, 2012). Metals are toxic to microbes due to (i) the chemical affinity of the heavy metal to the thiol groups found within the cell, (ii) the affinity to macro-biomolecules and (iii) the solubility of the heavy metal (Seiler and Berendonk, 2012). The most common mechanisms in which metals exert injury to microbial cells relate to oxidative stress, protein damage, or membrane alterations which disrupts the normal functionality of the cell (Lemire *et al*., 2013). Metals specific to this study, their mode of action and mechanisms by which microbes become resistant to each are discussed in Section 1.6.

1.4.2 Antibiotics and their modes of action

Infectious diseases have been the leading cause of mortality in almost all of human's existence (Aminov, 2010). The discovery of antibiotics which kill bacteria that cause infectious disease, was seen as a "wonder discovery" (Davies and Davies, 2010). Since the discovery of penicillin in 1928 antibiotics have become an important part in life on earth and have led to great advances in the effective treatment of diseases (Martinez, 2009). Davies and Davies (2010) defined the term antibiotic as "any class of organic molecule that inhibits or kills microbes by specific interactions with bacterial targets, without any consideration of the source of the particular compound or class".

Antibiotics are grouped into classes according to their mode of action in bacterial cells (Kohanski *et al*., 2010). Kohanski and co-workers (2010) described cell death due to antibiotics as a complex process which firstly involves a physical interaction between molecule and target site, followed by alterations of the cell at biochemical, molecular and structural levels. The eukaryotic and prokaryotic cell differs in many ways and antibiotics target these structural differences in order to treat bacterial infections without affecting the eukaryotic cell. These structural differences include the presence of a peptidoglycan layer in prokaryotic cell which is absent in the eukaryotic cell and the bacterial ribosome differs from its counterparts in eukaryotic cells, as they are much smaller in prokaryotic cells (Kaufman, 2011; Tenover, 2006).

Antibiotics that interfere with bacterial cell wall synthesis do so in two ways, both of which focus on interference with the synthesis of the peptidoglycan layer. The first is involves an interference with the enzymes necessary for peptidoglycan layer synthesis (Tenover, 2006). The second prevents the cross-linking steps by binding to the terminal D- alanine residues of the emerging peptidoglycan chain, therefore making cell wall synthesis unstable (Reynolds, 1989). Antibiotics which exert an action against protein synthesis usually bind to the 30S or 50S ribosomal sub-units (Tenover, 2006). Some antibiotics disrupt DNA synthesis and cause lethal double-strand DNA breaks during replication (Kohanski *et al*., 2010). Others exert their antibacterial effect by blocking the folic acid synthesis pathway, ultimately inhibiting DNA synthesis (Tenover, 2006). Details of the antibiotics relevant to this study are discussed in Section 1.6.

1.5 Antimicrobial resistance

Microbes have developed the ability to withstand toxic attacks, a term commonly known as antimicrobial resistance. However, antimicrobial resistance is a complex concept as there are many factors that contribute to the phenomenon such as the variety of antimicrobials that exert an effect and different environments and taxonomic groups in which this phenomenon is present (Pal *et al*., 2015; Chudobova *et al*., 2014).

1.5.1 Microbial metal resistance mechanisms

Microbes respond to excess metal concentrations by becoming resistant to toxic levels of heavy metals. There are three possible mechanisms by which organisms become resistant to toxic levels of metals. The first mechanism is an active efflux of the of the heavy metal ion out of the cell (Spain and Alm, 2003; Bruins *et al*., 2000). It is the most cost effective and popular way of detoxification (Nies, 1999). The second mechanism involves complexation of the heavy metal ion within the cell. This usually occurs with metals which have a high affinity for sulfurs and is changed to complexes by thiol-containing molecules. This is the most "expensive" way of detoxification in terms of ATP used by the cell (Spain and Alm, 2003; Nies, 1999). The third mechanism involves the reduction of the heavy metal to a less toxic oxidative state (Spain and Alm, 2003; Bruins *et al.*, 2000; Nies, 1999). The reduced products should be removed from the cell and this is usually done by an efflux system that is most effective at low concentrations of the metal (Nies, 1999).

1.5.2 Microbial antibiotic resistance mechanisms

An increase in usage of antibiotics has led to an increase in pollution and bio-availability thereof in the environment and microbes have developed ways to resist the effect of antibiotics (Amalesh *et al.,* 2012; Martinez, 2009). Rapid dissemination of antibiotic resistance have raised concerns on the part that water environments play in the occurrence of this phenomenon (Berglund, 2015; Chudobova *et al*., 2014; Lachmayr *et al*., 2009; Kümmerer, 2003). Studies have further shown that little of the antibiotics found in the environment are as a result of naturally occurring antibiotic-producing strains, but rather as result of human application. Some microbes are not only resistant to one class of antibiotics, but have developed resistance to a number of antibiotic classes, thus elevating their level of resistance to antibiotic treatment (Davies and Davies, 2010).

Susceptible populations become antibiotic resistant by mutation and selection or by acquiring resistance encoding genetic information from other bacteria (Davies and Davies, 2010). Antibiotic resistance due to mutations is caused by use of antimicrobials, in which susceptible strains were killed and newly resistant strains were allowed to survive and grow. This is termed vertical evolution (Madhavan and Maruli, 2011 & Tenover, 2006). Mutations can cause resistance in four ways; the first is an alteration of the target protein of the antibiotic which involves modification or elimination of the binding site. The second is an up regulation of enzymes which inactivates the antibiotic. The third mechanism involves an alteration of a protein channel on the outer membrane required by the substance to enter the cell (example: OmpF in *E. coli).* A fourth mechanism involves an efflux pump which expel the antibiotic out of the cell (Madhavan and Maruli, 2011, Davies and Davies, 2010; Tenover, 2006).

A great concern lies in acquired bacterial antibiotic resistance and the readily dissemination thereof between susceptible populations (Tenover, 2006). Genes for antibiotic resistance have been found on both chromosomes and plasmids (Amalesh *et al*., 2012). The genes can be transferred via horizontal gene transfer (HGT) pathways (Martinez, 2009). Mechanisms involved in acquiring genetic information include transformation, conjugation or transduction, all of which may be facilitated by mobile genetic elements (MGEs) transposons, integrons and plasmids with regards to transfer and incorporation (Marti *et al*., 2013; Alekshun and Levy, 2007). These mechanisms allow bacteria to become resistant to multiple antibiotic classes and may occur between different bacterial species and genera. This is termed horizontal evolution (Madhavan and Maruli, 2011; Tenover, 2006). During cell lysis of resistant bacteria, resistance genes are released into the environment. These resistance genes can also be acquired by other bacteria and incorporated, thus transforming the previously susceptible strain into a resistant strain (Tenover, 2010).

1.5.3 Co-selection of metal and antibiotic resistance

A study by Alonso *et al*. (2001) found antibiotic resistant bacteria in a chemically polluted environment without antibiotics as a selective pressure. This observation suggested that there are other factors selecting for antibiotic resistance in the natural environment. Recent global studies have suggested that metal contamination can directly select for metal resistance and co-select for antibiotic resistance in the environment (Amalesh *et al*., 2012; Martinez, 2009; McArthur *et al*., 2012; Nies, 1999). This co-selection phenomenon has been found in a variety of natural environments. Henriques and co-workers (2016) recently found that bacteria on salt marsh plants that sequestrate and accumulate heavy metals, in contaminated environments, were resistant to both heavy metals and antibiotics, while there exists no antibiotic pressures. Pal and co-workers (2015) highlighted that data related to co-selection of metal and antibiotic resistance is still lacking and that it is difficult to say which metals are likely to co-select for which antibiotic resistance. However, they investigated a large range of completely sequenced genomes from over 565 different bacterial genera and found that genomes with metal or biocide resistance genes (BRGs) carried antimicrobial resistance genes more frequently than those without. Seiler and Berendonk (2012) specified minimum co-selective concentrations (MCC) of certain metals that result in a co-selection of antibiotic resistance, in their review of recent studies investigating this phenomenon.

1.6 Antimicrobials of the current study and resistance mechanisms

The antimicrobials of interest included four heavy metals (Cu, Fe, Pb, and Zn) and seven antibiotics (ampicillin, amoxicillin, tetracycline, erythromycin, streptomycin, trimethoprim and chloramphenicol) from six classes.

1.6.1 Copper (Cu)

Cu is an essential trace element and important redox co-factor in catalytic activities of enzymes (Argüello *et al*., 2013; Nies, 1999). Proteins incorporate Cu to aid with cell structure and catalytic processes (Flemming and Trevors, 1989). It is one of the oldest materials used in industries such as architecture, electricity, coinage, biochemical as well as chemical applications (Richardson, 1997). In agriculture, Cu is a growth promotor that is commonly added as a feed additive (Wales and Davies, 2015). Cu interacts with radicals easily especially oxygen where it forms hydroperoxide radicals which lead to protein damage, making it toxic to microbes in the environment (Argüello *et al*., 2013; Dupont *et al*., 2011 & Nies, 1999).

Resistance for Cu is encoded on both plasmids and chromosomes (Pal *et al*., 2015; Altimira *et al*., 2012; Nies, 1999). The detoxification and efflux of Cu is often mediated by the CopB system (Nies, 1999). Altimera *et al.* (2012) isolated five strains of Cu resistant bacteria belonging to *Stenotrophomonas, Sphingomonas* and *Arthrobacter* genera in Cu-polluted agricultural soils with MICs ranging from 3.1 - 4.7 mM for Cu. Pal and co-workers (2015) found that Cu resistance genes on chromosomes associated with many ARG's. Previous studies have measured Cu concentrations that exceeded worldwide levels in both the MR and WFS systems (Hamman, 2012; Van Aard and Erdman, 2004).

1.6.2 Iron (Fe)

Fe plays an important part in many biochemical processes in the biological cell such as electron transfer, cell structure, photosynthesis, oxygen transfer, gene regulation (Kalantari, 2008; Krewulak and Vogel, 2008). Most of the common ore and rock forming minerals contain large amounts of Fe. Gangue most commonly associated with Fe-ore is dolomite, quartz, calcite and feldspar, carbonaceous matter and clay substances (Taylor *et al*., 1988). Detrimental effects of Fe depends on the pH, the amount and type of dissolved organic matter and the redox conditions. Phosphates, trace elements and fluoride are known to enhance Fe (II) oxidation in the environment. Kimiran-Erdem *et al*. (2015) recently found Fe concentrations in surface water to correlate with antibiotic resistance which includes it as a potential selector for antibiotic resistance.

A lack of Fe is more commonly the reason for bacterial mortality rather than an excess thereof (Kim *et al*., 2009). However, high concentrations of Fe has shown to result in a decrease of bacterial growth and thus it still poses a toxic threat to bacteria in high concentrations (Mgbemena *et al*., 2012). This could be attributed to Fe that catalyze the Fenton reaction which produce a highly reactive hydroxyl radical, which in turn damages the cell membrane (Kim *et al*., 2009).

In 2011, high concentrations of Fe was measured in the Tweelopiespruit, Rietspruit and Bloubankspruit river systems which form part of the Witwatersrand mining area (Durand, 2012). The Tweelopiespruit converges with the WFS and thus a high concentration of Fe is expected in the WFS.

1.6.3 Lead (Pb)

Pb is a toxic metal that is found naturally in the environment. During melting, smelting and recycling activities Pb is also released into the environment where it deposits in water, dust, food and soil (Zhang *et al*., 2012; Von Schirnding *et al*., 2003). Pb contamination is often associated with gold mining activities (Sabah and Fouzul, 2012). High levels of Pb was previously measured in Klerkskraal-, Potchefstroom- and Boskop dams. These were attributed to alkyls released by motorboat exhausts (Van Aard and Erdmann, 2004). Hamman (2012) measured concentrations of Pb in the lower WFS at 1.32 times higher than that of the MR and attributed it to gold mining in the vicinity of the WFS.

There are several ways in which bacterial cells become resistant to the toxic effect of Pb (Naik and Dubey, 2013). These mechanisms include PIB –type ATPases transport proteins that cause an efflux of Pb out of the bacterial cell by ATPases transport proteins, induction of metallothioneins that immobilize Pb by sequestration thereof and changes to cell morphology (Jaroslawiecka and Piotrowska-Seget, 2014; Naik and Dubey, 2013; Liu *et al*., 2003; Blindauer *et al*., 2002). Roane and Kellogg (1996) found Pb resistant bacteria in soils that have had no previous Pb exposure, suggesting that Pb resistance is wide spread in the environment. Additional to Pb resistance these bacteria showed multiple resistance to antibiotics. Drudge and co-workers (2012) found a Pb resistance gene cluster alongside genes encoding multiple antibiotic resistance on transferable plasmids in floc bacteria influenced by trace elements.

1.6.4 Zinc (Zn)

Life is impossible without Zn and it is therefore an important heavy metal to investigate in biological processes (Zhang *et al*., 2012; Nies, 1999). However, both a deficiency and an excess of Zn can be fatal to bacterial cells (Coudhury and Srivastava, 2001). There are four ways in which bacteria resist the inhibitory effect of Zn: (i) through extracellular accumulation, (ii) efflux systems, (iii) intracellular sequestration and (iv) metallothionein sequestration (Coudhury and Srivastava, 2001).

Zn holds the highest concentration in animal manure compared to any other heavy metal (Zhang *et al*., 2012). Zn often makes its way to water environments where it has a high affinity for organic matter in sediment (Seiler and Berendonk, 2012). Hamman (2012) determined the Zn

concentrations of the WFS to be 103.49 times higher than the levels measured in the MR. This was again attributed to the upstream gold mining activities.

1.6.5 β-lactams (Ampicillin and Amoxicillin)

B-lactams are one of the oldest known and popular classes of antibiotics with over 50 antibiotics representing this class (Lewis, 2013, Lachmayr *et al*., 2009; Poole, 2004). They dominate the world antibiotic market as two thirds of the antibiotics administered to humans worldwide belong to this class (Lachmayr *et al*., 2009; Poole, 2004). Their mode of action is to target the enzymes (Penicillin-Binding proteins) involved in cell wall synthesis (Lewis, 2013; Poole, 2004). However, shortly after the introduction of penicillin in 1945, bacteria showed resistance towards this antibiotic class which involves hydrolysis of the amide bond in the four- member β-lactam ring by β-lactamase (Lewis, 2013, Lachmayr *et al*., 2009; Poole, 2004). Previous studies investigating the co-selection of metals and antibiotics found resistance to this class of antibiotics more frequently than any other, in isolates that were resistant to multiple antibiotics and metals (Chudobova *et al*., 2014; Hwang *et al*., 2005).

1.6.6 Aminoglycosides (Streptomycin)

The discovery of streptomycin produced by *Streptomyces griseus* in the early 1940's gave rise to the group aminoglycosides that include streptomycin, neomycin, kanamycin and gentamycin (Van Hoek *et al*., 2011). This drug was the first to successfully treat tuberculosis (TB) and has since become the drug of choice in treatment of TB (Jagielski *et al*., 2014). Aminoglycosides exert their effect by binding to the 30S ribosomal sub-unit and inhibit protein synthesis (Jagielski *et al*., 2014; Springer *et al*., 2001). Some resistance mechanisms to streptomycin involves mutations in the *rpsL* and *rrs* genes which ultimately reduces the affinity for streptomycin (Nhu *et al*., 2012). The most common resistance mechanism to streptomycin is enzymatic modification of the agent by any of a number of aminoglycoside modifying enzymes (Ramirez and Tolmasky, 2010). Recent studies have shown that Cu and to a lesser extend Zn inhibit aminoglycoside acetyltransferase (AAC) resistance enzymes, thus enhancing bacterial susceptibility towards aminoglycoside antibiotics in environments with high concentrations of these metals (Henriques *et al*., 2016).

1.6.7 Tetracycline

Tetracycline is used to treat bacterial infections of both Gram-positive and Gram-negative bacteria (Ullah *et al*., 2012). They inhibit protein synthesis by binding to the 30S ribosome sub-unit and interfering with the association of the bacterial ribosome with aminoacyl-tRNA (Adesoji *et al*., 2015). Over 40 tetracycline resistance genes are known of which most (60%) code for efflux pumps, the others protect ribosomes by decreasing the affinity of the tetracycline to the ribosome or inactivate tetracycline by enzymatic activity (Sun *et al*., 2014; Ullah *et al*., 2012). The same

tetracycline resistance genes are found across different bacterial genera, indicating a transfer of these genes amongst the bacterial population (Ullah *et al*., 2012; Auerbach *et al*., 2007). The most commonly found tetracycline resistance genes are associated with plasmids and transposons which indicate that tetracycline resistance is acquired (Adesoji *et al*., 2015; Ullah *et al*., 2012).

1.6.8 Macrolides (Erythromycin)

Macrolides are drugs that contain a 12- or more element macrocyclic lactone ring. A variety of bioactive agents form part of the macrolide class of compounds. These include antifungal drugs, prokinetics, immune-suppressants and antibiotics. Antibiotics form part of 14-, 15- and 16-membered macrolides. They penetrate tissue easily and have effective antimicrobial activity once inside the cell. The first macrolide antibiotic introduced to clinical practice was Erythromycin A, isolated from *Streptomyces* more than 50 years ago (Kanoch and Rubin, 2010).

Erythromycin resistance in especially streptococci have been widely investigated in clinical samples (Juda *et al*., 2016; Schroeder and Stephens, 2016; Veraldo *et al*., 2009). Resistance mechanisms consist either of an efflux of erythromycin or by a methylase-mediated modification of a ribosomal target site (Veraldo *et al.*, 2009). The *erm* class gene-encode methylases associated with ribosomal target site modification. It causes an uncommon mutation of 23S rRNA or ribosomal proteins, when an adenine residue in 23S rRNA is methylated post-transcriptionally. This may then lead to co-resistance to macrolide, lancosamide and streptogramin B (MLSB) resistance as their ability to bind to the ribosome is reduced (Juda *et al*., 2016; Schroeder and Stephens, 2016; Veraldo *et al*., 2009).

1.6.9 Trimethoprim

Trimethoprim and sulfonamides are often combined in treatment in order to have a broader spectrum of inhibition (Eliopoulos and Huovinen, 2001). Trimethoprim exerts its antibacterial effect by binding to the dihydrofolate reductase enzyme which prevent bacteria from forming tetrahydrofolic acid which is required for the synthesis of thymidine. Thus, trimethoprim inhibits DNA or RNA synthesis (Brolund *et al*., 2010; Quinlivan *et al*., 2000). Bacterial resistance to trimethoprim usually relate to the function of the outer membrane, by for example permeability barriers or efflux pumps (Eliopoulos and Huovinen, 2001). Trimethoprim resistance genes that belong to the *dfr* (dihydrofolate reductase) gene family are often found on class 1 and class 2 integrons and are widespread in the environment (Berglund, 2015). *Bacteriodetes*-, *Clostridium*- and *Neiserria* spp have natural insensitivity to this antibiotic (Eliopoulos and Huovinen, 2001). Co-resistance of trimethoprim and heavy metals generally found on class 1 integrons have been observed in metal polluted environments (Nageswaran *et al.*, 2012; Akinbowale *et al*., 2007).

1.6.10 Chloramphenicol

Chloramphenicol is a broad spectrum antibiotic and popular due to easy storage. This antibiotic inhibits protein synthesis by binding to the 50S ribosomal sub-unit. However, its usage has decreased dramatically due to its adverse side effects and antibiotic resistance (Lopez-Perez *et al*., 2013; Fernandez *et al*., 2011). MGEs carry chloramphenicol resistance genes which are easily spread in the environment via vertical- / horizontal transfer (Berglund, 2015). The three major mechanisms leading to chloramphenicol resistance include (i) chloramphenicol acetyl-transferases, (ii) efflux of chloramphenicol by specific- or multidrug transporters, and (iii) rRNA methylase (*cfr*- gene) which confers resistance to chloramphenicols, oxazolidinones, lincosamides, pleuromutilins, and streptogramin A antibiotics all at the same time (Fernandez *et al*., 2011).

1.7 Antibiotic resistance genes

Antibiotic resistant genes (ARGs) are increasingly being released into the natural environment (Berglund, 2015; Capkin *et al.*, 2015; Marti *et al*., 2013; Biyela *et al*., 2004; Alonso *et al*., 2001.). These genes are found in almost all environments where they are able to persist. An increasing amount of focus is now being placed on investigating antibiotic resistance and the dissemination thereof in the natural environment (Molale and Bezuidenhout, 2016; Berglund, 2015; Drudge *et al*., 2012; Lachmayr *et al*., 2009, Martinez, 2009). ARGs are also seen as environmental pollutants and thus a key factor in investigating human impacts on antibiotic resistance (Marti *et al*., 2013).

1.7.1 β-lactamase resistance genes (*amp*C and *bla*TEM)

The first gene found to destroy penicillin was the β-lactamase encoding gene *amp*C (Jacoby, 2009). It is estimated that β-lactamase enzymes existed for the past 2 billion years. Furthermore, their epidemiology has had considerable correlation to anthropogenic and the prolificacy of resistance to this class over the past 60 years (Lachmayr *et al*., 2009). Continued selective pressures resulted in the selection of common enzymes such as *bla*TEM which can now hydrolyze third and fourth generation cephalosporins (Nikaido, 2009).

The *bla*TEM gene has been shown to be the dominant ampicillin resistant gene in previous studies (Bora *et al*., 2014; Bailey *et al*., 2011; Lachmayr *et al*., 2009). These genes are commonly found on class 1 intergrons with resistance determinants for a number of other antibiotics and found on the TnA group of transposons (Berglund, 2015; Bailey *et al*., 2011). Amino acid substitution of parent BlaTEM enzymes often result in extended spectrum β-lactamases (ESBL) (Lacmayr *et al*., 2009). Genes encoding ESBL are generally found on plasmids that confer resistance to multiple antibiotic classes (Bora *et al*., 2014). Thus, the mobility of these genes are considerable and expected to be abundant in the natural environment.

1.7.2 Tetracycline resistance efflux pumps (*tet*A, *tet*L, *tet*K)

Of the four general mechanisms for antibiotic resistance (Section 1.5.2), extrusion of antibiotics by efflux pumps is seen as one of the most important with regards to multidrug resistance (Sun *et al*., 2014). *tet*A, *tet*L and *tet*K are some of the important efflux pumps that belong to the major facilitator superfamily (MFS) and associate with multidrug resistance. They encode for energy dependent membrane associated efflux proteins (Sun *et al*., 2014; Ullah *et al*., 2012). *tet*A was reported for the first time in isolates from the *Alcaligene* genus and members of this genus has been isolated from a range of clinical and environmental water sources (Adesoji *et al.*, 2015). Previous studies investigating tetracycline efflux pumps most frequently detected *tet*A from the range of *tet*-genes screened for (Adesoji *et al*., 2015; Li *et al*., 2010a). These genes are commonly found on MGEs that carry resistance genes for a number of antibiotics (Pal *et al*., 2015, Li *et al*., 2010a)

1.8 Phenotypic methods for characterization of antimicrobial resistance in surface water

Metagenomic approaches have proved to provide a broader range of information regarding the structure of the bacterial community compared to biased results provided by culture dependent methods (Jordaan and Bezuidenhout, 2016). However, gaining insight into antibiotic resistance of the bacterial community by means of culture independent methods requires the application of advanced and expensive approaches. Routine implementation of such methods are not always feasible in developing countries (Bora *et al*., 2014).

Heterotrophic bacteria are frequently used to investigate human impact factors, such as antibiotic resistance, in environmental settings (Patel *et al*., 2014). Their short generation times, high diversity and rapid recovery from environmental changes make them ideal indicators of stressors present in surface waters (Jordaan and Bezuidenhout, 2016). Antimicrobial resistance genes are rapidly disseminated among heterotrophic bacteria in different environmental settings making them important hosts of this clinically important phenomenon (Madhavan and Maruli, 2011; Lachmayr *et al*., 2009; Tenover, 2006). They are therefore increasingly being used in studies investigating antimicrobial resistance (Zhang *et al*., 2015). Thus, for screening purposes and identifying hot-spots for antibiotic resistance it is more feasible to screen for antimicrobial resistance in HPC bacteria and then to implement advanced approaches in such environments.

Low nutrient media such as R2A are usually used in water-based studies to isolate HPC bacteria, as it represents the nutrient availability in water resources best (Allen *et al*., 2004). Culturing HPC bacteria in the presence of antimicrobials on nutrient media selects for antimicrobial resistant bacteria (Mezger *et al.*, 2015). The level to which a culture is resistant to an antibiotic can be determined by detecting the minimal inhibitory concentration (MIC) by agar dilution as described

by Wiegand *et al*. (2008). Direct comparison of resistance phenotypes from contaminated and reference sites have successfully linked elevated antibiotic resistance to contaminated exposure (Baker-Austin *et al*., 2006).

1.9 Molecular approaches used for the identification and characterization of multiple antibiotic resistant bacteria

The identification of microorganisms is one of the cornerstones in microbiology which aid to predict possible outcomes of an observation (Janda and Abbott, 2002). Comparing cell morphology and complementary biochemical criteria has allowed biologists to formally describe an estimated 5000 prokaryotic organisms (Kellenberger, 2001). These descriptions have been organized into a reference book titled “Bergey’s Manual of Systematic Bacteriology” (Whitman *et al*., 2012) which can be used to classify unknown strains. However, not all known bacteria are fully described in this manual and human error with notation of biochemical results have proved this technique to be limiting (Ryu *et al*., 2013; Petti *et al*., 2005). Molecular methods such as PCR amplification and sequencing allows for highly specific and sensitive identification of bacteria or genes.

The starting point for any molecular based method involves the extraction of good quality DNA. Tan and Yiap (2009) summarized the most common used chemical approaches for DNA extraction. Other approaches include heat treatment (Dashti *et al*., 2014) or commercial kits such as those produced by Macherey-Nagel (Germany). The isoamyl:chloroform:phenol liquid-liquid extraction method of DNA has proven to be a feasible and effective method in extraction of DNA. This method gets rid of any cell debris, proteins, lipids and carbohydrates while also inhibiting RNAse activity and removes RNA (Tan and Yiap, 2009).

A number of phylogenetic markers that include specific protein coding or structural genes have been identified which can distinguish between different species (Srinivasan *et al*., 2015). The most popular of these are the 16S and 23S rRNA genes as numerous copies of these genes are present in the bacterial genome. However, gene databases contain more representative sequences of the 16S rRNA gene compared to the 23S rRNA gene, thus 16S rRNA is incorporated for identification more often (Ryu *et al*., 2013).

Metagenomics and next generation sequencing (NGS) are exciting new technologies which offer rapid methods to fully investigate the entire collection of resistance genes within a genome or directly from the environment (Pal *et al*., 2015; Tan *et al*., 2015). The availability of people with the skills to analyze bioinformatics data and the costs involved are limiting factors for these approaches. However, since ARGs are seen as environmental pollutants, effort should be made to identify prevalent genes. A feasible method is PCR amplification of specific markers for selected

genes of which antibiotic resistance was prevalent (Bora *et al*., 2014; Bailey *et al*., 2011; Nikaido, 2009; Li *et al*., 2010a).

1.10 Outline of the dissertation

Chapter 1 provides an overview on concepts relevant to the safety of surface water in South Africa and the NWP with a focus on antimicrobial resistance found in surface waters. The concepts focused on are: (i) the historical and current state and impacts on the WFS and the MR; (ii) physico-chemical quality parameters that need to be monitored; (iii) antimicrobial substances found in the environment and their modes of action on bacteria; (iv) resistance mechanisms of bacteria to these antimicrobial attacks and (v) descriptions of specific element of the methodological approaches are provided.

Chapter 2 reports on the physico-chemical state of sites surrounding the WFS and MR in addition to the levels of antimicrobial resistant bacteria detected at each site. The antibiotic resistance phenotypes of the isolated antimicrobial resistant bacteria was also reported. This was a culture based study that directly compared results of impacted sites with that of the reference sites in order to confer resistance selection to the impacts found at impacted sites

Chapter 3 characterizes multiple antibiotic resistant (MAR) bacteria that was isolated from each of the sites based on their molecular identity, the MICs of each to selected antimicrobials and the presence of selected ARG's. The MICs were determined by agar dilution and the molecular classification was performed by PCR amplification of specific genes and sequencing of the amplicons.

Chapter 4 provides a summary of the conclusions and limitations of the current study. In addition, recommendations for inclusion in future studies on the topic at hand is provided in this chapter.

CHAPTER 2

Antimicrobial resistant HPC bacteria and relation to physico-chemical quality of the water in selected surface water of the North West Province

2.1 Introduction

Water sources are scarce in South Africa and the growing population places the available resource under enhanced pressure (DWA, 2013). Water pollution is the leading cause for death and disease globally (Mgbemena *et al*., 2012). Proper management of available natural water resources in South Africa is therefore an inevitable task. In order to manage any system, problem or phenomenon one needs information. In South Africa records with data on trends and history is still lacking, which places natural water resources in great danger (Fatoki *et al*., 2001). In addition to domestic and municipal usage of the resource, water is also a necessity for economic growth (Durand, 2012). The major economic users of water in the NWP are the agricultural and mining industries (DWAF, 2008). Both of these economically relevant industries are documented to release heavy metals and other chemicals into the natural environment. These chemicals leach to aquatic environments where they are able to persist and disseminate to downstream consumers (Barnard *et al*., 2013; Seiler and Berendonk, 2012; NWPG, 2002; Van der Walt *et al*., 2002).

Bacteria are frequently used as indicators of environmental physical and chemical stress due to their short generation time, high diversity and quick reaction to changes (Jordaan and Bezuidenhout, 2016). Levels and diversity of HPC bacteria are used as an indirect measure of bacteriological quality of water. High biological diversity is normally indicative of healthy aquatic systems. Such systems are impacted by various physical and chemical parameters such as temperature, pH, dissolved oxygen, electrical conductivity and nutrient fluctuations (Jordaan and Bezuidenhout, 2013). These parameters are influenced by immediate or past pollution events. They are thus used to indicate whether the water is safe/suitable for specific use, but most importantly suitable to sustain a healthy environmental water reservoir (DWAF, 1996). Such parameters were also used to set RWQOs (Table 2.1) specific for the utility of water from the MR WMA (DWAF, 2009).

Heavy metals are part of the natural environment through geology. However, above certain critical concentrations most heavy metals are toxic to bacterial cells (Nies, 1999). As a natural response, bacteria have been able to develop mechanism to resist this toxicity (Lemire *et al*., 2013; Spain and Alm, 2003; Coudhury and Srivastava, 2001; Nies, 1999). Recent studies have found strong correlations between the existence of metal and antibiotic resistance in bacteria (Henriques *et al*.,

2016; Chen *et al*., 2015; Pal *et al*., 2015; Seiler and Berendonk, 2012; Wright *et al*., 2006). Both these substance are used in treatment of various infectious diseases or as feed additives in agriculture (Seiler and Berendonk, 2012; Martinez, 2009). It was demonstrated that the resistance mechanisms of bacteria to metals and antibiotics are similar (Davies and Davies, 2010). This phenomenon has extensively been studied in clinical settings. However, the rapid dissemination of antibiotic resistance and the correlation with heavy metal resistance, has since shifted the focus to the natural environment (Di Cesare *et al.*, 2016; Kümmerer, 2003).

A century of gold mining activities in the West Rand of Gauteng has severely impacted water quality of the WFS which converges with the MR upstream from Potchefstroom (Durand, 2012; Coetzee *et al.*, 2006). The MR is the major source of drinking water to this town (Barnard, *et al*., 2013). There had been concerns about the influence of the proximity of the water sources of the town to the mining fields (Winde, 2010; Van der Walt *et al*., 2002). This is because during the processing of ores vast amounts of metal contaminated water are released into the natural environment (Durand, 2012). Even when this water is treated to specific standards, it will negatively impact the quality of the receiving water. It is thus expected that water from mines that decant treated water, runoff from the slime dams and other mining activities will impact on the metal levels detected in the water of the WFS and thus also on the MR (DEAT, 2008). Elevated metals in these important water sources could select for and maintain a metal resistant bacterial population as well as speed up selection for antibiotic resistance (Di Cesare, 2016).

Seiler and Berendonk (2012) reviewed a range of studies that investigated environmental metal- and antibiotic resistance co-selection. They specified the minimum MCC of metals in water that would co-select for metal- and antibiotic resistance (Appendix A). Studies have linked co-resistance for multiple antibiotics to metals such as Cu, Pb, Fe and Zn among others (Kimiran-Erdem *et al*., 2015; Naik *et al.*, 2013; Seiler and Berendonk, 2012; Dupont *et al*., 2011). Tlokwe Municipality of Potchefstroom regularly measures heavy metal concentrations in WFS and MR. Data (Appendix A) provided by the Municipality indicate that concentrations of these metals exceeded the MCC levels. Thus, metal pollution of the WFS and MR systems may also be selecting for an antibiotic resistant bacterial population. This is of concern considering the health impacts it may pose to the downstream consumer (Chudobova *at el*., 2014). Furthermore, both the WFS and MR are also impacted by agriculture, urbanization and rural settlements (Jordaan and Bezuidenhout, 2016; Van der Walt *et al.*, 2002). Diverse bacterial communities have been detected in the MR (Jordaan and Bezuidenhout, 2016) and MAR enterococci had also been isolated from this river (Molale and Bezuidenhout, 2016).

The aim of this study was to investigate antimicrobial resistant HPC bacteria at sites surrounding the MR and WFS confluence and to determine if these could be linked to physico-chemical parameters. The specific objectives were thus to (i) enumerate the HPC bacteria and antimicrobial (ampicillin, Cu, Fe, Pb and Zn) tolerant HPC bacteria by plate counts; (ii) determine antibiotic resistance phenotypes of the antimicrobial tolerant isolates found at each site by a disc diffusion method and (iii) determine whether physico-chemical properties of surface water at sites surrounding the WFS and MR confluence impact on the occurrence of antibiotic resistant bacteria.

2.2 Materials and methods

2.2.1 Study area

The surface water at two sites from the WFS and four in MR was of interest (Figure 2.1). Selected sites represent a diverse range anthropogenic activities impacting the water quality. The WFS converges with the MR 20 km north of Potchefstroom, before the Boskop dam. Two sites are located in the lower WFS just outside Carltonville (WFS 1) and Welverdiend (WFS 2) respectively and have been recorded to be directly impacted by gold mining activities (Coetzee *et al*., 2006). The four sites in the MR were distributed among two sites before the confluence (MR BC 1 & MR BC 2) that have no recorded gold mining impacts (Jordaan and Bezuidenhout, 2016) and two sites after the confluence (MR AC 1 & MR AC 2). The geographical co-ordinates (Appendix B) for each site was determined with the Garmin nüvi 1310 a Global Positioning System (GPS) from Garmin, US. The sites at which Tlokwe Municipality measured the metal concentrations are also indicated on the map (Figure 2.1) and their geographical co-ordinates are listed in Appendix B.

2.2.2 Sample collection

Surface water samples were collected for each site according to the South African National Standard ISO 5667-6:2005 (SANS, 2006). Sampling took place on three dates in 2015. The first sampling date was on 09/03/2015 in the warm-rainy time of year. The second sampling date was on 12/05/2015 and was still a rainy time in the year, but temperatures were colder. The last sampling date was on 27/07/2015 which was a cold-dry time of year. A direct sampling procedure took place at most sites, thus the sterile sampling bottle was submerged by hand under the water and filled to the brim for further analysis (Jordaan and Bezuidenhout, 2016). The two sites in the MR after the confluence were hard to reach and sampling took place from a bridge. At these sites a technique involving dipping of the sterile sampling bottle into the river by rope was employed. Gloves were worn throughout the sampling procedure and samples were placed on ice in sealed cooler boxes for transport to the laboratory. All samples were analyzed within 6 hours after sampling commenced.

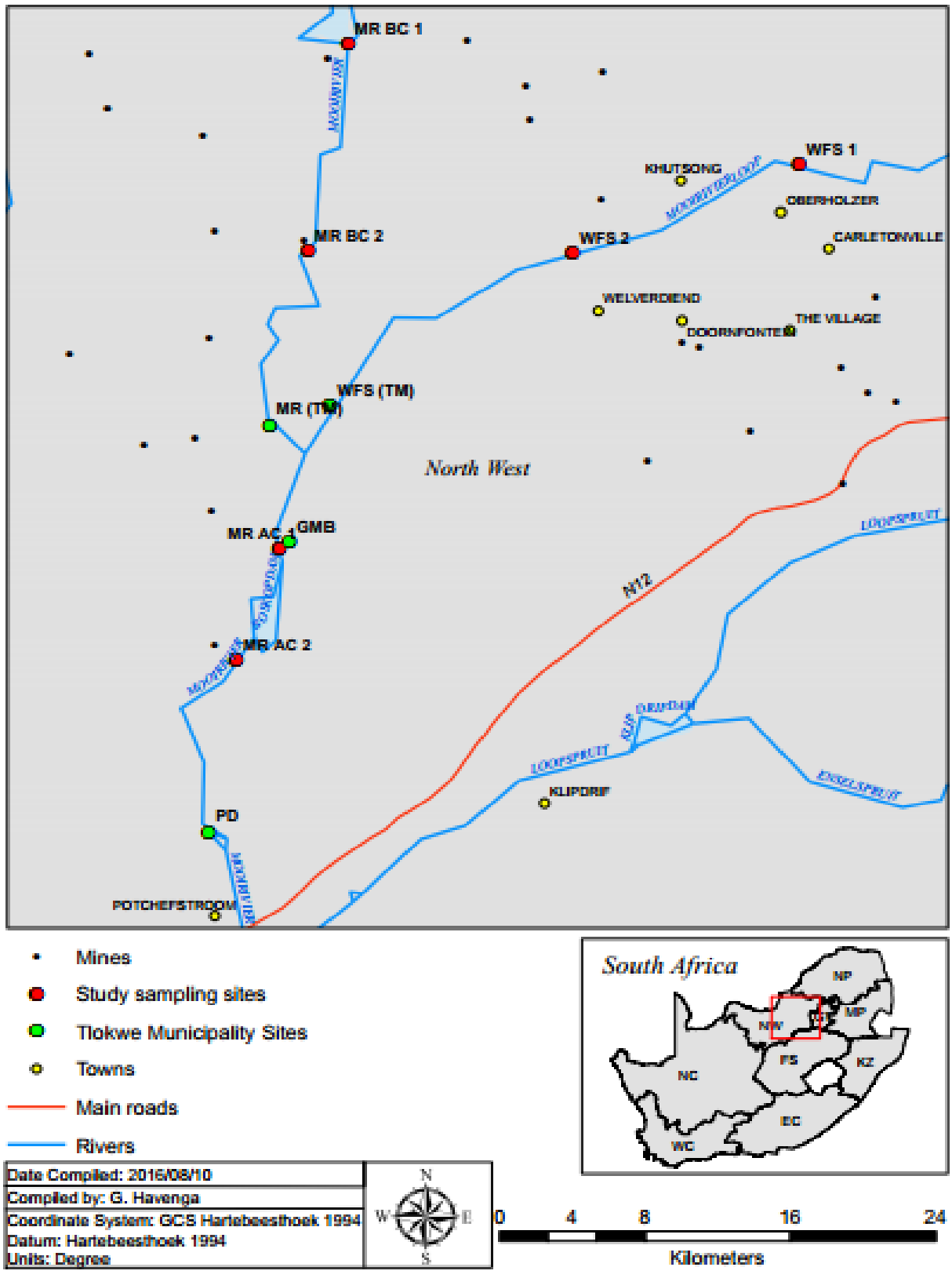


Figure 2.1: Map indicating the specific sites of the current study and the sites at which Tlokwe Municipality measured heavy metal concentrations.

MR- Mooi River, WFS- Wonderfonteinspruit, BC- before confluence, AC- after confluence, (TM) - of Tlokwe Municipality, GMB- Gerhard minnebron, PD- Potchefstroom dam.

2.2.3 Physico-chemical parameters

The physical water quality parameters were measured in situ in triplicate. A Multi-Parameter Testr 35 Series (Eutech Instruments, Singapore) was used for temperature (ºC), pH and TDS (mg/L) measurement and a Series 150 Multi-Parameter (Orbeco Hellige E-Chem instrument, US) for dissolved oxygen (mg/L), both according to the manufacturers' instructions. Briefly, probes were submerged in the water source until readings on the instrument settled and were possible to note. The probes of both instruments were rinsed with distilled water before and after each measurement. The surface water samples were transported to the laboratory where chemical analysis of the phosphates (PO_4^{2-}) (method 8153), sulphates (SO_4) (method 8051), nitrates (NO^{3-}) (method 8039) and COD (method 8000) were measured in duplicate as milligrams per liter (mg/L) according to manufacturer's instructions (Hach Company, 2007) with a HACH DR 2800TM (HACH, US).

The physico-chemical and general microbiological data form part of a WRC funded research project (K5/2347//3). The candidate was one of the members of the research team that collected some of the data. It was agreed that all participants would use data from the set and it is thus unavoidable that overlaps of the actual data in this dissertation, some other M.Sc dissertations and the WRC final report will exist.

2.2.4 Enumeration of heterotrophic plate count bacteria on selective media

The colony forming units (CFUs) of the HPC bacteria found in the surface water of each of the sites were determined on R2A agar (Sigma Aldrich, US). R2A agar supplemented with different antimicrobials (pH 8.0), were incorporated into the study to determine the presence- and amount of antimicrobial resistant bacteria at each of the sampling sites.

Stock solutions of all the antimicrobials (ampicillin, Cu, Fe, Pb and Zn) were prepared with ultra-pure water and filter sterilized through a 0.22 µm membrane prior to supplementation as described by Wiegand *et al*. (2008). Concentrations for each stock solution was x 1000 the desired final concentration. R2A agar was prepared according to manufacturer's instructions and autoclaved. One milliliter of each sterilized antimicrobial stock solution was supplemented aseptically to separate 999 mL autoclaved and cooled (± 60°C) R2A agar, in order to gain the desired final concentrations, before it was poured into sterile petri dishes.

R2A supplemented with ampicillin to the final concentration of 100 µg/L was incorporated to screen for, quantify and isolate antibiotic- or more specifically β-lactam resistant HPC bacteria from the surface water at each site. R2A agar was also supplemented separately with different metals Cu, Fe, Pb and Zn to screen for metal resistant bacteria among the HPC bacteria, present

at each of the sampling sites. The metal containing R2A agar were made up to two final concentrations namely 1 mM and 2.5 mM for each of the metals screened for in the study.

A dilution series was prepared aseptically in the laboratory within 6 hours after sampling from each surface water sample as described by Jordaan and Bezuidenhout (2016). Briefly, dilutions up to 10^{-5} were prepared in triplicate for each sample. These were used as inoculum that was spread aseptically on R2A media with and without antimicrobial agent. The petri dishes were then incubated at room temperature for 5 days after which the CFUs were counted and recorded.

2.2.5 Isolation and purification of antimicrobial resistant colonies

Morphologically distinct colonies on the antimicrobial containing plates were selected for further analysis. These were sub-cultured onto R2A agar containing the original antimicrobial it was isolated from. This was done aseptically by the 4-quadrant streak plate method described by Pelczar and Reid (1958). Sub-culturing was repeated at least three times before the purity of the culture was determined by Gram staining. The Gram staining procedure was followed as described by Brucker (1986). The culture was viewed under the microscope to determine purity of the isolate.

2.2.6 Antibiotic resistance profiles (ARPs) of purified isolates

The purified isolates were tested for their susceptibility to seven (7) antibiotics by using a modified version of the Kirby-Bauer disk diffusion method (Bauer *et al*., 1966). Isolates were grown overnight in nutrient broth at 26°C and spread aseptically onto Meuller Hinton agar (Merck, Germany). The following antibiotic disks (Mast diagnostics, UK) were used: ampicillin (10 µg), amoxicillin (10 µg), tetracycline (30 µg), erythromycin (15 µg), streptomycin (20 µg), trimethoprim (5 µg) and chloramphenicol (30 µg). The plates were incubated at 26°C for 24 - 48 hours after which inhibition zones were measured in millimeters.

The standards set out by the manufacturer for some specific species that cause infectious disease were taken into consideration. Since these were environmental isolates in the current study, a “universal standard” for all of the antibiotics was determined and applied. This was done by determining the averages of the different standards stipulated by the manufacturer for resistance, intermediate resistance and susceptibility. The standard as applied to this study was thus as follow: inhibition zone Resistant ® ≤ 13 mm; Intermediate resistant (IR) = > 13 to < 17 mm; Susceptible (S) ≥ 17 mm. These values were used only to screen for possible resistance.

In order to make the data of the Antimicrobial Resistance Phenotypes (ARPs) more homogenous for statistical analysis (agglomerative hierarchical cluster analysis) and to avoid unnecessary

minor sub-clustering an additional scoring system was implemented. For example: if one isolate has an inhibition zone of 18 mm to ampicillin while another isolate has an inhibition zone of 19 mm to ampicillin, the program will divide these two isolates into separate minor sub-clusters. When this happens for 7 antibiotics among 50+ isolates the data becomes a mess. Thus, to clean-up the data, instead of inhibition zone diameters consisting of any score between 1 to 45 mm they were assigned a score of 10 or 8 or 5 or 2 or 1 based on the inhibition zone size measured per antibiotic. Larger inhibition zones were penalized more harshly. A table with specific scores assigned to specific diameters is summarized in Appendix C.

2.2.7 Statistical analysis

All averages and standard deviations were calculated using Microsoft Office Excel 2013. RDA multivariate analysis were performed by Canoco for Windows Version 4.0, GLW-CPRO©, and visualized by a correlation bi-plot (Ter Braak and Smilauer, 1998). The RDA was used to illustrate the relationship between the average physico-chemical parameters and HPC/antimicrobial resistant HPC levels at sites surrounding the MR and WFS confluence. This was performed using the average from all three sampling occasions for each parameter. Data was log transformed prior to analysis and the Monte Carlo permutation test was performed to test significance (499 permutations; $p \leq 0.05$). A probability level of 0.05 or less was considered to be statistically significant. Data from antibiotic resistance profiles of all the purified isolates at each site was subject to agglomerative hierarchical clustering (AHC) using Ward's method and Euclidean distances in XLSTAT (v 2013.5.00).

2.3 Results

2.3.1 Physico-chemical results of surface water at WFS and MR sites during 2015

Averages of the physico-chemical surface water measurements recorded at four sites in the MR and two sites in the WFS, on three sampling occasions in 2015, are summarized in Table 2.1 and Table 2.2 respectively. RWQOs for the MR catchment set out by the Department of Water Affairs (DWA), relevant to the current study, are also included in these tables (2.1 and 2.2). The site MRBC 2 was dry during the May and July sampling occasions and therefore results on these dates, at this site, are not included in the table.

Table 2.1: Average physico-chemical variables recorded for the Wonderfonteinspruit sites on during 2015

Parameter	RWQO	WFS 1			WFS 2		
		March	May	July	March	May	July
Temp °C	**N/A**	22.10 ± 0.00	18.43 ± 0.37	14.90 ± 0.08	21.57 ± 0.05	15.17 ± 0.24	11.03 ± 0.05
pH	**8.0**	8.34 ± 0.01	8.36 ± 0.06	8.31 ± 0.01	8.35 ± 0.01	8.07 ± 0.10	8.23 ± 0.01
DO mg/L	**N/A**	7.43 ± 0.12	14.43 ± 0.19	8.33 ± 0.37	7.13 ± 0.26	8.47 ± 0.52	14.87 ± 0.65
TDS mg/L	**370.0**	***639.00*** ± 4.55	***753.33*** ± 2.49	***838.00*** ± 1.63	***585.00*** ± 17.91	***753.67*** ± 1.25	***846.00*** ± 2.94
NO_3 mg/L	**0.3**	0.30 ± 0.00	***1.40*** ± 0.00	0.00 ± 0.00	0.20 ± 0.00	***12.0*** ± 0.00	0.00 ± 0.00
PO_4^{2-} mg/L	**0.4**	NA	***4.53*** ± 0.07	***7.24*** ± 0.18	NA	***2.46*** ± 0.03	***3.40*** ± 0.05
SO_4 mg/L	**75.0**	***150.00*** ± 38.00	***139.00*** ± 4.50	***122.50*** ± 1.50	***90.50*** ± 13.50	***102.00*** ± 2.50	***109.00*** ± 5.00
COD mg/L	**N/A**	2.00 ± 1.00	0.00 ± 0.00	7.50 ± 0.50	20.50 ± 5.50	0.00 ± 0.00	9.00 ± 0.00

RWQO- Resource water quality objective for the Mooi River catchment (DWAF, 2009), MR- Mooi River; BC- before confluence; AC- after confluence; Temp- Temperature; TDS-total dissolved solids; DO- dissolved oxygen; COD- chemical oxygen demand, N/A- not available; ± values after this symbol indicates the standard deviation from the average; Values in ***red italics*** exceeded the RWQOs set for the Mooi River catchment.

Table 2.2: Average physico-chemical variables recorded for the Mooi River sites during 2015

Parameter	RWQO	MR BC 1			MR BC 2		MR AC 1			MR AC 2	
		March	May	July	March	March	May	July	March	May	July
Temp °C	**N/A**	22.20 ± 0.08	17.20 ± 0.43	14.33 ± 0.12	27.77 ± 0.05	20.50 ± 0.08	17.77 ± 0.45	12.60 ± 0.45	21.68 ± 0.25	20.83 ± 0.42	14.10 ± 0.08
pH	**8.0**	8.18 ± 0.01	8.28 ± 0.02	8.40 ± 0.01	7.50 ± 0.07	7.78 ± 0.02	6.90 ± 2.81	8.05 ± 0.01	8.13 ± 0.00	8.32 ± 0.03	8.50 ± 0.01
DO mg/L	**N/A**	7.23 ± 0.95	13.63 ± 1.18	10.90 ± 0.29	6.77 ± 0.12	6.87 ± 0.52	13.87 ± 0.76	9.77 ± 0.19	6.30 ± 0.16	13.20 ± 0.29	10.60 ± 0.33
TDS mg/L	**370.0**	274.0 ± 0.00	350.00 ± 3.74	354.00 ± 1.41	***430.07*** ± 0.95	***504.33*** ± 3.09	***565.67*** ± 5.79	***603.00*** ± 2.16	***426.33*** ± 9.39	***548.33*** ± 18.86	***577.67*** ± 2.62
NO_3 mg/L	**0.3**	***0.50*** ± 0.20	***0.85*** ± 0.05	0.20 ± 0.10	***51.25*** ± 12.85	***1.05*** ± 0.05	***1.11*** ± 0.00	***1.60*** ± 0.10	***0.45*** ± 0.05	***0.45*** ± 0.05	***0.85*** ± 0.25
PO_4^{2-} mg/L	**0.4**	NA	0.07 ± 0.02	***0.55*** ± 0.23	NA	NA	0.21 ± 0.02	0.34 ± 0.11	NA	***0.42*** ± 0.01	***0.57*** ± 0.07
SO_4 mg/L	**75.0**	1.50 ± 0.50	4.0 ± 3.00	75.00 ± 10.50	0.00 ± 0.00	***89.00*** ± 1.00	***80.0*** ± 0.00	***93.50*** ± 2.50	***85.00*** ± 2.00	***80.0*** ± 0.00	***90.00*** ± 1.00
COD mg/L	**N/A**	22.50 ± 4.50	0.00 ± 0.00	3.00 ± 0.00	45.50 ± 3.50	8.00 ± 2.00	0.00 ± 0.00	0.00 ± 0.00	7.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00

RWQO- Resource water quality objective for the Mooi River catchment (DWAF,2009), MR- Mooi River; BC- before confluence; AC- after confluence; Temp- Temperature; TDS-total dissolved solids; DO- dissolved oxygen; COD- chemical oxygen demand, NA- not available; ± values after this symbol indicates the standard deviation from the average; Values in ***red italics*** exceeded the RWQOs set for the Mooi River catchment

Average river water temperatures consistently decreased between sampling occasions (Tables 2.1 and 2.2). This is ascribed to the change from summer to winter. Temperatures in March ranged between 20.50 and 27.80°C, in May between 15.17 and 20.83°C and in July colder temperatures between 11.00 and 14.90°C were recorded. A striking result observed is that the temperature of MR BC 2 was at least 5.22°C warmer compared to measured temperatures at the other sites. pH of the surface water generally varied between 7.50 and 8.50 for all three occasions, with the exception of MR AC 1 on the May occasion, where a lower pH average of 6.90 was recorded. Dissolved oxygen recorded in March (6.30 to 7.43 mg/L) was lower compared to May (8.47 to 14.43 mg/L) and July (8.33 to 14.87 mg/L). TDS consistently, with the exception of site MR BC 1, exceeded the RWQO (370.00 mg/L) at all sites. Average TDS concentrations in the WFS (585.0 to 846.00 mg/L) was consistently higher than the MR (274.00 to 603.00 mg/L) and an increased trend from MR before confluence to MR after confluence sites for this parameter is observed.

Nitrate concentrations of the MR consistently, except MR BC 1 on July, exceeded the RWQO (0.30 mg/L) with some concentrations of up to 51.25 mg/L recorded at MR-BC 2. Nitrate levels exceeding the RWQO (0.30 mg/L) in the WFS (1.40 and 12.0 mg/L) were only recorded during the May sampling occasion. Phosphates were included for May and July. In May phosphate concentrations exceeding the RWQO (0.40 mg/L) was recorded at MR AC 1 and at both of the WFS sites. This trend continued in July with the inclusion of the MR BC 1 site. The highest phosphate concentrations (2.46 to 7.43 mg/L) were recorded in the WFS sites. Average sulphate levels consistently exceeded the RWQO (75.0 mg/L) at both the WFS and the two MR-AC sites (>85 mg/L). Both phosphates and sulphates showed a general increase in concentrations from the warmer to the colder sampling occasion.

2.3.2 Heterotrophic plate count bacteria levels

The average HPCs enumerated on R2A and R2A supplemented with different antimicrobials from MR and WFS sites, on three sampling occasions in 2015, are summarized in Table 2.3 and 2.4 respectively. There are no specific water quality guidelines set out for HPC bacteria in surface water. No HPC bacteria were detected at MR BC 1 during the March sampling occasion and no water was found at MR BC 2 (May and July), thus these results are excluded from Table 2.3. HPC levels on un-supplemented R2A media ranged between 6.66×10^2 to 2.75×10^6 CFU/mL. The levels of these bacteria were one or more logs lower in July compared to March, with a general decrease in levels from warmer to colder sampling occasions.

Table 2.3: Heterotrophic plate counts (HPCs) and antimicrobial resistant HPCs of the Wonderfonteinspruit sites on three sampling occasions in 2015 (CFU/mL)

Plate Counts	Am. Cons.	WFS 1			WFS 2		
		March	May	July	March	May	July
HPC		2.75 x10^6	1.53 x10^6	1.32 x10^5	5.47 x10^6	1.31 x10^5	9.10 x10^3
***HPC-Ap**	**100 µg/ml**	2.62 x10^3	4.72 x10^3	4.11 x10^3	3.31 x10^3	3.55 x10^3	5.20 x10^2
****HPC-Cu**	**1 mM**	1.14 x10^3	1.17 x10^3	2.40 x10^2	9.10 x10^2	1.55 x10^3	ND
****HPC-Fe**	**1 mM**	5.15 x10^3	2.50 x10^3	8.20 x10^3	9.44 x10^3	2.38 x10^3	1.04 x10^3
	2.5 mM	2.13 x10^1	6.00 x10^1	1.40 x10^1	3.58 x10^2	ND	ND
****HPC-Pb**	**1 mM**	1.10 x10^5	6.42 x10^5	2.24 x10^4	4.76 x10^4	8.62 x10^4	1.06 x10^4
	2.5 mM	1.47 x10^3	4.93 x10^3	7.25 x10^3	2.26 x10^3	9.58 x10^2	1.08 x10^2
****HPC-Zn**	**1 mM**	1.08 x10^3	2.80 x10^2	3.00 x10^2	2.23 x10^3	2.08 x10^2	6.50 x10^1
	2.5 mM	2.80 x10^2	4.00 x10^1	3.00 x10^1	1.55 x10^2	ND	ND

Am.Cons.- Concentration of the antimicrobial in the media; WFS- Wonderfonteinspruit; HPC- heterotrophic plate count; Ap- Ampicillin, ND- None detected

* HPC from antibiotic containing media

** HPC from metal containing media

Table 2.4: Heterotrophic plate counts (HPCs) and antimicrobial resistant HPCs of the Mooi River sites on three sampling occasions in 2015

Plate Counts	Am. Cons.	MR BC 1		MR BC 2		MR AC 1			MR AC 2	
		May	July	March	March	May	July	March	May	July
HPC		4.75×10^5	3.40×10^4	2.30×10^6	1.47×10^5	5.16×10^5	2.65×10^4	1.02×10^5	6.83×10^4	2.0×10^3
***HPC-Ap**	**100 µg/ml**	1.52×10^3	1.50×10^1	1.27×10^5	4.31×10^3	2.00×10^3	9.50×10^1	2.63×10^3	2.62×10^3	4.00×10^1
****HPC-Cu**	**1 mM**	ND	ND	2.83×10^3	ND	ND	ND	ND	1.56×10^2	ND
****HPC-Fe**	**1 mM**	2.25×10^2	9.70×10^1	2.20×10^5	1.00×10^3	4.85×10^3	2.50×10^2	1.78×10^3	3.01×10^3	7.50×10^1
	2.5 mM	1.00×10^1	ND	2.42×10^3	1.30×10^2	2.31×10^1	ND	1.80×10^2	5.82×10^1	ND
****HPC-Pb**	**1 mM**	8.33×10^2	7.70×10^1	2.80×10^5	2.00×10^4	2.58×10^4	2.93×10^2	2.09×10^4	4.85×10^4	3.00×10^2
	2.5 mM	1.95×10^1	ND	1.75×10^5	1.05×10^3	4.69×10^2	ND	4.00×10^4	2.91×10^3	ND
****HPC-Zn**	**1 mM**	5.70×10^1	ND	4.15×10^4	7.40×10^2	2.65×10^2	6.70×10^1	1.25×10^2	2.50×10^2	ND
	2.5 mM	2.28×10^1	ND	2.00×10^4	3.18×10^2	1.40×10^2	ND	7.00×10^1	4.84×10^1	ND

Am.Cons.- Concentration of the antimicrobial in the media; MR- Mooi River; BC- before confluence; AC- after confluence; HPC- heterotrophic plate count; Ap- Ampicillin, ND – None detected

* HPC from antibiotic containing media

** HPC from metal containing media

HPC bacteria were found on at least the lowest concentration of all the antimicrobial (ampicillin, Cu, Fe, Pb and Zn) containing R2A media (Tables 2.3 and 2.4). Ampicillin, Fe and Pb resistant bacteria was detected at all the sites on all three occasions.

The metal resistant HPC levels were generally one to two logs higher on the 1 mM than on the 2.5 mM metal concentration. An exception to this was the HPC levels on the two concentrations of Zn at MR BC 2 and MR AC 1 sites in March and July (MR AC 1). In this case there was no log difference (Table 2.4). Overall the HPC bacteria was most susceptible to Cu with levels ranging between 0.00 – 2.83 x 10^3 on 1mM Cu plates. No HPCs were detected on 2.5 mM Cu containing R2A and therefore results at this concentration was not included in Tables 2.3 and 2.4. The HPC bacteria were least susceptible to Pb with levels ranging between 3.00 x 10^1 to 2.80 x 10^5 CFU/mL.

The March occasion showed MR BC 2 to have the highest number of resistant HPC bacteria to all the antimicrobials when compared to the levels of all the other sites. However, in general, across the different sampling dates, the two WFS had higher levels of resistant bacteria to the antimicrobials when compared to the MR sites. The WFS 1 site generally had higher levels of these antimicrobial resistant HPC bacteria compared to the WFS 2.

2.3.3 Relationship between physico-chemical and enumerated HPC bacteria

Figure 2.2 display a RDA correlation bi-plot of the overall relationship between average physico-chemical parameters and enumerated HPC bacteria at the five sites. Averages for each parameter from the different sampling occasions were determined and analyzed. The data from MR BC 2 was not included in the analysis, as only one data set was available for this site. Furthermore, only HPC results from the 1 mM concentration of metal containing media was included in the analysis

The HPC levels (antimicrobial resistance HPC included) correlated with TDS, sulphates and phosphates. Based on the angle of vectors, it can be concluded that these correlations were strong with the exception of HPC-Cu resistant levels. Strong correlations between sulphates and HPC-Zn, HPC-Pb and, to a lesser extent, HPC-Fe resistant bacteria was found. HPC-Fe resistant bacteria correlated stronger with TDS compared to the other HPC levels. HPC-ampicillin resistant bacteria correlated strongest with phosphate levels. These parameters correlated the strongest with WFS 1. The WFS 2 and MR AC 1 site correlated strongly with nitrate levels. COD and DO had strong negative correlation with the HPC bacteria levels. These two parameters had strong correlations with MR BC 1.

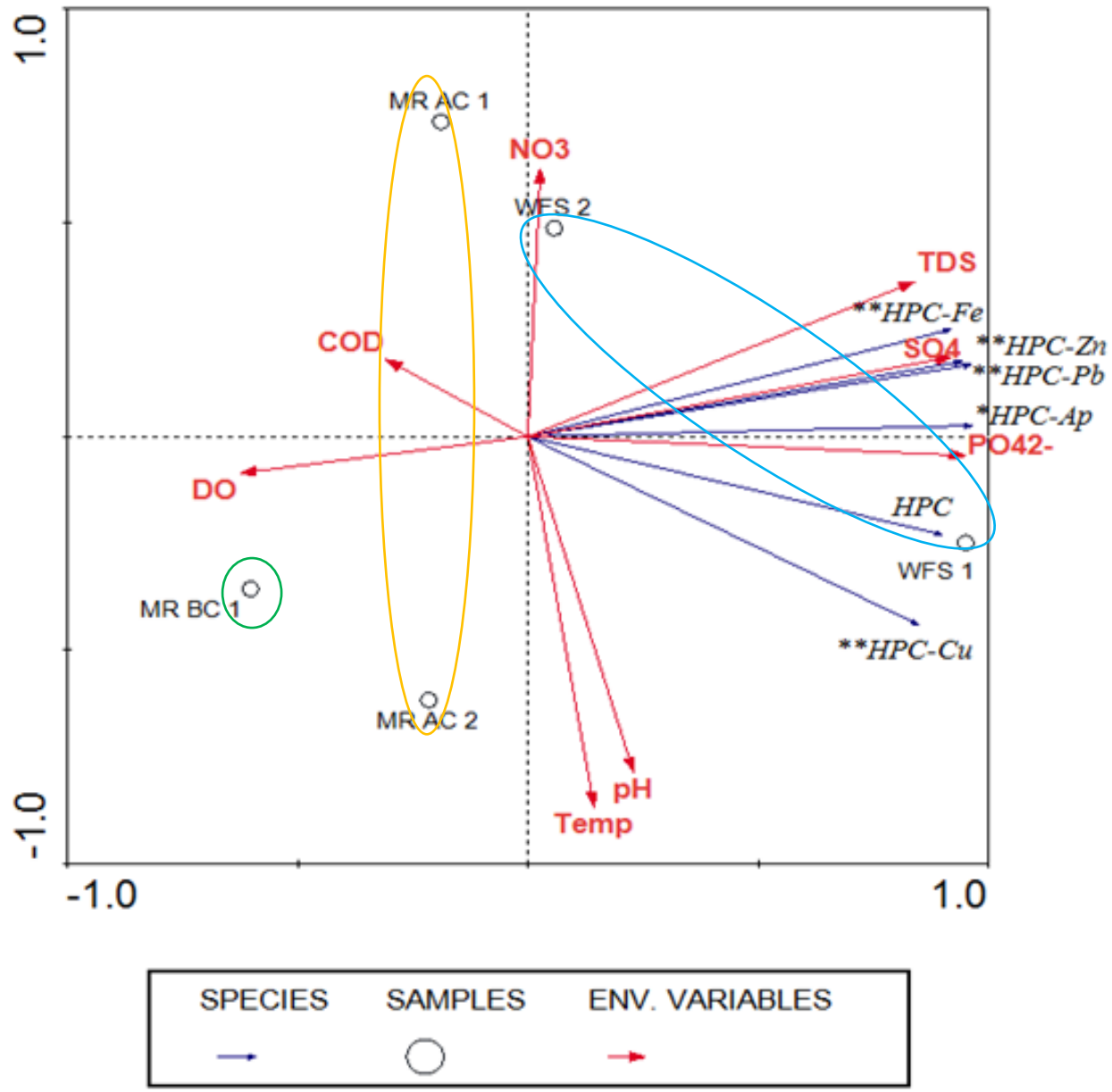


Figure 2.2: Redundancy analysis (RDA) correlation bi-plot indicating the statistical relationship between the average physico-chemical and HPC/ antimicrobial resistant HPC results from five sites of the overall averages of 2015.

MR- Mooi River, WFS- Wonderfonteinspruit, BC- before confluence, AC- after confluence, Temp- Temperature; TDS- total dissolved solids; DO- dissolved oxygen; COD- chemical oxygen demand, ; HPC- heterotrophic plate count
* HPC from 100 µg/mL ampicillin containing media
** HPC from 1 mM metal containing media

Temperature and pH showed positive correlation with the MR AC 2 site (Figure 2.2). From the RDA analysis of the physico-chemical and HPC levels presented in Figure 2.2 it is evident that the observed trends could be ascribed to the relationship between these two data sets (physico-chemical parameter levels on the one hand and HPC levels on the other).

2.3.4 Percentage of purified antimicrobial resistant isolates resistant to selected antibiotics

Morphologically distinct isolates (n=321) were purified and screened for antibiotic resistance by disc diffusion. The March sampling occasion accounted for 129 purified and screened isolates, 128 isolates were purified and screened during the May sampling occasion and 76 during July. Seven antibiotics belonging to six antibiotic classes were incorporated in the screening. Figure 2.3 illustrates the overall percentage from all the purified isolates from three sampling occasions that were resistant to each of the seven antibiotics screened for per site. Also included in the charts are the percentages of isolates found at each site that had multiple antibiotic resistance (MAR). These MAR isolates were resistant to three or more classes of antibiotics. The total number of isolates screened for at each site are indicated in the brackets next to the site name.

Figure 2.3 indicates that resistance to each of the antibiotics were found at all the sites. The two β-lactam antibiotics amoxicillin (Ax) and ampicillin (Ap) consistently showed the highest percentages (58.0 to 65.5% and 58.0 to 63.1% respectively) of resistant bacteria, followed by trimethoprim (Tm) (41.9 to 54.4%) for most sites from the total pool of isolates. Percentages of isolates resistant to Ax and Ap differed from one another indicating a variance in resistance phenotypes and potentially also genotypes. Tetracycline (Tt) (30µg) had the lowest percentages (7.4 to 28.2%) of resistant isolates at most sites followed by streptomycin (St) (12.9 to 46.2%). Percentages of isolates resistant to erythromycin (Er) ranged between 23.4 to 35.1%. Between 25.6 to 47.7% of isolates from the various sites were resistant to chloramphenicol (Cp) (30µg).

MAR percentages of more than 35% was found at all the sites of the total purified. WFS 1 site indicated the highest percentages of isolates resistant to most antibiotics compared to the other sites, except for St and Cp. The highest percentage of MAR isolates was also found at this site. MR BC 2 and MR AC 2 had higher percentages of isolates resistant to St. The highest percentage of Cp resistant isolates was observed at MR BC 1 and MR AC 2.

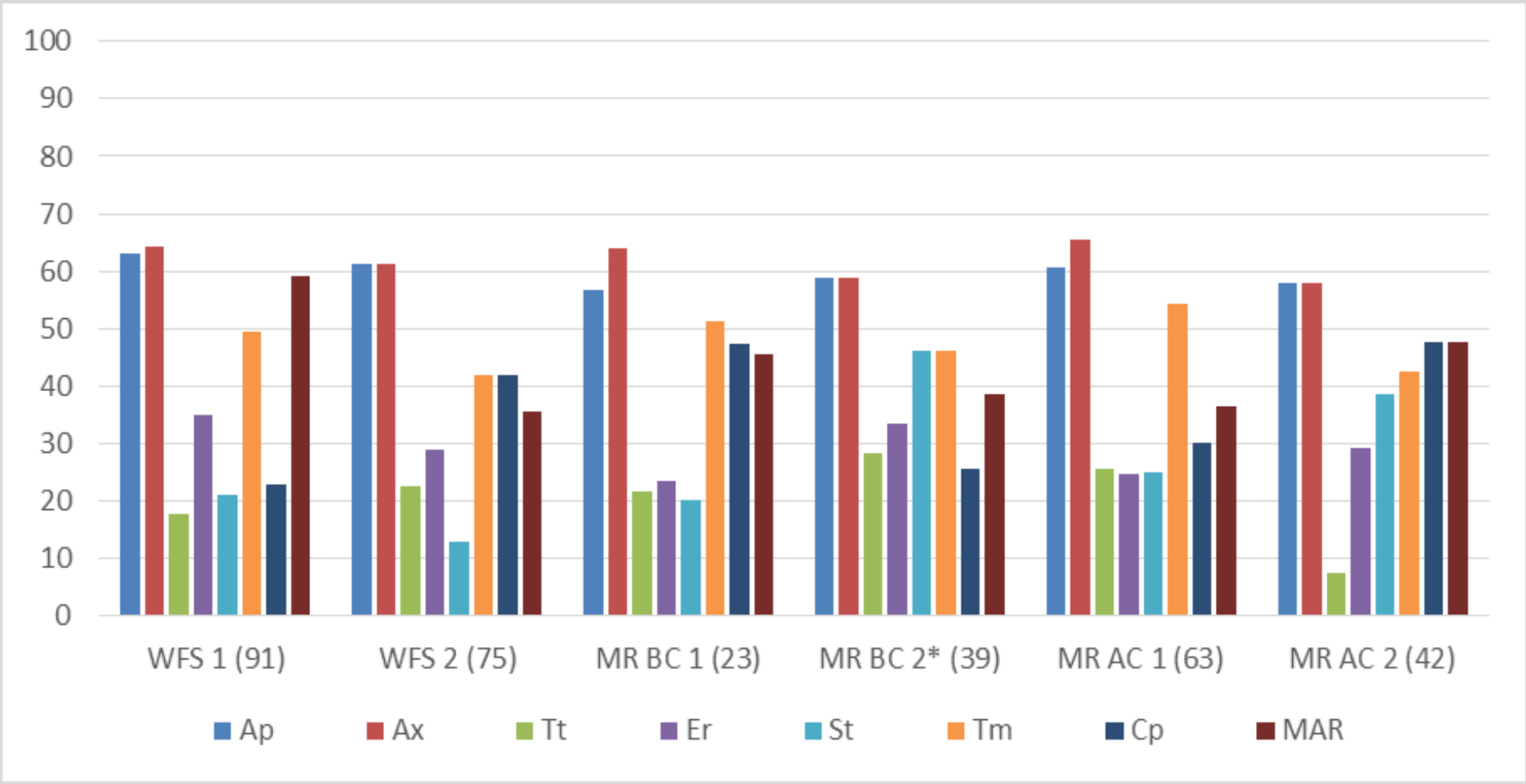


Figure 2.3: Bar chart illustrating the percentages of purified HPC isolates resistant to different antibiotics and percentage multiple antibiotic resistant HPC at sites surrounding the WFS and MR confluence

WFS- Wonderfonteinspruit; MR- Mooi River; BC- before confluence; AC- after confluence; Ap- ampicillin; Ax- amoxicillin; Tt tetracycline; Er- erythromycin; St-streptomycin; Tm- trimethoprim; Cp-chloramphenicol; MAR- multiple antibiotic resistant

(#) Numbers in brackets after site names indicate the number of isolates that the percentage was calculated from for that specific site

* MR BC 2 data indicated on the chart only represents one sampling occasion

Site MR BC 1 also had higher percentages of isolates resistant to Ax, St, Tm, Cp and MAR compared to WFS 2. However, the number of isolates included in the analyses differed considerably. Only 23 isolates were screened at MR BC 1 compared to the 75 at WFS 2. An increase in percentage isolates resistant to most antibiotics is observed from the MR AC 1 to MR AC 2. Eighty two percent of the metal resistant isolates were resistant to at least one antibiotic that it was screened for. Thus, this potentially points towards co-resistance to antibiotics and metals among these isolates.

2.3.5 Multiple antibiotic resistant phenotypes

Thirty two MAR phenotypes were detected from 133 MAR isolates analyzed. A summary of all of the MAR phenotypes and the number of isolates that displayed the specific phenotype per site is summarized in Table 2.5. The MAR phenotype in which isolates were resistant to all of the antibiotics (Ax, Ap, Tt, Er, St, Tm, Cp) was observed among 29 isolates and was observed at all the sites, but was present more frequently among isolates from MR BC 2 (n=8), WFS 2 and MR AC 2 (both n=7). Only 3 isolates each from WFS1 and MR AC 1 were resistant to all of the antibiotics even though 37 and 24 of the total MAR isolates were from these sites respectively. Ten of the MAR isolates were from the control site MR BC 1 of which only 1 was resistant to all seven antibiotics. Furthermore, 35.1 % of the MAR isolates were resistant to at least five classes of antibiotics that were screened for.

The results of the AHC analysis for the three sampling occasions are illustrated in Figures 2.4 to 2.6. Cluster centroid (CC) scores and the distances between clusters that were generated by the software XLSTAT (v 2013.5.00) are summarized in Appendix E. CC-scores provide the average score that the members of that cluster had for a specific antibiotic. A CC- score above 7 indicated that the most of the isolates in that cluster were resistant to the antibiotic, a CC-score between 4 and 7 indicated that some isolates in the cluster were resistant or intermediate resistant to the antibiotic and a score below 4 indicated that most/all of the isolates of that cluster were susceptible to the antibiotic.

a) Cluster analysis of MAR isolates from the March sampling period

A total of 54 of the March MAR HPC isolates were subjected to AHC analysis of the inhibition zone data. In Figure 2.4 three branches are formed above the automatic truncation point (dotted line) calculated by the software (XLSTAT v 2013.5.00). Thus, the antibiotic resistance profiles of the MAR isolates from this sampling period grouped into three clusters (A, B and C).

Table 2.5: Multiple antibiotic resistant phenotypes

	Multiple antibiotic resistant phenotype	Number of isolates observed with MAR phenotype / site					
		WFS1	WFS2	MRBC1	MRBC2	MRAC1	MRAC2
1	Ax, Ap, Tt, Er, St, Tm, Cp	3	7	1	8	3	7
2	Ax, Ap, Er, Tm, Cp	2	3	1	1	2	4
	Ax, Ap, Tt, Tm	4	3	1	1	2	2
4	Ax, Ap, Er, Tm	6			2	1	
5	Ax, Ap, St, Tm	3			1	2	2
	Ax, Ap, Tt, Er, St, Tm	2	3	1		2	
7	Ax, Ap, Tt, Er, Tm, Cp	3	2	1			1
8	Ax, Ap, Tt, St, Cp	1	1			3	
9	Ax, Ap, St, Tm, Cp			2			2
10	Ax, Ap, Er, St, Tm	1				1	1
	Ax, Ap, St, Cp	1				1	1
	Ax, Ap, Tm, Cp	1		1		1	
13	Ax, Ap, Tt, St, Tm	1					1
	Ax, Ap, Tt, Tm, Cp	1					1
	Ax, Ap, Er, St,	2					
	Ax, Ap, Tt, Er, Tm	1				1	
	Ax, Ap, Tt, Cp	1	1				
	Ax, Ap, Tt, Er, St	1			1		
	Ap, St, Tm				1	1	
	Ax, Ap, Er, St, Tm, Cp						2
	St, Tm, Cp				1		1
22	Ax, Ap, Er, Cp	1					
	Tt, Er, St, Tm	1					
	Tt, Er, Tm	1					
	Ap, Er, Tm			1			
	Er, Tm, Cp			1			
	Ax, St, Cp				1		
	Ap, Tm, Cp					1	
	Tt, Tm, Cp					1	
	Ax, Er, St, Tm, Cp					1	
	Tt, St, Tm						1
	Ax, Ap, Tt, Er, St, Cp,					1	

WFS- Wonderfonteinspruit, MR- Mooi River, BC- before confluence, AC- after confluence, Ax- amoxicillin, Ap- ampicillin, Tt- tetracycline, Er- erythromycin, St- streptomycin, Tm- trimethoprim, Cp- chloramphenicol

Cluster A consists of 21 isolates divided into 2 sub-clusters (A1 and A2). CC data suggests that the average member of cluster A is resistant to Ax (CC-score: 7.95), Ap (CC-score: 8.24) and Tm (CC score: 7.62). Some members are predicted to be resistant or intermediate to Tt (CC-score: 6.05), St (CC-score: 5.57) and Cp (CC-score: 4.26). Furthermore, members in this cluster in general have no resistance to Er. Thus, the general antibiotic resistance pattern of this cluster is Ap > Ax > Tm > (Tt) > (St) > (Cp) (antibiotics in brackets had an intermediate CC score in the specific cluster).

Cluster B is composed of 12 isolates divided into two sub-clusters (B1 and B2). The general antibiotic resistance pattern for major cluster B predicted from CC data is Ax = Ap = Tm (CC-score: 10.00) > Er (CC-score: 9.25). Some members are resistant to Tt (CC-score: 5.750) and Cp (CC-score: 5.58). Furthermore, the CC data suggests that generally members of Cluster B is not resistant to St. There is less dissimilarity between the sub-clusters of major cluster B compared to the sub-clusters of major clusters A and C, as they are joined together at a lower point on the dendrogram. Isolates of cluster B were isolated from Ap, Cu, Zn and Pb at the WFS 1, MR BC 2 and MR AC 1 sites.

CC data suggest that the average member of the 21 isolates from major cluster C, are resistant to all of the antibiotics screened for. The general antibiotic resistance pattern of cluster C is Ap (CC-score: 10.00) > Ax = St (CC-score: 9.91) > Tt (CC-score: 9.76) > Er (CC-score: 9.52) > Tm (CC-score: 9.38) > Cp (CC-score: 7.14). However, in-cluster variance is observed with the cluster splitting into two sub-clusters. Sub-cluster C1 consist of the members that were resistant to all of the antibiotics screened for. The members of sub-cluster C2 were resistant to many of the antibiotics with different scores for each. The isolates from major cluster C were isolated from the two WFS sites, MR BC 2 and MR AC 1, indicating that isolates at these sites were generally resistant to more antibiotics compared to the MR BC 1 and MR AC 2 sites.

Additional clusters within the sub-clusters of the dendrogram, indicate variance from the general antibiotic resistance pattern of the major cluster and variance in resistance scores towards the separate antibiotics. Major clusters B and C were more similar to one another than to cluster A, as the point where they join are lower on the dendrogram compared to cluster A. Members of these two clusters (B and C) were generally resistant to more antibiotics, compared to the members of cluster A. A greater number of isolates clustered into major clusters B and C indicating that antibiotic resistance patterns that are composed of larger numbers of antibiotics were more prevalent compared to antibiotic resistance patterns with fewer antibiotics.

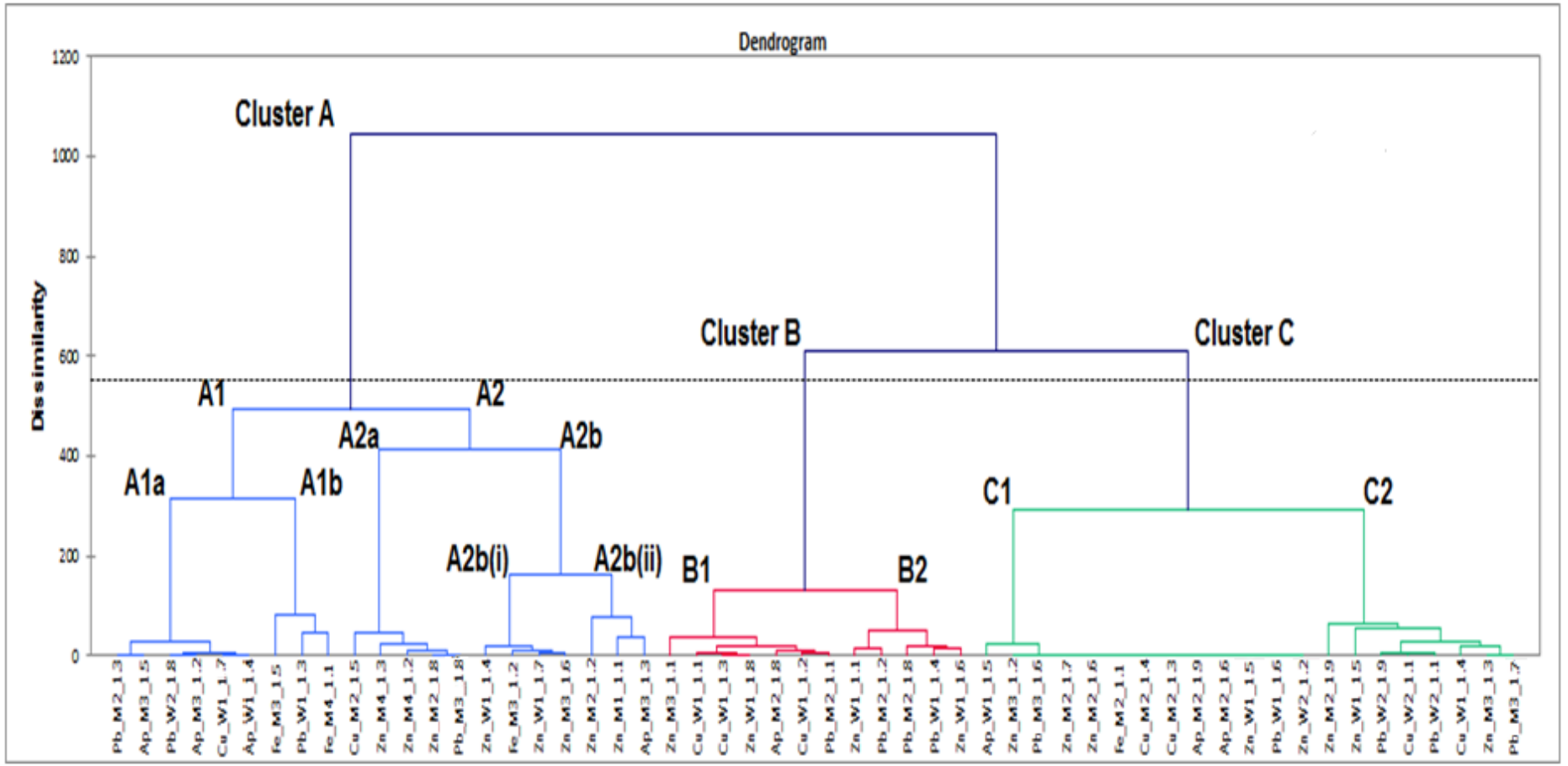


Figure 2.4: Dendrogram illustrating the relationship of 54 multiple antibiotic resistant heterotrophic plate count bacteria isolated from antimicrobial supplemented R2A media from 6 surface water sites surrounding the MR and WFS confluence, on the March sampling period. Antibiotic inhibition zone diameter data was used to compile the cluster formation using Ward's method and Euclidean distances in XLSTAT (v 2013.5.00). The isolate label indicates the **antimicrobial** it was isolated from**,** the **site** at which it was isolated**,** the **sampling occasion** and the **sample** number (Appendix D).

b) Cluster analysis of MAR isolates from the May sampling period

A total of 51 MAR HPC isolates from the May sampling were subjected to AHC analysis. The dendrogram in Figure 2.5 illustrate that the tree branched into three clusters (A, B and C) above the automatic truncation point. In Figure 2.5, twenty eight of the MAR isolates group into cluster A, seven into cluster B and sixteen into cluster C. The general ARP of cluster A is predicted to be Tm (CC-score: 9.86) > Cp (CC-score: 9.64) > Ax (CC-score: 9.43) > Ap (CC-score: 9.07) > Er (CC-score: 8.46) > St (CC-score: 7.54). Some isolates of cluster A were also resistant to Tt which has a CC-score of 5.71. All of the members of minor sub-cluster A1a were resistant to all of the antibiotics screened for. In general isolates from this cluster had ARPs consisting of more antibiotics (at once) compared to clusters B and C. Furthermore, the cluster represented isolates from all of the sampling sites and antimicrobial containing media. Thus, the general antibiotic resistance pattern of this cluster is widely disseminated among the sites and accompanied by resistance to a range of antimicrobials.

Cluster B consists of 7 isolates with a general ARP of Ax = Ap (CC-score: 10.00) > Tt (CC-score: 9.43) > Cp (CC-score: 8.86) > St (CC score: 6.86). The members of cluster B were not considerably dissimilar as the two sub-clusters and minor sub-clusters are joined very low on the dendrogram. Therefore, in general there is not much variance in antibiotic resistance patterns of the isolates that is predicted by the CC data. Members of this cluster were isolated from all of the antimicrobial containing media, except Fe and were isolated at the two WFS and two MR AC sites.

Cluster C is more similar to cluster B due to members of these two clusters displaying resistance to fewer antibiotics compared to those in cluster A. However, the antibiotics to which they display resistance differs considerably, hence the split of the two clusters above the automatic truncation point. The general antibiotic resistance pattern of the 16 isolates of Cluster C, according to the CC data, is: Ap (CC-score: 9.44) > Ax (CC- score: 9.13) > Tm (CC-score: 8.88) > Tt (CC-score: 7.19), with some isolates being resistant to Er (CC-score: 4.63) and St (CC-score: 4.63). Cluster C is sub-divided into 2 sub-clusters C1 and C2. Based on the height on the dendrogram at which these two sub-clusters are joint, it is evident that the members of these sub-clusters are considerably dissimilar.

Important trends highlighted in Figure 2.5 are that as was observed for the March isolates (Figure 2.4) most of the isolates were resistant to not only three or four antibiotic classes but to five or six. Furthermore, major clusters did not differentiate between isolates from different sites or isolation media, however partial groupings of these features was evident in minor sub-clustering.

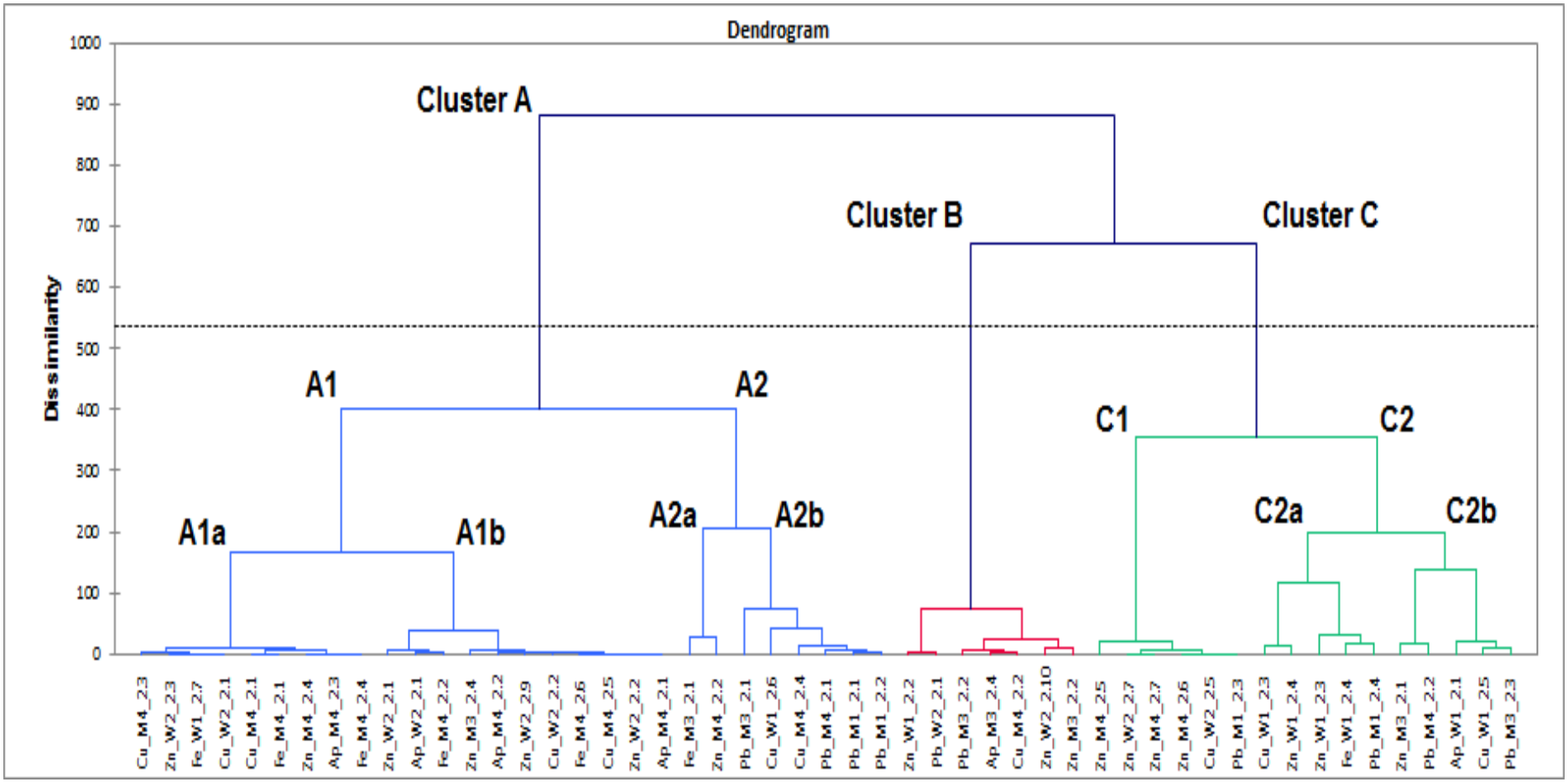


Figure 2.5: Dendrogram illustrating the relationship of 51 multiple antibiotic resistant heterotrophic plate count bacteria isolated from antimicrobial supplemented R2A media from 5 surface water sites, surrounding the MR and WFS confluence, on the May sampling period. Antibiotic inhibition zone diameter data was used to compile the cluster formation using Ward's method and Euclidean distances in XLSTAT (v 2013.5.00). The isolate label indicates the antimicrobial it was isolated from, the site at which it was isolated, the sampling occasion and the sample number (Appendix D).

c) Cluster analysis of MAR isolates from the July sampling period

Twenty eight MAR HPC isolates from the July sampling period were subjected to AHC analysis and is illustrated in (Figure 2.6). Once again the dendrogram branched into three clusters above the automatic truncation line (dotted line).

In Figure 2.6, cluster A consists of 9 isolates with a general antibiotic resistance profile of Ax = Ap (CC-score: 9.56) > Tm (CC-score: 8.89) > St (CC-score: 7.00). However, the cluster sub-divides into two branches just below the automatic truncation line, indicating that there is still a substantial degree of dissimilarity between the antibiotic resistance profiles of the members in cluster A. Most of the isolates from this cluster, except Pb_M4_3.1, were isolated from Ap and Zn containing media. In cluster A there were six isolates from the WFS 1 site while the other three were from the two MR AC sites.

Fifteen isolates formed cluster B and they have a general antibiotic resistance pattern of Ax = Ap = Tm (CC-score: 9.73) > Er (CC-score: 9.53) > Cp (CC-score: 8.67) > St (CC-score: 7.40). Some of the isolates of cluster B were also resistant to Tt (CC-score: 6.67). Antibiotic resistance patterns of Isolates of the two sub-clusters (B1 and B2) are more similar to one another than the isolates of sub-clusters in the other two major clusters of the dendrogram (Figure 2.6), since the sub-clusters B1 and B2 are joined at a lower point on the dendrogram. The members of minor sub-cluster B2a were resistant to all of the antibiotics screened for, except Fe_M4_3.1 that is intermediate for Tt. The two isolates of minor sub-cluster B2b were resistant to all but Cp. Three isolates (Zn_W2_3.1, Zn_W1_3.4 and Ap_M1_3.1) of minor sub-cluster B1a were resistant to all but St. Isolates from all the sampling sites clustered in B.

Four isolates group into cluster C. Three isolates were isolated from Pb containing media and one from Ap containing media. Two isolates from cluster C (Pb_M1_3.3 and Pb_M1 _3.4) were isolated at the control site (MR BC 1). CC data suggest that the isolates in cluster C were resistant to Cp (CC-score: 9.50) > Er (CC-score: 7.50) > Ap (CC-score: 7.00). CC-scores from 4.00 to 6.25 for Ax, St and Tm indicate that some isolates may also be resistant to these antibiotics. The absence of a flat line at the base of the dendrogram indicate that each of the isolates in this cluster has a unique ARP.

The overall observation from figures 2.4 to 2.6 is that site or antimicrobial specific selection media could not conclusively predict the antimicrobial resistance patterns. However, partial grouping of isolates from the same sample site/media in minor clusters were observed. This particular method/approach is quite crude, but it shows potential in source tracking and may also have some other applications in antibiotic resistance studies.

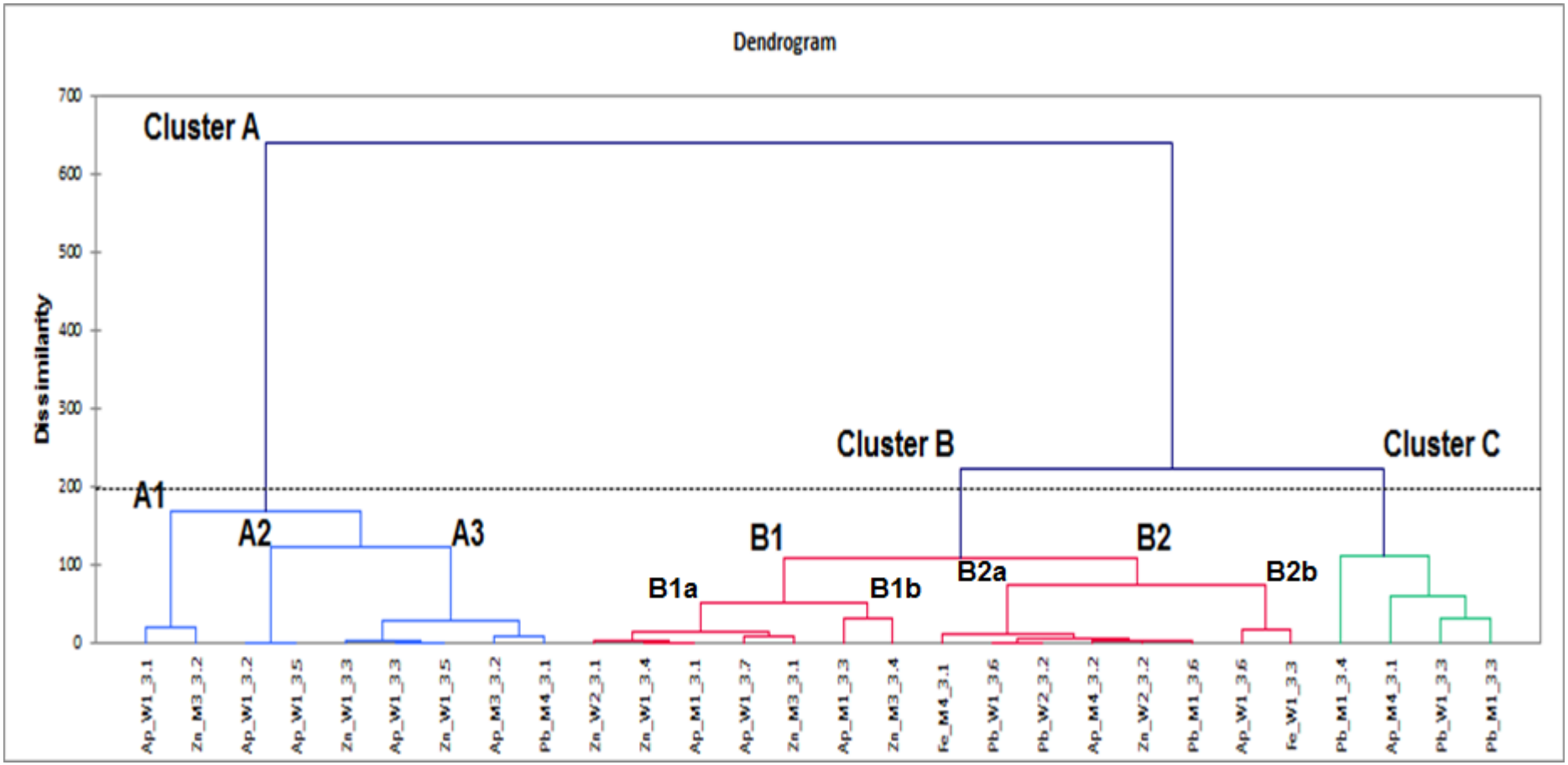


Figure 2.6: Dendrogram illustrating the relationship of 28 multiple antibiotic resistant heterotrophic plate count bacteria isolated from antimicrobial supplemented R2A media from 5 surface water sites, surrounding the MR and WFS confluence, on the July sampling period. Antibiotic inhibition zone diameter data was used to compile the cluster formation using Ward's method and Euclidean distances in XLSTAT (v 2013.5.00). The isolate label indicates the antimicrobial it was isolated from, the site at which it was isolated, the sampling occasion and the sample number (Appendix D).

2.4 Discussion

The natural environment, and water sources in particular have enjoyed increased attention as important hosts and disseminators of antibiotic resistance (Berglund, 2015; Chudobova *et al*., 2014; Biyela *et al*., 2004). Various anthropogenic and biological activities have been documented to be involve in selection of this phenomenon. The aim of this study was to investigate antimicrobial resistant HPC bacteria at sites surrounding the WFS and MR confluence and to determine if these could be linked to physico-chemical parameters.

2.4.1 Physico-chemical quality of surface water at WFS and MR sites in 2015

Water temperatures are influenced by hydrological (e.g. effluent temperatures, source of water, water volume, tributaries and groundwater contribution), climatic (e.g. cloud cover, solar radiation, air temperatures, precipitation and evaporation), regional (e.g. longitude and latitude) and structural (topography, slope, riparian cover, geology, water depth and turbidity) factors (Dallas, 2008; Makhlough, 2008; Ahipathy and Puttaiah, 2006; DWAF, 1996). Seasonal changes are reflected in the temperature results of the current study, which corresponds with the sinusoidal pattern explained by Dallas (2008). There is no specific resource water quality objective (RWQO) set out for surface water temperature for the MR catchment, however temperature influences most of the other parameters (Delpla *et al*., 2009). The high temperatures of the MR BC 2 site could be attributed to the low water depth observed (visually) on the day of sampling (Dallas, 2008).

pH levels of the sampling sites of the current study was expected to be more acidic due to mining impacts (DWAF, 1996). However, slight alkaline pH levels consistently detected across all the sites, are attributed to the dolomitic nature of the environment causing a buffering equilibrium (Barnard *et al*., 2013; Dallas and Day, 2004). Higher pH may cause particles to be less soluble in water and to cleave to the sediment (Venkatesharaju *et al*., 2010; Banks *et al*., 2004). Lower pH levels at the MR BC 2 and MR AC 1 sites could possibly indicate leaching of water, with a lower pH, from the water table (Winde, 2010). The water table in the vicinity of these sites are relatively close to the surface and it has been reported that mining polluted water recharge to the surface via karst springs (Durand, 2012; Coetzee *et al*., 2006; Van der Walt *et al.*, 2002).

High TDS levels indicate the presence of inorganic salts and organic materials in the surface water. This could possibly be attributed to industrial run-off, non-point source pollution or agricultural run-off (WHO, 2003). TDS levels were measured as higher on the colder occasions compared to warm sampling occasions. This is attributed to increased atmospheric temperatures combined with low

precipitation rates that lead to an increase in evaporation and transpiration rates that ultimately result in higher ionic concentrations (Ideriah *et al*., 2010). Considering results of the chemical analysis, certain assumptions of activities that contribute to the varying TDS levels can be made. In addition to the chemical parameters measured in the current study, TDS levels exceeding the RWQOs could be attributed to the presence of other harmful contaminants or metals, as was measured by Tlokwe Municipality in the vicinity around the same time as the current study (Dallas and Day, 2004; Appendix A).

High sulphate levels in the WFS could be an indication of mining pollution, as vast amounts of sulphate salts are released during the processing of ores (Barnard *et al*., 2013; Durand, 2012; DWAF 2009; Van der Walt *et al.*, 2002). Furthermore, a reaction between sulphuric acid and dolomite or limestone yields sulphate salts in the environment (Durand, 2012). The lower levels of sulphates detected in the water at MR sites before the WFS confluence compared to MR sites after the confluence could be used to argue for mining impacts on the sulphate levels. Similar trends were observed by Barnard and co-workers (2013). In their study, Barnard *et al*. (2013) measured higher sulphate levels in the Boskop and Potchefstroom dams (after the confluence) compared to Klerkskraal dam. They attributed this to mining pollution. Results of the current and previous studies suggest that the sulphate pollution in the MR is as result of the highly polluted WFS that converge with it. The slightly lower sulphate concentrations at the two MR AC sites when compared to the WFS sites represents a diluting effect after the addition of water from the upper MR (Klerkskraal dam).

Nitrate levels of the WFS indicate that agricultural run-off may impact the water quality (Suthar *et al*., 2009; Yang *et al*., 2007). However, this was seen more prevalently in the MR as nitrate levels frequently exceeded the RWQOs at all the sites. Recorded nitrate concentration at the MR BC 2 (March) site was approximately 128 times higher than the RWQOs set out by the DWA (DWAF, 2009) for the MR catchment. The high nitrate levels at this site may thus also contribute towards the high TDS levels. These results contradicted that recorded by Jordaan and Bezuidenhout (2016), where the specific site (Muiskraal) was their control site in a study investigating the bacterial community and physico-chemical quality of the greater MR in 2012. On the day of sampling cattle were grazing close to this site and the water level was low. It is possible that the cattle may have disturbed the sediment. The latter activity could have released nutrients and bacteria into the water column similar to that found by Line and co-workers (1998), which could explain the recorded nitrate and TDS levels at this site. Due to the drought conditions for the region this site was dry during the July sampling run and comparative data were not available. The elevated nitrate levels at the MR-BC 2 site, was not

disseminated to downstream sites, also indicating that this was a site specific occurrence and that the disturbance did not have a downstream impact. Other possible culprits of nitrate contamination include animal waste, pesticides, poor managed sewage water (septic tanks) and detergents (Dallas and Day, 2004; Hill and Olckers, 2001).

Elevated phosphate levels may also be attributed to agricultural practices across the entire system, which release phosphates and contribute to poor water quality of all the sites (Zhang *et al*., 2012; Razak *et al*., 2009). Considerably higher phosphate levels were measured in the WFS compared to the MR. This could also be attributed to Pb resistant bacteria occurring in the system. Such bacteria could precipitate Pb as a phosphate salt (Chudobova et *al.*, 2014). Continuous elevated nitrate and phosphate levels may result into eutrophication. This becomes even more apparent when high phosphate concentrations are present, as phosphate is seen as a limiting factor for eutrophication (Oberholster and Ashton, 2008).

2.4.2 Heterotrophic plate count (HPC) assays

a. HPC bacteria as an indirect indicator of water quality

The constant decrease in HPC bacteria from warmer river temperatures to colder temperatures may be attributed to temperature induced stress that result in reduced bacterial growth rates and survival of certain species (Jordaan and Bezuidenhout, 2016). In comparison to the HPC results of Jordaan and Bezuidenhout (2016) the total HPC bacteria at the MR AC 2 site (only overlapping site) enumerated in the current study was considerably (2 logs) lower. There is no standard set for the number of HPC bacteria in the RWQOs for the MR (DWAF, 2009). Lower bacterial loads generally indicate better water quality (Allen *et al*., 2004), however this observation may be a matter of concern in surface water environments. It may indicate that major changes in bacterial structure occurred over the past three years. This may result in functional changes that alter ecosystem properties by metabolic feedback as explained by Jordaan and Bezuidenhout (2016). A lack in data since 2012 makes evaluation of this observation difficult as many factors could have influenced the system resulting in a decline in bacterial load (Zarraonaindia *et al*., 2013). This highlights the importance of regular or annual monitoring in order to identify environmental or ecosystem changes and to evaluate the causes and consequences of such changes (Fatoki *et al*., 2001).

b. Direct assessment of anthropogenic activities in selection of antimicrobial resistance

The number of HPC bacteria on the media containing no antimicrobial were consistently exponentially higher compared to that of the antimicrobials. This demonstrates that an exponential number of the HPC bacteria present at each of the sites were susceptible to the specific antimicrobial screened for. However, HPC results in the current study confirmed that HPC bacteria were able to grow on antimicrobial containing media at all of the sites. No HPC bacteria were detected on any of the media for the MR BC 1 site on the March sampling occasion. Environmental or methodological reasons could not be found for this and it was accepted that the sample was compromised by contamination in the sample bottle that resulted in the mortality of the HPC bacteria. Furthermore, due to low rainfall and drought conditions of 2015, the MR BC 2 site had no water available on both the May and July sampling periods.

Growth on the antimicrobial containing media was generally more prevalent at the anthropogenic impacted sites (WFS 1 & 2, MR BC 2, MR AC 1 and MR AC 2) compared to the minimal impacted site (MR BC 1). This trend was also found by numerous recent studies investigating different metal polluted aquatic environments (Henriques *et al*., 2016; Pal *et al*., 2015; Wales and Davies, 2015; Chudobova *et al*., 2014; Naik *et al*., 2013; Wright *et al.*, 2006). These studies all concluded that environmental antimicrobial pollution (i) selects for resistance to specific antimicrobials and (ii) it may co-select for multiple resistance to a range of antibiotics. This phenomenon was ascribed to coupling of heavy metal and antibiotic resistance (Seiler and Berendonk, 2012). Coupling can be physiological (cross-resistance) and/or genetic (co-resistance). Cross-resistance refers to a single mechanism that provide resistance towards different antimicrobials. For example efflux pumps that extrude different toxins from the cell (Pal *et al*., 2015; Baker-Austin *et al*., 2006). Co-resistance refers to different resistance genes located on a single genetic element (Pal *et al*., 2015; Seiler and Berendonk, 2012).

c. The case of MR BC 2

The MR BC 2 site had the highest HPC counts on most of the antimicrobial containing media and especially on the plates with high concentrations. This is the same site of which the sediment could potentially have been re-suspended into the water column by cattle. A greater selection pressure exists in alkaline sediments as they act as a sink for most heavy metals (Sparks, 2005). This is due to the decrease of solubility of metals with an increase in pH as well as the affinity of metals to organic matter found in the sediment (Seiler and Berendonk, 2012). Higher concentrations of metals would directly select for more metal resistant bacteria and indirectly also select for more antibiotic resistant bacteria (Di Cesare *et al*., 2016). Thus, the high levels of HPC bacteria on all of the antimicrobial

containing media (particularly on the higher concentrations of metals) substantiates the assumption that mixing of the sediment did occur at this site on the day of sampling. This deduction is supported by the nitrate and TDS data. Results also suggest that the sedimentary environment hosts a greater antimicrobial resistance population (Tuckfield and McArthur, 2008). The consequential relevance of the sediment in the selection of antimicrobial resistance in the current study area should be investigated further as suggested by Wright *et al*. (2006).

The origin of metals that potentially exerts the selective pressure on the HPC bacteria in the sediments and bulk water at site MR BC 2 is unknown. The specific site is not directly impacted by any mining activities that would result in a metal load. It has been documented that underground karst springs may recharge metal polluted groundwater to the surface at sites that are in close vicinity to a site under investigation (Coetzee *et al*., 2006; Van der Walt *et al*., 2002). However, no sulphates were detected at the MR BC 2 site, whereas the WFS sites and the downstream MR sites had considerable levels of sulphate, thus indicating that mining pollution might not be the culprit at this site.

It would be more appropriate to suggest that agricultural practices contributed to the potential metal resistant bacterial load at this site. Animal manures may also contain high concentrations of metals and therefore these could accumulate in the sediment over time (Zhang *et al*., 2012). Zn has a high affinity for organic matter and may bind to organic material in aquatic systems and then find its way into the sediment (Seiler and Berendonk, 2012). Results of the current study found the highest number of Zn resistant HPC levels at MR BC 2. Cu is commonly used as a growth promotor and therapeutic agent for livestock (Wales and Davies, 2015). The results from the present study showed the highest abundance of Cu-resistant HPC bacteria were enumerated from water obtained from site MR BC 2. Thus, it is suggested that the high levels of HPC bacteria found on the antimicrobial containing media at this site could be attributed to metals from agricultural run-off (especially animal manure), that settled in the sediment. These metals selected for metal resistance (Altimera *et al.*, 2012) among sedimentary bacteria, which were then re-suspended into the water column prior to sampling. Comparative data for this site is unavailable due to drought conditions on the other sampling occasions.

d. General trends observed for specific antimicrobials from remainder of the sites

HPC bacteria was enumerated on ampicillin containing media from all water samples obtained from all sites and for all sampling periods. Henriques and co-workers (2016) also found abundant levels of ampicillin resistant bacteria at all their sites including the control site. These authors investigated the

co-selection of metal and antibiotic resistance in an epiphytic bacterial community in contaminated salt marshes. They ascribed this finding to be representative of the extensive dissemination of β-lactam resistance determinants in the environment. Widespread application of antibiotics from the β-lactam antibiotic class, could mean that this antibiotic run-off and land in the freshwater environment, where it directly selects for resistance to members of the entire class (Poole, 2004). Furthermore, *Pseudomonas* spp. host intrinsic abilities to produce β-lactamases (Luczkiewicz *et al*., 2015). These species were abundantly detected in environmental studies that investigated antimicrobial resistance phenomena (Hennriques *et al*., 2016; Icgen and Yilmaz, 2014). *Pseudomonas* spp. were some of the dominant identified HPC bacteria that were present in the MR in 2012 (Jordaan and Bezuidenhout, 2016). This could explain the presence of bacteria on media containing ampicillin at the reference site, in the present study. However, the number of HPC bacteria counted on the ampicillin containing media are higher at the impacted sites. This indicates that in addition to intrinsic resistance mechanisms there is also a selection processes from anthropogenic impacts that contribute to resistance to the antibiotic.

HPC counts on the metal containing media were generally lower on the media containing higher metal concentrations. Metal resistant bacteria were isolated from most sites on media containing 1 mM of most of the metals. This was not the case for Cu. For most of the sites no HPC bacteria resistant to Cu at a concentration of 1 mM could be detected. Pal and co-workers investigated co-occurrence patterns of metal resistance genes (MRGs), BRGs and ARGs from publicly available fully sequenced plasmids (n=4582) and bacterial genomes (n=2522). They found that the plasmid bound Cu resistance genes did not connect with any ARGs, whereas analysis of the genes irrespective of their location on plasmids or chromosomes yielded opposite results. In the present study most of the bacteria isolated from the Cu containing media were resistant to at least one antibiotic and many were resistant to a range of antibiotics (Section 2.3.5). Thus, it could be speculated that Cu resistance genes of the current study are bound to chromosomes and therefore not readily disseminated as for plasmid bound genes (Alekshun and Levy, 2007). However, there are many factors that could influence these results and other approaches such as whole genome sequencing could provide more accurate insight into specific selection mechanisms (Schwartz *et al*., 2015; Tan *et al*., 2015). Cu tolerant bacteria were detected at WFS 1 and 2 (latter only March and May) as well as MR BC 2 (March) and MR AC 2 (May). In addition to livestock farming as was ascribed to MR BC 2 (Wales and Davies, 2015), mining activities in the vicinity (WFS sites) of the sampling points also attribute to Cu in the system (Hamman, 2012; Van der Walt *et al.*, 2002) that select for Cu resistance (Altimera *et al*., 2012).

The toxic effect of Fe most commonly lies in the limited availability thereof rather than the abundance. It is an essential element for all life forms and most commonly the absence thereof exerts a toxic effect to microbes (Kim *et al*., 2009). There was considerable growth on 1 mM containing Fe media for all sites and levels ranged from 10^1 to 10^5 CFU/mL. The microbial levels were higher on 1 mM Fe containing media compared to those containing 2.5 mM. This observation indicates that a high concentration Fe does have an effect on survival similar to the other metals. Similar observations were made by Mgbemena and co-workers (2012). This could be attributed to Fe that catalyzes the Fenton reaction (Kim *et al*., 2009) producing a highly reactive hydroxyl radical which in turn damages cell membranes. Fe concentrations have also been found to correlate with antibiotic resistance indicating that Fe pollution is a possible selector for antibiotic resistance (Kimiran-Erdem *et al*., 2015).

The largest loads of potentially metal tolerant HPC bacteria were enumerated on Pb containing media for all sites and during all sampling periods. Furthermore, HPC bacterial growth were frequently detected on the 2.5 mM Pb containing media (including MR BC1). The exception was for the MR sites during July. Thus, the bacterial community in this system has a higher tolerance to Pb compared to the other metals tested for. This high tolerance to Pb might be attributed to the low solubility of the metal and ultimately low bioavailability (Nies, 1999). However, Pb resistance should not be ignored as a possible driver for antibiotic resistance selection in the current study as the concentration for Pb in the surface water of anthropogenic impacted sites exceeded the MCC values set (Seiler and Berendonk, 2012). Though Pb concentrations at the MR BC 1 site was not measured by the Municipality, the metal could be present from motorboat fuels that pollute Klerkskraal dam (Van Aard and Erdmann, 2004). Several P-type ATPase efflux systems have been associated with plasmid encoded Pb resistance (Naik *et al*., 2013; Nies 1999). Drudge and co-workers (2012) found a Pb resistance gene cluster alongside genes encoding multiple antibiotic resistance on transferable plasmids in floc bacteria influenced by many trace elements. The authors (Drudge *et al*., 2012) explained that the presence of trace elements activates the SOS response in bacteria. This could stimulate the exchange of gene cassettes. Thus, plasmids, transposons and integrons containing these determinants could be readily disseminated among the bacterial community during stress conditions leading to rapid spread of multiple resistance determinants (Alekshun and Levy, 2007).

Zn tolerant bacteria were enumerated on the 1 mM Zn containing media at all of the sites, except MR BC 1 and MR AC 2 on the July occasion. This metal is common in sulphate ores and elevated concentrations measured at sites with high sulphate levels may be due to release by mining activities (Spitz and Trudinger, 2009). However, the possibility exist that animal manure may attribute to Zn

concentrations in the environment as explained for the MR BC 2 site (Zhang *et al*., 2012). Our results exposed considerable levels HPC bacteria on Zn containing media with HPC levels ranging from 10^2 to 10^4 at anthropogenic impacted sites on the 1 mM concentration of Zn. Limited loss in bacterial load is found from the 1 mM to the 2.5 mM concentration of this metal in the media at most of the sites. This indicates that, even though the numbers of HPC bacteria weren't the highest detected from all of the metals screened for, the bacteria that were able to grow on this metal can generally tolerate higher concentrations of Zn compared to bacteria found on the 1 mM concentrations of the other metals. Determinants of Zn resistance can be plasmid or chromosomal bound (Pal *et al*., 2015). Resistance mechanisms towards Zn include extracellular accumulation, efflux of the metal, metallothionein sequestration and intracellular sequestration (Coudhury and Srivastava, 2001). Several studies have also found that cadmium, which has no biological function, is a look-alike of Zn. Bacteria use the same resistance mechanism to resist the inhibiting effect of Cadmium as for Zn (Prapagdee and Watcharamusik, 2009). Considerable levels of cadmium have also been detected in the WFS (Hamman, 2012) which could select for cadmium resistance that supplement Zn resistance. The high tolerance of bacteria to Zn could be attributed to bacteria using more than one of these mechanisms at the same time, thus enhancing their survival potential (Banjerdkij *et al*., 2003).

2.4.3 Relationship of physico-chemical parameters and enumerated HPC bacteria of surface water at WFS and MR sites in 2015

From the RDA results it is evident that the six sites of the current study are all impacted on by different factors. The WFS 1 site correlated with all of the HPC bacterial-, TDS-, sulphate- and phosphate-levels compared to the other sites. This highlights that mining impacts are influencing the water quality at his site as mentioned previously. The observation that most of the HPC bacteria enumerated from the metal containing media correlated with this site suggest that metal pollution from mining activities have a greater selection pressure for resistance towards these antimicrobials compared to the impacts of the other sites. This support previous observations that metal polluted areas directly select for metal and antibiotic resistance (Di Cesare *et al*., 2016; Henriques *et al.*, 2016). The weak relationship between nitrates and phosphates in the RDA indicate that different impacts are releasing these nutrients into the environment. Phosphate related more closely to the WFS 1 (mining impacted site) and all of the HPC bacteria. This corresponds with the previously mentioned observation of Chudobova and co-workers (2014) in which Pb resistant bacteria are said to precipitate Pb as a phosphate salt in the environment.

The WFS 2 and MR AC 1 sites are grouped together on the RDA suggesting that they are impacted on in a similar manner. These two sites correlate strongly with nitrates suggesting that they are highly impacted on by agricultural activities that release nitrates into the environment. The weak relationship between the WFS 1 and WFS 2 sites indicate that there is a form of "dilution" of the mining impact from the upstream to the downstream site. However, the WFS 2 site correlates with the same parameters as the WFS 1 site, only in a weaker manner. Another possible reason for the higher nitrate levels at WFS 2 compared to WFS 1, could be formal or informal urbanization and anthropogenic activities in Carltonville. An article in the local newspaper, the Carltonville Herald, highlighted that sewage water are streaming from the Khutsong informal settlements to surrounding canals and the environment (http://carletonvilleherald.com/2791, 2015). The MR AC 1 site is downstream of the WFS 2 site which indicate that pollution of the WFS sites have impacts on the downstream MR site. The control site (MR BC 1) as well as the MR AC 2 site had the weakest correlations to all of the chemicals analyzed, and were rather influenced by the physical parameters. The observation that the MR AC 2 site correlated best with the control site (MR BC 1) might suggest that there is a form of remediation taking place between the MR AC 1 and MR AC 2 sites, which almost restores the water quality to baseline quality. The observation could be attributed to Boskop Dam that might dilute the contamination and act as a sink for substances, which ultimately remediates the water quality of the MR. However, there is still a closer, though very weak, relationship between the MR AC 2 site and the two WFS sites compared to MR BC 1. This indicate that the confluence of the WFS ultimately has a deteriorating effect on the downstream surface water quality of the MR as was also observed by Barnard and co-workers (2013) in relatively the same area.

2.4.4 Antibiotic resistant profiles of isolated antimicrobial tolerant HPC bacteria

In the current study resistance was assigned conservatively. Almost all the metal resistant isolates were also tolerant to one or more antibiotic class, indicating there is a co-occurrence of resistance to metal and antibiotic resistance (Chen *et al*., 2015). Although the present study screened only for β-lactam resistant bacteria directly from the water, evidence (Figure 2.3) is provided that a large percentage of the ampicillin and metal tolerant HPC bacteria were also resistant to other antibiotic classes. Resistance to the two β-lactam antibiotics (Ampicillin/Amoxicillin) was found most frequently among the isolates. The abundance of β-lactam resistance determinants present in the natural environment and its relationship to metal resistance was also demonstrated in a number of previous studies (Henriques *et al*., 2016; Luczkiewicz *et al*., 2015; Chudobova *et al*., 2014; Allen *et al*., 1977). In the present study, tetracycline resistance was less common since only a limited number of isolates were resistant to this antibiotic. Similar results were found in a previous study (Chudobova *et al*.,

2014). This is in contrast to that found by Chen and Co-workers (2015) which found that tetracycline resistance was ubiquitous among the metal tolerant bacteria in their study. This indicate that the co-selection of metal and antibiotic resistance is complex and different factors influence the phenomenon. However, tetracycline resistant bacteria were detected at all of the sites. More than a third of the antimicrobial tolerant isolates from all of the sites, were MAR. This indicates that pollution in the area of the current study is not only co-selecting for one or two antibiotics, but an entire range of antibiotic classes and therefore, present health risks especially for the immunocompromised (Chudobova *et al*., 2014; Mulamattathil *et al*., 2014; Baker-Austin *et al*, 2006).

Antimicrobial resistance patterns have been frequently used to track sources of especially human fecal pollution. This is due to geographic specific patterns that evolve from different impacts (Mthembu *et al*., 2010; Burnes, 2003). The use of HPC bacteria (unknown) instead of a single known specie complicates the demographics of such analysis. It should be taken into consideration that morphologically distinct isolates were selected for further analysis from each site. Thus, it is expected that different species should have different phenotypes.

In the current study partial clustering of isolates from specific sites and from specific antimicrobial containing media was found for the three sampling occasions in 2015 (Figures 2.4 – 2.6). Thus, it appears that there is a degree of specificity in selection for antibiotic resistance phenotype related to sites and isolation antimicrobials. Interestingly, the most prevalent resistance phenotype (included all antibiotic classes: Ax, Ap, Tt, Er, St, Tm, Cp) were found more abundantly among isolates from media that were supplemented with metals compared to the isolates enumerated from ampicillin containing media. Furthermore, the observation that the highest percentage of MAR resistant bacteria was found at the WFS 1 site confirms that heavy metal pollution has a considerable selective impact for antibiotic resistance at that study site.

The concentrations of Cu, Pb and Zn measured in the surrounding area of the current study were exceeding the MCC levels (Seiler and Berendonk, 2012). It therefore supports the assumption that metal pollution is a potential driver of the co-occurrence of metal and antibiotic resistance in the current study. For example Pal and co-workers (2015) found that Cu, Fe and Zn were co-selectors for resistance to β-lactam, tetracycline and aminoglycosides among others. Seiler and Berendonk (2012) reviewed a range of studies that found MAR isolates that were resistant to Cu, Zn and Pb. The occurrence of MAR bacteria at the control site could be attributed to intrinsic antibiotic resistance or

it could indicate that there are anthropogenic impacts that influence the bacterial community at this site. These factors should be investigated further.

2.5 Conclusion

The current study is contributing to understanding the potential compounded impacts of metals and antibiotics from mining, agriculture and urbanization on heterotrophic bacteria in the aquatic environment in gold mining impacted aquatic systems of the NWP of South Africa. This was done by means of a direct approach which involves evaluating observations from highly impacted sites to that of minimally impacted sites. This a very complex phenomenon which requires more advanced approaches to fully understand all the mechanisms involved. However, the study could demonstrated that different anthropogenic activities surrounding the system is degrading the water quality served to the downstream town of Potchefstroom. Observations such as higher levels of antimicrobial resistant bacteria at mining impacted sites compared to minimally impacted sites proves that metals in the environment are selecting for metal resistance and co-selecting for antibiotic resistance. The study could also conclude that there is a co-occurrence of metal and antibiotic resistant bacteria present in the water and that some metal resistant isolates were resistant not only to one or two antibiotics, but to multiple antibiotic classes.

CHAPTER 3

Antimicrobial resistance and molecular characterization of multiple antibiotic resistant HPC bacteria

3.1 Introduction

A range of anthropogenic activities are impacting surface water sources where they are aiding in the dissemination of antibiotic resistant bacteria (ARB) and genes (ARGs). Some of these anthropogenic impacts include the mis- and over- use of antibiotics in medicine (human and animal) or as feed additives in agriculture (Capkin *et al*., 2015; Nikaido, 2009; Sengeløv *et al*., 2003), failure of treatment plants to effectively remove antibiotics / ARB (Adesoji *et al.*, 2015; Bergeron *et al*., 2015), and chemical applications or effluents containing compounds (e.g. metals and biocides) that co-select for antibiotic resistance (Capkin *et al.*, 2015; Pal *et al*., 2015). ARGs are increasingly seen as clinical relevant environmental pollutants due to their part in the etiology, prolificacy and epidemiology of antibiotic resistance and particularly multiple antibiotic resistance in the global context (Li *et al*., 2010a). Limited data on the presence of ARGs in the natural environment is available in developing countries such as South Africa (Laffite *et al*., 2016; Molale and Bezuidenhout, 2016; Biyela *et al*., 2004).

There are four general mechanisms encoded that provide bacteria with the ability to resist the inhibiting effect of antibiotics. These mechanisms include (i) alterations or elimination of binding sites of the antibiotic target proteins; (ii) production of enzymes that inactivates antibiotic activity; (iii) reduced uptake due to alterations of protein channels of the outer membrane; and (iv) active extrusion of the antibiotic from the cell by efflux pumps (Madhavan and Maruli, 2011; Davies and Davies, 2010; Tenover, 2006). These mechanisms are all mediated by ARGs. Though most HPC bacteria found in the environment are harmless, their ability to act as reservoirs for genes that can be transferred to pathogenic strains is inevitable (Thompson *et al.*, 2007). Previously susceptible strains can acquire ARGs by vertical evolution or by horizontal transfer of MGEs from resistant strains (Li *et al*., 2010a).

A number of MAR HPC bacteria were isolated from anthropogenic impacted sites in the WFS and MR in this study (Chapter 2). Resistance to two β-lactam antibiotics (ampicillin and amoxicillin) was most frequent among these HPC isolates. Resistance to β-lactam antibiotics are most frequently attributed to the production of β-lactamase which hydrolyze the four member β-lactam ring (Palzkill, 2013; Jacoby, 2009; Nikaido, 2009). Genes frequently found by previous environmental studies to

encode for β-lactamase include *amp*C and *bla*TEM. It has been documented that efflux pumps, such as those belonging to the MFS, particularly the *tet*-class, associate with multidrug resistance (Sun *et al*., 2014). In the current study, tetracycline resistance was least frequently detected compared to the other antibiotics (Chapter 2). However, tetracycline resistance was frequent among the MAR isolates. The presence of MAR bacteria is a cause for concern and indicate the presence of ARGs that can be rapidly disseminated. Many recent studies have turned to the natural environment in order to get better insight into how antibiotic resistance is disseminated (Berglund, 2015; Drudge *et al*., 2012; Lachmayr *et al*., 2009, Martinez, 2009). It is therefore important to get deeper insight into the resistance levels of the present MAR isolates, to determine which species are dominant and could carry these resistance traits. It is also important to determine which ARGs are present in this population. This type of data will aid to predict possible threats to humans, plants and animals that are dependent on the source.

The aim of the current study was to identify selected MAR isolates among HPC bacteria isolated from water sources, and characterize the isolates based on MIC and genes (*bla*TEM, *amp*C, *tet*A, *tet*L, *tet*K) present in their genomes.

3.2 Materials and methods

3.2.1 Isolate collection

Seventy two isolates were selected from the MAR pool of isolates (Chapter 2) that were extracted from surface water at sites in the WFS and MR. Details of sample collection, colony isolation purification and antibiotic resistant profiling are stipulated in Section 2.2 of this dissertation. The isolates of the current study were selected at random while being cautious not to select morphologically similar isolates from the same isolation antimicrobial at the same site. Isolate labels are the same as that used in Chapter 2 and is summarized in Appendix D.

3.2.2 Detection of minimal inhibitory concentration (MIC) ranges

An agar dilution method was used to determine the MIC of the MAR isolates to certain antibiotics. The antibiotics included in this assay were amoxicillin, tetracycline, erythromycin and streptomycin. Antimicrobials (ampicillin, Cu, Fe, Pb or Zn) from which the isolate was originally isolated was also included in the assay.

3.2.2.1 Media preparation

Concentration gradient stock solutions of x1000 the desired final concentrations were prepared for each of the antimicrobials. A stock solution gradient was used instead of a single stock solution as this kept the nutrient concentration homogenous in the media at the different concentrations. To R2A media (999 mL) that was autoclaved and cooled (± 65°C) 1 ml of stock solution was added to yield the final concentrations for each antibiotic (50 mg/L, 100 mg/L, 130 mg/L, 150 mg/L, 180 mg/L and 200 mg/L; and for the metals: 1.0 mM, 1.5 mM, 2.5 mM, 5.0 mM and 6.5 mM). The media was then poured into sterile petri dishes and cooled.

3.2.2.2 MIC assay

Pure cultures were inoculated onto different concentrations of the metal it was originally isolated from, and on different concentrations of the specific antibiotics it had previously shown resistance to, that was part of the MIC assay. Each isolate was also inoculated onto R2A without any antimicrobial to serve as a negative control. Plates were incubated for 36-48 hours at 26°C. After incubation inoculated areas were evaluated for growth. The MIC was then assigned to be between the highest concentration on which growth was observed and the first concentration where there was no growth; or potentially values higher than the maximum concentration.

3.2.3 Genomic DNA Isolation

The total genomic DNA of selected isolates were extracted by using a slightly altered version of the Phenol:Chloroform:Isoamyl alcohol protocol adapted from Ausubel *et al*. (1995). In brief, the pure isolates were cultured overnight in nutrient broth at 26°C and pelleted by centrifugation (12 000g for 2 minutes). The pellet of each sample was lysed in a lysis buffer [50 mM TrisHCl pH 8.0 (Sigma Aldrich, US), 100 mM NaCl, 10 mM EDTA (Merck, Germany) and 1% SDS (Thermo Fisher Scientific, US)], 10 mg/ml lysozyme solution (Thermo Fisher Scientific, US) and 10 mg/ml Rnase A (Macherey-Nagel, Germany) solution. The lysis solution was then vortexed and incubated at 37°C for 30 minutes. Proteinase K (10 mg/ml) (Macherey-Nagel, Germany) was added and the solution was incubated at 56°C for 1-3 hours. DNA was then isolated from the solution by adding 0.2 M NaCl and chloroform: isoamyl (24:1) and mixed by inverting the tubes several times. After centrifugation (12 000 g for 3 minutes) the top aqueous phase containing the DNA was transferred to a fresh tube. Two volumes of ice cold absolute ethanol was added and the mixture incubated overnight in at -20°C. This was followed by centrifugation (12 000 g for 30 minutes), the ethanol was decanted and the pellet was again washed in two steps with ice cold 70% ethanol (centrifuged by 12 000 g for 10 minutes) and

the supernatant was decanted. The pellet was left to air dry at room temperature for 1 hour. The DNA that remained in the tube was dissolved in nuclease free sterile MiliQ water.

3.2.4 Quality control of DNA and PCR products

Agarose gel electrophoresis and NanoDrop spectrophotometry (Thermo Fischer Scientific, US) was performed to evaluate the quality, quantity and integrity of the genomic DNA. Agarose was also used to determine if the PCRs worked. For the genomic DNA A260nm/A280nm ratios of between 1.7 and 2.0 were considered as good quality DNA for use in subsequent assays. Concentrations were adjusted according to requirements for subsequent PCR.

For agarose gel electrophoresis: a 1.5% (w/v) agarose gel (peqLab, Germany) with loading wells was prepared in 1 x TAE buffer [40 mM Tris (Sigma Aldrich, US), 20 mM Acetic acid (Merck, Germany), 1 mM EDTA (Merck, Germany), at pH 8.0 (Merck, Germany)]. The gel was placed in a Bio-Rad electrophoresis system and covered with 1 x TAE buffer. GelRed (Biotium, US) was added to 6x Orange Loading Dye (Fermentas Life Sciences, US) prior to electrophoresis. 3 µL DNA/PCR product was mixed with 2 µL gelRed-Orange Loading dye mix (for visualization under ultra-violet (UV) light) and loaded to the gel. A 1kb, O"GeneRuler™ (Fermentas Life Sciences, US) was included to estimate amplicon size. Electrophoresis was performed for 55 min at 80 volts. The gel was captured with a ChemiDoc™ MP (BioRad, US) UV imager and visualized with Image Lab™ software (BioRad, US).

3.2.5 Polymerase chain reaction (PCR) gene amplification

All PCR reactions were prepared aseptically and on ice in a laminar flow cabinet. PCR assays were performed using a Techne® Prime Elite thermo cycler (Cambridge, UK). A negative control was included for each PCR run, which contained no DNA template. The primer sets (Applied Biosystems, UK) and thermocycler conditions for each of the genes amplified are summarized in Table 3.1. For the 16S rRNA, *amp*C, *bla*TEM and *tet*A genes the PCR reaction mixtures had a final volume of 25 µL which consisted of double strength DreamTaq PCR Master Mix (0.05 U/µL Taq DNA polymerase in reaction buffer, 0.4 mM of each dNTP and 4 mM MgCl2)12.5 µL) (Fermentas Life Sciences, UK), 0.5 µL of each primer (10 µM), 1 µL of DNA template (50-100 ng/µL) and PCR grade water. For the *tet*L and *tet*K genes the reaction mixture had a final volume of 20 µL which was made up of 10 µL of 2x DreamTaq PCR Master Mix (0.05 U/µL Taq DNA polymerase in reaction buffer, 0.4 mM of each dNTP and 4 mM MgCl2) (Fermentas Life Sciences, UK), 0.2 µM primer (mixed), 1 µL DNA (50-100 ng/µL) and PCR grade water.

Table 3.1: Oligonucleotide primers and thermocycler conditions used

	Primer	Sequence (5'-3')	Size (Bp)	PCR conditions	Reference
16 S	16S rRNA: 27-F	AGAGTTTGATCMTGGCTCAG[a]	1465	(i) Denaturation at 95^0 C for 300 seconds. (ii) 30 cycles: 95^0 C for 30 seconds, 52^0 C for 30 seconds, 72^0 C for 60 seconds. (iii) Final extension at 72^0 C for 300 seconds.	Lane (1991)
	16S rRNA: 1492-R	TACGGYTACCTTGTTACGACTT[a]			
β-Lactamase antibiotics	*amp*C-F	TTCTATCAAMACTGGCARCC[a]	530	(i) 35 cycles 94°C for 30 seconds 49°C for 30 seconds and 72°C for 60 seconds at. (ii) Final extension at 72°C for 420 seconds.	Schwartz *et al*. (2003)
	*amp*C-R	CCYTTTTATGTACCCAYGA[a]			
	*bla*TEM-F	AAAATTCTTGAAGACG	1080	(i) Denaturation at 95°C for 300 seconds. (ii) 35 cycles: 94°C for 60 seconds, 63°C for 30 seconds, 72°C for 60 seconds. (iii) Final extension at 72°C for 600 seconds.	Sharma *et al*. (2010)
	*bla*TEM-R	TTACCAATGCTTAATCA			
Tetracycline efflux pumps	*tet*A-F	GTAATTCTGAGCACTGTCGC	957	(i) Denaturation at 94°C for 180 seconds, (ii) 25 cycles at 94°C for 60 seconds, 57°C for 60 seconds, 72°C for 60 seconds. (iii) Final extension at 72°C for 600 seconds.	Sengeløv *et al*. (2003)
	*tet*A-R	CTGCCTGGACAACATTGCTT			
	*tet*L-F	ATAAATTGTTTCGGGTCGGTAT	1077	(i) 35 cycles at 95^0 C for 60 seconds, 50^0 C for 60 seconds 72^0 C for 30 seconds. (ii) Final extension 72^0 C for 300	Trzcinski *et al*. (2000)
	*tet*L-R	AACCAGCCAACTAATGACAATGAT			
	*tet*K-F	TATTTTGGCTTTGTATTCTTTCAT	1159		
	*tet*K-R	GCTATACCTGTTCCCTCTGATAA			

a- Wobbles according to IUPAC: M= A or C, R= A or G, Y= C or T.

3.2.6 Sequencing of amplicons

PCR amplicons were purified with a silica resin as described by Li and co-workers (2010b). In brief, 500 µL NaI (6 M) was added to the PCR product and inverted. Silica matrix [SiO_2 in PCR grade water (Sigma Aldrich, US)] was added, mixed and then incubated at room temperature for 5 minutes. The mixture was then centrifuged (14 500 g for 10 seconds) and the supernatant was removed. The pellet was washed with a washing buffer (50% ethanol, 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA). After a second repeat of the washing step the mixture was centrifuged (14 500 g for 10 seconds) and the supernatant removed. The tube was left to air dry. After drying, the pellet was re-suspended in 20µL ultra-pure water and centrifuged (14 500 g for 2 minutes). The supernatant containing the DNA was transferred to a clean tube. The DNA concentration was determined by NanoDrop spectrophotometry (Section 3.2.4).

After the first purification of the PCR product, the BigDye Terminator version 3.1 Sequencing kit PCR was performed as described by the protocol of the manufacturer (Applied Biosystems, UK). These products were then subjected to another purification step. The second purification step was performed with a ZR DNA Sequencing Clean-up kit (Zymo Research, US) as descried by the manufacturer.

HiDi Formamide (Thermo Fisher Scientific, US) was added to the purified DNA and loaded to a sequencing plate for sequencing. Sequencing was performed with an ABI 3100 sequencer and data was analyzed with Sequencing Analysis v5.3.1 software. Geospiza Finch TV (version 1.4) software was used to view chromatograms of the raw sequence data. BLASTn searches (http;//www.ncbi.nlm.nih.gov/BLASTn) were performed on all the amplified DNA sequences to compare with previous sequences on databases and to finally identify and verify the sequence. Only matches of above 98% similar identity were considered as positive identity of the amplified gene.

3.3 Results

The DNA quality extracted from all of the isolates part of this study were within the acceptable A260/A280 ratios (1.7 - 2.0). The DNA quality was also confirmed by agarose gel electrophoresis and subsequent PCRs could be performed on all of the isolates. The identity of the 72 MAR HPC isolates, their antibiotic resistance patterns (from Chapter 2), and their resistance towards selected antimicrobials and genes that were detected for each are summarized in Table 3.1.

3.3.1 Identified isolates

Isolates were identified to species (or at least genus) level belonging to 4 phyla (Proteobacteria, Bacteriodetes, Firmicutes and Actinobacteria). The majority of the isolates were from the phylum Proteobacteria. Of these most belonged to the Gammaproteobacteria class. Fourteen were identified as species of the *Pseudomonas* genus and one isolate was a *Cellvibrio* sp. also from the Pseudomonadaceae family. Another 14 isolates belonged to the genus *Acinetobacter* from the Moraxellaceae family. One *Xanthomonas* sp. and three *Stenotrophomonas maltophilia* isolates represented the Xantomonadaceae family. Finally, two *Klebsiella pneumonia* isolates were identified as well as one *Serratia marcescens* from the Enterobacteriaceae family. These Gammaproteobacteria species were from most of all the sampling sites and represented 15 of the multiple antimicrobial resistant phenotypes. The phenotypes include each of the 8 most prevalent phenotypes.

Among the 9 Alphaproteobacteria isolates there were representatives of the Sphingomonadacea (*Sphingobium yanoikuyae*, *Novosphingobium* sp. and Sphingomonas *aerolata*), Rhizobiaceae (*Rhizobium* spp. and *Agrobacterium vitis*) and Caulobacteriaceae (*Brevundimonas nasdae*) families that were from the WFS 1, WFS 2, MR BC 2 and MR AC 1 sites. These isolates represented six of the multiple ARPs.

Two isolates, *Massilia* sp. and *Acidovorax* sp. isolated from WFS 1, were identified only to genus levels and represented the Betaproteobacteria class. Both of these isolates had an antibiotic resistant profile of Ax, Ap, Tt, Er, St, Tm, with the *Massillia* sp. isolate additionally being resistant to Cp, whereas the *Acidovorax* sp. was intermediate for this antibiotic.

The Bacteriodetes phylum had representatives of the Flavobacteria (*Flavobacterium* spp. and *Chryseobacterium lactis*) and Sphingobacteria (*Sphingobacterium* spp. and *Chitinophaga oryziterrae*) classes. The seven isolates were predominantly from the two MR AC sites with the exception of one *Flavobacterium* sp. that was isolated from Pb containing media at the control site (MR BC 1). Isolates in this phylum had fewer antibiotics in their resistance patterns and were resistant to only 3 or 4 antibiotic classes. However, some were intermediate resistant to additional antibiotics.

Table 3.2: Summary of identified surface water HPC isolates their resistance to selected antimicrobials and the resistance genes detected for each

Identified species	Site of isolation	Antibiotic resistant phenotype	MIC to Isol.Am*	MIC to different antibiotics (mg/L)				Resistance genes detected
				Ax	Tt	Er	St	
Proteobacteria								
Gammaproteobacteria								
Pseudomonas mosselii	Ap_M2_1.9	Ax, Ap, Tt, Er, St, Tm, Cp	> 200	> 200	50 - 100	>200	<50	*bla*TEM, *tet*A
Pseudomonas chloraphis	Zn_M4_2.4	Ax, Ap, Tt, Er, St, Tm, Cp	2.5 - 5.0	>200	<50	180 - 200	<50	*bla*TEM
Pseudomonas fluorescens	Fe_M4_2.1	Ax, Ap, Tt, Er, St, Tm, Cp	5.0 - 6.5	>200	50 - 100	>200	<50	*bla*TEM
	Cu_M4_2.1	Ax, Ap, Tt, Er, St, Tm, Cp	1.5 - 2.5	>200	50 - 100	>200	<50	*bla*TEM
	Zn_M3_1.3	Ax, Ap, Tt, Er, St, Tm, (Cp)	2.5 - 5.0	>200	<50	>200	50 - 100	*bla*TEM
	Pb_M4_2.1	Ax, Ap, Er, St, Tm, Cp	2.5 - 5.0	>200	-	>200	<50	*bla*TEM
	Cu_W1_2.3	Ax, Ap, Er, St, (Tt)	1.5 - 2.5	>200	-	50 - 100	<50	*bla*TEM
Pseudomonas protogens	Ap_M4_2.3	Ax, Ap, Tt, Er, St, Tm, Cp	>200	>200	50-100	>200	>200	*bla*TEM
	Zn_W2_2.1	Ax, Ap, Tt, Er, Tm, Cp	2.5 - 5.0	>200	50-100	>200	-	*bla*TEM
Pseudomonas moraviensis	Fe_M4_2.6	Ax, Ap, Er, Tm, Cp, (Tt), (St)	5.0 - 6.5	>200	<50	>200	<50	*bla*TEM
	Pb_W2_1.1	Ax, Ap, Tt, Er, St, Tm,	2.5 - 5.0	>200	50 - 100	>200	50 - 100	*bla*TEM
Pseudomonas psychrophilla	Cu_W2_2.2	Ax, Ap, Er, Tm, Cp, (Tt)	1.5 - 2.5	>200	-	50 - 100	<50	*amp*C, *bla*TEM
Pseudomonas constantinii	Cu_M4_2.4	Ax, Ap, Er, St, Tm, Cp, (Tt)	1.0 - 1.5	>200	-	50 - 100		*bla*TEM
Pseudomonas extremorientalis	Zn_W2_3.1	Ax, Ap, Tt, Er, Tm, Cp, (St)	5.0 - 6.5	>200	50 - 100	>200	-	*bla*TEM
Pseudomonas gessardii	Zn_W1_3.4	Ax, Ap, Tt, Er, Tm, Cp, (St)	2.5 - 5.0	>200	<50	>200	<50	ampC,
	Zn_M3_2.4	Ax, Ap, Er, Tm, Cp, (St)	2.5 - 5.0	>200	-	>200	<50	*bla*TEM
Pseudomonas rhodesiae	Cu_W1_1.3	Ax, Ap, Er, Tm, (Tt), (Cp)	1.0 - 1.5	>200	-	>200	-	*bla*TEM
Pseudomonas putida	Pb_W1_1.4	Ax, Ap, Tt, Er, Tm, Cp	2.5 - 5.0	>200	50 - 100	>200	-	*bla*TEM
	Pb_M2_1.3	Ax, Ap, Tt, Tm, (Cp)	2.5 - 5.0	>200	<50	>200	-	*bla*TEM
	Cu_W1_1.1	Ax, Ap, Er, Tm	1.5 - 2.5	>200	-	50 - 100	-	*bla*TEM
Pseudomonas syringae	Pb_M2_1.1	Ax, Ap, Er, Tm	2.5 - 5.0	>200	-	>200	-	*bla*TEM
Pseudomonas fragi	Cu_W1_2.5	Ax, Ap, Er, Tm	1.5 - 2.5	>200	-	>200	-	*bla*TEM
Pseudomonas composti	Pb_M3_1.8	Ax, Ap, St, Tm	1.5 - 2.5	>200	-	-	<50	*bla*TEM

Pseudomonas cichorii	Cu_W1_1.7	Ax, Ap, Tt, Tm	1.0 - 1.5	>200	< 50	-	<50	*bla*TEM
Cellvibrio sp.	Ap_W1_1.4	Ax, Ap, Tt, Tm	>200	>200	< 50	-	-	*bla*TEM
Acinetobacter beijerinckii	Zn_M2_1.6	Ax, Ap, Tt, Er, St, Tm, Cp	5.0 - 6.5	>200	130 - 150	>200	50 -100	*bla*TEM
	Cu_M2_1.4	Ax, Ap, Tt, Er, St, Tm, Cp	1.5 - 2.5	>200	100 - 130	>200	<50	*bla*TEM
Acinetobacter haemolyticus	Cu_M2_1.3	Ax, Ap, Tt, Er, St, Tm, Cp	1.0 - 1.5	180 - 200	50 - 100	>200	<50	*bla*TEM
	Pb_W1_1.6	Ax, Ap, Tt, Er, St, Tm, Cp	5.0 - 6.5	>200	50 - 100	>200	50 - 100	*bla*TEM
	Cu_W2_1.1	Ax, Ap, Tt, Er, St, Tm	1.0 - 1.5	>200	50 - 100	>200	<50	*bla*TEM
	Cu_W1_1.2	Ax, Ap, Er, Tm, (Tt)	1.5 - 2.5	>200	-	>200	-	*bla*TEM
	Zn_W1_2.4	Ax, Ap, Tt, Er, St	2.5 - 5.0	>200	50 - 100	150 - 180	<50	*bla*TEM
	Ap_M3_2.4	Ax, Ap, Tt, St, Cp	>200	>200	<50	-	<50	*bla*TEM
Acinetobacter johnsonii	Ap_W1_3.7	Ax, Ap, Er, Tm, Cp, (Tt), (St)	>200	>200	-	>200	-	*bla*TEM
Acinetobacter calcoaceticus	Pb_M2_1.8	Ax, Ap, Tt, Er, St, Tm, Cp	5.0 - 6.5	100 - 130	< 50	<50	<50	*bla*TEM
	Zn_W1_1.7	Ax, Ap, St, Cp, (Tt), (Er)	1.5 - 2.5	>200	-	-	-	*bla*TEM
Acinetobacter sp.	Ap_W1_3.6	Ax, Ap, Tt, Er, St, Tm	>200	>200	100 - 130	>200	>200	*bla*TEM, *tet*A
	Zn_W2_1.2	Ax, Ap, Tt, Er, St, Tm	2.5 - 5.0	100 - 130	50 - 100	>200	<50	
	Zn_W1_1.5	Ax, Ap, Tt, St, Tm	2.5 - 5.0	100 - 130	<50	-	<50	*bla*TEM
Stenotrophomonas maltophilia	Ap_M2_1.6	Ax, Ap, Tt, Er, St, Tm, Cp	> 200	>200	>200	>200	>200	*amp*C, *bla*TEM, *tet*A
	Zn_W2_1.5	Ax, Ap, Tt, Er, St, Tm, Cp	2.5 - 5.0	>200	100 - 130	>200	<50	*bla*TEM
	Zn_M2_1.9	Ax, Ap, Tt, Er, St	2.5 - 5.0	>200	50 - 100	-	<50	*bla*TEM
Xantomonas sp.	Ap_M3_1.5	Ax, Ap, Tt, Tm, (Cp)	> 200	>200	<50	-	-	*bla*TEM
Klebsiella pneumoniae	Zn_W1_1.6	Ax, Ap, Tt, Tm, (Er)	1.5 - 2.5	130 - 150	>200	50 - 100	-	*bla*TEM, *tet*A
	Pb_W1_1.3	Tt, Er, Tm, (Ap), (St)	2.5 - 5.0	-	50 - 100	<50	-	
Serratia marcescens	Zn_M3_1.1	Ax, Ap, Tt, Er, Tm	1.5 - 2.5	>200	<50	>200	-	*bla*TEM
Alphaproteobacteria								
Sphingobium yanoikuyae	Cu_W1_1.4	Ax, Ap, Er, St, Tm, (Tt), (Cp)	1.5 - 2.5	>200	-	<50	<50	*amp*C, *bla*TEM
Novosphingobium sp.	Ap_M2_1.8	Ax, Ap, Er, Tm	>200	>200	-	50 - 100	-	*bla*TEM
Sphingomonas aerolata	Cu_M2_1.5	Ap, St, Tm	1.5 - 2.5	>200	-	-	<50	*bla*TEM
Rhizobium massilae	Zn_M2_1.7	Ax, Ap, Tt, Er, St, Tm, Cp	1.0 - 1.5	>200	<50	< 50	<50	
Rhizobium sp	Fe_W1_2.7	Ax, Ap, Tt, Er, St, Tm, Cp	2.5 - 5.0	>200	-	50 - 100	50 - 100	
Rhizobium mongolense	Pb_M3_1.7	Ax, Ap, Tt, Er, St, Tm	1.5 - 2.5	>200	<50	<50	50 - 100	
Agrobacterium vitis	Fe_M3_1.5	Tt, Tm, Cp	1.5 - 2.5	-	<50	-	-	
Brevundimonas nasdae	Pb_W2_3.2	Ax, Ap, Tt, Er, St, Tm, Cp	2.5 - 5.0	100 - 130	<50	>200		*bla*TEM

	Zn_M2_1.8	Ax, Ap, St, Tm	2.5 - 5.0	50 - 100	-	-		*bla*TEM
Betaproteobacteria								
Massilia sp	Pb_W1_3.6	Ax, Ap, Tt, Er, St, Tm, Cp	2.5 - 5.0	>200	50 - 100	50 - 100		
Acidovorax sp	Fe_W1_3.3	Ax, Ap, Tt, Er, St, Tm, (Cp)	5.0 - 6.5	>200	<50	50 - 100	50 - 100	*bla*TEM
Bacteriodetes								
Flavobacteria								
Flavobacterium sp.	Pb_M1_2.2	Ax, Ap, St, Tm, Cp, (Er)	1.0 - 1.5	180 - 200	-	-	<50	*bla*TEM
	Ap_M3_3.2	Ax, Ap, St, Tm, (Tt)	>200	180 - 200	-	-	<50	*bla*TEM
	Zn_M4_2.7	Ax, Ap, Tt, Tm	2.5 - 5.0	>200	50 - 100	-	-	
Chryseobacterium lactis	Cu_M4_2.2	Ax, Ap, Tt, St, Tm	1.5 - 2.5	>200	<50	-		*bla*TEM
Sphingobacteria								
Chitinophaga oryziterrae	Zn_M3_3.2	Ax, Ap, Er, St, Tm	2.5 - 5.0	>200	-	>200	50 - 100	
Sphingobacterium sp	Fe_M3_1.2	Ax, Ap, Tt, St, Cp	5.0 - 6.5	>200	<50	-	<50	*bla*TEM
	Fe_M3_2.1	St, Tm, Cp	2.5 - 5.0	-	-	-	<50	
Firmicutes								
Bacilli								
Bacillus cereus	Fe_M2_1.1	Ax, Ap, Tt, Er, St, Tm, Cp	2.5 - 5.0	>200	>200	>200	>200	*bla*TEM
	Zn_M3_2.2	Ax, Ap, Tt, Er, St, Cp	2.5 - 5.0	>200	50 - 100	>200	<50	
	Cu_W2_2.5	Ax, Ap, Tt, Tm	1.0 - 1.5	>200	<50	-	-	*bla*TEM
	Ap_W1_3.5	Ax, Ap, Tt, Tm	>200	>200	<50	-		*bla*TEM
Bacillus thuringiensis	Pb_M2_1.2	Ax, Ap, Er, Tm, Cp	5.0 - 6.5	>200	-	<50	-	*bla*TEM
Staphylococcus sp.	Pb_W2_1.9	Ax, Ap, Tt, Er, St, Tm	2.5 - 5.0	>200	100 - 130	>200	50 - 100	
Enterococcus hirae	Pb_M3_1.6	Ax, Ap, Tt, Er, St, Tm, Cp	2.5 - 5.0	>200	50 - 100	>200	50 - 100	
	Zn_W1_1.1	Ax, Ap, Er, Tm, Cp	2.5 - 5.0	>200	-	50 - 100	-	
Actinobacteria								
Curtobacterium flaccumfaciens	Zn_M2_1.2	Ax, St, Cp,	2.5 - 5.0	>200	-	-	<50	*bla*TEM

MIC- Minimum inhibitory concentration; Isol.am- Isolation antimicrobial, isolates were intermediate resistant to the antibiotics in brackets (); Ax- amoxicillin, Ap- ampicillin, Tt- tetracycline, Er- erythromycin, St- streptomycin, Tm- trimethoprim, Cp- chloramphenicol

Eight isolates from the two WFS and two MR AC (MR BC 2 and MR AC 1) sites belonged to the Firmicutes phylum, with species representing the *Bacillus*, *Staphylococcus* and *Enterococcus* genera. These isolates represented five of the multiple antibiotic resistance phenotypes. Finally, a single isolate from the MR BC 2 site (*Curtobacterium flaccumfaciens*) represented the Actinobacteria phylum. This isolate was resistant to Ax, Ap, St, Tm, Cp and intermediate resistant to Cp.

3.3.2 Minimal inhibitory concentrations (MICs)

The MICs of MAR isolates was determined as explained in Section 3.2.2. From Table 3.2 it is evident that all isolates that were originally obtained from ampicillin containing media had Ap MICs greater than 200 mg/L. In general, with few exceptions, the isolates from the Pb, Zn and Fe containing media had MICs that were greater than 2.5 mM. In some cases this was above 5.0 mM. Isolates from the Cu containing media generally had MICs ranging from 1.0 to 2.5 mM for this metal.

High resistance levels to amoxicillin among all of the isolates that were resistant to the β-lactams antibiotics were also observed in the current study. This was regardless of the media that the isolate was originally isolated from or the identity of the isolate. Eighty four percent of isolates from the class Gammaproteobacteria and 50% from the Bacilli class that were resistant to erythromycin had MIC levels exceeding 100 mg/L for this antibiotic. Moderate to high MIC levels (50 to >200mg/L) were observed for tetracycline among 57% of the isolates that were resistant to tetracycline. The streptomycin resistant bacteria of the current study generally had low tolerance to streptomycin. Only 31% of the isolates that were resistant to streptomycin had MICs exceeding 50 mg/L. Of these only 4% exceeded 200 mg/L.

3.3.3 Antibiotic resistant genes

The presences of five ARGs were screened for by PCR amplification and sequencing verification. This was performed for selected MAR isolates that were resistant to the antibiotic for which the specific gene encoded resistance to.

Both the β-lactamase encoding genes (*amp*C and *bla*TEM) were successfully amplified from particular isolates (Table 3.1), with amplicon sizes of approximately 530 and 1080 bp respectively. Additionally, four representative amplicons (Figure 3.1 A & B) of each gene were subjected to sequencing and their identities were confirmed by BLASTn searches. The *amp*C gene was only detected in the four isolates represented in Figure 3.1A, whereas *bla*TEM was detected in 76% of the isolates. Two of the isolates that contained the *amp*C gene were originally isolated from Cu-containing

media (Cu_W2_2.2: *Pseudomonas psychrophila* and Cu_W1_1.4: *Sphingobium yanoikuyae*), one was isolated from Zn containing media (Zn_W1_3.4: *Pseudomonas gessardi*) the other was isolated from ampicillin containing media (Ap_M2_1.6: *Stenotrophomonas maltophilia*). The isolates in which *bla*Tem was present represented each of the antimicrobial containing isolation media, sampling sites, and phyla detected in the current study.

Of the three (*tet*A, *tet*L and *tet*K) tetracycline efflux pumps screened for, only *tet*A was successfully detected and verified by sequencing. The *tet*A gene was present in four isolates and the amplicon sizes of each were approximately 956bp (Figure 3.4). Three of the isolates to host this gene was originally isolated from Ampicillin containing media (Ap_M2_1.9: *Pseudomonas mosselii*; Ap_W1_3.6: *Acinetobacter* sp. and Ap_M2_1.6: *Stenotrophomonas maltophilia*) and one was originally isolated from Zn containing media (Zn_W1_1.6: *Klebsiella pneumoniae*)

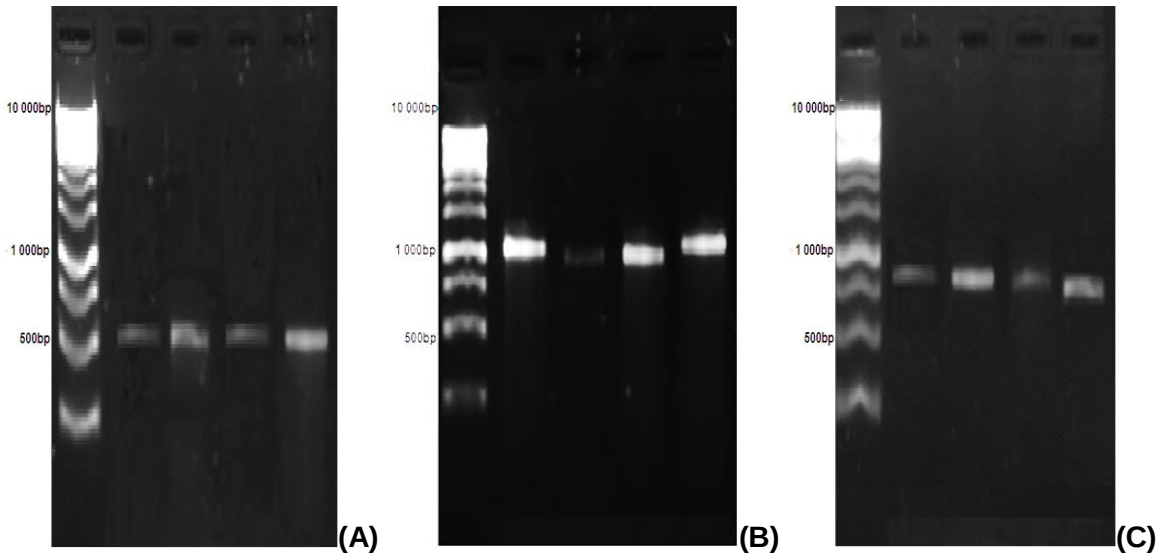


Figure 3.1: Images of agarose gel after electrophoresis of representative amplified ARGs dyed with gelRed and visualized under UV light.

(A): *amp*C (amplicon size: ± 530bp) - **L1:** 1kb Ladder; **A1:** Cu_W2_2.2; **A2:** Cu_W1_1.4; A3: Ap_M2_1.6; **A4:** Zn_W1_3.4. **(B) *bla*TEM** (amplicon size: ±1080bp) - **L2:** 1kb Ladder; **B1:** Fe_M4_2.1; **B2:** Cu_W1_1.7; **B3:** Cu_M4_2.2; **B4:** Ap_W1_3.5. **(C) *tet*A** (amplicon size: ± 957bp) - **L3:** 1kb Ladder; **C1:** Ap_M2_1.9; **C2:** Zn_W1_1.6; **C3:** Ap_M2_1.6; **C4:** Ap_W1_3.6

Amplicon bands were detected for *tet*L (Appendix F) PCRs, however, amplicon sizes varied among isolates and sequencing of the amplicons had no significant matches on the genBank database. No amplicons for *tet*K were detected in any of the isolates. No results for these were thus included.

3.4 Discussion

The aim of the current study was to identify selected MAR isolates among HPC isolated from water sources, and characterize the isolates based on MIC and genes (*bla*TEM, *amp*C, *tet*A, *tet*L, *tet*K) present in their genomes.

3.4.1 General overview: Identification of MAR isolates

Most of the selected MAR isolates were identified to species level (some only genus level) by analysis of their bacterial 16S rRNA gene sequence. The four phyla (Proteobacteria, Bacteriodetes, Firmicutes and Actinobacteria) detected in the current study were also of the most frequently phyla that were enumerated and detected by Jordaan and Bezuidenhout (2016) in the MR. Furthermore, these phyla were part of the most abundantly found phyla in a couple of recent studies that were similar to the current study (Henriques *et al*., 2016; Vaz Moreira *et al*., 2014; Li *et al.*, 2010a). The current detected phyla did not fully represent all of the phyla detected by Jordaan and Bezuidenhout (2016). Their study took place in the cold and dry season of 2012 and focused on the total bacterial community using a culture independent DNA based method (454 pyrosequencing). The current study was concerned with the identification of antimicrobial resistant HPC bacteria (culture dependent). Thus, it was expected that less inclusive results would be found. However, resistance traits found in the cultured isolates of the current study could be shared with the total bacterial community (Li *et al*., 2010a; Thompson *et al*., 2007). A greater abundance of Proteobacteria was identified compared to the three other phyla. This could be attributed to the favored growth of Gammaproteobacteria (most abundant bacterial Class detected) on R2A media (Li *et al*., 2010a). Almost all the bacterial species or genera were common residents of wastewater or freshwater environments. The individual species detected and their clinical or agricultural relevance are further discussed in Section 3.4.4.

3.4.2 General trends of the minimum inhibitory concentrations (MICs) detected

The MIC results of the current study reflected some of the general trends observed for the HPC results from the antimicrobial containing media (Chapter 2). Considerably high MIC levels to the β-lactam antibiotics were prevalent among all of the identified species and at all of the sampling sites, irrespective of which antimicrobial agent these were originally isolated from. Results in Chapter 2 study suggested that the prevalence of β-lactam resistance could be attributed to the wide

dissemination of β-lactamase determinants in the environment as well as the intrinsic resistance of commonly detected *Pseudomonas* spp. (Henriques *et al*., 2016; Luczkiewicz *et al*., 2015; Poole, 2004). In this Chapter, results presented confirmed that both of these scenarios could true for the MR and WFS confluence. Firstly, a considerable number of β- lactam resistant *Pseudomonas* spp. were isolated and identified. Additionally, *bla*TEM, a gene encoding for the enzyme β-lactamase was found in 78% of the isolates representing each of the phyla and sites of the current study. The relevance of the presence of these genes is further discussed in Sections 3.4.3 and 3.4.4.

Isolates originally obtained from the Cu containing media had lower tolerance to the metal compared to isolates that were isolated from other metals. It is speculated that low tolerance to Cu is potentially attributed to resistance associating with chromosomes instead of plasmids. Thus, determinants for Cu resistance are not as readily disseminated as the determinants for resistance to the other metals (Pal *et al*., 2015). Altimera *et al* (2012) detected higher MICs for Cu resistant isolates from Cu polluted agricultural soils. This could be defended as bacteria from agricultural soils may be impacted more directly by Cu in their immediate environment compared to bacteria in surface water. Most of the isolates from Cu containing media of the current study were still resistant to other antibiotics (amoxicillin, tetracycline and erythromycin) with all of the isolates (from Cu containing media) having MICs above 180 mg/L for amoxicillin. This could indicate that lower concentrations of Cu potentially co-select for antibiotic resistance. The observation could also be attributed to other impacts selecting for additional antibiotic resistance that do not related to Cu. Either way, the co-occurrence of Cu- and multiple antibiotic resistances were observed and specific causes and mechanisms should be investigated further.

Fe availability is generally a limiting factor for bacterial growth. In the present study it is observed that all of the MAR isolates originally isolated on Fe-containing media, except one (*Agrobacterium vitis* (Fe_M3_1.5)), had MIC levels exceeding 2.5 mM for this metal. Some had MIC levels to Fe of above 5.0 mM. *Agrobacterium vitis* was resistant to three antibiotic classes (Tt, Tm, Cp) compared to the larger antibiotic resistance pattern spectrums of the remaining isolates enumerated on media containing this metal (Fe). Furthermore, this isolate did not show high resistance to tetracycline in the MIC assay. This suggests that isolates with resistance to more antibiotics were less inclined to catalyze the Fenton reaction. Considering the results suggests that resistance to Fe may relate to antibiotic resistance mechanisms that entail active efflux. These may also be involved in pumping Fe out of the cell. Therefore, less Fe is present inside the cell and the Fenton reaction is not catalyzed. This is speculation and has not yet been documented in previous studies. It should be further

investigated. Resistance mechanisms to Fe are not well described for environmental isolates. This is probably due to Fe not traditionally being seen as a toxic element even though it has been found (Kimiran-Erdem *et al*., 2015). On the other hand, mechanisms for microbes to resist Fe depletion, especially in mammalian hosts, are well described (Cherayil, 2011).

High concentrations of Pb are known to either inhibit cell growth or select for Pb resistance mechanisms, such as the activation of efflux pumps (Naik and Dubey, 2013). These P-type ATPase efflux systems may be located on plasmids that may also host genes for a range of antibiotic resistance mechanisms (Drudge *et al*., 2012). This may explain the observation that isolates exhibiting higher MIC levels for Pb were generally also resistant to several antibiotic classes. These isolates generally had higher MICs to these antibiotics compared to the isolates with lower MICs for this metal. In this study the highest HPC bacterial growth occurred on Pb containing media compared to media containing other metals. This was attributed to potential factors such as low bioavailability of the metal (Roane and Kellog, 1996) or P-type ATPase efflux systems encoded on plasmids in the presence of high concentrations of Pb (Drudge *et al*., 2012). The observation of Pb resistant bacteria at the control site could be attributed to Pb being released by motorboat exhausts in the Klerkskraal Dam which might be contaminating the MR (Van Aard and Erdmann, 2004)

The isolates from Zn containing media had high MICs >2.5 mM for Zn. This could be attributed to a number of resistance mechanisms as described in Section 2.4.2d (Prapagdee and Watcharamusik, 2009; Banjerdkij *et al*., 2003; Coudhury and Srivastava, 2001). In addition to resistance to Zn, a number of MAR isolates originally isolated from media containing Zn had reduced susceptibility to amoxicillin, tetracycline and in to lesser extend also streptomycin. This indicates that there could be co-selective pressures responsible for Zn and antibiotic resistance.

In addition to the high MIC levels detected for metals and the β-lactam antibiotics, high resistance (MIC above 100 mg/L) to tetracycline and erythromycin were detected in at least one isolate representative from each of the anthropogenic impacted sites. The high MIC levels for these antibiotics are of clinical concern as treatment of possible infectious diseases caused by bacteria that are resistant to these antibiotics will be difficult (Chudobova *et al.*, 2014). Even though tetracycline resistance was the least abundant phenotype observed among al HPC isolated tested (Chapter 2), some MAR isolates identified were highly resistant to this antibiotic. The high MICs detected for tetracycline could be attributed to any one of the 40 genetic tetracycline resistance determinants (Li *et al.*, 2010a). Though not abundant, *tet*A genes were detected in the chromosomes of four isolates

in the current study. This confirms that there are genetic determinants present in the surface water of the current study, which could be responsible for the high tetracycline MIC levels (Wang *et al.*, 2014). Only three of the identified isolates showed extreme resistance to streptomycin (MIC >200 mg/L). A larger number were generally resistant to this antibiotic according to the results of the disc diffusion method.

3.4.3 Antibiotic resistance genes detected

Various studies have reported the presence of ARGs in the natural environment and especially in surface water sources (Molale and Bezuidenhout, 2016; Adesoji *et al*., 2015; Bergeron *et al*., 2015; Zhang *et al*., 2015; Marti *et al*., 2013; Coutinho *et al*., 2014; Thompson *et al.*, 2007; Biyela *et al*., 2004).

The surface water systems of the current study support a number of agricultural, recreational (fishing, swimming and picnic activities) and religious (baptism) activities (Molale and Bezuidenhout, 2016). The presence of ARGs in these waters could readily disseminate from the environment to humans and animals (Molale and Bezuidenhout, 2016). Furthermore, drinking water purification systems have not been designed to remove ARGs even if they remove the bacteria containing the genes (Adesoji *et al*., 2015). Thus, these genes may pass though drinking water production facilities and land in bulk water as well as biofilms. Such genes may be passed directly to the human biota through consumption, where they are able to confer resistance to clinical relevant bacteria (Bergeron *et al*., 2015). This concern is relevant to the current study as the MR is the major source for drinking water to the downstream town of Potchefstroom (Jordaan and Bezuidenhout, 2013). Sequencing verification of PCR amplicons indicated that three of the five resistance genes screened for, were present in genomes of some MAR isolates.

*bla*TEM was the most frequently detected ARG and was found in 78% of the isolates. Previous studies have also found this gene to be the most prevalent resistance gene detected in environmental isolates (Bailey *et al*., 2011; Lachmayr *et al*., 2009). The gene was detected in isolates from all of the sites. All but one (Zn_M2_1.8: *Brevundimonas nasdae*) of the isolates that hosted this gene had high levels of resistance (MIC >100 mg/L) to the two β-lactam antibiotics. In addition, *ampC* was detected in four isolates of which three were isolated from the WFS sites and were originally isolated on metal containing media. The presence of both these genes on metal containing media indicates that β-lactam resistance may co-occur with metal resistance in these metal impacted surface waters. These results could indicate that the MAR isolates of the current study may host determinants for ESBL or

metallo-β-lactamase (MBL). These ESBLs provide bacteria with the ability of resistance to a broader spectrum of this class of antibiotics (Bora *et al*., 2014; Palzkill, 2013). Both genes (*bla*TEM and *ampC*) are often found on transposons and plasmids which may give reason to the prevalence of resistance to antibiotics of the β-lactam class in this aquatic ecosystem (Bailey *et al*., 2011). Some isolates in the current study were resistant to the β-lactam antibiotics, but *bla*TEM and *ampC* could not be detected. This could potentially indicate that other mechanisms are responsible for the observed resistance (Palzkill, 2013) or plasmids containing these genes might have been lost by the methodological approaches taken.

Efflux pumps are generally found in all bacterial species either on chromosomes or plasmids (Sun *et al*., 2014). Both *tet*L and *tet*K are efflux pumps associated with tetracycline resistance. However, *tet*K was also not detected in tetracycline resistant *Enterococcus* isolates from surface waters of the NWP of South Africa (Molale and Bezuidenhout, 2016). These authors did detect *tet*L in 17% of their *Enterococcus* isolates. The exact primers and PCR conditions of Molale and Bezuidenhout (2016) for *tet*L were applied to the tetracycline resistant isolates of the current study. The amplicons were not of expected size and sequencing of these gave non-specific results. The lack of a positive control made optimization of the protocol difficult. Further optimization and the inclusion of a positive control are required in order to confirm the gene as absent from the aquatic system of the NWP. Li and co-workers (2010a) screened for a range of tetracycline resistance (*tet*) genes from wastewaters and receiving bodies of an oxytetracycline production plant. Their results found that in an environment with such a prominent presence of the selector (oxytetracycline), *tet*K was the least abundant *tet* gene detected among 23 *tet* genes screened for. Furthermore, *tet*L genes were detected in 34.1% of their isolates whereas *tet*A was most abundant (detected in 67.0% of the isolates) among all of their isolates.

The *tet*A gene was detected in the genomes of four MAR isolates of the current study, confirming their presence in the surface water of the specific study area. However, they were not detected in all of the isolates that were resistant to tetracycline. The following suggestion could be made as to why this gene was not as widely disseminated among the tetracycline resistant isolates and to why there was high resistance (MICs) for tetracycline in isolates: (i) It could be that the specific genes searched for are not present and that other tetracycline resistance genes are conferring resistance (Molale and Bezuidenhout, 2016); (ii) Instead of being part of the chromosome, the gene (or other *tet* genes) may rather be situated on plasmids (Sun *et al*., 2014; Nikaido *et al*., 2009) and that these plasmids were lost during culturing or DNA isolation. Table 3.1 shows that *tet*A was detected alongside one or both

of the β-lactamase resistance genes. This provides evidence that some HPC bacteria in the environment of the current study host multiple ARGs in their chromosomes. This is an observation that has been made in previous studies (Capkin *et al*., 2015; Pal *et al*., 2015). One isolate that hosted *tet*A gene was originally isolated from Zn containing media, illustrating that not only does the gene associate with resistance to other antibiotics it also associates with metal resistance. The *tet*A gene belongs to the MFS of the multidrug transporters (Sun et al., 2014; Nikaido *et al*., 2009). Thus, it was expected to find this gene among the MAR isolates and it may be conferring resistance to more than one antimicrobial substance in the particular surface water body.

3.4.4 Evaluation of the clinical and agricultural relevant species detected

Most of the 73 MAR isolates identified belonged to the genera *Pseudomonas* and *Acinetobacter*. A range of other genera were also detected and most of these were also prevalent in previous studies of aquatic systems (Di Cesare *et al*., 2016; Henriques *et al*., 2016; Icgen and Yilmaz, 2014; Zhang *et al*., 2009). The current study did not focus on screening for pathogenic bacteria as HPC bacteria irrespective of their pathogenicity have the ability to host ARGs (Thompson *et al*., 2007). However, a concerning observation was the MAR representatives that were identified as species or genera previously documented to be pathogenic to humans, animals and plants. Implications of finding these species in the MR system thus need to be further explored. Some of these clinical relevant species are further described here.

The abundance of *Pseudomonas species* among the MAR pool of the current study could be attributed to a wide range of intrinsic resistance determinants (Henriques *et al*., 2016; Luczkskiewicz *et al*., 2015; Hwang *et al*., 2005). The plasticity of the *Pseudomonas* genome allows members of the genus to acquire all known antibiotic resistance genetic mechanisms (Luczkskiewicz *et al*., 2015). However, the current study did not detect all of the genes screened for in the genomes of all of the *Pseudomonas* isolates. Henriques and co-workers (2016) found *Pseudomonas species* with the antibiotic resistance phenotype Ax, Ap, Tt, Tm, Cp among Cu and Zn resistant isolates. The *Pseudomonas species* isolated from Zn in the current study were also resistant to these antibiotics, with the addition of others. In the case of the Henriques *et al*. (2016) study, this resistance phenotype was associated with the well described mercury reductase *mer*A plasmid gene. This particular gene may be wide spread in nature and particularly the geographical location of the current study. This was not determined in the current study and should be included in future studies. Some members of the *Pseudomonas* genus documented to be pathogenic include *P. putida*, *P. mosseilii, P. fluorescens, P. syringae* and *P. cichorii.* *Pseudomonas putida* is a well-documented human pathogen that have

been found to cause nosocomial infections and bacteremia from skin tissue infections among others (Fernández *et al*., 2015; Thomas *et al*., 2013). *Pseudomonas mosseilii* and *Klebsiella pneumonia* have cytotoxic effects on human cells (Leneveu-Jenvrin *et al*., 2013; Cano *et al.,* 2009). *Pseudomonas syringae* and *Pseudomonas cichorii* are plant pathogens that primarily cause leaf spot in different plant species (Marques *et al*., 2016; Morris *et al*., 2008).

In the present study, several MAR *Acinetobacter* sp. were isolated from a number of sites. Three isolates could only be identified to genus level while the others were identified as strains belonging to *Acinetobacter beijerinckii*, *Acinetobacter haemolyticus*, *Acinetobacter johnsonii* and *Acinetobacter calcoaceticus*. This genus is clinically relevant as some strains are able to lyse mammalian red blood cells and to disrupt gelatin (Nemec *et al*., 2009). A number of recent studies have detected members of this genus to be resistant to both metal and antibiotics from the natural environment (Di Cesare *et al*., 2016; Kimiran-Erdem *et al*., 2015; Pal *et al*., 2015; Icgen and Yilmaz, 2014). Akbulut and co-workers (2014) also found multiple drug and heavy metal resistance in hemolytic and non-hemolytic *Acinetobacter* isolates. As its name suggests *Acinetobacter haemolyticus* is a heamolytic pathogen that have been associated with clinical infections (Doughari *et al*., 2011). Acinetobacter beijerincii is a recently classified heamolytic agent in humans and its major habitat is soil and water (Visca *et al*., 2011; Nemec *et al*., 2009). *Acinetobacter johnsonnii* is an agent for catheter related blood stream infections (Pindi, 2013). The soil organism *Acinetobacter calcoaceticus* has also been associated with rare cases of nosocomial infections (Glew *et al*., 1977). The *Acinetobacter* genus are gaining increased attention due to their potential to cause severe infections and their profundity to develop multidrug resistance (Doughari *et al*., 2011). Based on the frequent detection of members of this genus from the sites of the current study and the possible health impacts that their presence pose, it is recommended that this genus is explored in more detail in future studies.

Stenotrophomonas maltophilia is related to *Pseudomonas* sp., but is increasingly seen as a global multiple-drug resistant opportunistic pathogen, known to colonize with *Pseudomonas aeruginosa* in the respiratory tract of cystic fibrosis patients (Brooke, 2012). A concerning observation is that in the present study, one isolate with this identity was resistant to all of the antibiotics screened for. In this case the MICs of >200 mg/L were observed. This isolate was positive for all three resistance genes tested for. Previous studies have also found *Stenotrophomonas maltophilia* to be involved in the genetic transfer of resistance determinants. Plasmids with B-lactamases and gene clusters hosting antibiotic and heavy metal resistance determinants have been found in strains of this species (Pages *et al*., 2008; Barbolla *et al*., 2004). Finding these pathogenic *Pseudomonas spp* that are resistant to

multiple antibiotics is a concern for human and animals as well as plants that are exposed to this water. The implications of these findings need further investigation.

The two Enterobacteriaceae species detected (*Klebsiella pneumoniae* and *Serratia marcescens*) are considered human health threats as both cause community acquired infections and some strains are multidrug resistant (Li *et al*., 2014; Ojdana *et al*., 2014; Kurz *et al*., 2003). This multi-drug resistance trait was also found in the current study. Both of these species are widespread in the environment where they are able to colonize and infect animals and plants (Holt *et al*., 2015; Kurz *et al*., 2003). Common life threatening infections caused by *Klebsiella pneumoniae* include meningitis, severe pneumoniae, endophthalmitis, necrotizing fasciitis and pyogenic liver abscess (Li *et al*., 2014). *Serratia marcescens* are cytotoxic *in vitro* and associate with certain lung infections (Kurz *et al*., 2003).

In another recent study, two *Chitinophaga* sp. have been isolated from metal contaminated soils (Chanda *et al*., 2014). These authors showed that isolates were also resistant to a range of antibiotics (Chanda *et al*., 2014). The *Chitinophaga* sp. representative species from the present study was resistant to all of the antibiotics screened for. It also had extreme MICs for some of the antibiotics. The finding of this species is similar to the observations of Jordaan and Bezuidenhout (2016) for the same study area. Some species in the *Sphingobacterium* genus are also known to cause human respiratory tract infections (Lambiase *et al*., 2009).

Of the Firmicutes phylum *Bacillus cereus* is a well described human pathogen that is commonly associated with food poisoning (Bottone, 2010). *Enterococcus hirae* is a well described zoonotic pathogen, but have been found to cause human urinary tract infections (Bourafa *et al*., 2015). However, it is usually associated with growth depression in young chickens (Farrow and Collins, 1985).The ARPs of the two *Enterococcus hirae* isolates of the current study were resistant to more antibiotics than the single isolate of this species that Molale and Bezuidenhout (2016) isolated from the MR system. The two *E. hirae* isolates of the current study were isolated on metal containing media and each had their own antibiotic resistance phenotype. A single staphylococcal isolate was only identified to genus level. A range of species in this genus are known human pathogens and numerous recent studies have also found metal and multidrug resistant strains of this genus in surface waters (Pal *et al*., 2015; Xue *et al*., 2015; Yilmaz *et al*., 2013). The isolate with this identity from the current study was isolated from Pb (MIC 2.5 – 5.0 mM) and was resistant to 5 antibiotic classes with high MICs towards the four antibiotics part of the MIC analysis. Thus, the frequent presence of isolates

from the Bacteriodetes phylum in the MR as also detected by Jordaan and Bezuidenhout (2016), pose considerable health impacts. Not only were potentially pathogenic bacteria present in the water, they were also multidrug resistant.

Genera of which some members are also known plant pathogens that cause leaf spot include *Rhizobium* sp., *Acidovorax* sp. and some *Xanthomonas* species (Morris *et al*., 2008; Marques *et al*., 2016; Coutingo and Wallis, 1991). *Xantomonas campestris* pathovars, for one, are leading causative agents of bacterial streak disease of maize, which is the staple food of South Africa (Coutingo and Wallis, 1991). In the present study, *Flavobacterium* sp. was isolated. This species have been associated with algal blooms or high nutrient and organic carbon levels (Jordaan and Bezuidenhout, 2016). The presence of these species pose environmental risks and may cause damage to crop farming if the water is used for irrigation.

Finding these potential pathogenic species that are resistant to multiple antibiotics is a concern for human and animals as well as plants that are exposed to this water. The MR is used for recreation as well as agricultural purposes. Treatment of infection caused by such pathogenic/opportunistic pathogens in consumers in general and especially the immune-compromised will be challenging (Jordaan and Bezuidenhout, 2016; Mulamattathil *et al*., 2014). The implications of these findings need further investigation.

3.5 Conclusion

This study contributes by identifying MAR species from surface water and provides detail on the different resistance profiles and resistance levels observed among different species from various sites. Though the natural environment is seen as an important host and disseminator of antibiotic resistance data, on the presence of antibiotic resistance determinants are still lacking in South Africa, which places the country in a dangerous situation. The current study was able to detect three resistance genes as present in MAR bacteria from surface water that is impacted on by a range of anthropogenic activities. A considerable number of these MAR bacteria were also resistant to metals at high concentrations which indicates that there is a co-occurrence metal and antibiotic resistance. Furthermore, the current situation raises concerns about human and animal health. Among the bacteria multiple antibiotic resistance phenotypes was common and high resistance levels were present among pathogenic/ opportunistic pathogenic bacteria.

CHAPTER 4

Conclusions, Limitations and Recommendations

4.1 Conclusions

The aim of this study was to determine levels of antimicrobial resistant bacteria and genes in selected surface water bodies of the NWP. In order to achieve this, two major objectives were formalized each with its own sub-objectives. Major objective one was to determine the antimicrobial resistant HPC bacteria levels in relation to physico-chemical quality of the water in selected surface water of the NWP. Major objective two was to identify and characterize MAR HPC bacteria using PCR and sequencing of amplicons (16S rRNA genes and selected ARGs) and micro dilution broth assay for MIC determination. The outcomes of these two major objectives are briefly highlighted in the sections that follow:

4.1.1 Antimicrobial resistant HPC bacteria levels in relation to physico-chemical quality of the water

The results demonstrated that gold mining activities in the WFS has a detrimental impact on the downstream physico-chemical and bacteriological quality of water to the rural communities around Carltonville. Furthermore, this source also impact on the water quality of the MR. A decline in the physico-chemical water quality from before the confluence (MR) to after the confluence of the two rivers was observed. It was also determined that agriculture was impacting the entire system which added to the nutrient load of the surface water. Antimicrobial resistant HPC bacteria were isolated from all of the sites investigated. Amongst these a large number were resistant to ampicillin. Of the metals screened for, the HPC bacterial communities were most susceptible to Cu and most resistant to Pb. Bacteria resistant to Zn and Fe were also detected at all of the sites. A general trend was that there is a greater selection for antimicrobial resistance in the WFS compared to the MR. This deduction was supported by the observation that the antimicrobial resistant bacterial levels at sites before the confluence of the MR and WFS is higher in the WFS segment compared to the levels at MR sites before and after the confluence of these two river segments. Furthermore, many of the isolates from metal containing media were resistant to antibiotics, of which a large number were resistant to multiple antibiotics.

4.1.2 Identity and characteristics of MAR-HPC and detection of ARGs

A total of 72 MAR isolates were successfully identified using 16S rRNA gene sequences. Most of the isolates belonged to the phylum Proteobacteria while the remainder belonged to the Bacteriodetes, Firmicutes and Actinobacteria phyla. Some of the genera and species detected have been previously documented to be potential pathogens. This is of concern considering the number of immunocompromised in South Africa and particularly the NWP that could be directly exposed to the surface waters. The minimal inhibitory concentration (MIC) of each identified isolate was determined for the antimicrobial that they were initially isolated from and for some antibiotics they showed resistance to. From the results it could be concluded that generally the isolates that were initially from Cu-containing media did not have extremely high MICs to this metal. The isolates obtained from the other metals (Pb, Zn) had very high MICs to the respective metal that they were isolated from. These isolates also had high MICs against the various antibiotics. It can thus be concluded that the pollution of the environmental setting may be playing a key role in the selection for antibiotic and metal resistance. ARGs (*bla*TEM, *amp*C and *tet*A) especially *blaTEM* were detected in the genomes of isolates from all of the phyla and sampling sites. These genes are present in the bacteria from the surface water of the MR and WFS and may be transferred by MGEs among different species.

In general, it is concluded that antimicrobial resistant bacteria and more specifically MAR bacteria are present at all of the sites of the current study. However, the numbers of these bacteria are considerably higher at the sites with greater mining impacts. Thus, it is concluded that the mining activity upstream of the WFS is selecting for more antibiotic resistant bacteria and the WFS should be considered a hotspot for co-selection of metal and antibiotic resistance. However, antibiotic resistance and genes are widespread in the environment as it was also found in the MR sites upstream of the confluence. These findings host considerable healthcare concerns as these surface waters are used for a number of recreational activities and as the major source of drinking water to the towns of Carltonville and Potchefstroom. This water is also used for agricultural production and may thus have impacts on livestock watering and irrigation. Finally, it can be concluded that the aim and objectives of the study were successfully achieved.

4.2 Limitations and Recommendations

The current study used a general culture based approach targeting HPC bacteria. Data is thus limited to this particular group only. Considering that most bacteria in the various niches on this planet is non culturable, a large cross-section of the microbial population may have been missed. However, the approach and various concepts used and demonstrated is still valid. Including more metals and

antibiotics for enrichment could provide some more details, but those used for the present study were sufficient to prove the original hypothesis.

Singleplex PCR method for detection of some antibiotic resistant genes are tedious and expensive considering bench time, reagents and controls. However, for screening purposes this approach can indicate the presence of genes and identify hotspots at which more expensive analysis should be considered. What this approach did was to link certain antibiotic phenotypes to genotypes.

Though a general conclusion could be made that metal and antibiotic resistance existed among HPC bacteria in the current study area, the study could not fully conclude on the specific mechanisms of resistance and which metals selected for which antibiotic resistance. The lack of technical standards and historic information made interpretation of the results even more difficult.

Another limitation was the unavailability of antibiotic concentrations in the water. Thus, statistical analysis could not pinpoint whether antibiotic resistance was rather selected by metal concentrations or antibiotic concentrations. None the less the study serves as a baseline and the WFS was identified as a hotspot for co-occurrence of metal and antibiotic resistance which was disseminated to downstream sites. Future studies should therefore build on the limitations of the current study to fully investigate the phenomenon in this system as it poses health risks to the consumer. The following recommendations are therefore proposed:

- **Screening for a larger range of ARGs by metagenomics and next generation sequencing (NGS) would provide more accurate information**

  Though routine implementation of molecular approaches are expensive and therefore not always possible in developing countries such as South Africa, effort should be made to determine the genes relevant to antibiotic resistance in the environment as suggested by Bora *et al*. (2014). Metagenomics and next generation sequencing offer rapid methods to investigate the entire collection of genes responsible and to determine how genes from different antimicrobials relate to one another (Pal *et al*., 2015). It is suggested that this type of analysis be performed at least once in hotspot areas in order to determine problematic or prominent genes and to predict the possible consequences. This will aid in developing accurate surveillance plans. In addition to the advantage of comprehensive information on ARGs, NGS can be applied in a feasible manner in order to perform other water quality assessments such as microbial source tracking of bioindicators and investigation of

biodegradation (Tan *et al*., 2015). Whole genome sequencing of isolate Ap_M2_1.6 from which three ARGs were detected or metal resistant isolates Zn_W1_1.6 and Cu_W1_1.4 from which two ARGs for each were detected, could bring greater insight into more resistance mechanisms present in the impacted system (Tan *et al*., 2015; Schwartz *et al*., 2015).

- **Re-evaluation of allowable metal concentrations in surface water after investigation of more surface water systems in South Africa**

  Metal concentrations have been under routine surveillance in surface waters due to their direct toxic effects to humans and animals and their general detrimental impact on water quality (DWAF, 1996). Recent studies have indicated that metal concentrations lower than that of the standards set out by DWAF for surface water are selecting for metal resistance (Seiler and Berendonk, 2012). This observation was also observed in the current study. Thus, it is proposed that the phenomenon be investigated in more systems and that Target Water Quality Ranges that were set out by DWAF (1996) and the resource quality objectives (DWAF, 2009) be re-evaluated considering the health concern, especially in a country with high numbers of immunocompromised individuals.

- **Include routine antibiotic concentration measurements and quantification PCR of resistance genes**

  In addition to routine measurements of metal concentrations, routine measurements of antibiotics in the environment should also be considered. This will aid in tracking sources that release antibiotics into the environment which serves as a selective pressure for antibiotic resistance (Lubick, 2011).

  The inclusion of qPCR could aid future studies to pinpoint whether antibiotic resistance was due to antibiotic contamination or due to metal contamination by means of statistical analysis as was performed by Zhu *et al*. (2013) on swine farms. Thus, this approach involves: (i) measurement of antibiotic and metal concentrations (ii) quantification of ARGs and (iii) correlating these values measured in (i) and (ii) using statistical methods.

- **Development of standard protocols and routine surveillance of antibiotic resistance in the natural environment**

  Research has been done globally to indicate that the natural environment is a reservoir and probably one of the main disseminators of antibiotic resistance (Lachmayr *et al*., 2009; Biyela *et al*., 2004; Kümmerer, 2003). This phenomenon together with waterborne disease, is killing millions of people annually. South Africa has a considerable number of immunocompromised people and it is therefore suggested that antibiotic resistance in the environment be investigated and monitored adequately (Mgbemena *et al*., 2012). Eradicating antibiotic resistance from a surface water source is not an option at the moment, thus baseline levels should be determined for each system and monitored. If fluctuations are observed, causative agents should be searched for and handled in such a manner as to keep the levels as low as possible (Lubick, 2011). This type of data will aid to predict possible threats to humans, animals and plants that are dependent on the source. Routine gene monitoring as described by Michael *et al*. (2016) could also be considered.

- **Include different microhabitats and the drinking water system**

  Previous studies have indicated that the sediment may play an important part as reservoir for the co-selection of metal and antibiotic resistance, an observation partially observed in the current study (Tuckfield and McArthur, 2008; Wright *et al*., 2006). Furthermore, co-selection of metal and antibiotic resistance has frequently been observed in biofilms (Baker-Austin *et al*., 2006). Thus, as also suggested by Wright *et al*. (2006) different microhabitats within the system should be investigated in order to identify reservoirs for the phenomenon. This will aid to predict how genes are transferred between the microhabitats and disseminate throughout the system.

  Baker-Austin and co-workers (2006) reviewed the co-selection of antibiotic and metal resistance and found that studies indicated that isolates within the drinking water system were more resistant to Cu, Zn and Pb than those isolated from the raw water. In addition to these metals the isolates were also resistant to multiple antibiotic classes. The authors attributed this to the various metals that bacteria are exposed to within drinking water systems. Future studies should therefore include drinking water and distribution systems when investigating the co-selection of metal and antibiotic resistance. This will aid to predict the threats that the metals found in drinking water systems or those that line the distribution system pose to human health.

- **Determination of pathogenicity and screening for virulence genes will be advisable for clinically relevant isolates**

  Many of the identified isolates of the current study were strains of species or genera that have previously been documented to be pathogenic. Thus, as suggested by Molale and Bezuidenhout (2016) it is important to investigate the pathogenicity of clinical relevant organisms, together with antibiotic resistance, to link and predict possible community infections.

REFERENCES

Adesoji, A.T., Ogunjobi, A., Olatoye, I.O., Douglas, D.D. 2015. Prevalence of tetracycline resistance genes among multi-drug resistant bacteria from selected water distribution systems in Southwestern Nigeria. *Annals of Clinical Microbiology and Antimicrobials*, 14(1): 1-8.

Ahipathy, M.V., Puttaiah, E.T. 2006. Ecological characteristics of Vrishabhavathi River in Bangalore, India. *Environmental Geology*, 49: 1217-1222.

Akbulut, S., Yilmaz, F., Icgen, B. 2014. Surface water isolates of hemolytic and non-hemolytic *Acinetobacter* with multiple drug and heavy metal resistance ability. *Journal of Water and Health*, 12(1): 1–12.

Akinbowale, O.L., Peng, H., Grant, P., Barton, M.D. 2007. Antibiotic and heavy metal resistance in motile aeromonads and pseudomonads from rainbow trout (*Oncorhynchus mykiss*) farms in Australia. *International Journal of Antimicrobial Agents*, 30(2): 177-182.

Al-Bahry, S.N., Al-Zadjali, M.A., Mahmoud, I.Y., Elshafie, A.E. 2012. Biomonitoring marine habitats in reference to antibiotic resistant bacteria and ampicillin resistance determinants from oviductal fluid of the nesting green sea turtle, *Chelonia mydas*. *Chemosphere*, 87(11): 1308-1315.

Alekshun, M.N., Levy, S.B. 2007. Molecular mechanisms of antibacterial multidrug resistance. *Cell*, 128(6): 1037-2050.

Allen, D.A, Austin, B., Colwell, R.R. 1977. Antibiotic resistance patterns of metal-tolerant bacteria isolated from an estuary. *Antimicrobial Agents and Chemotherapy*, 12(4): 545-547.

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

Alonso, A., Sanchez, P., Martinez, J.L. 2001. Environmental selection of antibiotic resistance genes. *Environmental Microbiology*, 3: 1-9.

Altimira, F., Yáñez C, Bravo G, González M, Rojas LA, Seeger M. 2012. Characterization of copper-resistant bacteria and bacterial communities from copper-polluted agricultural soils of central Chile. *BMC Microbiology*, 12(1): 186-190.

Amalesh, S., Bera, Khatun, M., Sinha, C., Pal, P., Lalee, A., Mandal, A. 2012. An investigation on heavy metal tolerance and antibiotic resistance properties of bacterial strain *Bacillus* sp. isolated from municipal waste. *Journal of Microbiology and Biotechnology Research*, 2(1): 178-189.

Aminov, R.I. 2010. A brief history of the antibiotic era, lessons learned and challenges for the future. *Frontiers in Microbiology*, 1: 134.

Argüello, J.M., Raimunda, D., Padilla-Benavides, T. 2013. Mechanisms of copper homeostasis in bacteria. *Frontiers in Cellular and Infection Microbiology*, 3: 73.

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.

Auerbach, E. A., Seyfried, E.E., McMahon, K.D. 2007. Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Research*, 41(5): 1143-1151.

Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J.G., Smith, J., Struhl, K. 1995. Short Protocols in Molecular Biology. New York, USA: Wiley and Sons, Inc.

Bailey, J.K., Pinyon, A.L., Anantham, S., Hall, R.M. 2011. Distribution of *bla*$_{TEM}$ gene and *bla*$_{TEM}$-containing transposons in commensal *Escherichia coli*. *Journal of Antimicrobial Chemotherapy*, 66(4): 745-751.

Baker-Austin, C., Wright, M.S., Stepanauskas, R., McArthur, J.V. 2006. Co-selection of antibiotic and metal resistance. *Trends in Microbiology*, 14(4): 176-182.

Banjerdkij, P., Vattanaviboon, P., Mongkolsuk, S. 2003. Cadmium-induced adaptive resistance and cross-resistance to zinc in *Xantomonas campestris. Current Microbiology*, 47(3): 260-262.

Banks, D., Markland, H., Smith, P.V., Mendez, C., Rodrigueez, J., Huerta, A., Saether, O.M. 2004. Distribution, salinity and pH dependence of elements in surface waters of the catchment areas of the Salars of Coipasa and Uyini, Bolivian Altiplano. *Journal of Geometrical Exploration*, 84: 141 -166.

Barbolla, R., Catalano, M., Orman, B.E., Famiglietti, A., Vay, C., Smayevsky, J., Centrón, D., Piñeiro, S.A. 2004. Class 1 integrons increase trimethoprim-sulfamethoxazole MICs against epidemiologically unrelated *Stenotrophomonas maltophilia* isolates. *Antimicrobial Agents and Chemotherapy*, 48(2): 666-669.

Barnard, S., Venter, A., Van Ginkel, C.E. 2013. Overview of mining related pollution on the water quality of the Mooi River system's reservoirs using basic statistical analysis and self-organized mapping. *Water SA,* (35)5: 655-662.

Bauer, A. W., Kirby, W. M. M., Sherris, J. C., Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, 36:493-496.

Bergeron, S., Boopathy, R., Nathaniel, R., LaFleur, G. 2015. Presence of antibiotic resistant bacteria and antibiotic resistance genes in raw source water and treated drinking water. *International Biodeterioration and Biodegradation*, 102: 370-374.

Berglund, B. 2015. Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination to antibiotics. *Infection Ecology and Epidemiology*, 5: 28564.

Bezuidenhout, C.C. 2012. A large scale study on microbial and physico-chemical quality of selected groundwater and surface water in the North-West Province, South Africa. WRC Report no: 1966/1/13.

Biyela, P.T., Lin, J., Bezuidenhout, C.C. 2004. The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. *Water Science and Technology*, 50(1): 45-50.

Blindauer, C.A., Harrison, M.D., Robinson, A.K., Parkinson, J.A., Bowness, P.W., Sadler, P.J., Robinson, N.J. 2002. Multiple bacteria encode metallothioneiens and SmtA-like fingers. *Molecular Microbiology*, 45: 1421-1432.

Bora, A., Hazarika, N.K., Shukla, S.K., Prasad, K.N., Sarma, J.B., Ahmad, G. 2014. Prevalence of bla_{TEM}, bla_{SHV}, and bla_{CTX-M} genes in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from Northeast India. *Indian Journal of Pathology and Microbiology*, 57(2): 249-254.

Bottone, E.J. 2010. *Bacillus cereus*, a volatile human pathogen. *Clinical Microbiology Reviews*, 23 (2) 382-398.

Bourafa, N., Loucif, L., Boutefnouchet, N., Rolain, J.M. 2015. *Enterococcus hirae*, an unusual pathogen in humans causing urinary tract infection in a patient with benign prostatic hyperplasia: first case report in Algeria. *New Microbes and New Infections*, 8: 7-9.

Brolund, A., Sundqvist, M., Kahlmeter, G., Grape, M. 2010. Molecular characterization of trimethoprim resistance in *Esherichia coli* and *Klebsiella pneumoniae* during a two year intervention on trimethoprim use. *PLoS ONE*, 5(2): e9233.

Brooke, J.S. 2012. *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. *Clinical Microbiology Reviews*, 25(1): 2–41.

Brucker, M.C. 1986. Gram staining: A useful laboratory technique. *Journal of Nurse- Midwifery*, 3(3): 156-158.

Bruins, M.R., Kapil, S., Oehme, F.W. 2000. Microbial resistance to metals in the environment. *Ecotoxicology and Environmental Safety*, 45(3): 198-207.

Burnes, B.S. 2003. Antibiotic resistance analysis of faecal coliforms to determine faecal pollution sources in a mixed use watershed. *Environmental Monitoring and Assessment*, 85(1): 87-98.

Burridge, L., Weis, J.S., Cabello, F., Pizarro, J., Bostick, K. 2010. Chemical use in salmon aquaculture: a review of current practices and possible environmental effects. *Aquaculture*, 306: 7-23.

Cano, V., Moranta, D., Llobet-Brossa, E., Bengoechea J.A., Garmendia, J. 2009. *Klebsiella pneumoniae* triggers a cytotoxic effect on airway epithelial cells. *BMC Microbiology*, 9:156.

Capkin, E., Terzi, E., Altinok, I. 2015. Occurrence of antibiotic resistance genes in culturable bacteria isolated from Turkish trout farms and their local aquatic environment. *Disease of Aquatic Organisms*, 114: 127-137.

Chanda, D., Sharma, G.D., Jha, D.K., Hijri, M., Al-Otaibi, F .2014. Isolation and characterization of *Chitinophaga* sp. from paper mill polluted soil. *International Journal of Scientific Research*, 3 (12): 2277-8179.

Chen, S. Li, X., Sun, G., Zhang, Y., Su, j., Ye, J. 2015. Heavy metal induced antibiotic resistance in bacterium LSJC 7. *International Journal of Molecular Sciences*, 16: 23390- 23404.

Cherayil, B.J. 2011. The role of iron in the immune response to bacterial infection. *Immunologic Research*, 50(1): 1-9.

Cho, K.H., Han, D, Park, Y, Lee, S.Y., Cha, S.M., Kang, J.H., Kim, J.H. 2010. Evaluation of the relationship between two different methods for enumeration faecal indicator bacteria: Colony-forming unit and most probable number. *Journal of Environmental Sciences*, 22: 846-850.

Chudobova, D., Dostalova, S., Blazkova, I., Michalek, P., Ruttkay-Nedecky, B., Sklenar, M., Neidl, L., Kudr, J., Gumulec, J., Tmejova, K., Konecna, M., Vaculovicova, M., Heynek, D., Masarik, M., Kynicky, J., Kizek, R., Adam, V. 2014. Effect of ampicillin, streptomycin, penicillin and tetracycline on metal resistant and non-resistant *Staphylococcus aureus*. *International Journal of Environmental Research and Public Health*, 11: 3233-3255.

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.

Coudhury, R., Srivastava, S. 2001. Zinc resistance mechanisms in bacteria. *Current Science*, 81(7): 768-775.

Coutingo, T.A., Wallis, F.M. 1991. Bacterial streak disease of maize (*Zea mays L.*) in South Africa. *Journal of Phytopathology*, 133(2): 112–112.

Coutinho F.H., Pinto L.H., Silviera, C.B., Pinto, L.H., Salloto, G.R., Cardoso, A.M., Martins, O.B., Viera, R.P., Clementino, M.M. 2014. Antibiotic resistance widespread in urban aquatic environments of Rio de Janeiro, Brazil. *Microbial Ecology*, 68(3): 441-452.

Dallas, H. 2008. Water temperature and riverine ecosystems: An overview of knowledge and approaches for assessing biotic responses, with special reference to South Africa. *Water SA*, 34 (3): 393–404.

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.

Darakas, E., Koumoulidou, T., Lazaridou, D. 2009. Faecal indicator bacteria declines via a dilution of wastewater in seawater. *Desalination*, 248: 1008-1015.

Dashti, A.A., Jadaon, M.M., Abdulsamad, A.M., Dshti, H.M. 2014. Heat treatment of bacteria: A simple method of DNA extraction for molecular techniques. *Kuwait Medical Journal*, 41(2): 117-122.

Davies, J., Davies, D. 2010. Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology reviews*, 74(3): 417-433.

DEA: Department of Environmental Affairs. 2012. Environmental sustainability indicators, Technical report 2012. Department of Environmental Affairs. Pretoria, South Africa: Government printer.

DEAT: Department of Environmental Affairs and Tourism. 2008. Emerging issues paper: Mine water pollution. CSIR, Natural Resources and the Environment. Pretoria, South Africa: Government printer.

Delpla, I., Jung, A.V., Baures, E., Clement, M., Thomas, O. 2009. Impacts of climate change on surface water quality in relation to drinking water production. *Environmental International*, 35: 1225-1233.

Di Cesare, A., Eckert, E.M., Corno, G. 2016. Co-selection of antibiotic and heavy metal resistance in freshwater bacteria. *Journal of Limnology*, 75(2): 59-66.

Doughari, H.J., Ndakidemi, P.A., Human, I.S., Benade, S. 2011. The ecology, biology and pathogenesis of *Acinetobacter* spp.: An Overview. *Microbes and Environments*, 26(2): 101-112.

DPW: Department of Public Works. 2012. Small waste water treatment works- design guidelines. Pretoria, South Africa: Government Printer.

Drudge, C.N., Elliot, A.V.C., Plach, J.M., Ejim, L.J., Wright, G.D., Droppo, I.G., Warren, L.A. 2012. Diversity of integron- and culture- associated antibiotic resistance genes in freshwater floc. *Applied and Environmental* Microbiology, 78(12): 4367-4372.

Dunn, G., Harris, L., Cook, C., Prystajecky, N. 2014. A comparative analysis of current microbial water quality risk assessment and management practices in British Columbia and Ontario, Canada. *Science of the Total Environment*, 469: 544-552.

Dupont, C.L., Grass, G., Rensing, C. 2011. Copper toxicity and the origin of bacterial resistance - new insights and applications. *Metallomics*, 3: 1109-1118.

Durand, J.F. 2012. The impact of gold mining on the Witwatersrand on the rivers and karst system of Gauteng and North West Province, South Africa. *Journal of African Earth Sciences*, 68: 24-43.

DWA: Department of Water and Sanitation. 2013. National water resource strategy 2. Chapter 2. Pretoria, South Africa: Government Printer.

DWAF: Department of Water Affairs and Forestry. 1996. South African water quality guidelines. Volume 7: Aquatic Ecosystems. Pretoria, South Africa: Government Printer.

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

DWAF: Department of Water Affairs and Forestry. 2008. Water for growth and development frame work. Pretoria, South-Africa: Government Printer.

DWAF: Department of Water Affairs and Forestry. 2009. Integrated water quality management plan for the Vaal River System: task 2: water quality status assessment of the Vaal River system. Report No. P RSA C000/00/2305/1.

DWAF: Department of Water Affairs and Forestry. 2010. Map of average rainfall of the North West Province. Pretoria, South Africa: Government Printer.

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.

Eliopoulos, G.M., Huovinen, P. 2001. Resistance to trimethoprim-sulfamethoxazole. *Clinical Infectious Disease*, 32(11): 1608-1614.

Farrow, J. A. E., Collins, M. D. 1985. *Enterococcus hirae*, a new species that Includes amino acid assay strain NCDO 1258 and strains causing growth depression in young chickens. *International Journal of Systematic Bacteriology*, 35(1): 73–75.

Fatoki, S.O., Muyima, N.Y.O., Lujiza, N. 2001. Situation analysis of water quality in the Umtata River catchment. *Water SA*, 27(4): 467-474.

Fernandez, M., Conde, S., De la Torre, J., Molina-Santiago, C., Ramos, J., Duque. 2011. Mechanisms of resistance to chloramphenicol in *Pseudomonas putida* KT2440. *Antimicrobial Agents and Chemotherapy*, 56(2): 1001-1009

Fernández, M., Porcel, M., De la Torre, J., Molina-Henares, M.A., Daddaoua, A., Llamas, M.A., Roca, A.,Carriel, V., Garzón, I., Ramos, J.L., Alaminos, M., Duque, E. Fernandes. 2015. Analysis of the pathogenic potential of nosocomial *Pseudomonas putida* strains. *Frontiers in Microbiology*, 6: 871.

Flemming, C.A, Trevors, J.T. 1989. Copper toxicity and chemistry in the environment: a review. *Water, Air and Soil Pollution*, 44: 143-158.

Gatseva, P.D., Argirova, M.D. 2008. High-nitrate levels in drinking water may be a risk factor for thyroid dysfunction in children and pregnant women living in rural Bulgarian areas. *International Journal of Hygiene and Environmental Health*, 211: 555-559.

Glew, R.H., Moellering, R.C., Kunz, L.J. 1977. Infections with *Acinetobacter calcoaceticus* (*Herellea vaginicola*): clinical and laboratory studies. *Medicine*, 56 (2): 79–97.

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.

Hamman, D. 2012. A holistic view on the impact of gold and uranium mining of the Wonderfonteinspruit. North-West University (Dissertation- M.Sc.).

Henne, K., Kahlsh, L., Hofle, M.G., Brettar, I. 2013. Seasonal dynamics of bacterial community structure and composition in cold and drinking water derived from surface water reservoirs. *Water Research*, 47: 5614-5630.

Henriques, I., Tacau, M., Leite, L., Fidalgo, C., Araujo, S., Oliviera, C., Alves, A. 2016. Co-selection of antibiotic and metal(loid) resistance in Gram-negative epiphytic bacteria from contaminated salt marshes. *Marine Pollution Bulletin*, 109(1): 427-434.

Hill, A.P., Olkers, T. 2001. Biological control against water hyacinth in South Africa: constraining factors success and new courses of action. (*In*: Julien, M.H., Hill, M.P., Center, T.D., DingJianqing, *eds*. Biological and integrated control of water hyacinth, *Eichhornia crassipes*. Beijing, China: ACIAR Proceedings. p. 33–38).

Holloway, J.M., Dahlgren, R.A. 2002. Nitrogen in rock: occurrences and biogeochemical implications. *Global Biogeochemical Cycles*, 16(4): 1118-1134.

Holt, K.E., Wertheim, H., Zadoks, R.N., Baker, S., Whitehouse, C.A., Dance, D., Jenney, A., Connor, T.R., Hsu, L.Y., Severin, J., Brisse, S., Cao, H., Wilksch, J., Gorrie, C., Schultz, M.B., Edwards, D.J., Nguyen, K.V., Nguyen, T.V., Dao, T.T., Mensink, M., Le Minch, V., Khu, N.T.K., Schultsz, C., Kuntamen, K., Newton, P.N., Moore, C.E., Strugnell, R.A., Thomas, N.R. 2015. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. *Proceedings of the National Academy of Science*, 112(27): 3574–3581.

Hur, J., Lee, B., Lee, T., Park, D. 2010. Estimation of biological oxygen demand and chemical oxygen demand for combined sewer systems using synchronous fluorescence spectra. *Sensors*, 10: 2460-2471.

Hwang, M.S.H., Morgan, R.L., Sarkar, S.F., Wang, P.W., Guttman, D.S. 2005. Phylogenetic characterization of virulence and resistance phenotypes. *Applied and Environmental Microbiology*, 71(9): 5182-5191.

Icgen, B., Yilmaz, F. 2014. Co-occurrence of antibiotic and heavy metal resistance in Kızılırmak River isolates. *Bulletin of Environmental Contamination and Toxicology*, 93: 735-743.

Ideriah, T.J.K., Amachree, O., Stanley, H.O. 2010. Assessment of water quality along AMADI Creek in Port Harourt Nigeria. *Scientia Africana*, 9(1): 150-162.

Jacoby, G.A., 2009. *AmpC* β-Lactamases. *Clinical Microbiology Reviews*, 22(1): 161-182.

Jagielski, T., Ignatowska, H., Bakuła, Z., Dziewit, Ł., Napiórkowska, A., Augustynowicz-Kopeć, E. 2014. Screening for streptomycin resistance-conferring mutations in *Mycobacterium tuberculosis* clinical isolates from Poland. *PLoS ONE* 9(6): e100078.

Janda, J.M., Abbott, S.L. 2002. Bacterial identification for publication: When is enough enough? *Journal of Clinical Microbiology*, 40(6): 1887-1891.

Jaroslawiecka, A., Piotrowska-Seget, Z. 2014. Lead resistance in micro-organisms. *Microbiology*, 160: 12-25.

Jordaan, K., Bezuidenhout, C.C. 2013. The impact of physico-chemical water parameters on bacterial diversity in the Vaal River, South Africa. *Water SA*, 39(3): 385-396.

Jordaan, K., Bezuidenhout, C.C. 2016. Bacterial community composition of an urban river in the North West Province, South Africa, in relation to physico-chemical water. *Environmental Science and Pollution Research International,* 23(6): 5868-5880.

Juda, M., Chudzik-Rzad, B., Malm, A. 2016. The prevalence of genotypes that determine resistance to macrolides, lincosamides and streptogramins B compared with spiramycin sisceptinility among erythromycin-resistant *Staphylococcus epidermidis. Memorias Do Instituto Oswaldo Cruz*, 111(3): 155-160.

Kalantari, N. 2008. Evaluation of toxicity of iron, chromium and cadmium on *Bacillus cereus* growth. *Iranian Journal of Basic Medical Sciences*, 10(4): 222-228.

Kalule-Sabiti M., Heath R. 2008. Underground water a key resource and the associated environmental challenges in North West Province of South Africa. Paper presented at the Water Institute of South Africa Biennial conference, Sun City, 18-22 May 2008. http://www.ewisa.co.za/literature/files/2008_018.pdf Date accessed: 07 Feb. 2015.

Kanoch, S., Rubin, B.K. 2010. Mechanisms of action and clinical application of macrolides as immunomodulatory medications. *Clinical Microbiology Reviews*, 23(3): 590-615.

Kaufman, G. 2011. Antibiotics mode of action and mechanisms of resistance. *Nursing Standard*, 25(42): 49-55.

Kellenberger, E. 2001. Exploring the unknown- The silent revolution of microbiology. *EMBO Reports*, 2(1): 5-7.

Kim, B.J., Park, J.H., Park, T.H., Bronstein, P.A., Schneider, D.J., Cartinhour, S.W., Shuler, M.L. 2009. Effect of iron concentration on the growth rate of *Pseudomonas syringae* and the expression of virulence factors in *hrp*-Inducing minimal medium. *Applied and Environmental Microbiology*, 75(9): 2720-2726.

Kimiran-Erdem, A., Arslan-Aydoğdu, E.O., Gürün, S., Altun, O. 2015. Determination of multiple antibiotic and heavy metal resistance of the bacteria isolated from the Küçükçekmece lagoon, Turkey. *Polish Journal of Environmental Studies*, 24(3): 1077-1084.

Koh, D.C., Ko, K.S., Kim, Y., Lee, S.G., Chang, H.W. 2007. Effect of agricultural land use on the chemistry of groundwater from basaltic aquifers, Jeju Island, South Korea. *Hydrogeology Journal*, 15: 727-743.

Kohanski, M.A., Dwyer, D.J., Collins, J.J. 2010. How antibiotics kill bacteria: from targets to networks. *Nature Reviews Microbiology*, 8(6): 423-435.

Krewulak, K.D., Vogel, H.J. 2008. Structural biology of bacterial iron uptake. *Biochimica et Biophysica Acta (BBA)- Biomembranes*, 1778(9): 1781- 1804.

Kume, T., Akca, E., Nakano, T., Kapur, S., Watanabe, T. 2010. Seasonal changes of fertilizers impacts on agricultural drainage in a salinized area in Adana, Turkey. *Science of the Total Environment*, 408: 3319-3326.

Kümmerer, K. 2003. The significance of antibiotics in the environment. *Journal of Antimicrobial Chemotherapy*, 52: 5–7.

Kurz, C.L., Cauvet, S., Andrès, E., Aurouze, M., Vallet, I., Michel, G.P.F., Uh, M., Celli, J., Filloux, A., De Bentzmann, S., Steinmetz, I., Hoffman, J., Finlay, B.B., Gorvel, J., Ferrandon, D., Ewbak, J.J. 2003. Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by *in vivo* screening. *The EMBO Journal*, 22(7): 1451-1460.

Lachmayr, K.L., Kerkhof, L.J., DiRienzo, A.G., Cavanaugh, C.M., Ford, T.M. 2009. Quantifying nonspecific TEM β-Lactamase (bla_{TEM}) genes in a wastewater stream. *Applied and Environmental Microbiology*, 75(1): 203-211.

Laffite, A., Kilunga, P.I., Kayembe, J.M., Devarajan, N., Mulaji, C.K., Giuliani, G., Sleveykova, A.I., Poté, J. 2016. Hospital effluents are one of several sources of metal, antibiotic resistance genes, and bacterial markers disseminated in Sub-Saharan urban rivers. *Frontiers in Microbiology*, 7: 1128-1142.

Lambiase, A., Rossano, F., Del Pezzo., M., Raia, V., Sepe, A., De Gregorio, F., Catania, M.R. 2009. *Sphingobacterium* respiratory tract infection in patients with cystic fibrosis. *BMC Research Notes* 2: 262.

Lane, D.J. 1991. 16S/23S rRNA sequencing. (*In*: Stackebrandt, E., Goodfellow, M, *eds*. Nucleic Acid Techniques in Bacterial Systematics. New York City, US: John Wiley & Sons. p. 125-175).

Leekha, S., Terrell, C.L., Edson, R.S. 2011. General principles of antimicrobial therapy. *Mayo Clinic Proceedings*, 86 (2): 156-167.

Lemire, J.A., Harrison, J.J., Turner, R. 2013. Antimicrobial activity of metals: mechanisms, molecular targets and applications. *Nature Reviews Microbiology*, 11: 371-384.

Leneveu-Jenvrin, C., Madi, A., Bouffartigues, E., Biaggini, K., Feuilloley, M., Chevalier, S., Connil, N. 2013. Cytotoxicity and inflammatory potential of two *Pseudomonas mosselii* strains isolated from clinical samples of hospitalized patients. *BMC Microbiology*, 13: 16-20.

Lewis, K. 2013. History of the β- lactams. *Nature Reviews Drug Discover*, 12: 371-387.

Li, B., Zhao, Y., Liu, C., Chen, Z., Zhou, D. 2014. Molecular pathogenesis of *Klebsiella pneumoniae. Future Microbiology*, 9(9): 1071-1081.

Li, D., Yu, T., Zhang, Y., Zhang, M., Li, Z., Liu, M., Qi, R. 2010a. Antibiotic resistance characteristics of environmental bacteria from an oxytetracycline production wastewater treatment plant and receiving river. *Applied and Environmental Microbiology*, 76(11): 3444-3451.

Li, J.F., Li, L., Sheen, J. 2010b. Protocol: a rapid and economical procedure for purification of plasmid or plant DNA with diverse applications in plant biology. *Plant Methods*, 6:1.

Line, D. E., Harman, W.A., Jennings, G.D. 1998. Comparing sampling schemes for monitoring pollutant export from dairy pasture. *Journal of American Water Resources Association*, 34: 1265-1273.

Liu, T., Nakashima, S., Hirosa, K., Uemura, Y., Shibasaka, M. 2003. A metallothionein and CPx-ATPase handle heavy metal tolerance in the filamentous cyanobacteria *Oscillatoria brevis. FEBS Letters,* 542: 159-163.

Lopez-Perez, M., Mirete, S., Valadez-Jardon, E., Gonzalez-Pastor, J.E. 2013. Identification and modeling of a novel chloramphenicol resistance protein detected by functional metagenomics in a wetland of Lerma, Mexico. *International Microbiology*, 16(2): 103-111.

Lubick, N. 2011. Tools for tracking antibiotic resistance. *Environmental Health Perspectives*, 119(5): 214-217.

Luczkiewicz, A., Kotlarska, E., Artichowicz, W., Tarasewicz, K., Fudala-Ksiazek. 2015. Antimicrobial resistance of *Pseudomonas* spp. isolated from wastewater and wastewater-impacted marine coastal zone. *Environmental Science and Pollution Research*, 22(24): 19823-19824.

Madhavan, H.N., Murali, S. 2011. Mechanisms of development of antibiotic resistance in bacteria among clinical specimens. *Journal of Clinical and Biochemical Sciences*, 1(2): 42-48.

Makhlough, A. 2008. Water quality characteristics of Mengkuang Reservoir based on phytoplankton community structure and physico-chemical analysis. Malaysia: University of Malaysia. (Thesis - M.Sc).

Marques, E., Borges, R.C.F., Uesugi, C.H. 2016. Identification and pathogenicity of *Pseudomonas cichorii* associated with a bacterial blight of gerbera in the Federal District. *Horticultura Brasileira*, 34(2): 244-248.

Marti, E., Jofre, J., Balcazar, J.L. 2013. Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment plant. *PLoS One*, 8(10): e78906.

Martinez, J.L. 2009. Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environmental Pollution*, 157(11): 2893-2902.

McArthur, J.V., Tuckfield, R.C., Baker-Austin, C. 2012. Antimicrobial textiles. (*In*: Coatees, A.R.M., *eds*. Antibiotic Resistance. Handbook of Experimental Pharmacology. New York, US: Springer. p. 211: 138).

Mezger, A., Gullberg, E., Göransson, J., Zorzet, A., Herthnek, D., Tano, E., Nilsson, M., Andersson, D.I. 2015. A general method for rapid determination of antibiotic susceptibility and species in bacterial infections. *Journal of Clinical Microbiology*, 53(2): 425-432.

Mgbemena, I.C., Nnokwe, J.C., Adjeroh,L.A., Onyemekara, N.N. 2012. Resistance of bacteria isolated from Otamiri River to heavy metals and some antibiotics. *Current Research Journal of Biological Sciences,* 4(5): 551-556.

Michael, C.A., Franks, A.E., Labbate, M. 2016. The antimicrobial resistance crisis: management through gene monitoring. *Open Biology*, 6: 160236.

Molale, L.G., Bezuidenhout, C.C. 2016. Antibiotic resistance, efflux pump genes and virulence determinants in *Enterococcus* spp. from surface water systems. *Environmental Science and Pollution Research*, 23(21): 21501-21510.

Moller, A.K., Barkay, T., Hansen, M.A., Norman, A., Hansen, L.H., Sorensen, S.J., Boyd, E.S., Kroer, N. 2014. Mercuric reductase genes (*mer*A) and mercury resistance plasmids in high arctic snow, freshwater and sea-ice brine. *FEMS Microbiological Ecology*, 87 (1): 52-63.

Monapathi, M.E. 2014. Diversity and antifungal susceptibility of yeast in the selected rivers of the North West Province. North-West University (Dissertation- M.Sc.).

Moniruzzman, M., Elabi, S.F., Jahangir, M.A.A. 2009. Study on temporal variation of physico-chemical parameters of Buriganga River water through GIS (Geographical Information Systems) technology. *Bangladesh Journal of Scientific and Industrial Research*, 44 (3): 327-334.

Morris, C.E., Sands, D.C., Vinatzer, B.A., Glaux, C., Guilbaud, C., Buffiere, A., Yan, S., Dominiquez, H., Thompson, B.M. 2008. The life history of the plant pathogen *Pseudomonas syringae* is linked to the water cycle. *The ISME Journal*, 2: 321-334.

Mthembu, M.S. Biyela, P.T., Djarova, T.G., Basson, A.K. 2010. The use of antibiotic resistance profiling as a means of tracing sources of faecal contamination in source water. *Water Science and Technology: Water Supply*, 10(2): 209–215.

Mulamattathil, S.G., Bezuidenhout, C.C., Mbewe, M., Ateba, C.N. 2014. Isolation of environmental bacteria from surface and drinking water in Mafikeng, South Africa, and characterization using their antibiotic resistance profiles. *Journal of Pathogens*, 2014: 1-11.

Nageswaran, N., Ramteke, P.W., Verma, O.P., Pandey, A. 2012. Antibiotic susceptibility and heavy metal tolerance pattern of *Serratia Marcescens* isolated from soil and water. *Bioremediation and Biodegradation*, 3(7): 1000158.

Naik, M.M., Dubey, S.K. 2013. Lead resistant bacteria: Lead resistance mechanisms, their applications in lead bioremediation and biomonitoring. *Ecotoxicology and Environmental Safety*, 98: 1-7.

Naik, M.M., Dubey, S.K., Khanolkar, D., D'Costa, B. 2013. P-type ATPase and *mdr*L efflux-pump mediated lead and multi-drug resistance in estuarine bacterial isolates. *Current Science*, 105(10): 1366-1372.

Nemec, A., Musılek, M., Maixnerova, M., De Baere, T., Van der Reijden, T.J.K, Vaneechoutte, M., Dijkshoorn, L. 2009. *Acinetobacter beijerinckii* sp. nov. and *Acinetobacter gyllenbergii* sp. nov., haemolytic organisms isolated from humans. *International Journal of Systematic and Evolutionary Microbiology*, 59: 118-124.

Nhu, N.T., Lan, N.T., Phuong, N.T., Chau, N, Farrar, J. 2012. Association of streptomycin resistance mutations with level of drug resistance and *Mycobacterium tuberculosis* genotypes. *International Journal of Tuberculosis and Lung Disease*, 16: 527-531.

Nies, D.H. 1999. Microbial heavy-metal resistance. *Applied Microbiology and Biotechnology*, 51: 730-750.

Nikaido, H. 2009. Multidrug resistance in bacteria. *Annual Review of Biochemistry*, 78: 119-146.

Noguerol-Arias, J., Rodríguez-Abalde, A., Romero-Merino, E., Flotats, X. 2012. Determination of chemical oxygen demand in heterogeneous solid or semisolid samples using a novel method combining solid dilutions as a preparation step followed by optimized closed reflux and colorimetric measurement. *Analytical Chemistry*, 84: 5548- 5555.

NWA: National Water Act. 1998. No. 36. Government Gazette No.19182. Cape Town, South Africa: Government Printer.

NWDACE: North West Department of Agriculture, Conservations and Environment. 2008. State of the Environment Report. North West Province, South Africa.

NWPG: North West Provincial Government. 2002. State of the Environment Report, South Africa. http://www.nwpg.gov.za/soer/fullreport/html Date accessed: 12 Aug. 2015.

Oberholster, P.J., Ashton, P.J. 2008. State of the Nation Report: An overview of the current status of water quality and eutrophication in South African rivers and reservoirs. CSIR Report No. CSIR/NRE/WR/IR/2008/0075/C. Council for Scientific and Industrial Research: Pretoria, South Africa.

Ojdana, D., Sacha, P., Wieczorek, P., Czaba, S., Michalska, A., Jaworowska, J., Jurczak, A., Poniatowski, B., Tryniszewska. 2014. The occurrence of *bla*CTX-M, *bla*SHV, and *bla*TEM genes in extended-spectrum β-lactamase-positive strains of *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* in Poland. *International Journal of Antibiotics*, 2014: 935842

Pages, D., Rose, J., Conrod, S., Cuine, S., Carrier, P., Heulin, T., Achouak, W. 2008. Heavy metal tolerance in *Stenotrophomonas maltophilia*. *PLoS One*, 3(2): e1539.

Pal, C., Bengtsson-Palme, J., Kridtiansson, E., Larsson, D.G.J. 2015. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. *BMC Genomics*, 16: 964.

Palzkill, T. 2013. Metallo-β-lactamase structure and function. *Antimicrobial Therapeutics Reviews*, 1277: 91-104.

Patel, M., Baxi, K., Dayma, P., Upadhyay, D., Parmar, N., Kundu, S., Haldar, S., Mody, K.H., Jha, B. 2014. Assessment of groundwater quality with respect to bacteriological contamination in Bhavnagar, Gujarat, India. *CLEAN- Soil, Air, Water*, 40(10): 1351-1362.

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.

Pelczar, M.J., Reid, R.D. 1958. Laboratory exercises in bacteriology and diagnostic methods. 7th eds. Lawrence, KS: The world Co.

Pereira, V.M. 2009. Occurrence of filamentous fungi and yeasts in three different drinking water sources. *Water Research*, 43: 3813-3819.

Petti, C. A., Polage, C.R., Schreckenberger, P. 2005. The Role of 16S rRNA gene sequencing in identification of microorganisms misidentified by conventional methods. *Journal of Clinical Microbiology,* 43(12): 6123–6125.

Pindi, P.K., Yadav, P.R., Shanker, A.S. 2013. Identification of opportunistic pathogenic bacteria in drinking water samples of different rural health centers and their clinical impacts on humans. *BioMed Research International*, 2013: 348250.

Poole, K. 2004. Resistance to β- lactam antibiotics. *Cellular and Molecular Life Sciences*, 61 2200-2223.

Prapagdee, B., Watcharamusik, A. 2009. Adaptive and cross-protective responses against cadmium-resistant bacterium isolated from a zinc mine. *Brazilian Journal of Microbiology*, 40(4): 838-845.

Quinlivan, E.P., McPartlin, J., Weir, D.G., Scott, J. 2000. Mechanism of antimicrobial drug trimethoprim revisited. *The FASEB Journal*, 14(15): 2519-2524.

Ramirez, M.S., Tolmasky, M.E. 2010. Aminoglycoside modifying enzymes. *Drug Resistance Updates*, 13(6): 151-171.

Razak, A., Asiedu, A.B., Entsua-Mensah, R.E.M., de Graft-Johnson, K.A.A. 2009. Assessment of water quality of the Oti River in Ghana. *West African Journal of Applied Ecology*, 15(1): 45-60.

Reynolds, P.E. 1989. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *European Journal of Clinical Microbiology and Infectious Diseases*, 8(11): 943-950.

Richardson, H.W. 1997. Handbook of copper compounds and applications. New York, US: Marcel Dekker.

Roane, T.M., Kellogg, S.T. 1996. Characterization of bacterial communities in heavy metal contaminated soils. *Canadian Journal of Microbiology*, 42(6): 593-603.

Ryu, H, Henson M, Elk, M, Toledo-Hernandez, C, Griffith, J, Blackwood, D. Noble, R., Gourmelon, M., Glassmeyer, S., Jorge, W., Domingo, S. 2013. Development of quantitative PCR assays

targeting the 16S rRNA genes of *Enterococcus* spp. and their application to the identification of *Enterococcus* species in environmental samples. *Applied Environmental Microbiology*, 79:196–204.

Sabah, A.A., Fouzul, A.M. 2012. The environmental impact of gold mines: pollution by heavy metals. *Central European Journal of Engineering*, 2(2): 304-313.

SANS: South African National Standards. 2006. Water quality- Sampling. Part 6: Guidance on sampling of rivers and streams. Pretoria, South Africa: SABS Standards Division.

Schroeder, M. R., Stephens, D.S. 2016. Macrolide resistance in *Streptococcus pneumoniae*. *Frontiers in Cellular and Infection Microbiology*, 6: 98.

Schwartz, T., Armant, O., Bretschneider, N., Hahn, A., Kirchen, S., Seifert, M., Dötsch, A. 2015. Whole genome and transcriptome analyses of environmental antibiotic sensitive and multi-resistant *Pseudomonas aeruginosa* isolates exposed to waste water and tap water. *Microbial Biotechnology*, 8(1): 116-130.

Schwartz, T., Kohnen, W., Jansen, B., Obst, U. 2003. Detection of antibiotic resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiology and Ecology*, 43: 325-335.

Seiler, C., Berendonk, T.U. 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Frontiers in Microbiology*, 3: 399.

Sengeløv, G., Agerso, Y., Halling-Sørensen, B., Baloda, S.B., Andersen, J.S., Jensen, L.B. 2003. Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. *Environment International*, 28(7): 587-595.

Sharma, J., Sharma, M., Ray., P. 2010. Detection of TEM & SHV genes in *Esherichia coli* and *Klebsiella pneumoniae* isolates in a tertiary care hospital from India. *India Journal of Medical Research*, 132: 332-336.

Silver. S., Pung, L.T. 2009. Heavy metals, bacterial resistance. (*In*: Schaechter, M., *eds*. Encyclopedia of microbiology. Oxford, England: Elsevier. p. 220-227).

Spain, A., Alm, E. 2003. Implications of microbial heavy metal tolerance in the environment. *Reviews in Undergraduate Research*, 2: 1-6.

Sparks, D.L. 2005. Toxic metals in the environment: The role of surfaces. *Elements*, 1: 193-197.

Spitz, K., Trudinger, J. 2009. Mining and the environment. London: CRC Press/Balkema. p. 69-65.

Springer, B., Kidan, Y.G., Prammananan, T., Ellrott, K., Böttger, E.C, Sander, P. 2001. Mechanisms of streptomycin resistance: selection of mutations in the 16S rRNA gene conferring resistance. *Antimicrobial Agents and Chemotherapy*, 45(10): 2877-2884.

Srinivasan, R., Karaoz, U., Volegova, M., MacKichan, J., Kato-Maeda, M., Miller, S., Nadarajan, R., Brodie, E.L., Lynch, S.V. 2015. Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. *PLoS One*, 10(2): e0117617.

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.

Sun, J., Deng,Z., Yan, A. 2014. Bacterial multidrug efflux pumps: mechanism, physiology and pharmacological exploitations. *Biochemical and Biophysical Research Communications*, 453(2): 254-267.

Sutcliff, D. 1998. The ionic composition of surface waters in the English Lake District, related to bedrock geology, with some singular facts and speculations on the existence of mineral-rich ground waters. *Freshwater Forum*, 11(1): 30-51.

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.

Sutton, M.A., Howard, C.M., Erisman, J.W., Billen, G., Bleeker, A., Grennfelt, P., Van Grisven, H., Grizzetti, B. 2011. The European nitrogen assessment: sources, effects and policy perspectives. Cambridge, England: Cambridge University Press.

Swamy, T.A., Kipngetich, T.E., Hillary, M. 2013. Determination of levels of phosphates and sulphates in domestic water from three selected springs in Nandi County, Kenya. *International Journal of Pharmacy and Life Sciences*, 4(7): 2828-2833.

Tan, B., Ng, C., Nshimyimana, J.P., Loh, L.L., Gin, K.Y.H., Thompson, J.R. 2015. Next-generation sequencing (NGS) for assessment of microbial water quality: current progress, challenges, and future opportunities. *Frontiers in Microbiology*, 6: 1027.

Tan, S.C., Yiap, B.C. 2009. DNA, RNA, and protein extraction: The past and the present. *Journal of Biomedicine and Biotechnology*, 2009: 574398.

Taylor, D.J.C., Page, D.C., Geldenhuys, P. 1988. Iron and steel in South Africa. *Journal of the South African Institute of Mining and Metallurgy*, 88(3): 73-95.

Tenover, F.C. 2006. Mechanisms of antimicrobial resistance in bacteria. *The American Journal of Medicine*, 119(6): 3-10.

Ter Braak, C.F.J., Smilauer, P. 1998. CANOCO. Reference manual and user's guide to Canoco for Windows: Software for canonical community ordination (Version 4). New York, US: Microcomputer Power.

Thomas, B.S., Okamoto, K., Bankowski, M.J., Seto, T.B. 2013. A lethal case of *Pseudomonas putida* bacteremia due to soft tissue infection. *Infectious Diseases in Clinical Practice*, 21(3): 147-213.

Thompson, S.A., Maani, E.V., Lindell, A.H., King, C.J., McArthur, J.V. 2007. Novel tetracycline determinant isolated from an environmental strain of *Serratia marcescens. Applied and Environmental Microbiology*, 73(7): 2199-2206.

Trzcinski, K., Cooper, B.S., Hryniewicz, W., Dowson, C.G. 2000. Expression of resistance to tetracycline in strains of methicillin resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, 45: 763-770.

Tuckfield, R.C., McArthur, J.V. 2008. Spatial analysis of antibiotic resistance along metal contaminated streams. *Microbial Ecology*, 55(4): 595-607.

Ullah, F., Malik, S.A., Ahmed, J., Ullah, F., Shad, S.M., Ayaz, M., Hussain, S., Khatoon, L. 2012. Investigation of genetic basis of tetracycline resistance in *Staphylococcus aureus* from Pakistan. *Tropical Journal of Pharmaceutical Research*, 11(6): 925-931.

Van Aard, W.J., Erdmann, R. 2004. Heavy metals (Cd, Pb, Cu, Zn) in mudfish and sediments from three hard-water dams of the Mooi River catchment, South Africa. *Water SA*, 30(2): 211-218.

Van der Walt, I.J., Nell, B., Winde, J. 2002. Integrated catchment management: The Mooi River (Northwest Province, South Africa) as a case study. *Cuadernos de Investigación Geográfica*, 28: 109-126.

Van Hoek, A.H.A.M., Mevius, D., Guerra, B., Mullany, P., Roberts, A.P., Aarts, H.J.M. 2011. Acquired antibiotic resistance genes: an overview. *Frontiers in Microbiology*, 2: 203.

Van Wyk, D.A.B., Bezuidenhout, C.C., Rhode, O.H.J. 2012. Diversity and characteristics of yeasts from water resources in the North West Province, South Africa. *Water and Science Technology: Water Supply* 12(4): 422- 430.

Vaz Moreira, I., Nunes, O.C. Manaia, C.M. 2014. Bacterial diversity and antibiotic resistance in water habitats: searching the links with the human biome. *FEMS Microbiology Reviews*, 38(4): 761-778.

Venkatesharaju, K., Ravikumar, P., Somashekar, R. K., Prakash, K. L. 2010. Physico-chemical and bacteriological investigation on the river Cauvery of kollegal stretch in Karnataka. *Journal of Science, Engineering and Technology*, 6(1): 50-59.

Veraldo, P.E., Montanari, M.P., Giovanetti, E. 2009. Genetic elements responsible for erythromycin resistance in streptococci. *Antimicrobial Agents and Chemotherapy*, 53(2): 343-353.

Visca, P., Seifer, H., Towner, K.J. 2011. *Acinetobacter* infection: An emerging threat to human health. *IUBMB Life*, 63(12): 1048-1054.

Von Schirnding, Y., Mathee, A., Kibel, M., Robertson, P., Strauss, N., Blignaut, R. 2003. A study of pediatric blood lead levels in a lead mining area in South Africa. *Environmental Research*, 93: 259-263.

Wales, A.D., Davies, R.H. 2015. Co-selection of resistance to antibiotics, biocides and heavy metals and its relevance to foodborne pathogens. *Antibiotics*, 4(4): 567-604.

Walmsley, R.D. 2000. Perspectives on eutrophication of surface waters. WRC Report No. Kv 129/00. Water Research Commission: Pretoria, South Africa.

Wang, W., Guo, Q., Xu, X., Sheng, Z.K., Ye, X., Wang, M. 2014. High-level tetracycline resistance mediated by efflux pumps *tet*(A) and *tet*(A)-1 with two start codons. *Journal of Medical Microbiology*, 63(11): 1454–1459.

Whitman, W.B., Goodfellow, M., Kämpfer, P., Busse, H.-J., Trujillo, M.E., Ludwig, W., Suzuki, K. 2012. Bergey's Manual of Systematic Bacteriology. 2nd ed., vol. 5, parts A and B. New York, US: Springer-Verlag.

WHO: World Health Organization. 2003. Guidelines for drinking water quality: Total dissolved solids in Drinking-water. Geneva, Switzerland: World Health Organization.

Wiegand, I., Hilpert, K., Hancock, R.E.W. 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*, 3: 163-175.

Winde, F. 2010. Uranium pollution of the Wonderfonteinspruit, 1997-2008 Part 1: Toxicity, regional background and sources of uranium. *Water SA*, 36(3): 239-256.

Wright, M.S., Peltier, G.L., Stepanauskas, R., McArthur, J.V. 2006. Bacterial tolerances to metals and antibiotics in metal-contaminated and reference streams. *FEMS Microbiology Ecology*, 58(2): 293–302.

Xue, H., Wu, Z., Li, L., Li, F., Wang, Y., Zhao. 2015. Coexistence of heavy metal and antibiotic ressitance within a novel composite staphylococcal cassette chromosome in a *Staphylococcus haemolyticus* isolate from Bovine Mastitis milk. *Antimicrobial Agents and Chemotherapy*, 59(9): 5788-5792.

Yang, C., Wu, D., Chang, C. 2007. Nitrate in drinking water and risk of death from colon cancer in Taiwan. *Environmental International*, 33: 649-653.

Yilmaz, F., Orman, N., Serim, G., Kochan, C., Ergene, A., Icgen, B. 2013. Surface water-borne multidrug and heavy metal-resistant *Staphylococcus* isolates characterized by 16S rDNA sequencing. *Bulletin of Environmental Contamination and Toxicology*, 91(6): 697-703.

Zarraonaindia, I., Smith, D.P, Gilbert, J.A. 2013. Beyond the genome: community-level analysis of the microbial world. *Biology and Philosophy*, 28(2): 261-282.

Zhang, F., Li, Y., Yang, M., Li, W. 2012. Content of heavy metals in animal feeds and manures from farms of different scales in North East China. *International Journal of Environmental Research and Public Health*, 9(8): 2658-2668.

Zhang, S., Han, B., Gu, J., Wang, C., Whang, P., Ma, Y., Cao, J., He, Z. 2015. Fate of antibiotic resistant cultivable heterotrophic bacteria and antibiotic resistance genes in wastewater treatment processes. *Chemosphere*, 135: 138-145.

Zhang, Y.L., Marrs, C., Simon, C., Xi, C.W. 2009. Wastewater treatment contributes to selective increase of antibiotics among *Acinetobacter* spp. *Science of the Total Environment*, 407: 3702-3706.

Zhu, Y., Johnson, T.A., Su, J., Qiao, M., Guo, G., Stedtfeld, R.D., Hasham, S.A., Tiedje, J.M. 2013. Diverse and abundant antibiotic resistance genes in Chines swine farms. *Proceedings of the National Academy of Sciences*, 110(9): 3435-3440.

APPENDIX A

Minimum co-selective concentrations (MCC) of relevant metals and the measured concentrations by the Tlokwe Municipality at sites in the Mooi River catchment on relevant dates. All values are in µg/L.

Metals	MCC (Seiler and Berendonk, 2012)	WFS (TM)			MR (TM)			GMB			PD		
		March	May	July	March	May	July	March	May	July	March	May	July
Cu	0.03	28.0	6.00	1.00	18.0	3.00	2.00	8.00	1.00	32.0	3.00	2.00	12.0
Fe	NA	50.0	10.0	10.0	40.0	40.0	0.00	10.0	60.0	40.0	20.0	30.0	0.00
Pb	0.29	2.00	1.00	53.0	1.00	1.00	3.00	1.00	1.00	1.00	1.00	1.00	2.00
Zn	19.61	N/D	20.0	70.0	N/D	20.0	210.0	N/D	20.0	250.0	N/D	10.0	60.0

The March, May and July sampling dates of the Tlokwe sampling were 10/03/15, 12/05/15 and 28/07/15 respectively. WFS (TM) Wonderfonteinspruit, MR (TM) – Mooi River site, GMB- Gerhardminne Bron, BDI- Boskop Dam Inflow, BDC- Boskop Dam Canal, PD- Potchefstroom Dam, N/A – not applicable, N/D – not determined. Cu- copper, Fe-iron, Pb – lead, Zn – zinc

APPENDIX B

Geographical (GPS) co-ordinates of the sites of the current study and the sites at which Tlokwe Municipality measured metal concentrations.

Sites of the current study		
Site	**Longitude**	**Latitude**
WFS 1	27.382111	-26.315806
WFS 2	27.270500	-26.367417
MR BC 1	27.158722	-26.253167
MR BC 2	27.139083	-26.360111
MR AC 1	27.124584	-26.514528
MR AC 2	27.103614	-26.571806
Tlokwe Municipality sites		
WFS (TM)	27.150000	-26.440000
MR (TM)	27.120000	-26.450000
GMB	27.130000	-26.510000
BDI	27.070000	-26.670000
PD	27.090000	-26.660000

WFS-Wonderfonteinspruit, MR- Mooi River, BC- before confluence, AC- after confluence, (TM)-Tlokwe Municipality, GMB- Gerhardminne Bron, BDI- Boskop Dam Inflow, BDC- Boskop Dam Canal, PD- Potchefstroom Dam

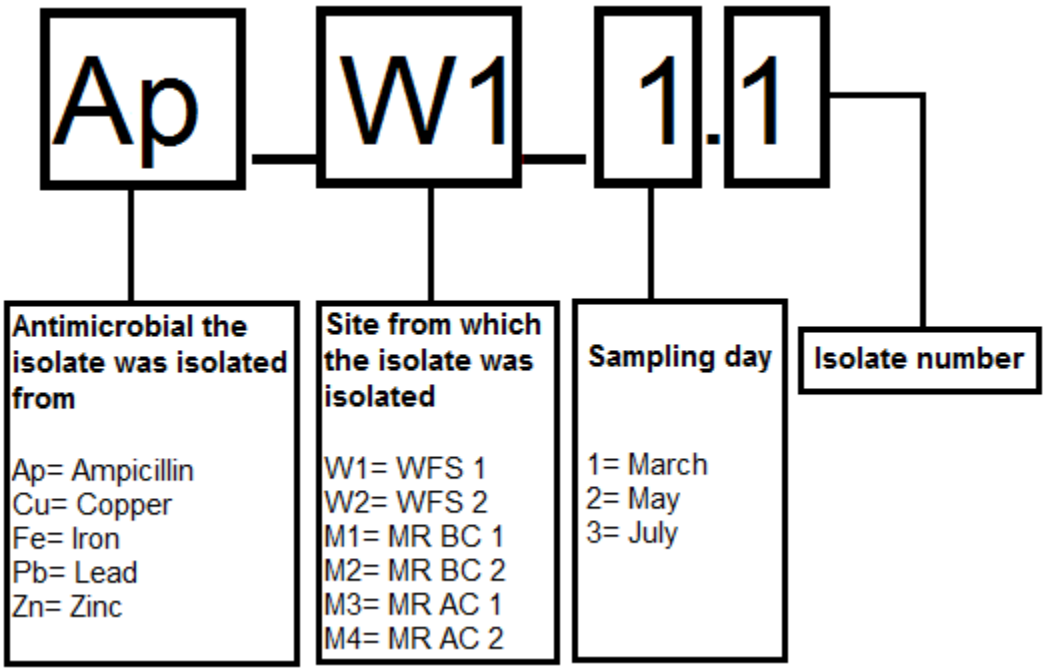
APPENDIX C

Scores used for Agglomerative hierarchical cluster analysis from inhibition zone data

Assigned score	**10**	**8**	**5**	**2**	**1**	**0**
Inhibition zone measurement (mm)	0 - 8	9 - 12	13 - 17	18 - 22	23 - 30	> 30

APPENDIX D

Description of the isolate labels with Ap_W1_1.1 as an example



APPENDIX E

E.1: AHC-Cluster Centroid scores for each antibiotic on three sampling occasions

March

Cluster	Ax	Ap	Tt	Er	St	Tm	Cp
A	7.952	8.238	6.048	1.810	5.571	7.619	4.286
B	10.000	10.000	5.750	9.250	2.083	10.000	5.583
C	9.905	10.000	9.762	9.524	9.905	9.381	7.143

May

Cluster	Ax	Ap	Tt	Er	St	Tm	Cp
A	9.429	9.071	5.714	8.464	7.536	9.857	9.643
B	10.000	10.000	9.429	3.143	6.857	0.286	8.857
C	9.125	9.438	7.188	5.188	4.625	8.875	1.875

July

Cluster	Ax	Ap	Tt	Er	St	Tm	Cp
A	9.556	9.556	3.111	3.556	7.000	8.889	0.778
B	9.733	9.733	6.667	9.533	7.400	9.733	8.667
C	6.250	7.000	3.500	7.500	4.000	4.750	9.500

E.2: Distances between clusters on the AHC dendrograms for three sampling dates

March

Cluster	A	B	C
A	0	9.070	10.501
B	9.070	0	8.954
C	10.501	8.954	0

May

Cluster	A	B	C
A	0	11.662	9.106
B	11.662	0	11.739
C	9.106	11.739	0

July

Cluster	A	B	C
A	0	10.562	11.635
B	10.562	0	8.418
C	11.635	8.418	0

APPENDIX F

Agarose gel image of *tet*L amplicons that were detected as non-specific