AN ASSESSMENT OF WATER QUALITY AND OCCURRENCE OF ANTIBIOTIC-RESISTANT BACTERIA IN NAAUWPOORTSPRUIT RIVER, MPUMALANGA PROVINCE, SOUTH AFRICA

By

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DEDICATION

To God Almighty and our saviour Jesus Christ, let all be the glory, I owe it all to you.

My Parents,

My Wife,

My Brothers,

My Sister,

My Daughter,

My Son,

Your unconditional love, admiration, and support you offered me through my studies. I thank you.

DECLARATION

I Khuthadzo Lunsford Mudau hereby declare that this dissertation, with the title: An assessment of water quality and occurrence of antibiotic-resistant bacteria in Naauwpoortspruit River, Mpumalanga province, South Africa which I hereby submit for the degree of Master of Environmental Science at the University of South Africa, is my work and has not previously been submitted by me for a degree at this or any other institution.

I declare that the dissertation does not contain any written work presented by other persons whether written, pictures, graphs or data, or any other information without acknowledging the source.

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I declare that I have not copied and pasted any information from the internet, without specifically acknowledging the source and have inserted appropriate references to these sources in the reference section of the thesis.

I declare that during my study I adhered to the Research Ethics Policy of the University of South Africa, received ethical approval for the duration of my study prior to the commencement of data gathering, and have not acted outside the approval conditions.

I declare that the content of my dissertation has been submitted through an electronic plagiarism detection program before the final submission for examination.

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LIST OF ACRONYMS

AMD	: Acid Mine Drainage
ANOVA	: Analysis of variance
AR	: Antibiotic resistance
ARB	: Antibiotic-resistant bacteria
BOD	: Biological Oxygen Demand
CLSI	: Clinical and Laboratory Standards Institute
COD	: Chemical Oxygen Demand
Conc	: Concentration
Cu	: Copper
DWA	: Department of Water Affairs
DWS	: Department of Water and Sanitation
DWAF	: Department of Water Affairs and Forestry
EC	: Electrical Conductivity
EPA	: Environmental Protection Agency
FC	: Faecal Coliform
Hg	: Mercury
HPC	: Heterotrophic Plate Counts
HMs	: Heavy Metals
IDP	: Integrated Development Plan
MAR	: Multiple antibiotic resistance

MIC	: Minimum Inhibitory Concentration
mL	: Millilitre
MRSA	: Methicillin-resistant *Staphylococcus aureus*
NCCLS	: National Committee for Clinical Laboratory Standards
NRF	: National Research Fund
RSA	: Republic of South Africa
SANS	: South African National Standards
SD	: Standard Deviation
SP	: Species
TC	: Total Coliform
TDS	: Total Dissolve Solids
HPC	: Heterotrophic Plate Count
WRC	: Water Research Council
WHO	: World Health Organization
WWTP	: Wastewater Treatment Plant
UNICEF	: United Nations International Children's Emergency Fund
UNEP	: United Nations for Environmental Protection Agency
ZID	: Zone Inhibition Diameter
ZN	: Zinc

ABSTRACT

Decreasing surface water quality in South Africa has become an issue of concern as the population grows, industrial and agricultural activities expand, and environmental pollution increases. Wastewater treatment plants and other anthropogenic activities are liable for releasing raw and inadequately treated effluents into the surface water. Extensive pollution accompanied by the use of disinfectants, pesticides, and other chemical pollutants has been attributed to increased antimicrobial resistance in bacteria such as *Escherichia coli* in surface water, increasing environmental antibiotic resistance spread. The research aimed to determine water quality and prevalence of antibiotic-resistant bacteria in Naauwpoortspruit River, eMalahleni, Mpumalanga Province. Five sampling sites were selected along the Naauwpoortspruit River and monitoring was done for seven consecutive months. Samples were collected and analysed for physicochemical, microbiological parameters, and susceptibility profile of antibiotic-resistant bacteria using standard methods. Pearson correlation analysis was used to assess the path and strength of the relationship between physicochemical and microbiological parameters in the study area.

Results of physicochemical and microbial parameters showed variation throughout the selected study sites. The results revealed a pH range of 4.45 – 7.9 and electrical conductivity levels range of 58.63 - 113.3 mS/m for the different sampling sites during the study period with lower levels detected during the winter period and higher levels in the summer period. Also, water samples showed a high total dissolved solids levels range of 381.1 – 736.45 mg/L and biochemical oxygen demand range of 67.1 – 168 mg/L for the different sampling sites during the study period. The Naauwpoortspruit River had higher levels of ammonia of 33.4 mg/L at Point A during the winter period as compared to 15 mg/L in the summer period. Heavy metals results showed that mercury range of 0.01 – 0.065 mg/L and copper range of 0.001 – 0.0035 mg/L were not compliant with aquatic ecosystem guidelines at all selected sites throughout the study period. The foremost finding of this study was that *E. coli* were present in all the selected sites at concentrations (>100 cfu/100ml). Elevated concentrations of 5.4 x 10^3 and 4.2 x 10^3 cfu/100ml for the total and faecal indicator bacteria were detected from sites downstream to 2.2 x10^3 and 2.35 x10^3 cfu/100ml for sites upstream river, in the rainy months. During the dry season, total coliforms, and faecal coliforms concentration of 0.4 x10^3 to 0.65 x 10^3 cfu/100ml were detected downstream and 0.25 x 10^3 and 0.5 x 10^3 cfu/100ml from upstream, respectively. The physicochemical and microbiological parameters measured at selected sites exceeded acceptable limits and proved unsuitable for applications such as full and intermediate recreational activities, and aquatic ecosystems. The variation in physicochemical parameters results was influenced by both natural processes and human activities such as salinity and Acid Mine Drainage (AMD) within the Naauwpoortspruit River.

Using the Kirby-Bauer disc diffusion method, *E. coli* and faecal coliforms were tested for resistance to antibiotics; ampicillin (10 μg/ml), kanamycin (30 μg), streptomycin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), ox tetracycline (30 μg), erythromycin (15 μg/ml) and norfloxacin (10 μg). More than 60% of faecal coliform were resistant to at least four of the tested antibiotics and between 60 - 80% of the *E. coli* isolates were resistant to β-lactam. The highest microbial antibiotic resistance (MAR) index value was observed at Site D (0.38 for *E. coli)* which showed multi-antibiotic resistance. Site D is characterized by wastewater treatment, power generation industries, and agriculture activities. The highest level of MAR observed at Site D indicates the need to control extensive pollution and constantly monitor the changing trends in antimicrobial resistance patterns of these waterborne pathogens. Statistical analysis showed that the development of microbiological parameters loads has a strong correlation with physicochemical parameters due to the association of sampling sites in the river environment. This study shows that the aquatic ecosystem needs constant monitoring to establish their conditions, impacts of pollution activities within the catchment, and input information into sustainable management of the water resources.

Keywords: Antibiotic resistant-bacteria, *Escherichia coli,* multiple antibiotic resistance, pathogenic bacteria, water quality, wastewater treatment.

CHAPTER ONE

INTRODUCTION AND STUDY BACKGROUND

1.1. Introduction and study background

South Africa is regarded as a semi-arid country, which makes water availability one of the key limitations to the country's economic freedom and social services (DWA, 2012; Donnenfeld *et al.,* 2018). Clean water supplies in South Africa rely mainly on seasonal rivers to fill dams, but due to lack of rainfall, overuse, and water contamination, surface water is becoming more strained both in terms of quality and quantity (Morokong *et al.,* 2016; De Klerk, 2016; DWA, 2018). In South Africa, water is a scarce commodity, and thus water quality is of critical importance for domestic, agricultural, and industrial use (De Klerk, 2016; Traore *et al.,* 2016; Mkhulisa, 2017). Recent studies have shown that rivers in South Africa are deteriorating as a result of anthropogenic surface water pollution (Bester, 2015; Traore *et al.,* 2016; Du Plessis *et al.,* 2017). Sources of water pollution include Wastewater Treatment Plants (WWTPs), industries, informal settlements, mining, and agricultural activities. Wastewater effluent pollution causes the manifestation of faecal pollution in an aquatic environment especially through untreated effluent discharge (Britz *et al*., 2012; Bester, 2015; Hobbie *et al*., 2018). Faecal pollution is prevalent in areas where there is a lack of sanitation facilities, for example: in human settlements, intensive livestock farming as well as partially or untreated sewage effluent discharging into surface water. The presence of pathogens in water causes waterborne diseases such as cholera, gastroenteritis, and typhoid fever (Donnenfeld *et al.,* 2018; WHO, 2018).

Surface water resources in catchments that are ridden with industrial, urban, and human settlement activities are prone to extensive pollution. South African economy is known to be dominated by mining activities while at the same time economic inequality impacts service delivery resulting in poor sanitation. This has the impact of resulting in rampant environmental pollution, with aquatic environments being much on the receiving end of the pollutants through both point and nonpoint sources. Changes in microbial community structures with pollution have been reported with the potential of antibiotic resistant strains proliferating (Davis *et al.,* 2016; Alonso *et al.,* 2017; Ateba *et al.,* 2020). It has been noted that heavy metal pollution and the input of synthetic organic pollutants such as pharmaceuticals contribute/induce microbial resistance to antimicrobials (Ebenebe *et al.,* 2017; Donnenfeld *et al.,* 2018; Herbig *et al.,* 2019).

Naauwpoortspruit River is the focus of the study and it is one of the tributaries from upper Olifant's River catchment. Olifant's River is considered one of the most polluted catchments in South Africa due to diverse pollutants and land use activities (Dabrowski, 2013; DWA, 2016; Pollard *et al*., 2017). Notable anthropogenic activities contributing to major pollution in the catchment include abandoned and active mines, agriculture runoff, and wastewater treatment plants (Dabrowski and Klerk, 2013; Mathebula, 2015; DWS, 2018). WWTWs also release heavily laden waste effluent containing high nutrient levels such as nitrogen, phosphorous and microbiological loads (Le roux, 2012; Dabrowski, 2013; Di Cesare *et al.,* 2017). These pollution sources are contributing to severe impacts on surface water quality. Based on Di Cesare *et al.* (2017) and Elbossaty (2017), high levels of phosphorus and nitrogen in water cause nutrient enrichment. This promotes algal development, such as cyanobacteria e.g., *Microcystis aeruginosa* and macrophytes, leading to oxygen depletion in surface waters causing death in the aquatic ecosystems (Self *et al.,* 2013; Mathebula, 2015; Retief *et al.,* 2019).

Based on current water trends, the Naauwpoortspruit River has reported high levels of sulphate and mercury resulting from mining and agricultural activities (DWS, 2016; Schreiner *et al.,* 2018). Maya *et al.* (2015) indicated that the nearby area of the Naauwpoortspruit River is a significant source of coal, and various studies have reported acid mine drainage (AMD) from both active and abandoned coal mines in the area. The presence of substantial levels of these toxic elements from AMD in the environment leads to both contaminations of the aquatic and terrestrial ecosystem (Oberholster *et al.,* 2017; Retief *et al.,* 2019). Malherbe *et al.* (2011) reported that aquatic benthic macroinvertebrates such *Odonata*, *Ephemeroptera,* and *Plecoptera* mortality in the Naauwpoortspruit River, has been related to the cumulative influences of AMD, WWTPs, and agricultural activities into the river system. Heavy metals from mining activities are a threat to several aquatic environments within the study area and these elements cannot be degraded and are ultimately indestructible (Ahirwar *et al.,* 2016; Benmalek *et al.,* 2016; Islam *et al.,* 2018).

Due to the elevated heavy metals levels in the aquatic environment, heavy metal resistance and adaptation mechanisms can be formed in microbial communities that allow successful detoxification and transformation of heavy metals from their toxic into non-toxic forms (EPA, 2012: Ahirwar *et al.,* 2016; Wen *et al.,* 2017). Heavy metal tolerance processes include accumulation of metals such as copper (Cu), iron (Fe),cadmium (Cd), zinc (Zn) and lead (Pb), enzymatic oxidation or reduction of toxic metals, physical exclusion of electronegative elements in membranes, metal ion efflux systems outside the cell (Berendonk *et al.,* 2015; Frieden, 2015; Chen *et al.,* 2019). Microbial pathogens can acquire and transfer resistance genes and virulence factors to one another by co-selection and sharing overlapping genetic mechanisms in the environment (Berendonk et al., 2015; Wen *et al.,* 2017). Factors such as activated sludge from wastewater treatment and heavy metals contribute to input and dissemination of antibiotic resistance in surface water (Li *et al.,* 2017; Wen *et al.,* 2017). Humans may also be subject to ARB and ARG by practices like aquatic sports, occupational exposure during agricultural irrigation, and ingestion of food produce irrigated with reclaimed water (Jones *et al.,* 2014; Hatosy and Martiny, 2015). Consumption of water contaminated by antibiotics can provide a selective pressure (a biotic or abiotic factor that alters the behaviour and fitness of an organism in each environment) within the gut, resulting in the development of antibiotic resistance in enteric bacteria (Li *et al.,* 2017; Amarasiri *et al.,* 2020). For example, it is estimated that death is 64 percent more likely in people infected with methicillin-resistant *Staphylococcus aureus* (MRSA) compared to people infected with a sensitive form of the bacteria (WHO, 2019). Acknowledging that the occurrence of antibiotic pathogenic microorganisms can cause major challenges in public health, this study intended to determine susceptibility profiles of ARB in the Naauwpoortspruit River. The other objective was to assess the physicochemical and microbiological water quality at different river sites using selected physicochemical and bacterial level indicators.

1.2. Problem statement

The increasing pollution of water and persistence of ARB has raised concerns over an increase in infections and related morbidity in humans (Frieden, 2015; Li *et al.,* 2017; Nyandjou *et al.,* 2019). Over the previous decade, there has been a significant reported increase of ARB in the environment with several organisms developing and gaining resistance to *β*-lactams, glycopeptides, tetracyclines, macrolides, and ketolides (Wen *et al*., 2014; Di Cesare *et al.,* 2015; Frieden, 2015). According to Mutuku *et al.* (2014) and Pal *et al.* (2015), the need for an antibiotic therapy analysis was stressed by a lack of clinical effectiveness of current antibiotics and others, and diminished performance of the approaches used to mitigate the spread of antibiotic resistance. Even though there have different attempts aiming to reduce antibiotic usage, promoting antimicrobial stewardship, and monitoring the spread of antimicrobial resistance, the results are minimal (Czekalski, 2012; Vital *et al.,* 2018; Wen *et al.,* 2020). Evidence advocates that the dissemination and proliferation of antimicrobial resistance are shaped by a diverse variety of factors, such as the use of antibiotics in various environments, stormwater drainage, and HMs in the environment (Frieden, 2015; Pal *et al.,* 2015; BengtssonPalme *et al.,* 2018).

Anthropogenic activities (e.g., mining, wastewater treatment, and agriculture) are liable for the widespread release of antibiotics and the distribution of ARB in the environment (especially into surface water) (Klein *et al.,* 2018; Nyandjou *et al.,* 2019; Sabri *et al.,* 2020). Direct discharge of antibiotics and heavy metals on the environment has a bigger impact on surface water and the aquatic ecosystem (Li *et al.,* 2017; Chen *et al.,* 2017). Various studies have reported on the co-selection of antibiotic resistance genes and metal resistance genes in a range of contaminated environments (Bengtsson-Palme *et al.,* 2018; Yu *et al.,* 2019; Felis *et al.,* 2020). Microbial organisms exposed to high levels of heavy metals in the river can develop and acquire a variety of mechanisms for adaptation and tolerance to these toxic elements.

These pathways include bioaccumulation involving complexing of metal ions within and outside the biosorption cell, mineralization and precipitation, enzymatic oxidation or reduction of toxic metals, and efflux systems of metal ions outside the cell (Srivasta *et al.,* 2013; Benmalek *et al.,* 2015). The selective pressure exerted by water pollution allows bacteria that are immune to heavy metals to thrive and maintain the genetic heritage of ARGs. For example *S. aureus* is an important human pathogen and penicillin had been a drug of choice for treatment of infections caused by this organism (ref). However, *Staphylococcus aureus allows* acquisition of a new penicillin binding protein, PBP2a, to create methicillin resistant *S. aureus* strains (MRSA) which are not inhibited by most β-lactam antibiotics (Deurenberg *et al.,* 2007; Cox and Wright, 2013). Methicillin-resistant *Staphylococcus. aureus* (MRSA) can be found in clinical and environmental settings and encodes β-lactamase and inactivate penicillin by catalysing the hydrolysis of the β-lactam ring (Fishovitz *et al.,* 2014; Jones *et al.,* 2014; Robinson *et al.*, 2016). The *mec*A gene, which is found on the genetic loci called staphylococcal cassette chromosome *mec (SCCmec)*, is the main gene responsible for methicillin resistance. The expression of mecA gene depends on two genes: *mec*R1, which regulates transcription, and *mec*I, which encodes the repressor protein (Robinson *et al.,* 2016; Foster, 2017). Bacteria such as *Staphylococcus spp.* and *Pseudomonas* spp. can quickly acquire resistance to antibiotics (Mendes *et al.,* 2015). These began a few years ago when the first strains resistant to penicillin were identified. The successive introduction of other antibiotics, such as macrolides, tetracycline, and chloramphenicol, had a similar result, that is, the rapid appearance of resistant strains (Kaur and Chate, 2015; Grossman *et al.,* 2016). This led to the formation of bacteria with a variety of resistances and an exceptional ability to survive and spread in diverse environments (Foster, 2017; WHO, 2019).

Studies assessing water quality in terms of physicochemical and microbial parameters is of paramount importance in South Africa, especially in the eMalahleni Town. This is because the information on the potential risks associated with natural water contaminated with faecal contamination, heavy metal, and ARB in the country is parsimoniously available (Mendelson *et al.,* 2015). This is worrisome when considering that environmental and clinical studies indicate that waterborne disease and ARB rates in South Africa are high (Eager *et al.,* 2012; Frieden, 2015; Maphumulo *et al.,* 2019). Waterborne diseases are contracted via the faecal-oral route and are predominantly caused by pathogens associated with faecal contamination in water systems.

The management thereof may require antibiotic therapy, however; the overuse of antibiotics has led to numerous antibiotic resistance in normal enteric and pathogenic bacteria (Rahman *et al.,* 2016). The nonexistence of an effective ARB monitoring system makes it difficult to obtain data on the quantities of antimicrobials that are used in agriculture, households, and clinical settings. Eager *et al.* (2012) and Karzis *et al.* (2019) has reported that resistance to *Staphylococcus aureus mastitis* is a current issue in the South African agriculture industry, accompanied by improved resistance of *E. coli* and *Enterococcus spp.* to tetracycline, fluoroquinolone, sulphonamide, amoxicillin, and trimethoprim sulpha combinations. Significant resistance was also reported in *E. coli* isolates against nitrofurantoin and ampicillin in Apies River, South Africa, indicating that the riverbed sediments could serve as reservoirs for multiple antibiotic resistance (MAR) bacteria and pathogens under different climatic conditions (Abia *et al.,* 2015). Therefore, in-depth studies on water quality and development of antibiotic-resistant bacteria in mining and urban impacted surface water such as the Naauwpoortspruit River is vital in providing suggestion to control the resistant bacteria.

1.3. Hypothesis

Naauwpoortspruit River is anthropogenically polluted and there is a significant occurrence of antibiotic-resistant bacteria.

1.4. Aim and objectives

1.4.1. Research aim

This study aimed to assess physicochemical and microbiological water quality, and the occurrence of ARB in Naauwpoortspruit River, Mpumalanga, South Africa.

1.4.2. Study objectives

a. To determine the physicochemical and microbiological water quality using selected physicochemical parameters and level of microbial indicator bacteria at various sites in the river.
b. To determine the level of ARB in the River in relationship to anthropogenic activities.
c. To determine susceptibility profiles of antibiotic-resistant bacteria isolated from Naauwpoortspruit River.

1.5. Research Questions

An assessment of water quality and occurrence of antibiotic-resistant bacteria at Naauwpoortspruit River has led to the following research questions:

a. What is the water quality at various sites of Naauwpoortspruit River in terms of physicochemical and microbiological parameters?
b. What is the level of ARB in the River and how do anthropogenic activities relate to water quality and occurrence of antibiotic-resistant bacteria in Naauwpoortspruit River?
c. What are the susceptibility profiles of antibiotic-resistant bacteria from Naauwpoortspruit River?

1.6. Significance of the study

Declining water quality is a major problem experienced globally and with no immunity to South Africa. The enriched scale of socio-economic activities such as industries, WWTPs, and agriculture are sources of point and non-point pollutants which have elevated amounts of deposits, including high nutrients, bacterial and viral faecal indicators into the Naauwpoortspruit River (Frieden, 2015; Mathebula, 2015: DWS, 2016).

Assessment of water quality and the associated ARB in Naauwpoortspruit River will provide data sets that will inform the responsible authorities on the level of pollution in the area. The determination of microbial contamination through indicator microorganisms in water quality is important to assist with information that can be used in the control and prevention of waterborne diseases. To limit the prevalence and dissemination of ARB and ARGs, it is important to consider the relative contribution in terms of water quality and the quantity of various possible transmission paths. Antibiotic resistance patterns may be used to identify potential sources of pollution (Holcomb *et al*., 2020). Data from this study can be used to determine the risks of exposure to ARG and possible public health impacts associated with contaminated surface water. Therefore, more information could stimulate the development of cost-effective, practical ways to avert adverse impacts of water pollution. The greatest beneficiaries of this will be aquatic ecosystems, downstream communities that depend on good water quality for their livelihoods, and responsible authorities who do compliance monitoring along the river.

1.7. Delineation and limitations of the study

The research was conducted out at the Naauwpoortspruit River, eMalahleni, Mpumalanga Province. The study aimed to assess water quality and the occurrence of ARB within the

Naauwpoortspruit River. The aim was to determine physicochemical and microbiological parameters within selected sites and determined the antibiotic resistance pattern of each isolate.

Naauwpoortspruit River covers a large area and with limited time to sample, sampling was carried out on five sampling sites and the samples were collected for seven months only. Limited time also contributed to the lack of detailed ARB studies including failure to conduct molecular genetics studies for ARB bacteria.

1.8. Ethical consideration

Research ethical clearance was obtained from the University of South Africa College of Agriculture & Environmental Sciences, Ethics Review Committee. The permission was also obtained from eMalahleni Local Municipality to conduct research and sample from the wastewater treatment effluent and along the River study area (Appendix C).

1.9. Chapter breakdown

This dissertation comprises five chapters with the inclusion of summary, conclusion, and recommendations.

Chapter 1: Presents the background of the study and formulates the problem statement, research objectives, significance of the study, and study limitations. **Chapter 2:** Discusses the literature review carried out during this study and provides a theoretical background to water quality and guidelines, water pollution contribution of agriculture activities, urbanization, WWTPs, and disinfection process, occurrence, and the spread of ARB in the river environment. **Chapter 3:** Describes in detail the materials and methods employed during the study. The study area including selected sampling sites was discussed. Statistical analyses and ethical considerations for the study were discussed. **Chapter 4:** Discusses the findings of the study and relates these to the research objectives outlined in chapter 1. The physicochemical and microbial parameters were compared to various water quality guidelines and statistical analysis to establish the difference between the sites. **Chapter 5:** Features summary and conclusions derived from the research are discussed. This final chapter makes recommendations to enhance the water quality of the Naauwpoortspruit River.

CHAPTER TWO

LITERATURE REVIEW

2.1. Introduction

Water pollution induces water quality deterioration that leads to harmful pathogens and other environmental hazards (DWA, 2012). Anthropogenic activities, surface runoff, and discharge of wastewater are sources of contamination to freshwater bodies and is a threat to public water supplies. However, water pollution in certain conditions can be instigated by natural processes such as weathering and flooding. But often than not, human activities are responsible for the inputs of organic and inorganic contaminants that reach the aquatic ecosystem (Griffins *et al.,* 2014; Hemson, 2016; Walters *et al.,* 2017). Inputs of organic and inorganic contaminants include heavy metals, detergents, fertilizers, and inorganic nutrients, and waste effluent that is rich in carbon that impacts adversely on humans and the environment (Jan *et al.,* 2015; Khwidzhili and Worth, 2016).

Some organisms can experience drastic changes in water quality without affecting the ecological structure and functions while others are vulnerable to minor changes in the physical and chemical composition of water systems that can lead to habitat degradation and biodiversity loss (Britz *et al.,* 2012; Griffins *et al.,* 2014). Deterioration of water due to human activities affected movement and adaptation of biodiversity within the aquatic ecosystem and such impacts can be noticed once there are radical changes in the environment (Self *et al.,* 2013; Oberholster *et al.,* 2017; Rashid *et al.,* 2018). For instance, in surface water, elevated levels of trace metals may present a hazard to ecological health of aquatic organisms and contribute to decline in their populations (Malik and Maurya, 2014). According to Pollard *et al*. (2017), microbial contamination from poorly or untreated wastewater effluent puts humans at risk of contracting microbial waterborne diseases such as cholera, typhoid, infectious hepatitis, and other gastroenteritis infections (Bester, 2015; Pollard *et al.,* 2017; Nicholas *et al.*, 2018). Therefore, the literature review explores the issues of water quality, anthropogenic activities that affect water quality, defines and describes antibiotic-resistant bacteria, environmental concern, and causes for resistance in the Naauwpoortspruit river environment.

2.2. Water quality and guidelines

Water quality can be defined as the appropriateness of water to sustain many purposes of use (Naidoo, 2013; Oberholster *et al.*, 2013; DWS, 2016). According to DWS (2017), water quality is interpreted as mean fitness for use. The definition of water quality differs according to the end-user of water. Water users may have certain requirements for the fit of use, which include physical, chemical, or biological characteristics (Bester, 2015; DWS, 2018). The set limits on the concentrations of determinants in the aquatic ecosystem may define water quality after testing.

Internationally, the World Health Organization (WHO) standards are the benchmarks for drinking water and aquatic ecosystem guidelines (WHO, 2018). The requirements and criteria for aquatic water quality provide environmental guidelines required for the control of wastewater discharges and the determination of the extent of the clean-up process. However, at a national level, countries have a statutory right to establish their guidelines and legislation on water quality standards and regulations (USEPA, 2012; WHO, 2018). (USEPA, 2012; WHO, 2018). WHO requirements are focused on the determinants which characterize the water. The standards for a substance's concentration in water that is not toxic if the water is regularly used for a certain reason are placed at a maximum level. However, certain variables set a minimum appropriate concentration for sustaining and maintaining biological functions like the dissolved oxygen (DO). Not all parameters have been assigned a guideline value, for instance, ammonia does not have a guideline value due that it is well below the concentration that may cause health concerns. While an aquatic ecosystem is not defined (WHO, 2018).

In South Africa water authorities have developed water guidelines and regulations to protect water resources in the country (DWS, 2017), thus water quality can be determined by comparing physicochemical and microbiological assessments with the legislated water guidelines for a particular use (SANS, 2015; DWS, 2017). These guidelines are specific values for a suite of variables for different water use sectors (domestic, industrial, irrigation, livestock watering, aquatic ecosystems, recreation, and aquaculture) that depict the change from one category of fitness for use to another (DWAF, 1996a; DWS, 2017). Within these different categories, users of water can make a profound decision on the probable impacts on the health and integrity of aquatic ecosystems (DWS, 2017). However, within the use of DWS aquatic ecosystem guidelines, there has been acknowledgment on the user guidelines that no standards for chemical determinants such as boron, and nickel but the WHO requirements do include those guidelines. (DWS, 2017; WHO, 2017).

Natural water quality varies considerably from one country to another, based on seasons, temperature conditions, and soil compositions, rocks, and surfaces over which it passes. Therefore, the key to sustainable surface water is to ensure that water quality is not polluted to a certain degree that harms aquatic life and its intended users, and while at the same time allowing it to be used and developed to a certain extent (DWS, 2016a; Schreiner *et al.,* 2018).

2.3. Description of physicochemical, microbiological parameters and their sources into the river environment.

2.3.1. Water quality parameters: physicochemical parameters

The existence of physicochemical parameters in the river environment is due to either natural causes or anthropogenic activities. For instance, heavy metals in nature can occur due to natural phenomena such as volcanic activity, rock weathering, and metal liquidation (Srivasta *et al.,* 2013; Shirani *et al.,* 2020). Furthermore, trace metals can be added to the environment by the application of anthropogenic activities, including mining, industrial effluent, domestic waste, agricultural runoff, and pesticides containing heavy metal residues (Chen *et al.,* 2018; Agoro *et al.,* 2020). Some trace elements are naturally occurring elements of the earth, namely, zinc, nickel, copper, which are regarded as essential elements which are vital to living microorganisms. However, even small amounts are toxic to aquatic ecosystems and humans.

2.3.1.1. Water temperature

The temperature has a strong impact on many physical and chemical properties of water, including oxygen and another gas solubility, chemical reaction rate, toxicity, and microbial activity (Dallas and Day, 2004; US EPA, 2012). According to Dallas (2008), higher temperatures reduce the solubility of dissolved oxygen in water, decreasing its concentration and thus its availability to aquatic organisms. Chemical reaction rates and the toxicity of many substances such as cyanide, zinc, phenol, and xylenes are increased as their temperature rises and animals are vulnerable to these toxins (Ebenebe *et al.,* 2017). If the organic loading is high oxygen depletion is further accelerated by greater microbial activity at the higher temperature (US. EPA, 2012; Self *et al.,* 2013).

Changes in water temperature may have a direct or indirect impact, including thermal discharges, land use changes, agricultural return irrigation fluxes, fluid changes (river regulation), cross-cistern transfers of water, river vegetation changes and global warming (Dallas, 2008; Kabir, 2014). According to Harmony Gold (2014), power generating industries are the major contributors of heat and radioactivity in surface water. Power generating industries plants employ two main types of cooling systems, namely once-through and recirculation (tower) cooling (Raptis *et al.,* 2016). In once-through cooling systems, the heat absorbed by the cooling water during the steam cycle is directly rejected back into the river.

In a cooling tower setup, on the other hand, most of the absorbed heat is removed via evaporation and dissipated into the atmosphere. The heat contained in the periodic cooling tower blowdown is negligible compared to the heat released in once-through cooling emissions (Stewartet *et al.,* 2013). The electrical supply commission (Eskom, 2020) has estimated that 25 million tons of fly ash are produced by power stations annually. This substantial quantity of ash content is deposited by coal power stations runoff into surface water during weathering and erosion (Dabrowski and Klerk, 2013; Maya *et al.,* 2015).

Water with high temperature may affect the metabolism, growth, behaviour, food and feeding habits, reproduction and life histories, geographical distribution and community structure, movements, and migrations, and tolerance to parasites, diseases, and pollution, of aquatic organisms (Dallas, 2008; Kabir, 2014; DWS, 2016). According to US EPA (2012), the thermal balance hypothesis plays a dominant role in keeping the distinction between the niches in lotic assemblies and controlling large-scale species diversity and distribution patterns. Aquatic organisms can be widely categorized as cold thermal organisms (those with small tolerance ranges in the cold regions and warm thermal are those tolerate hot and tropical areas (thousands of tolerant organisms with narrow tolerance ranges) (Alonso *et al.,* 2017; Ebenebe *et al.,* 2017). The impact of water temperature on individuals can become apparent by physiological and behavioural results, population levels by individuals' growth, fertility, and survival; and community levels by favoured temperature-tolerant taxa over the thermal intolerant (Malherbe *et al.,* 2011; Oberholster *et al.,* 2017).

2.3.1.2. pH

Potential hydrogen is the measurement of hydrogen ions, or acidity in the water (Bester, 2015; DWS, 2018). Water has hydrogen ions and hydroxyl ions. When there are equal numbers of both, the water is neutral. As the hydrogen ions increase, the water becomes more acidic and when the hydroxyl ions increase, the water becomes more basic. pH is measured on a logarithmic scale of 0 – 14 and seven is neutral, below seven is acidic while above 7 is basic. According to DWS (2017), aquatic ecosystem supposed to have a pH that ranges from 6 - 9 which can support diverse aquatic biodiversity, however, most surface waters are becoming acidic due to AMD from mining activities and agriculture effluents affects water (Naidoo, 2013; Ngwenyama *et al.,* 2017; Retief *et al.,* 2019).

Important factors that may influence pH include geology, biotic activities, type of vegetation, atmospheric influences, acid-neutralizing or buffering capacity, and cation exchange capacity (Naidoo, 2013; DWS, 2017). According to Gonah (2016), there is AMD discharge from active and abandoned mines in Witwatersrand, South Africa which is starting to be felt now from century as it impacts the aquatic ecosystem. The effect of AMD pollution has been particularly pronounced in the case of the Blesbokspruit in Springs and the Klip River (which drains the southern portion of the Witwatersrand escarpment) because of tailings dumps abound in their upper catchments. In cases where the water table is near the surface, the upper 20 cm of soil profiles has shown severe contamination by heavy metals (McCarthy, 2011; Soleimani *et al.,* 2018). Maya (2015) has also learned that abandoned coal mines in eMalahleni are discharging approximately 62 ML/d of water and about 50 ML/d of that water is AMD into the Olifant's River catchment. This AMD has seen sulphate concentration in Witbank Dam now exceeds the 200 mg/L level, which is the recommended maximum in water for domestic use (DWS, 1996b; Agoro *et al.,* 2020). The quality of local water is so poor that ESKOM imports water from the eastern escarpment for use in the power stations in the Witbank-Middelburg area (Eskom, 2020).

Other aquatic ecosystems are alkaline with a pH ranging from 7 - 11 due to natural and anthropogenic activities. In the Luvuvhu river catchment, basic conditions are attributed to the geological formation of Sibasa basalt (Makhera *et al.,* 2011). The importance of pH as a controlling variable in chemical reactions at the cellular and subcellular levels has been well documented (Hamid *et al.,* 2017). However, as organisms become more complex, they are often able to adequately regulate their body chemistry despite unfavourable external conditions. Cyanobacteria usually grow within a pH range of 7.5 to 9.0 (Makhera *et al.,* 2011; Mwangi, 2014).

2.3.1.3. Electrical Conductivity

Conductivity is a measure of the ability of water to pass an electrical current (US EPA., 2012). Water conductivity is impaired by the presence of inorganic dissolved solids (negative charges), such as chloride, chloride, nitrate, sulphate and phosphate anion, magnesium, iron, and aluminium cation (ions that carry a positive charge). Organic compounds such as grease, phenol, alcohol, and sugar do not conduct electrical current very well and therefore have a low conductivity when in water. Temperature also affects the conductivity, the colder the water, the higher the conductivity. Therefore conductivity is shown as 25 degrees Celsius (25 °C) (U.S. EPA, 2012; Edokpayi *et al.,* 2017).

Water conductivity in streams and rivers is mainly affected by the geology of the area through which the water flows. Streams passing through granite-based areas appear to be less conductive as granite is made up of more inert, non-ironic materials (dissolved into ionic components) when washed into the water (Rashid *et al.,* 2018). However, streams passing through areas with clay soils appear to be more conducive due to the presence of materials that ionize when they are washed in the water. Urbanization industrialization and wastewater discharge in South Africa have detrimental effects on the conductivity of water through the high presence of chloride, phosphate, and nitrate (Britz *et al.,* 2012; Haarhoff *et al.,* 2020). The basic unit of measurement of conductivity is the mho or siemens. Conductivity is measured in micromhos per centimetre (µmhos/cm) or micro siemens per centimetre (mS/cm) (Abhineet and Dohare, 2014).

2.3.1.4. Total dissolved solids and suspended solids

TDS is the measure of the total concentration of solid particles which are present in water (US EPA, 2012; Mwangi, 2014). In the aquatic ecosystem, dissolved solids include silt and clay particles, plankton, algae, fine organic debris, and other particulate matter. Higher concentrations of suspended solids can serve as carriers of toxins, which readily cling to suspended particles (Dabrowski and Klerk, 2013; Oberholster *et al.,* 2017). This is particularly a concern where pesticides are being used on irrigated crops. Where there high TDS, pesticide concentrations may increase well beyond those of the original application as the irrigation water travels down irrigation ditches (Le Roux *et al.,* 2012; DWS, 2016). High levels of TDS in agriculture can clog irrigation devices, while in sewerage can be linked-to pipe burst and leakages which then water may run to surface water (Edwin *et al.,* 2014; De Klerk, 2016). elevated concentration of TDS can also reduce the efficiency of WWTPs, as well as the operation of industrial processes that use raw water.

Total solids also affect dissolved oxygen within the river environment by decreasing the passage of light through water, thereby slowing photosynthesis to aquatic plants (Dallas, 2008; Self *et al.,* 2013; Pour *et al.,* 2014). Water will heat up more rapidly and hold more heat; this, in turn, might adversely affect aquatic life that has adapted to a lower temperature regime. Sources of total solids include industrial discharges, sewage, fertilizers, road runoff, and soil erosion (Maya et al., 2015; Ebenebe *et al.,* 2017; Oberholster *et al.,* 2017). Total solids are measured in milligrams per litre (mg/L). Impacts of elevated levels of TDS can be water balance in the cells of aquatic organisms. For instance, an organism placed in water with a very low level of solids, such as distilled water, will swell up because water will tend to move into its cells, which have a higher concentration of solids (Frieden, 2015).

2.3.1.5. Dissolved oxygen (DO)

Assessment of DO is fundamental as it influences chemical and biological processes within the aquatic ecosystem (Oun *et al.,* 2014; Donoso *et al.,* 2017). Dissolved oxygen refers to the volume of oxygen that is contained in the water (US EPA, 2012). Oxygen in the aquatic environment is produced by photosynthesis of algae, plants and is removed by respiration of plants, animals, and bacteria, BOD degradation process, sediment oxygen demand, and oxidation (Pour *et al.,* 2014; Zaghloul *et al.,* 2019). Variations of DO can within a day or seasonally depending on relation to temperature and biological activity of photosynthesis and respiration (DWS, 2017; Gosch *et al.,* 2019).

During the day, surface water has high levels of dissolved oxygen due to oxygen generated from photosynthesis and when night falls, photosynthesis stops, and plants consume oxygen as they respire, decreasing the dissolved oxygen levels (Self *et al.,* 2013; Pour *et al.,* 2014). Besides biological influences, various physical parameters such as turbulence, atmospheric pressure, surface reaction, river flow, and estuarine circulation influence the distribution of dissolved oxygen in the aquatic ecosystem (Griffins, 2014; Olaolu *et al.,* 2014). The solubility of oxygen decreases as temperature and salinity increase and is more dependent on temperature variation than on salinity variation (Stewartet *et al.,* 2013).

The DO in the surface water is intimately linked to the organic matter cycle, the exchange fluxes between the atmosphere and the water surface, and the input fluxes from the river catchment (Dallas, 2018; Nel *et al.* 2013; Li *et al.,* 2017). In the Aquatic ecosystem, a point where DO concentration decreases and can affect aquatic flora and fauna is referred to as hypoxia (Dallas and Day, 2004; US. EPA, 2012). A strong correlation between hypoxia and human activity has been found in many areas such as the Gulf of Mexico, Texas–Louisiana; the Northern Adriatic Sea, and Sweden–Denmark (Sabri, 2020). Freshwater from surface water rivers is usually saturated with oxygen. In slow, stagnant waters, oxygen only enters the top layer of water, and deeper water is often low in DO concentration due to the decomposition of organic matter by bacteria that live on or near the bottom (Dallas, 2008). During rainy seasons, oxygen concentrations tend to be higher because the rain interacts with oxygen in the air as it falls. Whereas during dry seasons, water levels decrease and the flow rate of a river slows down.

2.3.1.6. Nitrates and nitrites

The determination of nitrates as nitrogen (mg/L NO_3-N) and nitrites as nitrogen (mg/L NO_2-N) levels in surface waters are indicators of the nutrient status and the degree of organic pollution in the aquatic ecosystem (Haller *et al.,* 2014; Mothetha, 2016). Nitrates are also used in the monitoring of drinking water due to potential health risks associated with its elevated levels, especially infants and animals (SANS, 2015; WHO, 2019).

Nitrate can reach both surface water and groundwater as a consequence of agricultural activity (including the excess application of inorganic nitrogenous fertilizers and manures), from wastewater treatment, and oxidation of nitrogenous waste products in human and animal excreta, including septic tanks (Drabowski *et al.,* 2013; Xue *et al.,* 2016; Pollard *et al.,* 2019). They are also used as an oxidizing agent and in the production of explosives, while purified potassium nitrate is used for glass making (DWAF, 1996a; Atashgahi *et al.,* 2015). Sodium nitrite is also used as a food preservative, especially in preserved meats (FAO, 2017). Nitrates occur naturally in plants, for which it is a key nutrient. Nitrate and nitrite are also formed endogenously in mammals, including humans. Nitrite can also be formed chemically in distribution pipes by Nitrosomonas bacteria during the stagnation of nitrate containing and oxygen in poorly treated drinking water in galvanized steel pipes or if chlorination is used to provide a residual disinfectant and the process is not sufficiently well controlled (Olujimi *et al.,* 2014; Oun *et al.,* 2016). Nitrates from different nitrogen sources have different nitrogen isotopic compositions, which can be used to identify the sources of nitrate and trace the nitrogen cycling process.

The nitrate concentration in the surface water is normally low (0 – 18 mg/l) but can reach high levels as a result of agricultural runoff, refuse dump runoff, or contamination with human or animal wastes (WHO, 2019). The concentration often fluctuates with the season and may increase when the river is fed by nitrate-rich aquifers. Nitrogen in surface waters has a variety of sources (Collivignarelli *et al.,* 2018), including atmosphere deposition, dust in rainwater, industrial wastewater, domestic sewage, urban garbage, nitrogen chemicals, fertilizers, livestock waste, and plant humus (D *et al.,* 2013; Mathebula, 2015; Wen *et al.,* 2017). The traditional method for identifying nitrate pollution sources in water bodies combines investigation of land use type of pollution area with analyses of concentrations of nitrogen compounds in water (Zhou, 2015; Singh, 2016).

2.3.1.7. Phosphate

In natural and treated water, phosphorus occurs roughly as sole dissolved orthophosphate (Griffins et al., 2014; Musyoki et al., 2016; Singh, 2016). Orthophosphate is the most thermodynamically balanced form of phosphate and is the form commonly identified in laboratory analysis. In aquatic ecosystems, sources of phosphorus include soil and rocks, wastewater treatment plants, runoff from fertilized lawns and cropland, runoff from animal manure storage areas, disturbed land areas, drained wetlands, water treatment, decomposition of organic matter, and commercial cleaning preparation (DWAF, 1996a; Naidoo, 2013; De Klerk, 2016). The addition of even a small amount of phosphorus to surface water can have negative consequences for water quality. Those adverse effects include algae blooms, accelerated plant growth, and low dissolved oxygen from the decomposition of additional vegetation. DWS aquatic ecosystem guidelines commends phosphorus concentrations of less than 0.005 mg/L to oligotrophic conditions to protect aquatic ecosystems; 0.005 – 0.025 mg/L is mesotrophic, and concentrations of 0.025 to 0.250 mg/L are eutrophic and <0.250 mg/L are hypertrophic (DWAF, 1996a; Naidoo, 2013; De Klerk, 2016; DWS, 2017).

Phosphate in nature is essential for plant growth but excessive amounts can lead to significant impacts on the ecological health of rivers. Phosphate can be introduced into waters from a variety of sources, primarily from industrial and sewage discharges and from losses from the application of animal manure and inorganic fertilizers to agricultural lands (DWAF, 1996a; DWS, 2016). High concentrations of phosphates and nitrates result in nutrient enrichment and eutrophication in the aquatic ecosystem (Dallas, 2008; Mwangi, 2014; Retief *et al.,* 2020).

2.3.1.8. Sulphate

Sulphates are found in almost all-natural water, where the raised concentration can originate from natural sources, mining activities, and landfill leaching (Mwangi, 2014; Mathebula, 2015; Gonah, 2016). Sulphur occurs mostly as sulphate ions resulting from the oxidation of elemental sulphur, sulphide minerals, or organic sulphur (Mathebula., 2015). In the aquatic ecosystem, sulphur occurs in low concentrations and if in abundance, it forms sulphuric acid which results is detrimental to aquatic organisms. Various concentrations of sulphate salts are used in foods (FAO, 2017), and ammonium sulphate is used in the fertilizer industry (Gosch *et al.,* 2019).

In aquatic ecosystems, sulphate is prevalent in the area located closer to industries that manufacture animal feeds, electronics, and metallic. In the mining area, sulphates are discharged into surface water through mining wastes and atmospheric deposition of sulphur dioxide (Dallas, 2008; Verlicchi *et al.,* 2020). The health effect is associated with ingestion of high levels of sulphate includes liver damage, hair and teeth problems, and weakness in a body (SANS, 2015; WHO, 2019). In South Africa's aquatic ecosystem, sulphate concentration recommended limit is 200 mg/L (DWAF, 1996a; DWS, 2017).

2.3.1.9. Ammonia

Ammonia concentration in surface water arises from the breakdown of nitrogenous compounds from organic and inorganic matters (U.S.EPA, 2012; Mathebula, 2015; Singh, 2016). In South Africa, ammonia is present in small amounts in air, soil, and water, and in large amounts in decomposing organic matter and its toxicity is affected by the concentrations of DO, carbon dioxide, and TDS, and the presence of other toxicants, such as metal ions. (DWAF, 1996a; DWS, 2017). It also may find its way to ground and surface waters through the discharge of industrial process wastes containing ammonia and fertilizers. Ammonia has been used in municipal WWTPs for over 70 years to prolong the effectiveness of disinfection chlorine added to drinking water (Griffin, 2014; Collivignarelli *et al.,* 2018).

The presence of elevated ammonia levels in raw water may interfere with the operation of manganese removal filters because too much oxygen is consumed by nitrification, resulting in mould, earthy tasting water. High ammonia concentration results in nitrite formation through the nitrification processes in which Nitrosomonas *spp* and Nitrobacter *spp* bacteria oxidize to form nitrite and nitrite being further oxidized to form nitrate (Zhang *et al.,* 2014; Mathebula, 2015; Wen *et al.,* 2017). High Nitrite and nitrate levels of greater than 1.0 mg/L in water, lead to low dissolved oxygen content, causing blue baby syndrome (Mwangi, 2014; Li *et al.,* 2018). To protect the aquatic ecosystem and water users, DWS recommends a range of 0 – 6 mg/L nitrate concentrations without adverse health effects and 0 – 100 mg/L with no adverse effects on livestock watering (DWAF, 1996b; DWAF,1996c).

2.3.1.10. Aluminium

Aluminium is abundant metal in the earth's crust and is its solubility in water depends on the pH. Aluminium can be selectively leached from rock and soil to enter any water sources. Aluminium in water can be present as aluminium hydroxide, a residual from the municipal feeding of alum (aluminium sulphate), or as sodium aluminate from clarification or precipitation softening (Dallas and Day, 2004). It has been known to cause deposits in cooling systems and contributes to the boiler scale. The high concentration of aluminium in water can neurotoxic to humans and animals and may cause Alzheimer's disease (WHO, 2019). Yet, it is beneficial in water treatment processes to reduce levels of organic matter, colour, turbidity, and microorganisms levels in water (Mathebula, 2015; WHO, 2019).

According to U.S. EPA (2012) and WHO (2019) aluminium above 0.1 ppm may impact colour but the level may not be appropriate in all water supplies. WHO (2019) accepts the aluminium of 0.2 ppm based on the importance of coagulant and that all municipal systems should be able to keep treated water below this value. The DWS aquatic ecosystem guidelines include other sources such as liquid effluents from metal construction, leather and textile industries, and paper industries (DWAF, 1996b).

2.3.1.11. Copper

The accumulation of copper into surface water can be by natural phenomenon such as weathering of rocks and runoff into the aquatic environment (Singh, 2016; DWS, 2017). According to WHO (2019), Cu is an essential nutrient to humans and organisms in small amounts and it is used for enzyme functioning and carbohydrate metabolism. Copper is slightly soluble in water and has a strong affinity for organic matter and sediments. Copper is most toxic in its cupric (Cu^{2+}) from (Lebepe *et al.,* 2016). Thus, it is found in lower concentrations in the water column compared to the sediments as it will bind with organic matter.

In the several studies of the USA, Cu concentrations in surface waters ranged between 0.0005 to 1 mg/L, the median value was 0.01 mg/litre (Oun *et al.,* 2014). Cu concentrations in aquatic ecosystems vary widely as a result of variations in water characteristics such as pH and TDS (Le Roux *et al.,* 2012; Wang *et al.,* 2014). After oral exposure in mammals, absorption of copper occurs primarily in the upper gastrointestinal tract and is controlled by a complex homeostatic process that involves both active and passive transport (Dallas, 2008; Olujimi *et al.,* 2015; Verlicchi *et al.,* 2020). In South Africa, recommended limit for dissolved copper at different water hardness, for instance: soft water concentrations <0.0003 mg/L, medium water concentrations 0.0008 mg/L, hard water concentrations 0.0012 mg/L, and very hard water concentrations of 0.0014 mg/L (DWAF, 1996a; DWS, 2017).

2.3.1.12. Iron

Iron in water may be present in varying quantities depending upon the geology of the area and other chemical components of the waterway (Haller *et al.,* 2014). Iron is an essential trace element required by both plants and animals (Frinden, 2015; Mathebula, 2015; Augustyn *et al.,* 2016). The Fe concentration in the natural environment must be low due to its solubility, with Fe concentrations expected to be ranging from 0.0001 - 0.5 mg/L in surface water and <0.002 mg/L in ocean water (DWAF 1996a, Self *et al.,* 2013; De Klerk, 2016). In some waters it may be a limiting factor for the growth of algae and other plants, especially it is precipitated by the highly alkaline conditions. It is also a vital oxygen transport mechanism in the blood of all vertebrates and some invertebrate animals.

The ferrous and the ferric irons are the primary forms of concern in the aquatic environment, although other forms may be in organic and inorganic wastewater streams. Iron is released into the environment by leaching from sandstones with iron oxides and hydroxides. Industrial discharge and AMD are responsible for high iron concentration in water (Gonah, 2014; Donoso *et al.,* 2017). Industrial sources discharge iron from petrochemical and iron smelting (Bilek *et al.,* 2016). Iron is toxic at high concentrations interfering with the function of several enzymes. Ingestion of water with a high concentration of iron causes tissue damage as a result of iron accumulation in the tissue cells. DWS guidelines to protect the aquatic ecosystem, recommend that Fe can range from 0.001 - 0.5 mg/L (DWAF, 1996a; Sibanda *et al.,* 2015; DWS, 2017).

2.3.1.13. Mercury

Mercury is a silver-white, liquid metal solidifying at 38.9° C to form a tin-white, ductile, malleable mass (U.S. EPA, 2012). Accumulation of Hg inputs to the environment is emitted result of natural and anthropogenic sources (Self *et al.,* 2013; Olujimi *et al.,* 2015; Walters *et al.,* 2017). Walters *et al.* (2017) has learned that mercury in surface water can occur through the accumulation of sediments that contain methylation and demethylation residuals. Mercury is widely distributed in the environment and biologically is the non-essential or non-beneficial element. Historically it was recognized to possess a high toxic potential and was used as a germicidal or fungicidal agent for medical and agricultural purposes.

Mercury intoxication may be acute or chronic and toxic effects vary with the form of Mercury and its mode of entry into the organism (SANS, 2015; DWS, 2017). Symptoms of acute, inorganic mercury poisoning include pharyngitis, gastroenteritis, vomiting followed by ulcerative hemorrhagic colitis, nephritis, hepatitis, and circulatory collapse. Chronic mercury poisoning results from exposure to small amounts of mercury over extended periods. Chronic poisoning from inorganic mercurials most often has been associated with industrial exposure, whereas poisoning from the organic derivatives has been the result of accidents or environmental contamination (DWAF, 1996b; Griffins, 2014). The mercury content of unpolluted USA rivers from 31 States where natural mercury deposits are unknown is less than 0.1 ug/l (Oun *et al.,* 2014). Klein *et al.* (2018) found also that the majority of U.S. waters contained less than 0.1 ug/l of mercury. In South Africa, DWS aquatic guidelines recommend that mercury range between 0.00004 – 0.0017 mg/L (DWAF, 1996a; DWS, 2017).

2.3.1.14. Zinc

Zinc is also a trace metal needed for biological growth in plants, animals, and humans. In surface water, it occurs in two oxidation states such as metal and zinc (II) with zinc (II) occurring in small concentrations. Within the environment, its sources are through the weathering of rocks, erosion, and industrial activities. Active mining activities and abandoned mines are chief causes of acid mine drainage into surface water in South Africa (Gonah, 2016; Edokpayi *et al.,* 2018). AMD may contain heavy metals such as zinc, iron, and mercury that are formed under natural conditions and are discharged into the environment through seepage, discharge from mines, and industrial activities (Dabrowski and Klerk, 2013; Musingwini 2014).

Zinc (as metal) is used in galvanizing, i.e., coating (hot dipping of various iron and steel surfaces with a thin layer of zinc to retard corrosion of the coated metal. In contact with iron, zinc is oxidized preferentially, thus protecting the iron. The second most important use of zinc, reaching major proportions in the last quarter-century, is in the preparation of alloys for dye casting. Zinc is used also in brass and bronze alloys, slush castings (in the rolled or extruded state), in the production of zinc oxide and other chemical products, and in photoengraving and printing plates.

Elevated zinc concentration in water can result in a bitter taste and exhibits a milky appearance thus affecting the aesthetic value of water (SANS, 2015; DWS, 2017). The toxicity of zinc compounds to aquatic animals is modified by several environmental factors, particularly hardness, dissolved oxygen, and temperature (Rashid *et al.,* 2018; Haarhoff *et al.,* 2020). Lebepe *et al.* (2016) in undertaking a review of the literature on the toxicity of zinc to fish, reported that salts of the alkaline-earth metals are antagonistic to the action of zinc salts, and salts of certain heavy metals are synergistic in soft water. Both an increase in temperature and a reduction in dissolved oxygen increase the toxicity of zinc. Acutely toxic concentrations induce the cellular breakdown of the gills, and possibly the clogging of the gills with mucous. In South Africa, to protect the environment aquatic guidelines recommend the Zn limit of 0.002 mg/L.

2.3.1.15. Manganese

Manganese sources into surface water can be from natural and anthropogenic activities and can be toxic over time (Mathebula, 2015; Retief *et al.,* 2020). Criteria for limiting manganese in surface water were based on determining dissolved manganese concentration (DWS, 2017; Griffin *et al.,* 2014). Manganese does not occur naturally as metal but is found in various salts and minerals, frequently in association with iron compounds (DWAF, 1996b; Musilova et al., 2016; Retief et al., 2020). Manganese is a vital micronutrient for both plants and animals. When manganese is not present in sufficient quantities, plants exhibit chlorosis (yellowing of the leaves) or failure of the leaves to develop properly (Mujuru *et al.,* 2016; Rodrigues *et al*., 2017). Inadequate quantities of manganese in domestic animal food result in reduced reproductive capabilities and deformed or poorly maturing young.

The principal manganese-containing substances are manganese dioxide, pyrolusite, manganese carbonate) and manganese silicate (Srivasta *et al.,* 2013; Shirani *et al.,* 2020). The primary uses of manganese are in metal alloys, dry cell batteries, micro-nutrient fertilizer additives, organic compounds used in paint driers, and chemical reagents (Rodrigues *et al.,* 2017). At concentrations of slightly less than 1 mg/L to a few milligrams per litre, manganese may be toxic to plants from irrigation water applied to soils with pH values lower than 6.0. The problem may be rectified by liming soils to increase the pH. Problems may develop with long-term continuous irrigation on other soils with water containing about 10 mg/l of manganese (Retief *et al.,* 2020). But as stated above, manganese rarely is found in surface waters at concentrations greater than 1 mg/l. To protect aquatic ecosystems, Department of Water and Sanitation guidelines recommends dissolved Mn level to be 0.18 mg/L.

2.3.2. Water quality parameters: Microbiological parameters

The microorganisms (bacteria, fungi, viruses, and protozoa) in water are habitual of faecal nature related to humans and animals (Zhang *et al.,* 2015; Edokpayi *et al.,* 2018; Haberecht *et al.,* 2019). The anthropogenic sources of microorganisms in surface water are mostly from WWTPs, agriculture, and domestic wastes (DWS, 2016; Ridanovic *et al.,* 2017; Wen *et al.,* 2020). According to Frieden (2015) and Elbossaty (2017), the quantities and significance of pathogens depend on components such as contamination level, pathogens tenacity in surface water, biological reservoirs, and dissemination of bacteria. Previous studies have demonstrated that land use activities and receiving environment can also influence the survival of microorganisms (Naidoo *et al.,* 2014; De Klerk, 2016; Wen *et al.,* 2020). Land uses practices such as informal settlements, WWTPs are reservoirs and disseminate a spectrum of pathogenic microorganisms (Mulamattahil, 2014). Exposure and use of microorganisms contaminated water may pose threat to human health with transmission of waterborne diseases e.g., typhoid fever and cholera (Edokpayi *et al.,* 2018; WHO, 2018; Wen *et al*., 2020). The assessment of water quality can depend on the physicochemical and microbiological organisms, which are associated with faecal origin (e.g., faecal coliform bacteria) which all play a part (Nguyen *et al.,* 2016; Ridanovic *et al.,* 2017).

2.3.2.1. Total coliforms (TC)

Total coliform bacteria are all bacteria that are gram-negative and rod-shaped (WHO, 2016; Seo *et al*., 2019). These bacteria may be living in vegetation, soil, and water (Elbossaty, 2017). Total coliform bacteria are an indicator of faecal contamination in water as their easy to detect (Islam *et al.,* 2018; Haberecht *et al.,* 2019; Wen *et al.*, 2020). Consistent use of faecal contaminated water is the major threat to human health and may lead to the dissemination of pathogens in the environment (Edokpayi *et al.*, 2018). Total coliforms include faecal and thermotolerant coliforms such as genus *Klebsiella a*nd they can be isolated from the aquatic environment without faecal pollution (Bohra *et al.,* 2012; Li *et al.,* 2017). There are also members of TC groups, *E. coli* strains that can be discovered in unpolluted water samples (Manegabe, 2015; Grossman *et al.,* 2016). According to Seo *et al*. (2019), total coliforms concentrations in surface water can be affected by organic matter. Borha *et al*. (2012) concluded that changing the aquatic environment due to rainfall and point source pollution plays a role in the proliferation and concentration of coliform bacteria in surface water.

2.3.2.2. Faecal coliforms

Faecal coliforms bacteria are non-spore-forming, gram-negative bacteria from total coliforms (Nguyen *et al*., 2016; Seo *et al.,* 2019). Faecal coliforms are also used as an indicator of faecal contamination and are easy to determine in the majority community of bacteria in the human gut. (Teklehaimanot *et al*., 2015; Cho *et al.,* 2018). Faecal coliforms are slightly specific indicators of faecal contamination than *E. coli*, as they can arise from non-faecal sources (Overbey *et al.,* 2015; Ridanovic *et al.,* 2017). Faecal contamination can come from municipal wastewater, industries, and septic tanks. Moreover, faecal contamination can arise from small sources such as greywater and rainwater each contributing to the overall problem (WHO, 2012). Whatsoever the source, when the faecal bacteria in water increases, the higher the risk of faecal contamination to humans and animals. Islam *et al*. (2017) highlighted that faecal contamination from agriculture, hospital waste, and wastewater treatment poses risk to human health. *E. coli* are from faecal coliforms and they cause intestinal illness in humans (Pal *et al.,* 2016; Islam *et al.,* 2018; WHO, 2018). Nguyen *et al*. (2016) have noted that faecal contamination levels increase in a tropical area with high temperature and increase rainfall. Untreated or partially wastewater discharges, agriculture effluents, stormwater runoff, water temperature are factors that control the faecal coliforms growth and concentrations in the river (Islam *et al*., 2017; WHO, 2018).

2.3.2.3. Heterotrophic plate count (HPC) bacteria

Heterotrophic plate count (HPC) bacteria refer to a wide range of microorganisms recovered from water that requires organic carbon for growth (Traoré *et al*., 2016; Elbossaty, 2017). HPC is used to test the efficiency and disinfection of water treatment (Frieden, 2015; WHO, 2018). There are high levels of HPC in treated water especially in stagnant parts of pipes or chambers and may harbour opportunistic pathogens with virulence factors (Mulamattahil, 2014; Wen *et al.,* 2014). According to Elbossaty (2017), the regrowth of heterotrophic bacteria in water pipelines or chambers can be influenced by high temperature, disposal of nutrients to bacteria, and lack of residual disinfectant. Municipal waste treatment, agriculture, and household effluents can be the source of heterotrophic bacteria in surface water (UNICEF, 2015; Rodrigues *et al.,* 2017). The study by WHO (2018) has found out that a high density of heterotrophic bacteria harbours opportunistic pathogens such as *Aeromonas, Salmonella,* and *Klebsiella* causes health complications in humans. Consumption of water polluted with these heterotrophic bacteria can lead to waterborne diseases in humans with the weakened immune system (WHO, 2018; Sabri *et al.,* 2020). Heterotrophic bacteria do not necessarily indicate faecal contamination. However, it also determines the quality of water and the disinfection process in water treatment (Lyn, 2017; Mulamattahil *et al*., 2015, WHO, 2012).

2.3.2.4. *Escherichia coli* (*E. coli*)

The major threat to freshwater supply in South Africa is water pollution (Edokpayi *et al*., 2016; DWS, 2016). In South Africa, many communities depend on untreated, faecal contaminated water from rivers and dams for domestic and agriculture use (Dabrowski and Klerk, 2013; DWS, 2016). These microbial pollutants penetrate the surface water through untreated or partial treated wastewater effluent or sewage leakages (Frieden, 2015; Malema *et al.,* 2018). Consumption of faecal contaminated water can lead to waterborne diseases e.g., diarrhoea and cholera (Cho *et al*., 2018; WHO, 2018). Faecal bacteria can be detected in a contaminated river environment, particularly from faeces of all warm-blooded animals (Marie *et al.,* 2018; Wen *et al.,* 2020). Their presence can be a suggestion of faecal pollution and may spread from one environment to the other (Tornevi *et al.,* 2014).

E. coli is a gram-negative motile facultatively anaerobic bacillus that may or may not be encapsulated (Khwidzhili *et al.,* 2016; Ridanovic *et al.,* 2017). *E. coli* bacteria are a subgroup of faecal coliforms, and they used as indicator organisms as they are easy to detect (Adefisoye and Okoh, 2016; Cho *et al.,* 2018). Different strains of *E. coli* are pathogenic, causes infections in humans and are mostly implicated in urinary tract infection (Thenmozhi, 2014; Park *et al.*, 2018;). Livestock and humans infected with *E. coli* strain 0157:H7 can contaminate rivers, and dams with faecal contamination (Nguyen *et al.,* 2016; Cho *et al*., 2018; Islam *et al.,* 2018). According to Ranjbar *et al*. (2016) pathogenic bacteria move into surface waters through faecal contamination from livestock and contaminated soil. Receiving surface water such as rivers and dams are constantly exposed to changing weather environments which may facilitate the spread of *E. coli* (Dabrowski and Klerk, 2013; Haberecht *et al.,* 2019). Tornevi *et al*. (2014) has learned that rainfall can increase *E. coli* concentration levels in surface water through runoffs and from sewerage leakages. Therefore, the influence of rainfall on the increased risk of faecal contamination should be monitored and be a continuous process.

2.4. The occurrence of water pollution and impacts on water quality

The rate of water pollution appears to be wide spreading in poor communities and developing countries associated with significant human settlements (Bester, 2015; Khatri *et al*., 2015; Herbig *et al.,* 2019). WHO (2017) has defined water pollution as changes in water properties that harm the aquatic ecosystem and the end user. Sources of pollution can be either point and non-point sources, where point source is a direct or specific point and non-point sources can be from natural factors that can cause greater impact on the environment (Martinez and Baquero, 2014; De Klerk, 2016; Verlicchi *et al.,* 2020). An example of a non-point source can be precipitation that can erode impurities from upstream to downstream (Pal *et al.,* 2015; Udall, 2018).

Naauwpoortspruit River has been significantly impacted by anthropogenic activities such as industries, agriculture, and wastewater treatment effluent containing nutrients and microbial contaminants (De Klerk, 2016; Elbossaty, 2017). Nkosi *et al.* (2014) highlighted that a big portion of the water pollution problem is partially or untreated sewage originating from urban areas discharging into water bodies. The foremost anthropogenic factors impacting the Naauwpoortspruit River are summarized in Table 1.

Table 1. Potential point and non-point sources of pollution at Naauwpoortspruit River from land-use practices (DWS, 2017).

Point Sources	• Steel Industries • Witbank Coalfields (site A) potential source of AMD into the catchment. • Naauwpoortspruit WWTP effluent (site D of this study) • Power generation - major coal-fired power stations and its largest industrial activity in the catchment.
Non-point Sources	• Formal and Informal settlements near Tasbet and Duvha Park (selected sampling site B of this study). • Leaking sewerage leakages especially at Tasbet extension (Site C of this study). • waste effluent from Car wash next to the bridge at Tasbet • Agricultural runoff - Dry-land cultivation of maize is practiced on 24 percent of the Naauwpoortspruit river catchment area. • Dumping site from municipality waste generation downstream next to selected site E of this study.

Surface water should be within acceptable standard limits to protect aquatic biodiversity and downstream users. With the increased concern for surface water quality, information regarding the physicochemical and bacteriological parameters continues to be of paramount importance to assess water quality and a threat to human health (Griffins, 2014; Bester, 2015; Pollard *et al.,* 2017). In Bangshi River, Bangladesh, a surface water study was conducted on drinking and agricultural development. The leading sources of water pollution varied from sewages, domestic wastes, industrial and agricultural runoff from farms using fertilizers (Kabir, 2014). In a similar study in the United States of America (USA) rural areas undertaken by Oun *et al.* (2014), the WWTPs, industrial effluents, and agricultural runoff are the main contributors to organic, nutrients parameters, and contributed to elevated levels of microbial load into surface water. Organic inputs from sludge can contribute to low dissolute oxygen levels in surface water, which can enable rapid growth of the blue-green algae (Olaolu *et al.,* 2014; Lee *et al.,* 2019). Municipal WWTPs are point and non-point sources for surface water contamination and nutrient enrichment. For example, in the Molopo river high bacterial levels were observed, e.g., faecal indicators such as *E. coli*, *Cryptosporidium spp*. and *Giardia* suggesting that untreated wastewater was dumped somewhere in the river (Mulamattahil, 2014). This was due to the WWTPs in the surrounding areas were loaded and discharging into the river (Mulamattahil, 2014).

Surface water can also be impacted by active and abandoned coal mining activities in a given area (Gonah, 2016). AMD is a significant environmental issue in South Africa, with high temperatures, radiation, and sulphide production associated with mining operations (Kotelo, 2013; Soleimani *et al.,* 2018). Active and abounded mines are releasing heavy metals, which seepage into water resources to cause AMD and heavy metal pollution (Self *et al.,* 2013; Ebenebe *et al.,* 2017). In a study by Parra *et al.* (2011) abounded mining operations have increased the generation of AMD with high iron and copper concentration found in the Andean tributaries of north-central Chile. The accumulation of heavy metals in surface water above tolerable concentrations has negative effects on the aquatic ecosystem and human health. Acid mine drainage is defined as the formation and movement of highly acidic water rich in heavy metals (Harmony Gold, 2014; Verlicchi *et al.,* 2020). This acidic water forms through the chemical reaction of surface water (rainwater, snowmelt, pond water) and shallow subsurface water with rocks that contain sulfur-bearing minerals, resulting in sulfuric acid (Gonah, 2016). Heavy metals can also leach from rocks that encounter the acid, a process that may be substantially enhanced by bacterial action. The resulting fluids may be highly toxic and, when mixed with groundwater, surface water, and soil, may have harmful effects on humans, animals, and plants. Active and abandoned coal mining activities in the Naauwpoortspruit River area, have changed the water chemistry of surface water 200km downstream (Dabrawoski *et al.,* 2013; Retief *et al.,* 2019). Threatening levels of heavy metals includes aluminium, manganese, molybdenum, and zinc were found in the Upper Olifant's

River catchment. The general results of pollution will be a high-cost implication in treating polluted water and in most cases, it requires modern methods for treatment like nanofiltration and reverses osmosis (Bester, 2015).

2.4.1. Water pollution contribution of agriculture

The agriculture sector is the largest water consumption sector worldwide (Karatas and Karatas, 2016; Wen *et al.,* 2017). According to FAO (2017) agriculture has evolved over the last decade with modern practices e.g., land preparation, new irrigation systems, fertilizer application, insecticides, pesticide application, and livestock handling. Through these practices, there is a high probability of agriculture runoff, soil erosion, nutrients (phosphates and nitrogen) runoff, insecticides, and other pathogens which flow into surface waters (Mwangi, 2014; FAO, 2017). In South Africa, the agriculture sector is categorized into two different sectors, the commercial and subsistence sector (Griffin *et al.,* 2014; Pall *et al.,* 2017). These different sectors consume water depending on the availability of water resources. According to Schreiner *et al.* (2018) agriculture sector in South Africa accounts for 60 percent of water use, followed by the municipal sector 27 percent. DWA (2013) reported that the main reason why the agriculture sector has high water demands is the 1.6 million hectares of land equipped for irrigation.

In South Africa, the agricultural production of maize crops is of strategic importance to the national food supply (Langner *et al.,* 2019; Haarhoff *et al.,* 2020). 24 percent of South Africa's dry-land cultivation of maize is practiced around the eMalahleni area (FAO, 2017; Haarhoff *et al.,* 2020). Phosphorous as phosphate is one of the limiting nutrients which increase in the aquatic environment after fertilization of agricultural lands resulting in eutrophication (Oberholster *et al.,* 2013). Elevated phosphate concentrations occur in waters that receive sewage, crop residues, leaching human and animal wastes as well as runoff from cultivated lands (Nguyen *et al.,* 2016). A study carried on the Lower Mississippi River, Louisiana, USA, has revealed that high nutrient input such as Zn and Fe from agricultural runoffs onto surface water is impaired by a high input of pesticides and fertilizers (Oun *et al.,* 2014). Nutrient enrichment has an impact on the surface as it allows organisms to proliferate (FAO, 2017). These organisms may be disease vectors and can also increase the abundance of cyanobacteria (blue-green algae). The proliferation of algae may slow the flow in watercourses, thus increasing the proliferation of organisms and sedimentation (Lee *et al.,* 2019).

There are loads of thousands of pesticides available in agricultural, forestry, and urban use, many of which are synthetically produced (FAO, 2017). Pesticide residues end up breaking down in the environment forming by-products, some of which are relatively toxic than the others (Naidoo, 2013; De Klerk, 2016). In animal farming, manures generated by livestock are frequently reused as organic fertilizers in farmlands and may be eroded to the aquatic ecosystem after digging the topsoil (Self *et al.,* 2013; Lee *et al.,* 2019). These waste components contain antibiotics such as tetracyclines and penicillin which are major sources of the increase in the concentration of the antibiotics in the aquatic system (Faleye *et al.,* 2018; Langner *et al.,* 2019). A study in the United States of America has found out that twelve classes of antimicrobials; arsenicals, polypeptides, glycolipids, tetracyclines, rifamycins, macrolides, lincosamides, polyethers, beta-lactams, quinoxalines, streptogramins, and sulphonamides are used at different times in the life cycle of poultry, cattle, and swine farming (Self *et al.,* 2013). These antibiotics residue and animal wastes from this farming may be a contributor to antibiotic resistance in aquatic ecosystems and can disperse over the long area (Griffin, 2014; Pall *et al.,* 2017; Retief *et al.,* 2019). Toxic pesticide effects are found to be influential on the reproduction of aquatic species leading to the disruption of symbiotic relationships and a loss of biodiversity in the Danube River, Hungary (Mann *et al.,* 2011). If aquatic organisms are not harmed immediately, they may accumulate chemicals from their environment into their tissues. This bio-concentration can lead to biomagnifications, a process in which the concentrations of pesticides and other chemicals are increasingly magnified in tissues and other organs as they have transferred up the food chain (Mann *et al.,* 2011; Britz *et al.,* 2012; Langner *et al.,* 2019). There is therefore a need to monitor surface water to protect and converse the resource from nutrients and other pollutants from the agriculture sector.

2.4.2. Urbanization

Aquatic environments are being exposed to noxious waste and contaminants from the domestic, and household waste produced day by day (Musingafi and Tom, 2014; Pollard *et al.,* 2017; Retief *et al.,* 2020). Densely inhabited urban areas are mostly dominated by poor, low-income residents, e.g., informal settlements and squatter camps, which do not have access to potable water and proper sanitation infrastructure. Mostly, informal settlements have open sewerage and drainage where rubbish waste and wastewater are dumped directly and these then discharges into rivers in that pollutant state (Mulamattahil, 2014; van Vuuren, 2015). Due to lack of proper or poor sanitation facilities such as toilets, people excrete in the bush, banks of the river, and next to sewerage pipes. During heavy rainfall periods, these drain and overflow into the surrounding river, increasing faecal contamination into surface water (Mwangi, 2014; Oberholster *et al.,* 2017). According to van Vuuren (2015), there is massive population growth and in-migration to eMalahleni town, with over 30 000 households being informal settlements. The high populated communities are associated with less sufficient sanitation infrastructure and sewerage directly discharging and seeping into the river (Drabowski *et al.,* 2013; Khatri *et al.,* 2014). Similar observations were discovered around Buffalo River, Eastern Cape Province, South Africa, where high populated communities are living in the area of less sufficient infrastructure. The communities that draw water from the river that is receiving sewerage runoff as their source of water, may pose a serious health threat to humans. According to Chigor *et al.* (2013), the Buffalo River is of poor quality, with high levels of pathogenic bacteria due to faecal contamination from the runoff, agriculture effluents, and informal settlements.

Increased populated urban areas are also linked to a rapid increase in clean water demand due to continues population growth (Teklehaimanot *et al.,* 2015; Rodrigues *et al.,* 2017) and increased burden on municipal water treatment infrastructure. For example, approximately 2,057 million cubic meters of water were required per annum in the year 2000 to meet local demand against a local reliable yield of 1,306 million cubic meters in the Vaal region (Haji, 2011). The deficit was therefore satisfied through numerous inter-basin water transfers in and out of the catchment (Haji, 2011; DWS, 2016; Mulamattahil, 2014). Hence the need to reuse treated wastewater proposed as a feasible alternative to overcome water shortage and involve numerous reuse options (Hemson, 2016). Nonetheless, if wastewater is not treated effectively, wastewater reuse can be harmful and poses potential health risks to the public (Cillers *et al.,* 2016; Hemson, 2016).

Domestic wastes from urban areas are potential point sources of contamination of both groundwater and surface water. Domestic wastes from the household are often comprised of cleaning products, fertilizers and medical waste which are synthetic from the petrochemical industries and health facilities, are dumped into surface water. These detergents and nutrients contain phosphates, nitrogen, and antibiotics which can pollute and still create secondary pollution in aquatic ecosystems (De Klerk, 2016; Rodrigues *et al.,* 2017). According to a study of Muzaffarabad City in Pakistan, human waste and improper sanitation systems are sources of faecal contamination into the environment (Muhammad *et al.,* 2018). Rivers are polluted by waste production from households and during rainy seasons, water erodes garbage’s in the adjacent areas to the surface water (Musingafi and Tom, 2014; Muhammad *et al.,* 2018). This is concerning as nutrients can exert long-standing to form eutrophication (Griffins *et al.,* 2014; DWS, 2016; Retief *et al.,* 2020). Therefore, an urgent need exists for improving the level of surface water and water monitoring to decrease the current negative impact which comes with the cost of treating water.

2.4.3. Rainfall and runoffs

Even though the degradation of water quality is almost invariably the result of human activities, certain natural phenomena can result in water quality falls below that required for purposes. Natural events such as rainfall and hurricanes events can lead to disturbances in water bodies by changing the hydrological conditions and influencing the thermal structure of reservoirs (Huang *et al.,* 2014). However, high amounts of particulate pollutants carried by runoff are brought into reservoirs. They may cause serious water pollution, which can conversely stimulate the production of nutrient enrichment and algae. The highly variable in rainfall and spatial distribution has an impact on the availability of water resources across South Africa (DWA, 2014). Moreover, global warming effects are showing variable weather patterns across South Africa, making it hard to predict rainfall patterns (Du Plessis, 2017). The occurrence of heavy rainfall and runoff has negative impacts on soil and surface water from a point and nonpoint pollutants due to runoff.

Agriculture activities, industries, highways, and bridges are non-point sources of pollutants such as nitrogen and phosphorus into surface water. Within the surface water, the sources of these nutrients are diverse and disseminated over great areas (Le roux, 2012; Mothetha, 2016). Discharge of oil and grease from highways and bridges can be a great example of a non-point source of contamination (U.S. EPA, 2012). According to Gössling *et al.* (2012), metal toxicity in aquatic environments is well documented and effects can be observed at different biotic levels: ranging from molecular and cellular level (protein damages, lipid peroxidation, chemosensory impairments, and osmoregulation failures); to organism level (change in behaviour, delayed growth and condition factor) and finally effects at a population level (alteration of the social hierarchies among fish). Impacts of water quality difference and rainfall-runoff on Jinpen reservoir were conducted in Northwest China. During heavy rainfall, surface water reached high turbidity of 130 NTU, which exceeded the limitation provided in environmental quality guidelines of China (Zhou *et al.,* 2015). Elevated turbidity from salinity and dissolve solids are transported by runoff to surface water, where high phosphorus concentration and faecal contamination were the noticeable pollutants into the surface water (Zhou *et al.,* 2015). A similar study in Antananarivo, Madagascar discovered that municipal WWTP and agriculture activities were the main contributor of microbial pathogens *Clostridia* and *intestinal enterococci* after heavy rainfall in drinking water drawn from the river source (Bastaraud et al., 2020). Islam *et al.* (2017) has revealed that surface water may be contaminated by *E. coli* and *enterococci* due to manure from livestock farming, associated ARB from runoff. Leakage from manure storage areas and septic tanks in Satkhira town of Bangladesh are contaminating surface water after rainfall (Kabir, 2014). Therefore, understanding seasonal variations with heavy rains and stormwater runoff impact on water quality becomes of paramount importance in providing data that could be used in planning and monitoring surface water.

2.4.4. Wastewater Treatment works and disinfection effects on water quality.

Each receiving body of water has a limited capacity to absorb pollutants without declining in quality. WWTPs aim to eliminate pollutants and bacteria from the wastewater to protect the environment and ensure effluent does not affect the public. It also provides suitable effluent quality for reuse (Iloms *et al*., 2020; Khatri *et al.,* 2015; Czekalski, 2012). Some of the pollutants that are of great concern are encountered in WWTPs with such influents derived from the abattoir, hospitals, and mining industries (Collivignarelli *et al*., 2018). Many research findings have discovered that WWTPs here in South Africa are not efficient, and their effluents are not in acceptable standards (Le roux, 2014; Harmony Gold, 2014; Naidoo, 2013; Edokpayi *et al*., 2016). Teklehaimanot *et al.* (2015) further highlighted that WWTPs in South Africa are not necessarily designed to eradicate non-biodegradable waste and remove antibiotic-resistant bacteria that are discharged onto the surface water.

In South Africa, discharging of industrial, WWTPs, and sewage leakages into rivers is a significant pathway of heavy metals into the aquatic ecosystem (Mwangi, 2014; Herbig *et al.,* 2019). According to Hemson (2016) the cholera outbreak that was experienced in Umfolozi River, KwaZulu-Natal from 2000-2001 illustrated the health risks to humans from using partially or untreated wastewater. High microbial pathogens such as faecal indicating bacteria e.g., *E. coli* were discovered in the Umfolozi River catchment, indicating that sewerage was being discharged at some point in the river (Hermoso, 2016). This was due to WWTPs effluent discharging into river catchment (Hermoso, 2016. DWS, 2017). In a similar study by DWS (2016), municipal WWTPs and agriculture were some of the practices found to be polluting Olifant's River catchment. WWTPs effluents contained nutrient concentration which was high beyond saturating concentration. The effect of nutrient enrichment includes an increase in deposit to cause eutrophication. High nitrogen and phosphates concentrations (including nitrate, ammonia, and organic forms) in surface water causes eutrophication, whereby increased production, and decomposition of algae, leads to reduced oxygen concentrations and kills aquatic biodiversity (Dabrowski and Klerk, 2013; DWS, 2016; Oun *et al.,* 2016).

In New Zealand, high quantities of certain metallic elements including cadmium, nickel, and zinc, were found in drainage leachates resulting from soil treated with sewage sludge (Agoro *et al.,* 2020). These heavy metals exhibit toxicity even at lower concentrations (Agoro *et al.,* 2020) and when released into surface water they build up over some time could be detrimental to human health and the aquatic ecosystem (Olujimi *et al.,* 2014; WHO, 2017). Oberholster *et al.* (2017) noted that municipal WWTPs, industrial manufacturing process, leaky sewers, and sewer overflow in the Olifant's River catchment contain trace organic chemicals such as Hg, Zn, and Cr into the surface water. Oberholster *et al*. (2017) further highlighted that the occurrence level of these trace metals is mostly influenced by physicochemical properties and their fate on the environment after discharge. Withers *et al.* (2014) restated that the accumulation of metals in an aquatic environment harms the human and aquatic ecosystem. Most metals are removed from the liquid effluents during the wastewater treatment process and end up in the solids such as sludge into the environment. However, if the treatment is not efficient, some of it is not completely removed and is released into surface water (Schreiner, 2018). Released heavy metals can be easily absorbed by other suspended particles in water, settling down in the riverbed, and are later released into the water column, where they become a potential secondary source of contamination, threatening ecosystems (Dabrowski and Klerk, 2013; Withers *et al.,* 2014; DWS, 2016).

2.4.4.1. Disinfection in wastewater treatment

The disinfection process is the last stage in wastewater treatment before effluent can be discharged into the environment (Netshidaulu, 2015; DWS, 2016). In wastewater treatment, disinfection is important to prevent bacteria outbreaks, particularly when downstream users use water for human consumption or other domestic uses (Collivignarelli *et al*., 2018). According to Bester (2015), the disinfection process involves chemicals such as chlorination, ozone, and physical disinfection e.g., ultraviolet rays, electric discharges in water, cavitation, and ultrasound and photochemical methods e.g., UV light, which become a priority in wastewater treatment. The chemical disinfection methods involve chlorination where it targets the cell walls of microorganisms and kills the pathogens (Devarajan *et al.,* 2016; Destiani *et al*., 2019). However, chlorine can leave behind a residual, which can generate harmful by-products upon reaction with organic particles (Mulamattahil, 2014; DWS, 2016; Hermson, 2016). Municipal WWTPs are supposed to uphold a minimum residual in the wastewater to ensure bacterial die-off during chemical disinfection. Being known that disinfection is the last process in a WWTP before discharge, the residual gets transferred with the treated discharge. The process of residual chlorine happens when chlorine reacts with natural organic compounds in water e.g., humic and fulvic acids to form a wide range of unwanted halogenated organic compounds including trihalomethanes (THMs), haloacetic acids (HAAs), chlorophenols, chloral hydrate, and haloacetonitriles (HANs) (Kralik *et al.,* 2017; Collivignarelli *et al.,* 2018; Li *et al.,* 2020). These unwanted halogenated organic compounds can cause hereditary malformations of the cardiovascular and neurological systems in humans (Li *et al.,* 2020).

Frieden (2015) and Collivignarelli *et al*. (2018) mentioned physical disinfection methods as another form of disinfection that can be utilized in water and wastewater treatment. These physical disinfections are centered on the use of different physical methods such as ultraviolet rays, electric discharges in water, cavitation, and ultrasound (Bester, 2015; Khatri *et al.,* 2015). According to Timmermann *et al*. (2015), ultraviolet rays can remove more than 99.99% of bacteria in a treated water sample. However, Destiani *et al*. (2019) has found out that antibiotic-resistant bacteria showed the potential for redevelopment in wastewater treatment after chlorination up to 5 mg/L and UV disinfection. Untreated or partially treated wastewater can shelter bacteria through turbidity or sludge from radiation disinfection and reduce the disinfection efficiency (Lee *et al*., 2015). Collivignarelli *et al.* (2018) has found out that ultraviolet radiations are very effective against bacteria (e.g., *Cryptosporidium* and *Giardia*) when effluent is properly treated. The disinfection process is the last process of wastewater treatment and the effectiveness of disinfection can mean effluent released has no pathogens (Bester, 2015; Devarajan *et al*., 2016).

The nature of organisms present in water influences the action of disinfectant and the disinfectant required in WWTPs (Netshidaulu, 2015). The presence of antibiotics and ARB in WWTPs is a concern on especially with current disinfection methods used in South Africa (Harmony Gold, 2014; Netshidaulu, 2015). The concern includes the existence of clinically relevant ARB and ARGs from influent into WWTPs, the potential for ineffective wastewater treatment and, the effects of effluent into the aquatic ecosystem (Fang *et al.,* 2014; Abia *et al.,* 2016; Collivignarelli *et al.,* 2018). Several studies have learned about the existence of ARB in wastewater effluents through the prevalent of ARGs and ARB downstream, which suggests that WWTPs are the source of ARB in receiving surface water (Faleye *et al.,* 2018; Gekenidis *et al.,* 2018). Currently, the effectiveness of the disinfection process in WWTPs, such as nanofiltration and chlorination on ARB and ARGs has not been well characterized.

2.4.5. Heavy metals in a river environment

The existence of heavy metals in the river environment is due to either natural causes or anthropogenic activities. In nature, heavy metals can occur due to natural phenomena such as volcanic activity, rock weathering, and metal liquidation (Srivasta *et al.,* 2013; Shirani *et al.,* 2020). Furthermore, heavy metals can be added to the environment by the application of anthropogenic activities, including mining, industrial effluent, domestic waste, agricultural runoff, and pesticides containing heavy metal residues (Chen *et al.,* 2018; Agoro *et al.,* 2020). Some trace elements are naturally occurring elements of the earth, namely, zinc, nickel, copper, which are regarded as essential elements which are vital to living microorganisms. However, even small amounts are toxic to aquatic ecosystems and humans.

Currently, heavy metal contamination is a challenge in many developing countries like South Africa and Bangladesh (Harmony Gold, 2014; Islam *et al.,* 2015). Urbanization and industrialization in South Africa have detrimental effects on the quality of water, sediment, and aquatic ecosystem. The disposal of urban wastes, untreated effluents from various industries, and agrochemicals in the open water bodies and rivers has reached an alarming situation in which are continually increasing the heavy metals level and deteriorating water quality. South Africa's economy is dependent on intensive mining and has seen the rise of abounded mines in the last decades (Gupta *et al.,* 2016; Gonah, 2016). Active and abounded mines are releasing acidic water, enriched with low pH, high levels of iron, and other heavy metals seepage into surface and groundwater (Naidoo, 2013; Ngwenyama *et al.,* 2017; Retief *et al.,* 2019). According to Mujuru *et al.* (2016) and Pollard *et al.* (2017), AMD has been exacerbated by the abandoned and active mine residues acting as point and non-point sources into surface water. For instance, in KwaZulu-Natal, South Africa, abandoned and defunct coal fields have started producing AMD, flowing into the Crocodile River (Mujuru *et al.,* 2016). Anyanwu *et al.* (2018) has stated that easy regulations and a lack of policy to address AMD, particularly at the mine closure stage in mining have formed an easy way out for mining companies to let the waste into the environment.

The Naauwpoortspruit River area has also been affected greatly by AMD from electrical generating industries. Coalfields for electricity generation has been the main activity in the eMalahleni town from the 1870s and had seen air quality deteriorate throughout the years due to power-stations burning coal, coal mining activities and uncontrollable ground fires (Ayanda et al, 2012; Maya et al., 2015; Benmalek *et al.,* 2015). Large coal stockpiles and significant amounts of ash generated during electrical generation are also sources of fugitive pollution in water. The effect of this pollution has been pronounced in the Naauwpoortspruit River catchment (which drains to Witbank Dam) (Orberhsolter *et al.,* 2017). Several mines in the eMalahleni coalfields have been closed over several years but have seen water accumulating from underground to its chambers and able to flow into neighbouring mines due to proximity with active mines (Soleimani *et al.,* 2018). Maya (2015) has revealed that in 2004, 62 ML/d of polluted mine water was discharged from abandoned coal mines into active mine before 50 ML/d of that water seepage into the Naauwpoortspruit River. The excess water had a very low pH and high iron content and therefore there was a need to lower the pH by adding limestone and precipitating the iron by blowing oxygen or air into the water. During the precipitation process, several heavy metals apart from iron were precipitated. The iron could settle and be separated and disposed of in tailing dumps while the water was discharged into local rivers. The discharged water was generally clear; however, a high sulphate content of 1500 mg/l was observed (Dabrowski and Klerk, 2013; Maya, 2015; Edokpayi *et al.,* 2016). The effect of the diffuse and point source pollution arising from eMalahleni coalfields is well illustrated by the elevated amounts of salinity levels at Witbank Dam and Olifant's River, which nearly doubles because of the inflow of water from the Naauwpoortspruit River and upper Olifant's River (DWS, 2016; Oberholster *et al.,* 2017; Retief *et al.,* 2020).

2.4.6. Microbiological indicators in a river environment

The microorganisms (bacteria, fungi, viruses, and protozoa) in water are habitual of faecal nature related to humans and animals (Zhang *et al.,* 2015; Edokpayi *et al.,* 2018; Haberecht *et al.,* 2019). The anthropogenic sources of microorganisms in surface water are mostly from WWTPs, agriculture, and domestic wastes (DWS, 2016; Ridanovic *et al.,* 2017; Wen *et al.,* 2020). According to Frieden (2015) and Elbossaty (2017), the quantities and significance of pathogens depend on components such as contamination level, pathogens tenacity in surface water, biological reservoirs, and dissemination of bacteria. Previous studies have demonstrated that land use activities and receiving environment can also influence the survival of microorganisms (Naidoo *et al.,* 2014; De Klerk, 2016; Wen *et al.,* 2020). Land uses practices such as informal settlements, WWTPs are reservoirs and disseminate a spectrum of pathogenic microorganisms (Mulamattahil, 2014). Exposure and use of microorganisms contaminated water may pose threat to human health with transmission of waterborne diseases e.g., typhoid fever and cholera (Edokpayi *et al.,* 2018; WHO, 2018; Wen *et al*., 2020). The assessment of water quality can depend on the physicochemical and microbiological organisms, which are associated with faecal origin (e.g., faecal coliform bacteria) which all play a part (Nguyen *et al.,* 2016; Ridanovic *et al.,* 2017).

2.4.6.1. Total coliforms (TC)

Total coliform bacteria are all bacteria that are gram-negative and rod-shaped (WHO, 2016; Seo *et al*., 2019). These bacteria may be living in vegetation, soil, and water (Elbossaty, 2017). Total coliform bacteria are an indicator of faecal contamination in water as their easy to detect (Islam *et al.,* 2018; Haberecht *et al.,* 2019; Wen *et al.*, 2020). Consistent use of faecal contaminated water is the major threat to human health and may lead to the dissemination of pathogens in the environment (Edokpayi *et al.*, 2018). Total coliforms include faecal and thermotolerant coliforms such as genus *Klebsiella a*nd they can be isolated from the aquatic environment without faecal pollution (Bohra *et al.,* 2012; Li *et al.,* 2017). There are also members of TC groups, *E. coli* strains that can be discovered in unpolluted water samples (Manegabe, 2015; Grossman *et al.,* 2016). According to Seo *et al*. (2019), total coliforms concentrations in surface water can be affected by organic matter. Borha *et al*. (2012) concluded that changing the aquatic environment due to rainfall and point source pollution plays a role in the proliferation and concentration of coliform bacteria in surface water.

2.4.6.2. Faecal coliforms

Faecal coliforms bacteria are non-spore-forming, gram-negative bacteria from total coliforms (Nguyen *et al*., 2016; Seo *et al.,* 2019). Faecal coliforms are also used as an indicator of faecal contamination and are easy to determine in the majority community of bacteria in the human gut. (Teklehaimanot *et al*., 2015; Cho *et al.,* 2018). Faecal coliforms are slightly specific indicators of faecal contamination than *E. coli*, as they can arise from non-faecal sources (Overbey *et al.,* 2015; Ridanovic *et al.,* 2017). Faecal contamination can come from municipal wastewater, industries, and septic tanks. Moreover, faecal contamination can arise from small sources such as greywater and rainwater each contributing to the overall problem (WHO, 2012). Whatsoever the source, when the faecal bacteria in water increases, the higher the risk of faecal contamination to humans and animals. Islam *et al*. (2017) highlighted that faecal contamination from agriculture, hospital waste, and wastewater treatment poses risk to human health. *E. coli* are from faecal coliforms and they cause intestinal illness in humans (Pal *et al.,* 2016; Islam *et al.,* 2018; WHO, 2018). Nguyen *et al*. (2016) have noted that faecal contamination levels increase in a tropical area with high temperature and increase rainfall. Untreated or partially wastewater discharges, agriculture effluents, stormwater runoff, water temperature are factors that control the faecal coliforms growth and concentrations in the river (Islam *et al*., 2017; WHO, 2018).

2.4.6.3. Total heterotrophic bacteria

Total heterotrophic bacteria (HPC) refer to a wide range of microorganisms recovered from water that requires organic carbon for growth (Traoré *et al*., 2016; Elbossaty, 2017). HPC is used to test the efficiency and disinfection of water treatment (Frieden, 2015; WHO, 2018). There are high levels of HPC in treated water especially in stagnant parts of pipes or chambers and may harbour opportunistic pathogens with virulence factors (Mulamattahil, 2014; Wen *et al.,* 2014). According to Elbossaty (2017), the regrowth of heterotrophic bacteria in water pipelines or chambers can be influenced by high temperature, disposal of nutrients to bacteria and lack of residual disinfectant. Municipal waste treatment, agriculture, and household effluents can be the source of heterotrophic bacteria in surface water (UNICEF, 2015; Rodrigues *et al.,* 2017). The study by WHO (2018) has found out that a high density of heterotrophic bacteria harbours opportunistic pathogens such as *Aeromonas, Salmonella,* and *Klebsiella* causes health complications in humans. Consumption of water polluted with these heterotrophic bacteria can lead to waterborne deceases in humans with the weakened immune system (WHO, 2018; Sabri *et al.,* 2020). Heterotrophic bacteria do not necessarily indicate faecal contamination. However, it also determines the quality of water and the disinfection process in water treatment (Lyn, 2017; Mulamattahil *et al*., 2015, WHO, 2012).

2.4.6.4. *Escherichia coli* (*E. coli*)

The major threat to freshwater supply in South Africa is water pollution (Edokpayi *et al*., 2016; DWS, 2016). In South Africa, many communities depend on untreated, faecal contaminated water from rivers and dams for domestic and agriculture use (Dabrowski and Klerk, 2013; DWS, 2016). These microbial pollutants penetrate the surface water through untreated or partial treated wastewater effluent or sewage leakages (Frieden, 2015; Malema *et al.,* 2018). Consumption of faecal contaminated water can lead to waterborne diseases e.g., diarrhoea and cholera (Cho *et al*., 2018; WHO, 2018). Faecal bacteria can be detected in a contaminated river environment, particular from faeces of all warm-blooded animals (Marie *et al.,* 2018; Wen *et al.,* 2020). Their presence can be a suggestion of faecal pollution and may spread from one environment to the other (Tornevi *et al.,* 2014).

E. coli is a gram-negative motile facultatively anaerobic bacillus that may or may not be encapsulated (Khwidzhili *et al.,* 2016; Ridanovic *et al.,* 2017). *E. coli* bacteria are a subgroup of faecal coliforms, and they used as indicator organisms as they are easy to detect (Adefisoye and Okoh, 2016; Cho *et al.,* 2018). Different strains of *E. coli* are pathogenic, causes infections in humans and are mostly implicated in urinary tract infection (Thenmozhi, 2014; Park *et al.*, 2018;). Livestock and humans infected with *E. coli* strain 0157:H7 can contaminate rivers, and dams with faecal contamination (Nguyen *et al.,* 2016; Cho *et al*., 2018; Islam *et al.,* 2018). According to Ranjbar *et al*. (2016) pathogenic bacteria move into surface waters through faecal contamination from livestock and contaminated soil. Receiving surface water such as rivers and dams are constantly exposed to changing weather environments which may facilitate the spread of *E. coli* (Dabrowski and Klerk, 2013; Haberecht *et al.,* 2019). Tornevi *et al*. (2014) has learned that rainfall can increase *E. coli* concentration levels in surface water through runoffs and from sewerage leakages. Therefore, the influence of rainfall on the increased risk of faecal contamination should be monitored and be a continuous process.

2.5. The occurrence of antibiotic resistance bacteria

The biological resistance of bacteria to antibiotics has become a universal problem and a threat to human health (UNICEF, 2015; WHO, 2018). Harnisz *et al*. (2015) and Felis *et al*. (2020) have described the increase in consumption of antibiotic drugs by humans have reflected in the presence of various residues of drugs on the environment, including the aquatic environment. Singer *et al.* (2016) and WHO (2018) presented that antibiotics and their transformation products are instigated into the environment through human waste such as water treatment, mining, and agriculture effluents. In the environment, some antibiotics are formed in nature and there are also synthetic (Munita *et al*., 2016). The pathways of antibiotics can be municipal wastewater treatment, households, and agriculture (Ramírez-Castillo *et al*., 2014). In wastewater treatment, antimicrobials mostly undergo biodegradation, absorption and transformation during activated sludge and precipitation, depending on the technology used (Sandhu *et al.,* 2016; Edokpayi *et al.,* 2017; Sabri *et al*., 2020). However, often the treatment of wastewater does not kill these contaminants, and then they end up in surface water through discharge (Bengtsson-Palme *et al.,* 2018; Wen *et al.,* 2020).

In the aquatic environment, antibiotic-resistant bacteria pose threat to living organisms that inhabit the environment (Yu *et al.,* 2019; Amarasiri *et al.,* 2020; Wen *et al.,* 2020). Firstly, antibiotics in health are created to fight infections in human and animals, and overuse leads to many antibiotics flushed into water treatment. Secondly, antibiotic drugs released into the aquatic environment accumulate and bacteria start to develop resistant to the antibiotics (Holcomb et al., 2020; Sabri *et al.,* 2020; Wen *et al.,* 2020). Most antibiotics used in hospitals and agriculture belong to the following classes: *β*-lactams, glycopeptides, macrolides, aminoglycosides, tetracyclines, quinolones, streptogramins (refer to Table 2).

Table 2. Classes of antibiotics

Class	Examples
Beta-lactams Glycopeptides Macrolides and ketolides Aminoglycosides Tetracyclines and Glycylcyclines Quinolones Lincosamides Streptogramins	Penicillin, Cephalosporins, Carbapenems, monobactams Vancomycin, Teicoplanin, Telavancin Gentamicin, Amikacin, Tobramycin, Netilmicin, Streptomycin Tetracycline, Tigecycline, Doxycycline, Minocycline, Clindamycin Quinupristin, dalfopristin

When resistant bacteria accumulate in the environment, they begin to infect other bacteria with their DNA using horizontal gene transfer (HGT) (Holcomb *et al*., 2020; Sabir *et al.,* 2020). When resistant bacteria genetic mutation process occurs, there are changes in the bacterial DNA resulting in newly acquired genes (Frieden, 2015; Price *et al*., 2017). These resistance genes can be transferred to other microbial pathogens through numerous ways such as conjugation, transformation, and transduction (Amarasiri *et al*., 2020; Ateba *et al.,* 2020). A study of rivers in northern Tanzania discovered that *E. coli* resistant to ampicillin, tetracycline, trimethoprim, sulfamethoxazole, and streptomycin was significantly higher (15 – 30 %) compared to other tested antibiotics (0 – 6 %; $p < 0.05$ (Lyimo *et al.,* 2016). Aquatic environments are storage or reservoir of resistance genes which makes it easy for bacteria to encounter these antibiotic resistance bacteria (Mothetha, 2016, WHO, 2016). The spread of antibiotics can be facilitated into the aquatic environment through wastewater treatment, storms water runoffs and irrigations (Traore *et al*., 2016).

2.6. The spread of resistant bacteria in an aquatic environment.

The proliferation of ARB can be through sewage from hospitals, wastewater treatment and agriculture effluents (WHO; 2018; Messina *et al.,* 2019; Sabri *et al.,* 2020). WWTPs are particularly rich in nutrients and bacteria-containing resistance genes which end up discharged into the receiving environment (Traore *et al.,* 2016; Sabri *et al.,* 2020). Water contaminated by pathogens is often discharged into the rivers which are used for irrigation, human consumption and used by livestock (Grossman *et al.,* 2016; Price *et al*., 2017). Furthermore, an environmental phenomenon like wind and stormwater runoff can transport bacteria over large distances and these can enable the spread of ARB (Bengtsson-Palme *et al.,* 2018).

Faecal contamination from wastewater treatment and sewerage leakages can harbour bacteria and may contribute to the outbreak of waterborne diseases in humans (Hobbie *et al*., 2017; Gaze, 2017; Berendonk *et al*., 2015). Humans are at risk of exposure to bacteria and continue to spread such pathogens without being aware (WHO, 2018; Park *et al.,* 2018; Sabir *et al.,* 2020). Carriers of resistance genes may spread within the vulnerable environment and instigate the occurrence of ARB (Munita *et al*., 2016; Zhang *et al*., 2016; Li *et al.,* 2017). When these bacteria are subsequently introduced into antibiotics, they may mutate, develop, and spread antibiotic resistant and multiple antibiotic resistance genes (Devarajan *et al.,* 2016; Singer *et al*., 2017; Holcomb *et al.,* 2020). Heavy metals pollution in the environment can also play an important role in the maintenance and prevalence of antibiotic resistance bacteria (Matjuda *et al.,* 2019; Wen *et al.,* 2020; Sabir *et al.,* 2020). This may arise from bacteria that share several overlapping genes due to metal tolerance and antibiotic resistance (Pal *et al.,* 2015; Li *et al.,* 2017; Klein *et al.,* 2018). Fletcher (2014) has emphasized that multiple antibiotic-resistant bacteria are liable for causing infectious diseases in humans and steadily proliferation to other bacteria. With Naauwpoortspruit River having many anthropogenic activities, pollutants such as metal ions, AMD, and wastewater treatment, antibiotic-resistant bacteria can spread and survive within such conditions.

2.7. The gaps in the literature review.

There is numerous literature on the assessment of water quality and prevalence of ARB in surface water. However, there is no study available on the prevalence and dissemination of antibiotic-resistant bacteria in the Naauwpoortspruit River. The current literature review focuses mainly on impacts made by anthropogenic activities to surface water quality but less on the occurrence of antibiotic-resistant bacteria. Even though the literature review has reported on microbial contamination within the Naauwpoortspruit River, Mpumalanga, there are no reports on the occurrence of antibiotic resistance bacteria (DWS, 2016; Verlicchi *et al.,* 2020).

DWS (2016) and Morokong *et al.* (2016) stated that there is a challenge within Olifant's River catchment, where Naauwpoortspruit River is a tributary, in terms of catchment receiving a combination of pollutants from mines, industrial waste runoff, partially treated municipal wastewater and urban runoff. AMD is a huge problem in South Africa that affects water quality in both surface and underground water. Heavy metals associated with AMD are subjected to widespread co-selection of antibiotic resistance genes (ARGs) and metal resistance genes in the environment (Harmony Gold, 2014; Sabri *et al.,* 2020). Considering the overwhelming worldwide health problem of antibiotic resistance, it is important to understand the prevalence and dissemination of antibiotics in the environment.

CHAPTER THREE

RESEARCH METHODOLOGY

3.1. Introduction

The purpose of this chapter is to present the research materials and methods used in conducting the study. It also offers specifics on the way this topic of the dissertation was studied based on the compilation and examination of information and data, within the framework of quantitative methods. It also addresses project-related risks, constraints, and ethical concerns.

3.2. Research design

The research aimed at assessing the water quality and prevalence of antibiotic-resistant bacteria in the Naauwpoortspruit River, Mpumalanga Province, South Africa. The research focused on quantitative research design. Vosloo (2014) describes a quantitative research design as a research methodology that focuses on gathering numerical data and statistical analysis to provide quantitative information. To achieve research objectives, antibiotic-resistant bacteria multiple water samples were collected over a period of seven months from five selected sites, and analysis was done.

3.3. Study area

Naauwpoortspruit River is in eMalahleni, Mpumalanga Province, South Africa with geographical coordinates 25.87’28° S and 29.25’53° E. Naauwpoortspruit River flows from uphill of Kromdraai area, then it passes through local mines and magisterial area of eMalahleni town which is shaped by urbanization. Between the river upstream and downstream, there are carwashes, Naauwpoort wastewater treatment works, farms, and new developments of Duvha Park and Tasbet residential area until the river reaches Witbank dam. Five sampling sites (Figure 2) were selected in the vicinity of Naauwpoortspruit River, eMalahleni. The sampling sites were chosen to cover the representative sections of the river and ensure an assessment of the land uses along the river course.

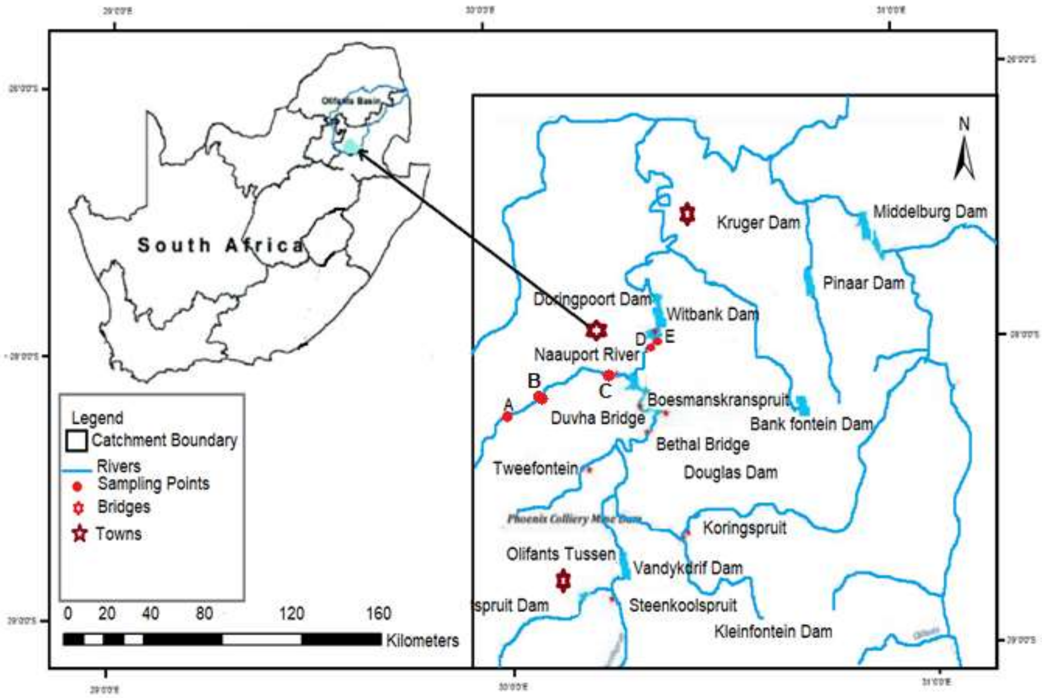

Figure 1. A map showing selected sampling sites at Naauwpoortspruit River, eMalahleni town

3.3.1. Climate

The weather in eMalahleni town is hot and humid (DWA, 2018). The average rainfall in eMalahleni ranges between 550 mm and 750, mostly from October to March. The average maximum temperature ranges between 16° C and 35°C from winter to summer and the minimum temperatures range between 0°C and 13°C from winter to summer. Hydrologically, the Naauwpoortspruit River belongs to the Olifant's River catchment (DWS, 2016).

3.3.2. Topography and Geology

The eMalahleni area is in a coalfield of Witbank (eMalahleni) in South Africa. The coalfields contain bands of coal within the sedimentary layers, Ecca Group of the Karoo Supergroup (van Vuuren, 2013). According to DWA (2016), the Naauwpoortspruit River is geographically situated in the highveld region of Mpumalanga which is comprised of a large flat area, referred to as the Springbok flats and rippling plains and pans. The soil formations are dominated by loamy soil which is crucial for agricultural activities and supports extensive irrigation (van Vuuren, 2013).

3.3.3. Population

It is estimated that 455 228 people in 2016 were living in eMalahleni local Municipality (StatsSA, 2016). According to eMalahleni IDP (2017), eMalahleni local municipality is experiencing population growth that has seen high demand for housing, growth of informal settlements, and greater demand for clean water (South African Cities Network, 2017). According to SA Cities (2014) eMalahleni Local Municipality water demand has increased by more than 40% from 2009 to 2014. This increase may be linked to population increase with 500 000 people now staying in eMalahleni, which exceeds the estimates from the 2011 census of 395 466 (Van Vuuren, 2013).

3.3.4. Land and water use

Different types of land and water users within eMalahleni Local Municipality affect water quality and water quantity (Oberholster *et al.,* 2017). Current land uses in and around the eMalahleni Local Municipality comprise of mining, agriculture, wastewater treatment, a residential area and a shopping centre. Coal mines have been operating in the eMalahleni area for over 100 years, supplying coal to the power station for generating electricity (South African Cities Network, 2017). According to Dabrowski (2013) and DWS (2016), mining, wastewater treatment, and agricultural activities emit large amounts of pollutants into the Olifants River and eMalahleni area.

3.4. Methodology

3.4.1. Sampling sites

Sampling was performed monthly at five selected sampling sites along the Naauwpoortspruit River and samples were collected from June – December 2019. Five sampling sites (Table 2) were selected, namely: Site A - by the Anglo - American mining activities; Site B – Tasbet residential; Site C – Duvha residential and car wash bridge; Site D - Naauwpoort WWTP site; Site E – Downstream of Naauwpoortspruit River. Satellite images of sampling sites are attached in appendix A.

Table 3. The detailed description of the sampling sites.

Characteristics	**Description**	**Location**	**Associated pollution activities**
Site A: River Upstream Site	Anglo- American mining activities	25°55'48.7"S 29°13'55.1"E	This site highlights the mining and agricultural activities along the upper Naauwpoortspruit River. (Appendix Figure 1A).

Site B: Upstream	Tasbet urban and Industrial area	25°56'05.7"S 29°14'40.7"E	This site highlights runoff from the urban area, industrial and agriculture activities (Appendix Figure 1B).
Site C: Downstream	Residential area, car wash, and bridge.	25°56'24.4"S 29°15'26.0"E	This site highlights runoff from urban and industrial areas. It also receives inorganic detritus from a car wash (Appendix Figure 1C).
Site D: Downstream	Naauwpoort Wastewater Treatment plant effluent discharge point and agriculture activities	25°56'24.2"S 29°15'38.5"E	This site highlights the point of discharge of WWTP effluent from residential and industrial areas and agriculture activities (Appendix Figure 1D).
Site E: Downstream	Residential and fishing activities	25°56'31.4"S 29°15'53.3"E	This site highlights small temporary effluent (tributary) from the residential area. With livestock farming and fishing activities around this site (Appendix Figure 1E).

3.4.2. Sampling technique

The sampling techniques used were according to the SANS 5667-6 method (SANS, 2015), which states the standard method of sampling for water treatment and water quality monitoring. Before sampling, all the containers were cleaned and sterilised by autoclaving to ensure that no microbial contaminants were present.

At the sampling sites, water samples were collected in duplicates for each site using 500 ml Schott and one-litre amber glass bottles, respectively. After sampling, the sample bottles were placed into a cooler box and carried to the Department of Environmental Science (UNISA) laboratory for analysis.

3.4.3. Analyses

Selected parameters were analysed in the laboratory to determine their concentrations and in relationship to South African Water Quality Guidelines for Aquatic Ecosystem, Agricultural Irrigation, Livestock And Watering (DWAF,1996a; 1996b;1996c), South African National Standard: Drinking water (SANS 241 :2015), World Health Organization (WHO) (2017) to give a picture of the requirements. These parameters are summarised in Table 4.

Table 4. List of selected determinants analysed during the study and the set DWS, SANS, and WHO limits.

Determinants	Units	Standard or guidelines				
		DWAF:			SANS: 241 (2015)	WHO: (2017)
		Aquatic Ecosystems (1996a)	Agricultural Irrigation (1996b)	livestock & watering (1996c)		
Physical parameters						
pH		5.5 – 11	6.5 - 8.4	**	5.0 – 9.7	***
Temperature	°C	***	***		***	***
Conductivity	mS/m	70 – 150		0 - 154	0 – 70	***
Total dissolve solids (TDS)	mg/L	**	0- 260	0 – 1000	0 – 1200	***
Chemical parameters						
Nitrate as Nitrogen	mg/L	<0.5 *	0 - 0.5	0 - 100	0 – 11	50
Chemical Oxygen Demand	mg/L	***	***	**		
Ammonia (NH3)	mg/L	<0.007	**	**	0 – 1.5	***
Phosphorus (Inorganic)	mg/L	<5	***	**	***	***
Sulphate (SO_4^-)	mg/L	***	***	0 - 100	500	***
Iron (Fe)	mg/L	***	0 - 5	0 -10	0 – 2	0.2
Zinc (Zn)	mg/L	<0.002	0 - 1	0 - 20	0.005	***
Mercury (Hg)	µg/L	<0.04	***	**	0 – 0.006	0.006
Manganese (Mn)	mg/L	<0.18	0-0.02	0 - 10	0 – 0.4	0.4
Copper (Cu)	mg/L	<0.0003	0 - 0.2	0 – 0.5	0 – 2	2
Microbiological parameters						

Total Coliforms	CFU/ 100 mL	1000			10	
Faecal Coliforms	CFU/ 100 mL	1000	0 – 10 000		0	0
E. coli	CFU/ 100mL	1000			0	0
heterotrophic plate count bacteria	CFU/ 100mL	1000			1.000	0

* A TWQR should only be derived after case and site-specific studies, the inorganic nitrogen of surface waters should not be changed more than 15%; (a) irrigation equipment

**TDS should not change by >15% from the normal cycles of the water body under impacted conditions at any time of the year.

*** Not stipulated

3.4.4. Physicochemical analysis

Various analytical methods were used to analyse and gather data on physicochemical determinants of the Naauwpoortspruit River.

3.4.4.1. Determination of Electrical Conductivity (EC).

EC is the ability of current conduction and it can estimate the amount of total dissolved salts or ions in surface water (U.S.EPA, 2012). EC has a well-established dependency on temperature and so the collected data are to be standardized to 25°C (Mathebula, 2015). The Multiparameter (HANNA Instruments, Johannesburg, South Africa) was used to measure electrical conductivity. A calibration standard solution was used to calibrate the meter according to the manufactures instructions.

3.4.4.2. Determination of Total Dissolved Solids (TDS).

TDS is described as the quantity of dissolved or soluble materials in surface water (Prabhakaran *et al.,* 2014; WHO, 2017). Water from sewage, stormwater runoffs, mining, and industrial wastewater effluent causes high TDS in freshwater (WHO, 2017). The Multiparameter (HANNA instruments, Johannesburg, RSA) was used to measure total dissolved solids. A calibration standard solution was used to calibrate the meter according to the manufactures instructions.

3.4.4.3. Determination of Potential Hydrogen (pH).

The water's pH is a measure of its hydrogen ions concentrations (U.S. EPA, 2012). The pH of water measures the degree of acidity or alkalinity (basicity). The Multiparameter (HANNA instruments, Johannesburg, (RSA) was used to measure pH.

3.4.4.4. Determination of Chemical Oxygen Demand (COD).

According to Li *et al.* (2017) COD is determined to measure variables from organic and inorganic contaminants in surface water. When organic pollution is discharged into the environment, their biological oxidation during biodegradation reduces oxygen levels and can lead to the development of septic conditions (Wen *et al.,* 2017). COD determination was done using photometric methods. The photometric methods starts by digestion of 2 mL of samples for 2 hours in an oven for culture-tube digestion with exposure at 150 °C. After cooling, the cuvette is inserted into the spectrophotometer and measured. The cuvette used for COD (0 to 150 mg/L) and spectrophotometer are from HANNA instruments (Johannesburg, RSA).

3.4.4.5. Determination of Total Phosphates (PO_4).

Singh (2013) has discovered that measuring phosphate in water indicates fertility or nutrient enrichment in water. Total phosphates in the environment are available in three forms that are organic phosphorous, orthophosphate, and polyphosphates (U.S. EPA, 2012). The recommended value of phosphates in an aquatic environment according to DWS: aquatic guideline is 10 mg/L. The method for the determination of total phosphates in wastewater samples using cuvette tests is based on the reaction between phosphate ions and molybdate ions and subsequent reduction by ascorbic acid. Phosphates determination was done using reagents, spectrophotometer and cuvette tests (0.5 to 5.0 mg/L) are from HANNA instruments (Johannesburg).

3.4.4.6. Determination of Biological Oxygen Demand (BOD_5).

Effluents discharged by wastewater treatment also contain organic materials that are decomposed by microorganisms that use oxygen in the process. The amount of oxygen used by these organisms in breaking down the waste is known as the biochemical oxygen demand or BOD (U.S.EPA, 2012). BOD_5 was measured using the BOD Oxidirect BOD Meter (HANNA instruments, Johannesburg (RSA). The initial concentration was labelled D_0. The sample was closed and incubated in a dark chamber for five days. On the fifth day of incubation, dissolved oxygen (DO) recordings were taken again using the same equipment and labelled D_2. Then BOD_5 was calculated by applying the following equation:

BOD_5, (mg/L) = [(Initial DO_0 - Final DO_5) x 300] ÷ mL sample *(P)*

P is the decimal volumetric of a sample.

D_0 is the initial DO of the sample

D_5 is the final DO of the sample after five days.

3.4.5. Trace metal analysis (TMs)

Five heavy metals were assessed in the study: Iron (Fe), Mercury (Hg), Copper (Cu), Manganese (Mn), and Zinc (Zn). Before each parameter analysis, 25 ml of each sample was filtered using a 0.45 µm membrane to eliminate any fibres. To preserve the samples, concentrated nitric acid (HNO_3) was added to prevent aging and immediate precipitation of metals. A concentration range of standards solution (0.001ppm to 1ppm) was prepared and analysis was done by using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) (Agilent Technologies Incorporated, USA).

3.4.6. Microbiological analysis

3.4.6.1. Culture media.

Culture media used consisted of m-Endo, m-Fc, m-Nutrient agar for standard Membrane Filtration Method (MFT). The media used was prepared according to the manufacturer's instructions (Sigma Aldrich, South Africa).

3.4.6.2. Total coliforms determination.

The MFT was utilized in the analysis of total coliform per sample and the result was calculated using an ideal range of 20 - 80 colonies X 100 CFU/100ml. A 100ml dilution of samples was filtered through 47 mm filter papers with a porous size of 0.45 µm. Following filtration, the filters were aseptically transferred to a sterile petri dish plates containing the culture medium of m-Endo agar. The plates were incubated for 24 hours at 37°C. After the incubation, the colonies were counted using a colony counter and the colonies were then grouped according to their morphological characteristics observed on the medium. Then total coliforms were calculated using the formula: Colony Forming Units (CFU)/100 mL = No of colonies X 100 ÷ mL sample filtered (Prathama, 2016, Shen *et al.,* 2019).

3.4.6.3. Faecal Coliforms determination.

For faecal coliforms determination, the MFT was utilized to determine the quantity of total coliform and faecal coliform per sample, the result was calculated using an ideal range of 2060 colonies X 100 CFU/100ml. A 100ml dilution of samples was filtered through 47 mm filter papers with a porous size of 0.45 µm. Following filtration, the filters were aseptically transferred to a sterile petri dish plates containing M-FC agar. The plates were then placed into an incubator and incubated at 45°C for 24 hours. As the faecal coliform colonies grow they produce an acid (through fermenting lactose) that reacts with the aniline dye in the agar thus giving the colonies their blue colour. After incubation, plates were observed for faecal coliform counts on a colony counter and the colonies were then grouped according to their morphological characteristics observed on the medium. The calculation was as follows: Number of colonies per 100ml = no of colonies ÷ sample volume filtered in mL x 100 (CFU = colony forming units) (Luyt *et al.,* 2012; Prathama, 2016).

3.4.6.4. Heterotrophic Plate Count

For Heterotrophic Plate Count, the MFT was used in calculating the quantity of heterotrophic plate count. A successive dilution of 10^{-1} up to 10^{-6} was prepared for each water sample. Using the spread plate technique, 0.1 mL of water samples was aseptically pipetted into standard plates containing nutrient agar. The medium was prepared according to the manufacturer's instructions. The inverted plates were then placed inside a bag and sealed before putting them into an incubator at 37°C for 48 hours. The plastic bag has to be sealed tightly all the time so that the agar stays moist. After incubation, plates were then observed for bacteria colonies on a colony counter and recorded. The average colony count were determined by adding the count from each plate of the same dilution, then divide by the number of plates to give the results in CFU/ml (Prathama, 2016, Shen *et al.,* 2019).

3.4.6.5. *E. coli* determination

The MFT was used for the detection of *E. coli* from water samples. The EZ-Stream water filtration system (Millipore SAS, France) was sterilized with 70% ethanol and washed with sterile distilled water. Sample volumes of 100 ml were filtered through 47 mm filter papers with a porous size of 0.45 µm. After filtration, membranes were aseptically placed on the surface of on 5 mL plates of Eosin Methylene Blue (EMB) Agar, and the plates were then placed into an incubator at 35°C for 24 hours. Most importantly, *E. coli* appears pal straw coloured on EMB agar, it is shiny green with a metallic sheen (Mulamattathil *et al.,* 2015: Shen *et al.,* 2019).

3.4.7. Antibiotic susceptibility testing

For antibiotic-resistant bacteria, disc diffusion was used to test for antimicrobial susceptibility testing (Table 5) (CLSI, 2014). Pure colonies were inoculated into R2A agar (Lab M Ltd., UK) and incubated at 30°C for 24 hours. After incubation, 0.1 mL of the culture was spread on Mueller-Hinton agar (MHA) (Merck, RSA). The medium was prepared according to the manufacturer's instructions and have a level depth 31 mL in a 100 mm circular plate. A total number of twelve commercially prepared antibiotic disks (Mast Diagnostics, UK) were placed on MHA that has a bacterial culture. Antibiotic discs were infused with different concentration; kanamycin (Kan) 30 µg, streptomycin (S) 30 µg, chloramphenicol (Chl) 30 µg; (ampillicin (AMP) 10 µg, erythromycin (Ery) 15 µg, norfloxacin (Nfx) 10 µg, oxytetracycline (Oxy-Tet) 30 µg. MHA plates with antibiotic disks were incubated at 37°C for 24 hours. After incubation, inhibition zones were measured (in millimetres). The measured values were compared to Performance Standards for Antimicrobial Susceptibility Testing (2019) provided by the Clinical and Laboratory Standards Institute (CLSI). This was done to determine whether Faecal and *E. coli* bacteria were susceptible, intermediate or resistant to antimicrobials (Mutuku *et al.,* 2014; CLSI, 2019).

Table 5. Description of antibiotics standards and inhibition zone diameter used in the study.

Antibiotic class	**Antibiotic**	**Abbrev.**	**Conc.**	**R**	**I**	**S**
Aminoglycosides	kanamycin	KAN	30µg	≤13	14-17	≥17
	streptomycin	STR	30µg	≤11	12-14	≥15
Chloramphenicol	chloramphenicol	CHL	30µg	≤12	13-17	≥18
β-lactams	ampicillin	AMP	10µg	≤11	12-13	≥14
Macrolides	erythromycin	ERY	15µg	≤13	14-22	≤23
Quinolone	norfloxacin	NFX	10µg	≤16	12-16	≥17
Tetracycline	Oxytetracycline	OXY-TET	30µg	≤14	15-18	≥19

3.4.8. Multiple antibiotic resistance index

Multiple antibiotic resistance (MAR) is when a single bacterium is resistant to more than one antibiotic (Sandhu *et al.,* 2016). This can occur in two distinct ways, such as, a bacterium can have several different resistance genes, each providing resistance to a particular antibiotic and the other possibility is that a single resistance mechanism gives resistance to more than one antibiotic (.Sandhu *et al.,* 2016; Nyandjou *et al.,* 2019). The presence of plasmids that contain one or more resistance genes, with each encoding a single antibiotic resistance (AR) phenotype, often causes the development of MAR in bacteria (Osundiya *et al.,* 2013). These AR genes can transfer to other bacteria of the same or different species.

MAR index is an effective, valid, and cost-effective method that is used in source tracking of antibiotic resistant organisms (Sandhu *et al.,* 2016). MAR index is calculated as the ratio between the number of antibiotics that an isolate is resistant to and the total number of antibiotics the organism is exposed to. A MAR greater than 0.2 means that the high risk source of contamination is where antibiotics are frequently used (Rotchell *et al.,* 2016). MAR index values > 0.2 indicate the existence of isolates from contaminated sources with frequent use of antibiotics while values ≤ 0.2 show bacteria from source with less antibiotic treatment (Thenmozhi *et al.,* 2014; Nyandjou *et al.,* 2019).

3.5. Statistical analysis

The mean values of results and standard deviation were calculated using Microsoft Excel 2014 edition. Tables and graphs were used to present the result obtained from the sample analysis. The antibiotic resistant patterns of different microorganisms were also shown graphically using percentage resistance per site. Pearson's correlation (a measure of linear coefficient (r) association) and hierarchical cluster analysis was applied to detect a correlation between pH, EC, TDS, COD, BOD, nitrates, sulphates, phosphates, and microbiological organisms at five selected sites per month, from June – December 2019 was determined using the SPSS 21.0 for windows program after standardization of tilted datasets. Correlations were considered statistically significant at p values of <0.05.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1. Introduction

This chapter presents and discusses the results of the study. The results for the physical, chemical and microbiological test results are presented and compared to the DWS water quality guidelines (DWAF, 1996a; DWS, 2017). The guidelines provide clarity and understanding for an acceptable range for water quality parameters when discharging into the aquatic environment (DWS, 2017). Results demonstrated some spatial and temporal variations throughout the study period. Statistical analysis on physicochemical and microbiological data was conducted using the t-test and Pearson's correlation to determine correlations amongst selected sites of the Naauwpoortspruit River.

4.2. Water quality trends along Naauwpoortspruit River

4.2.1. Physicochemical parameters

The results for the monthly assessment of physicochemical parameters are presented in Figures 2 to15.

4.2.1.1. pH

The determined pH values from all selected sites ranged from 4.45 – 7.9 and are presented in Figure 2. The mean pH of Site A was 6.52 ± 0.17 with a range of 4.45 to 7.33 over the study period. The lowest pH value of 4.45 was recorded in June 2019, which was below the acceptable DWS Aquatic Ecosystem limit, pH of 5.5 – 11 (DWAF, 1996a; DWS, 2017). Site A is located at short distance from mining and industrial activities in the upstream of the river and is prone to mining and manufacturing operations such as coal mining and steel industries. The industries release pollutants that contain metal ions, localised points of acidification, and other residual pollutants that contact water through AMD (Dabrowski and Klerk, 2013; Oberholster *et al.,* 2017). AMD is known to be a by-product of mining impacts on the environment, which negatively impacts the aquatic ecosystem and underground water reservoirs (Muruven, 2011; Neingo *et al.,* 2016; Retief *et al.,* 2020). The acidic water pH that was detected at Site A, therefore, reflected the impact of the surrounding mining and industrial activities on the Naauwpoortspruit (Mey and Van Niekerk, 2009; Dabrowski and Klerk, 2013). Once-off sampling study was conducted in Olifants river and its tributary which illustrated that abandoned mining areas are clearly the most important source of metals in the upper Olifants system (Dabrowski and Klerk, 2013; DWS, 2016). The study further highlighted that abandoned mining sites are concentrated within the Klipspruit, Kromdraaispruit, Saalboomspruit and Naauwpoortspruit catchment. According to Bell *et al.* (2001) and Muruven (2011) acid mine drainage is generally characterised by low pH water and high salt and metal concentrations.

Site B had a low pH of 5.01 detected in June and the highest pH level of 7.45 in August 2019. Site B location in an area that is associated with agriculture and industrial activities that can release nutrients and toxic organic chemicals associated with herbicides and pesticide pollutants leading to low pH levels in June. Runoff and wastewater from agriculture and industrial activities can also contain high sulphate and other heavy metals levels that through the reactions in the environments can cause lowering of the water pH thus rendering it unfit for domestic, irrigation, and livestock watering purposes (Sibanda *et al.,* 2015; DWS, 2017). Site C had a low pH level of 6.45 in June 2019 and the highest was recorded at 7.86 in November 2019. Site C is the starting point, downstream of Naauwpoortspruit associated with residential and car wash activities and wastewater runoff. Site D recorded a low pH level of 6.34 in July 2019 and a high level of 7.9 in December 2019 and Site E low level of 6.56 in July 2019 and a high level in 7.86 September 2019, respectively. Site D is located next to a WWTP and Site E further down is associated with fisheries and livestock farming. Based on the measured pH values from Site D and E, pH was within DWS Aquatic Ecosystem guidelines (pH between 5.5 and 11) (DWAF, 1996a; DWS, 2017).

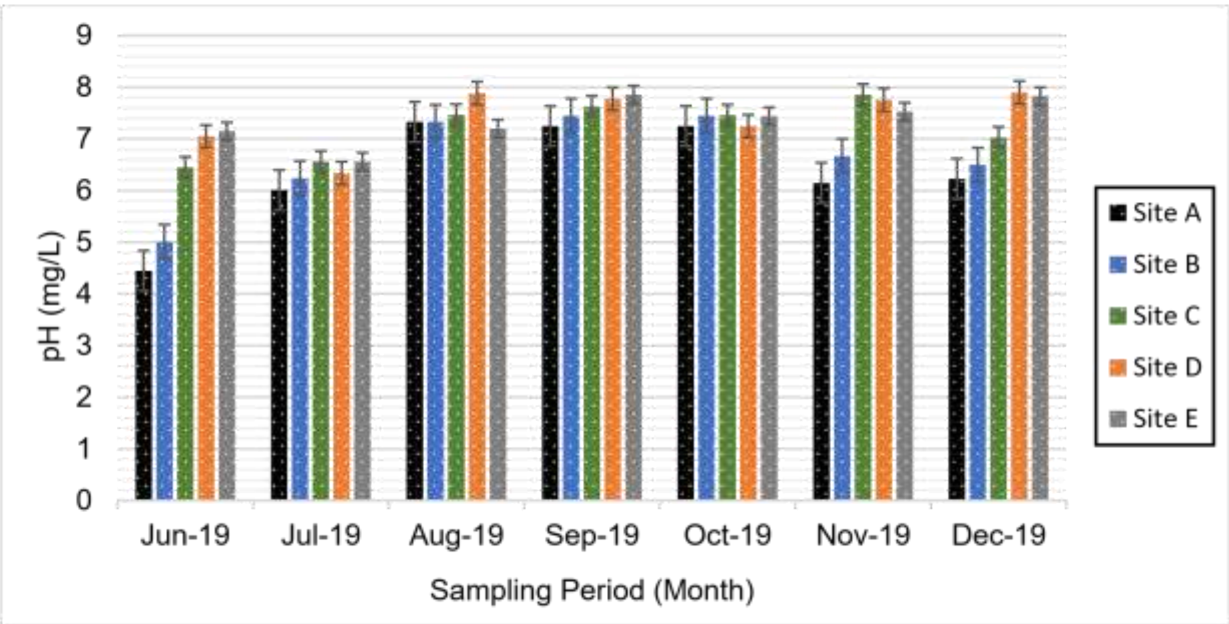

Figure 2. pH concentration during the study period (June – December 2019) for the Naauwpoortspruit River.

The pH of the surface water can be a good measure of the consistency of the water since it specifies the acidity and basic properties of the water (U.S.EPA, 2012). Anthropogenic activities which can influence a surface water pH are WWTPs and mining activities with discharge effluents containing high organic, nutrient, and microbiological loads to the river systems. The high nutrient concentrations lead to eutrophic conditions in the river systems

(Bester, 2015; De Klerk, 2016; Walters *et al.,* 2017). Hamid *et al.* (2017) has attributed that the variation of surface water pH values to soil characteristics or geology, inputs from the land use, and hydrogen ions from runoff. Low pH may adversely affect fish and other ecosystem based aquatic species (Verlicchi *et al.,* 2020).

4.2.1.2. Electrical Conductivity and Total Dissolved Solids

Figures 3 and 4 show EC and TDS data from the five selected sites of Naauwpoortspruit River. Both variables are considered similar hence they have been interpreted simultaneously. The EC and TDS concentrations for the five different sites in the study area ranged between 58.63 – 101.3 mS/m and 381.1 – 736.45 mg/L, respectively. These concentrations were within the limit suggested by South African guidelines. For instance, for drinking water, the guidelines suggest a range of 0 - 1200 mg/L for TDS (SANS, 2015) and 0 - 1000 mg/L to protect Livestock & Watering and from 200 to 1100 mg/L to protect Aquatic Ecosystem (Golder Associated, 2011; DWAF,1996a; 1996c).

Site A mean TDS concentration was 460.94 mg/L ± 70.46 with maximum concentration of 568.1 mg/L in December. Naturally, TDS are influenced by soil and geological characteristics of the catchment (Adelana et al. 2010; Dougall, 2007; Chapman, 1996). In the present study, maximum TDS concentration are attributed to coal mining activities that characterize the upper Naawupoortspruit river (DWS, 2018). The coalfields of eMalahleni consist bands of coal within the sedimentary layers, Ecca Group of the Karoo Supergroup (van Vuuren, 2013). These coal sedimentary rocks is composed mostly of carbon and hydrocarbons, which contain energy that can be released through combustion (burning) (Ayanda *et al.,* 2012; Maya *et al.,* 2015;). Studies conducted by McCarthy and Precious (2009) and Maya *et al.,* (2015) have found out that during mining and mineral extraction, the rock mass is extensively fragmented, thereby dramatically increasing the surface area and consequently the rate of acid production. Certain host rocks, particularly those containing large amounts of calcite or dolomite, are able to neutralise the acid but during coal and gold deposits natural neutralising processes are overwhelmed and large quantities of acidic water are released into the environment by mining activities (Gonah, 2014; Schutte, 2018).

The TDS is likely to increase in water as water moves downstream because salts are continuously being added through natural and manmade processes whilst very little is removed or diluted by precipitation or natural processes (Gonah, 2014; DWS, 2018). Domestic and industrial effluent discharges and surface runoff from urban, industrial and cultivated areas are examples of the types of return flows that contribute to increased TDS concentrations (Mwangi, 2014; De Klerk, 2016; Walters *et al.,* 2017). Agriculture releases nutrients and toxic organic chemicals associated with herbicides and pesticides while mines release coal residuals into the water and mine dust in the atmosphere which can be later deposited into surface water after a rainfall (DWS, 2016; Edokpayi *et al.,* 2016). This can be observed with elevated TDS concentration of 736.45 mg/L measured during December in Site E. The adverse impacts of elevated TDS levels are therefore mostly related to aesthetic (such as taste) impacts and economic impacts due to crop damage where elevated TDS concentrations may cause leaf burn, and decreased crop yields, caused by soil salinisation. Humans can tolerate considerable high levels of TDS (1 000 mg/L) but a TDS concentration of 120 mg/l can affect the macroinvertebrate's eggs and larvae, which result in mortality, community structure, and nutrient cycling (DWAF, 1996a; Mwangi, 2014; De Klerk, 2016). High TDS may also cause osmotic stress and affect the osmoregulatory ability of aquatic fauna (Gonah, 2014; Edokpayi *et al.,* 2017).

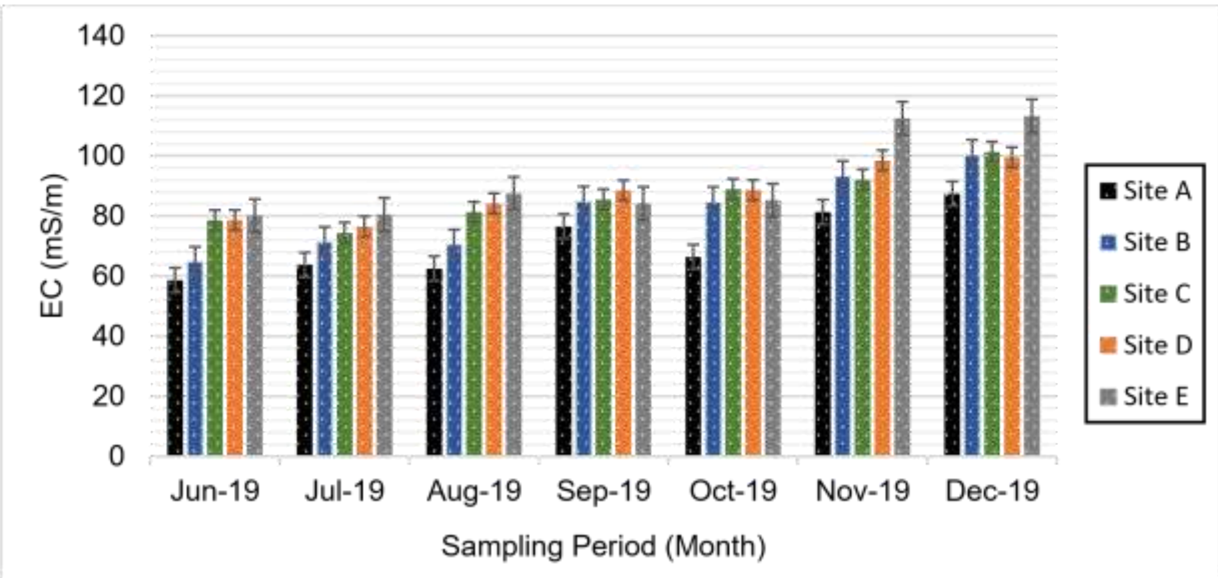

Figure 3. EC concentrations determined during the study period (June – December 2019) for the Naauwpoortspruit River.

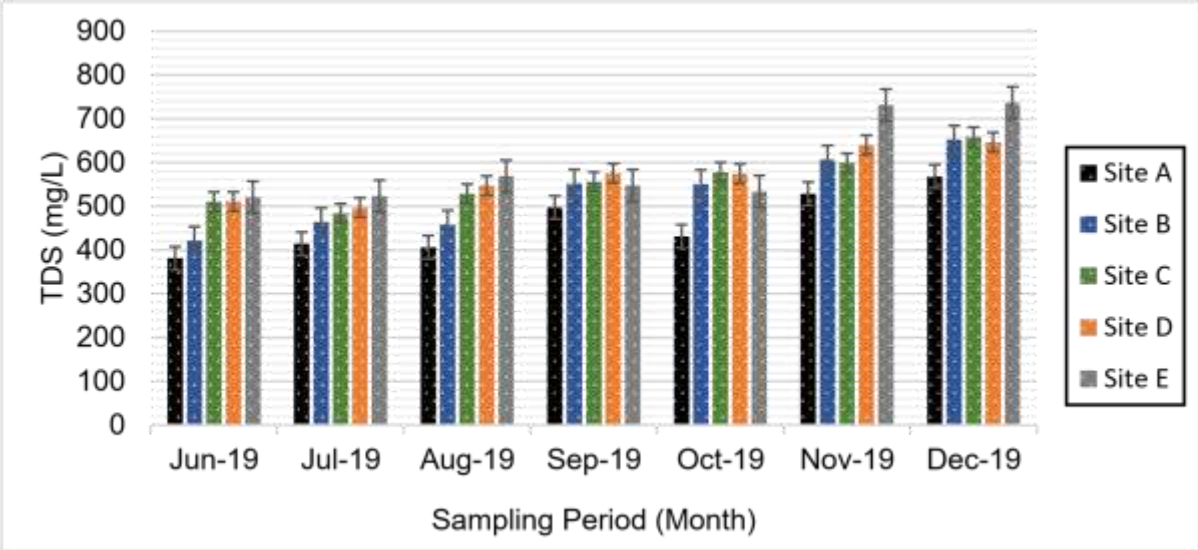

Figure 4. TDS concentrations during the study period (June – December 2019) for the Naauwpoortspruit River.

4.2.1.3. Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD)

Figure 5 shows the COD concentrations results from all five sampling sites ranging from 29 – 250.3 mg/L. A maximum COD concentration of 250.3 mg/L was observed at Site C (located downstream) during September and may be attributed to WWTPs effluent, stormwater runoff, sewerage leakages, and car wash effluent. According to DWS Aquatic Ecosystem guideline, the amount of suspended material in the water affects the dissolved oxygen saturation concentration, either chemically, by the oxygen-scavenging effects of the suspended particles, or physically, by reducing the volume of water available for the solution (DWAF, 1996a; DWS, 2017). WWTPs effluent, stormwater runoff, sewerage leakages, and car wash effluent have the effect of contributing to both physical and chemical effects to the river under study. The South African water quality guidelines do not specify the COD concentrations for domestic, recreational, aquatic ecosystems and agricultural purposes. The COD guidelines available are for industrial purpose with the values range between 0 - 10 mg/L and wastewater limit ranging between 0 – 75 mg/l (DWAF, 1996c;DWS, 2017).

Site A measured a mean COD concentration of 71.88 mg/L ± 41.58 throughout the study, with a maximum concentration of 133.4 mg/L in September 2019. Elevated COD can be attributed to high phosphate concentrations, decaying plant matter, stormwater, and surface runoff from the residential area. Oun *et al.* (2014) and Olujimi *et al.* (2015) stated that decay in oxygen in slow-flowing rivers is attributed to the action of rainfall and stormwater runoff in removing vegetation or to high decay of organic matter followed by an increasing water temperature. The discharge of WWTPs and car wash effluent in receiving surface water further decreases dissolved oxygen concentrations due to increased microbial activities occurring during the degradation of organic matter (Hobbie *et al.,* 2017; Oberholster *et al.,* 2017; Pollard *et al.,* 2017). High BOD and COD deplete oxygen because microorganisms are using up the dissolved oxygen in aquatic ecosystems (Oun *et al.,* 2014).

COD concentration showed temporal fluctuations within different sites of the Naauwpoortspruit River in September where site A was 133.4 mg/L, Site C was 250.3 mg/L. This fluctuation may be attributed to dry and hot periods, where water movement is slow causing limited mixing of water and air which leads to a decline in dissolved oxygen concentration at Site A. Site C COD concentrations increased due to effluent from the residential area and car wash activities, as also demonstrated in other studies (Ngang and Agbazue, 2016; Donoso *et al.,* 2017; Zaghloul *et al.,* 2019).

A maximum BOD concentration of 410.5 mg/L was reported at Site D in December 2019 and was not compliant with the industrial guideline (10 mg/L) and wastewater limit (75 mg/L) (DWAF, 1996a; DWS, 2017). The maximum BOD at Site D may be attributed to WWTP discharge which releases high levels of impurities that increase ozone-depleting substances in surface water (Britz *et al.,* 2012; Pour *et al.,* 2014; De Klerk, 2016). According to DWS (2017), the depletion of dissolved oxygen in conjunction with the presence of toxic substances can also lead to a compounded stress response in aquatic organisms. Elevated toxicity will then be detected for zinc, copper, cyanide, sulphide, and ammonia. Oxygen saturated conditions can also tend to suppress photosynthesis of green algae, benefit toxic blue-green algae, which are more tolerant of polluted water and may pose a danger to other water users (DWS, 2017; Li *et al.,* 2017; Gosch *et al.,* 2019). As a result, the maximum levels of BOD in the Naauwpoortspruit River can negatively influence biodiversity within the surface water and downstream users of water (Zaghloul *et al.,* 2019). Sources of BOD in the environment includes leaves and woody debris, dead plants and animals, animal manure, effluents from pulp and paper mills, wastewater treatment plants, feedlots, and food-processing plants; failing septic systems; and urban stormwater runoff (US EPA, 2012; Self *et al.,* 2013).

The minimum concentration of BOD at Site A (105.5 mg/L) and Site B (109 mg/L) in September 2019, could be attributed occasional precipitation which then the river water will experience dilution so it can lower the value of BOD (Pour *et al.,* 2014; Susilowati *et al.,* 2018). In an aquatic ecosystem, oxygen is a basic element that influences flora and fauna composition in the surface water. The low content of dissolved oxygen indicates the low quality and freshness of the water due to lack of oxygen in the water and will have an impact on aquatic life (Dallas, 2004; Gosch *et al.,* 2019). The amount of oxygen in the water depends also on the activity of photosynthesis of organisms in the water. On the surface of the water, oxygen levels will be higher, due to the process of diffusion between water with free air and the process of photosynthesis (US EPA, 2012; Griffins *et al.,* 2014; Zaghloul *et al.,* 2019). With increasing depth, there will be a decrease in dissolved oxygen levels due to the decreasing process of photosynthesis and oxygen levels that are widely used for respiration and oxidation of organic and inorganic materials (Dallas , 2004; Griffins *et al.,* 2014; Chatanga *et al.,* 2019). The oxygen requirements of benthic macroinvertebrates vary per species, life stages, and different life processes and sizes (Malherbe *et al.,* 2011; Ngwenyama *et al.,* 2017). Many species of mayfly nymphs, caddisfly larvae, and stonefly larvae are not very tolerant of pollution and can only survive in swift, cool, well-oxygenated water (Self *et al.,* 2013; Pour *et al.,* 2014; De Klerk, 2016).

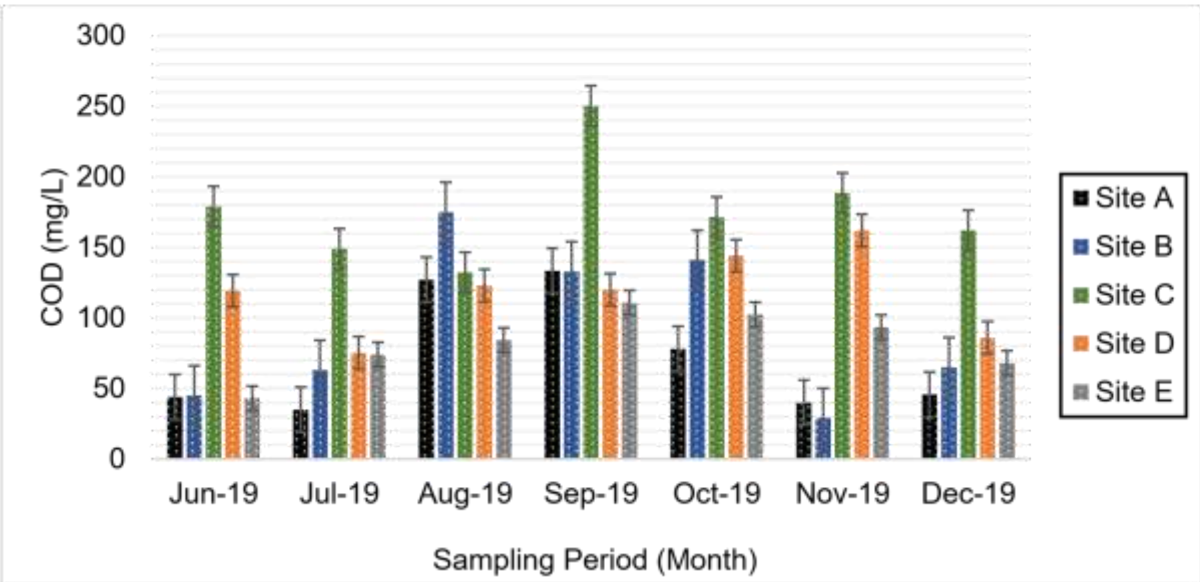

Figure 5. Shows COD concentrations during the study period (June – December 2019) for the Naauwpoortspruit River.

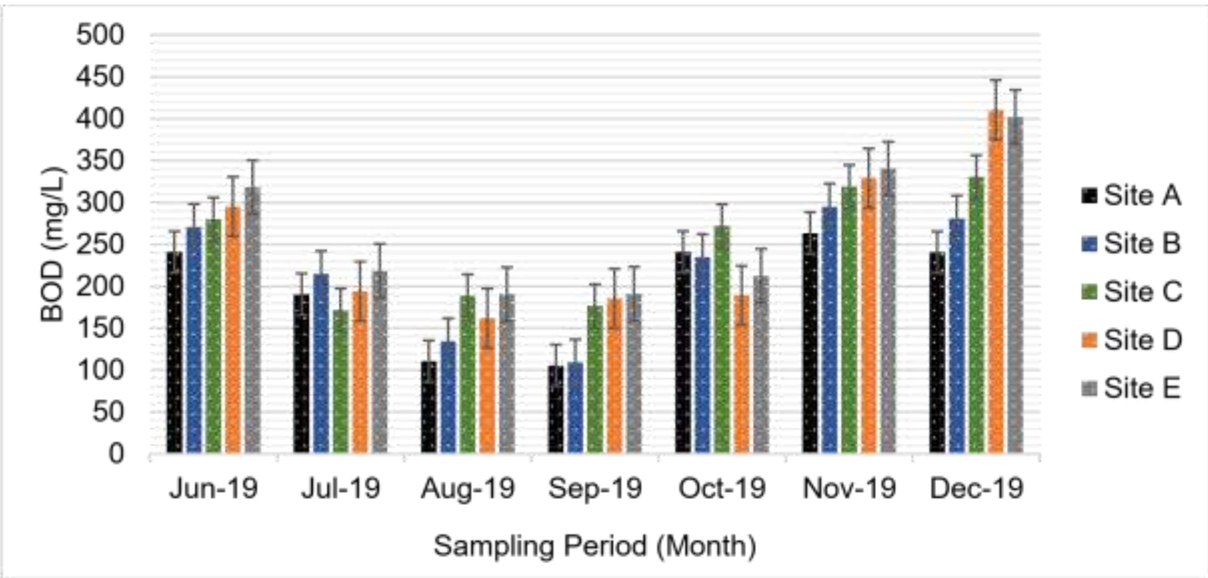

Figure 6. BOD concentrations were measured during the study period (June – December 2019) for the Naauwpoortspruit River.

4.2.1.4. Nitrates

The high concentration of nitrates and nitrite ions in surface water impact the environment negatively, with observed impacts of blooms of algae and eutrophication in the rivers and dams (Zhou, 2015; Singh, 2016; Xue *et al.,* 2016). In South Africa, natural surface water total nitrogen concentrations are less than 0.5 mg/L and may range between 5 – 10 mg/L when water is highly impacted (DWAF, 1996a; DWS, 2017). To protect human health, South Africa guidelines suggest a range of 0 – 6 mg/L as nitrate as nitrogen concentrations without adverse health effects, and from 0 – 100 mg/L with no adverse effects for livestock watering (DWAF, 1996b and 1996c). To protect aquatic ecosystems the South Africa guidelines propose the following ranges of nitrate concentrations: <0.5 mg/L as oligotrophic conditions and from 0.5 – 2.5 mg/L as mesotrophic conditions (DWAF, 1996a; 1996b, 1996c).

In the current study, nitrate concentrations across the selected sampling sites ranged from 3.39 – 8.9 mg/L (Figure 7). At downstream sites, Site D mean nitrates concentration was 6.12 mg/L ± 1.62, with a maximum of 8.3 mg/L and Site E mean concentration was 6.69 mg/L ± 1 .56, with a maximum of 8.9 mg/L) in November respectively. These elevated concentration are attributable to WWTP discharge and agricultural activities which can contribute to inorganic and organic compounds in surface water. Dabrowski and De Klerk (2013) found that the unacceptable category of water quality in the Naauwpoortspruit River was due to WWTPs, leakage of sewerage, land clearing, and feedlots. Various studies have found that high nitrate amounts are attributed to urban runoff, wastewater treatment, and industrial effluents into rivers (Zhang *et al.,* 2014; Mathebula, 2015; Wen *et al.,* 2017; Retief *et al.,* 2020). The study by

Mothetha (2016) further restated that wastewater treatment effluent contributes to high nitrates pollution that can be observed from downstream of the river. Rainfall runoff, chemical fertilizers in agricultural activities, sewage, and landfill by domestic waste are sources of nitrate in surface water which leads to eutrophication and salinity (Haller *et al.,* 2014; Mothetha, 2016).

Site A had a mean nitrate concentration of 5.14 mg/L ± 1.65, with a maximum of 7.2 in December and a minimum nitrate concentration of 3.39 mg/L in August 2019. The low nitrate concentration in the surface water can be attributed to less organic pollutants such as related to the use of fertilizers during August (Self *et al.,* 2013). According to Hobbie *et al.* (2017) the growth of macrophytes and algae, through increased evapotranspiration rates, can consume nitrates and leads to denitrification. Other studies showed that in winter high nitrate concentration may be attributed to higher biological performance and higher rates of evapotranspiration (Self *et al.,* 2013; WHO, 2017; Collivignarelli *et al.,* 2018). In contrast, Britz *et al.* (2012) revealed that nitrate levels are low during the summer, even though fertilizer is applied, because growing plants utilize nitrogen, and high rates of evaporation and transpiration are often observed.

Site C had a maximum concentration of 7.7 mg/L in December 2019 and a minimum concentration of 4.2 mg/L in September 2019. A maximum nitrates concentration of 8.3 mg/L was recorded at Site D in December 2019 while a minimum concentration of 4.15 mg/L was recorded in September 2019. Site E recorded a maximum nitrate concentration of 8.9 mg/L in November 2019 and a minimum concentration of 5.3 mg/L in September 2019. The nitrates concentration in all selected sampling sites of Naauwpoortspruit River was within acceptable DWS aquatic ecosystem of 5 – 10 mg/L when water is highly impacted during the study period (DWAF, 1996a; DWS, 2017).

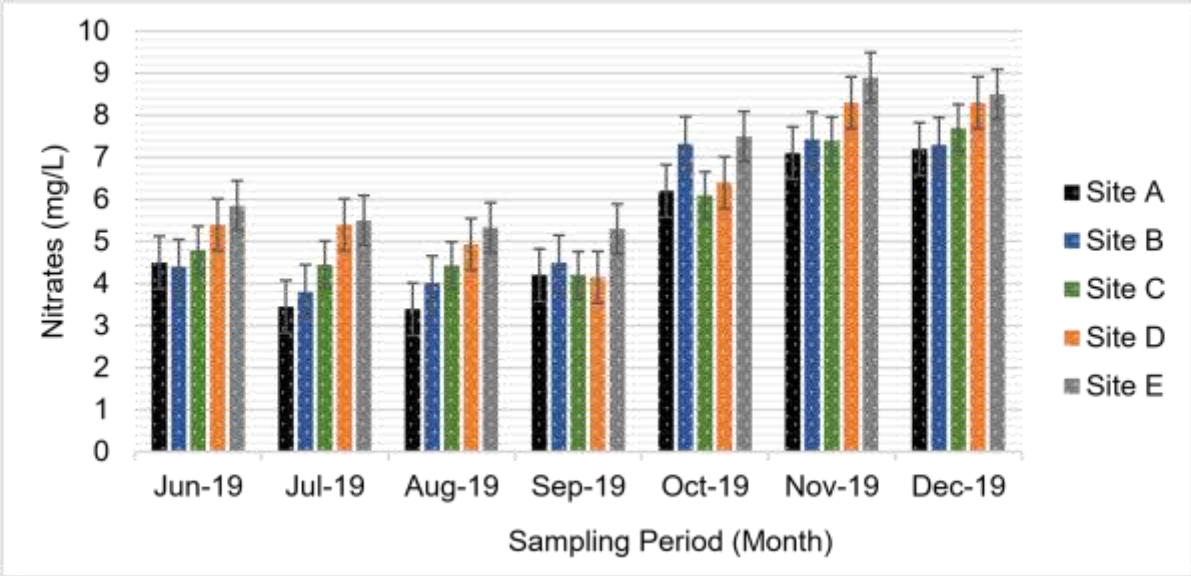

Figure 7. Nitrate concentrations during the study period (June – December 2019) for the Naauwpoortspruit River.

4.2.1.5. Sulphate

The total sulphates ranged from 73 – 124.3 mg/L (Figure 8). Comparing to DWS aquatic guidelines, sulphate concentration from selected sampling sites of Naauwpoortspruit River was within the acceptable limit of 200 mg/L (DWAF, 1996a; DWS, 2017). Sulphates are found in almost all-natural water, where the raised concentration can originate from natural sources such as mining activities and landfill leachates (Mwangi, 2014; Mathebula, 2015; Gonah, 2016). In the present study, maximum sulphates concentrations can be attributed to coal mining tailings and salinity from industrial activities finding their way into surface water (Dabrowski and Klerk, 2013; Mwangi, 2014; Verlicchi *et al.,* 2020). According to Li *et al.* (2014), water salinity refers to the amount of the dissolved salts in the surface water. These dissolved salts include sodium chloride, magnesium sulphates, potassium nitrates, and sodium bicarbonate may arise from industrial discharge and mining salts. Mining pollution may affect surface water through rainfall, rock weathering, and irrigations from high salts water (Dabrowski and Klerk, 2013; Khatri *et al.,* 2015). Site A mean total sulphate concentration was 112.72 mg/L ± 6.47, with a maximum concentration of 124 mg/L in December 2019 and a minimum concentration of 104.5 mg/L in September 2019. Site A is located upstream of Naauwpoortspruit River, with the area comprised of mining and industrial activities. Exposure to high sulphate levels from upstream can contribute to increased sulphur fluxes and high concentrations in receiving downstream (Gosch *et al.,* 2019; Agoro *et al.,* 2020; Verlicchi *et al.,* 2020). Moreover, levels of total sulphate in surface water can be the result of prolonged atmospheric deposition from industries and can be deposited into the water through precipitation and oxidation of pyrite deposits into surface water (Olujimi *et al.,* 2015; Singh, 2016; Edokpayi *et al.,* 2017).

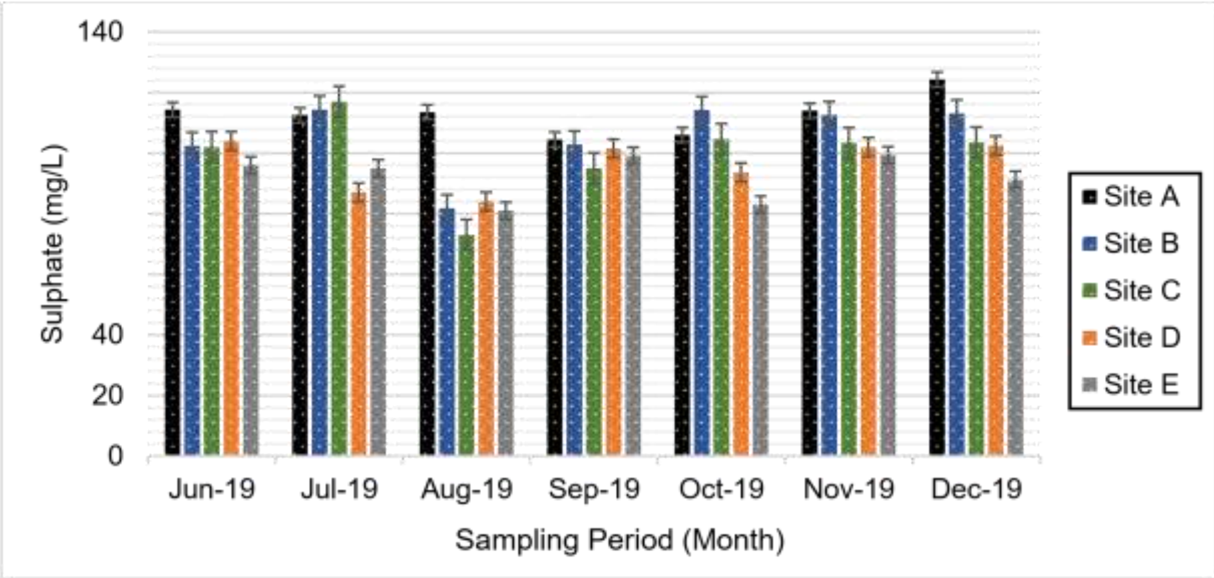

Figure 8. Sulphate concentrations during the study period (June – December 2019) for the Naauwpoortspruit River.

Site B mean concentration was 105.89 mg/L ± 11.83 with a maximum total sulphates concentration of 114.4 mg/L in October 2019. Sulphate from Site B is attributed to runoff from the natural occurring and anthropogenic activities such urbanisation, industrial and agriculture activities with the catchment. A concentration of 500 - 750 mg/L cause a temporary laxative effect on humans from drinking water (SANS, 2015), however, for industrial use such as sugar production and concrete manufacturing sulphate must be reduced below 20 mg/L (DWS, 2017; Ebenebe *et al.,* 2017; Retief *et al.,* 2019). In Upper Olifant's River which includes Naauwpoortspruit River, mining residuals containing sulphates were discovered to be a source of pollution after runoff eroded mining landfills sites (Oberholster *et al.,* 2013). Site D measured a mean concentration of 96.38 mg/L ± 8.18 with a maximum concentration of 104 mg/L in June and a minimum concentration of 84 mg/L in August 2019. Site D is adjacent to Naauwpoort WWTP and sulphate level may be attributed to wastewater effluent into surface water. The main problems related to the presence of high sulphate concentrations in wastewater can be the influent into the anaerobic reactors where there is competition between sulphate reducing bacteria (SRB) and methane producing archaea (MPA) for the same substrates (H2,acetate), sensitivity of MPA to sulphide, leading to methanogenesis inhibition when the sulphide concentration surpasses certain limits and precipitation of trace metals, causing nutritional deficiencies in the reactor. The production of sulphide during anaerobic treatment of sulphate containing wastewaters can reduce the efficiency of anaerobic treatment leading to release effluent containing methanogens and can precipitate nutrients essential to methanogens (Chelliapan and Sallis, 2015; Agoro *et al.,* 2020). Site E recorded a mean sulphate concentration was 92.08 mg/L ± 7.43 with a maximum concentration of 99.3 mg/L in November 2019. This concentration was complaint to drinking water limit of 500 – 700 mg/L. Site E located at the downstream of Naauwpoortspruit River and receives dilution of sulphate concentrate after precipitation, and runoff from upstream. Therefore, elevated concentration of sulphate can be harmful humans and to salmon fish species, egg developments and toxic to human health (DWAF, 1996a; Edokpayi *et al.,* 2017; Chatanga *et al.,* 2019).

4.2.1.6. Phosphate

In natural and treated water, phosphorus occurs roughly as sole dissolved orthophosphate (Griffins, 2014; Musyoki *et al.,* 2016; Singh, 2016). Orthophosphate is the most thermodynamically balanced form of phosphate and is the form commonly identified in laboratory analysis. South African water guidelines recommends that orthophosphate levels of less than 0.005 mg/L to oligotrophic conditions to protect aquatic ecosystems; 0.005 – 0.025 mg/L is mesotrophic, and concentrations of 0.025 to 0.250 mg/L are eutrophic and <0.250 mg-L are hypertrophic (DWAF, 1996a; Naidoo, 2013, De Klerk, 2016; DWS, 2017). In the current study, the highest phosphates concentration of 2.2 mg/L at Site D (downstream) in December exceeded the Aquatic Ecosystem guideline of 0.005 mg/L.

The maximum level of phosphates were measured during the rainy periods at sites located in regions of intensive agricultural activities. This sites are, Site B (1.4 mg/L), Site D (2.2 mg/L) and Site E (2.1 mg/L) along the Naauwpoortspruit River (Figure 9). This could indicate that there was possible chemical pollution due to farmers applying fertilizers and insecticides during this period, leading to an increase of these nutrients in the surface water and soil (Mwangi, 2014; DWS, 2016; Retief *et al.,* 2020). Several fertilizers used for modern farming contain plant nutrients that increase crop yield, especially nitrogen and phosphorus (Haarhoff *et al.,* 2020). However, these nutrients can lead to eutrophication, structural modification, and the functioning of biotic communities at high concentrations in the water system (Oberholster *et al.,* 2013; Oun *et al.,* 2014). High nutrient content has an impact on the surface as it contributes to eutrophication and the growth of algae in surface water (FAO, 2017). Nutrient enrichment can influence the growth of cyanobacteria (blue-green algae). The proliferation of algae can reduce water flow, thus decreasing oxygen levels and poor light penetration (Lee *et al.,* 2019). In accordance with Dabrowski *et al.* (2013), in South Africa, there are substantial eutrophication problems, especially in rivers and dams. Therefore, where high phosphorus levels from agricultural activities and discharge of raw sewage into surface water, surface water may end up being eutrophic and may result in the breeding of aquatic organisms that interferes with symbiosis and the depletion of biodiversity (Mann *et al.,* 2011; Langner *et al.,* 2019).

The phosphates at Site A measured a maximum concentration of 1.4 mg/L in December 2019, which surpassed the orthophosphate levels of 0.005 mg/L acceptable DWS aquatic ecosystem set limit (DWAF, 1996a, DWS, 2017). Site B recorded a maximum concentration of 1.4 mg/L in November 2019 and a minimum concentration of 0.2 mg/L in June 2019. Maximum phosphate concentration of 1.6 mg/L was detected at Site C in December 2019 and a minimum concentration of 0.4 mg/L in June 2019. The phosphate of Site D showed an elevated concentration of 2.2 mg/L in December 2019 and a minimum concentration of 0.6 mg/L in August 2019. Site E had recorded a maximum phosphate concentration of 2.1 mg/L in December 2019 and a minimum concentration of 0.47 mg/L in September 2019. In a study of Upper Kuils River in South Africa, the mean level of phosphates of 0.28 mg/L – 5.27 mg/L was higher than the current study, with a mean of 0.12 to 2.2 mg/L and much higher than DWS: aquatic guidelines limit of 0.25 to 250 mg/L (Mwangi, 2014; DWS, 2015; Retief *et al.,* 2020).

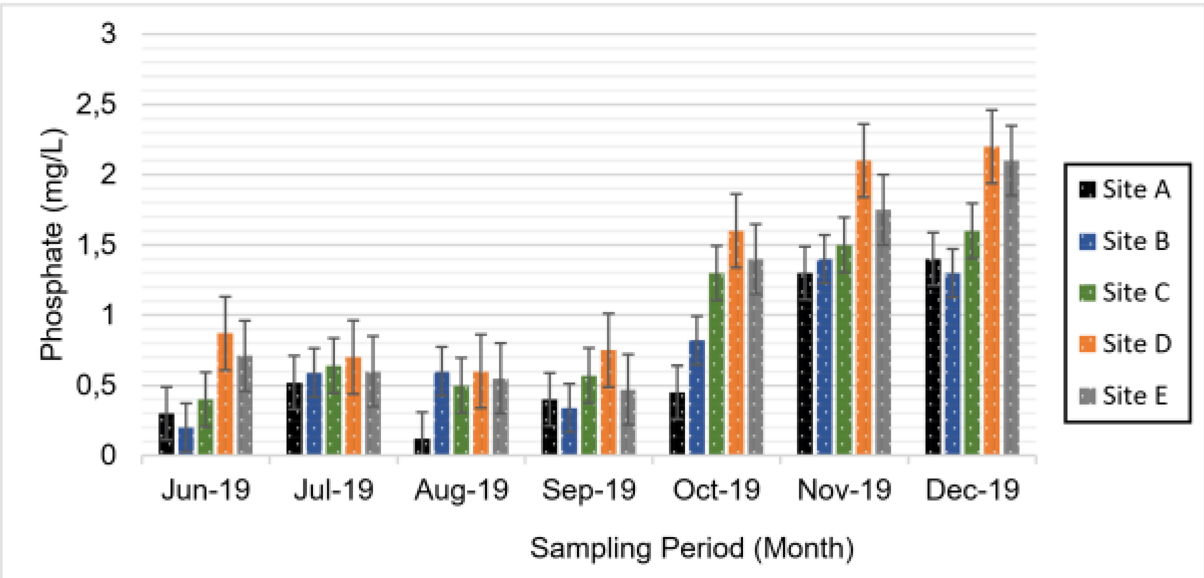

Figure 9. Phosphate concentration during the study period (June – December 2019) for the Naauwpoortspruit River.

4.2.1.7. Ammonia (NH_3-N)

Ammonia concentration from all selected sites ranged from 6.4 - 33.4 mg/L with a maximum concentration of 33.4 mg/L detected at Site A (upstream) in June 2019 (Figure 10). This concentration is non-compliant with the drinking water of 1.5 mg/L (SANS, 2015) and DWS Aquaculture of 0.025 mg/L making it less fit to for drinking and aquaculture (DWAF, 1996d; DWS, 2017). Site A recorded a mean ammonia concentration of 22.41 gm/L ± 6.86 with a maximum concentration of 34 mg/L in June 2019. The elevated ammonia concentration at Site A is attributed to industrial effluent from which ammonia is released as a by-product from the destructive distillation of coal in the manufacture of metallurgical coke and coal-gas (US EPA, 2012; Du *et al.,* 2017). Ammonia can also be from mining leachate especially prevalent in anoxic sediments because nitrification (the oxidation of ammonia to nitrite (NO_2^-) and nitrate (NO_3^-) is inhibited (Mulamattahil, 2015; Mujuru *et al.,* 2016). The aquatic ecosystems guideline recommends ammonium concentration of less than 0.007 mg/L to preserve aquatic habitats (DWAF, 1996a). The sources of ammonium in the environment are diverse , ranging from industrial, agriculture, and metabolic process and their detection in water indicates sewerage and animal feeding pollution (WHO, 2011).

In September 2019, Site A and Site B measured higher concentration of ammonia, 25.3 mg/L and 23.3 mg/L than recommended DWS Aquatic Ecosystem limit of 0.007 mg/L (DWAF, 1996a), respectively. The interchange of higher concentration may be attributed by mining and industrial discharge into surface water as both sites found in mining and industries area, upper stream of Naauwpoortspruit river (Frieden, 2015; Elbossaty, 2017). Poorly treated wastewater effluent and agriculture fertilizers contamination can lead to salinity and nutrients causing eutrophication and algae downstream (Dabrowski and Klerk, 2013; Griffin, 2014). Site D and Site E are found downstream of Naauwpoortspruit River, with the former receiving point of discharge of WWTP effluent from residential and industrial areas and agriculture activities. According to Sibanda *et al.* (2015) and Mothetha (2016) discharge of partially treated wastewater treatment effluents and sewerage leakages can also contribute to loads of faecal contamination and allow favourable conditions for other microbial organisms to grow. Agoro *et al*. (2020) and Holcomb *et al.* (2020) have reported that WWTPs and sewerage contamination in water supplies has risen at an unprecedented pace worldwide. Besides, Oberholster *et al.* (2017) indicated that there could be significant correlations between sewerage leakages, WWTPs effluent from rainfall-runoff. Consequently, this allows microorganisms from WWTPs, sewerage leakages to easily be transferred from Site A (upstream) to Site E (downstream). Excessive algal growth from nutrient enrichment results in the utilisation of available dissolved CO_2, which reduces the carbonic acid content of the water, thus resulting in anoxic and hypoxic environments (Self *et al.,* 2013; Oberholster *et al.,* 2017). Dabrowski et al. (2013) and Elbossaty (2017) have indicated that floating algal blooms are one of the most visible features of eutrophication.

The trend of sites shows a variation of ammonia concentration within the Naauwpoortspruit River, with Site C recording a mean of 14.3 mg/L ± 2.16 and a maximum ammonia concentration of 16.7 mg/L was recorded in September 2019 and a minimum concentration of 10.5 mg/L was observed in November 2019. In Site D, the mean ammonia concentration was 13.17 mg/L ± 3.19 with a maximum of 18.4 mg/L in September 2019 and a minimum concentration of 8.4 mg/L in November 2019. Site E reached a mean of 9.72 mg/L ± 2.37 with maximum concentration of 13.4 mg/L in June 2019 and a minimum concentration of 6.4 mg/L in November 2019. The visual trend was documented during the research, particularly downstream, for animals drinking and getting closer to the river. According to U.S. EPA (2012) and Griffin (2014) drinking highly contaminated surface water with ammonia concentration may have effects on the respiratory systems of animals and health risks to humans.

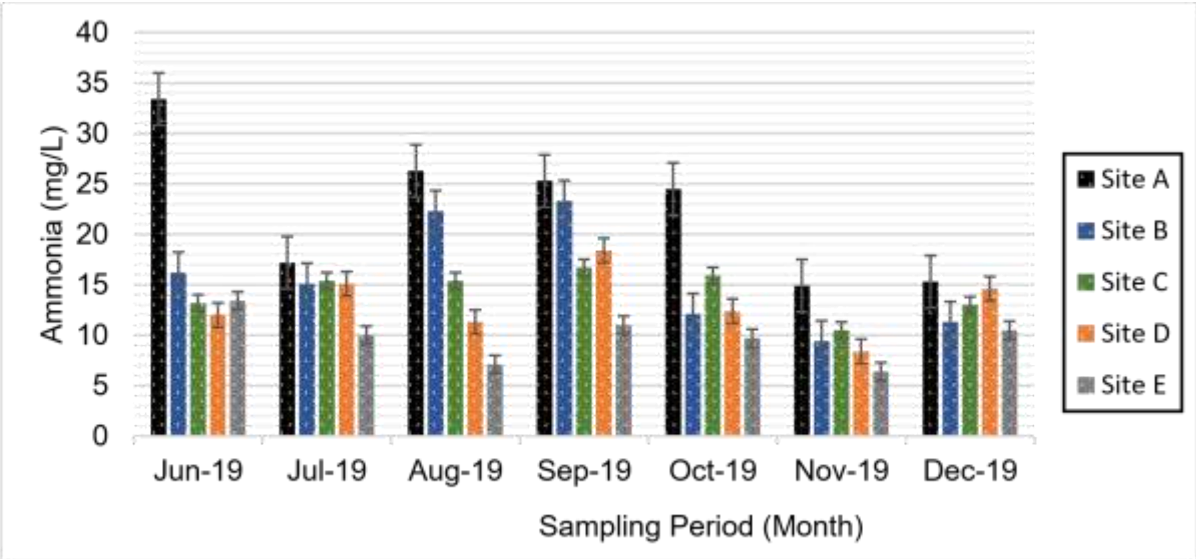

Figure 10. Ammonia concentrations during the study period (June – December 2019) for the Naauwpoortspruit River.

4.2.1.8. Mercury (Hg)

The seasonal variation in Mercury concentrations from Naauwpoortspruit river is presented in Figure 11. The level of mercury varied from 0 to 0.04 µg/L during the study area. To protect the aquatic ecosystem, DWS recommended limit for mercury is 0.04 µg/L (DWAF, 1996a; DWS, 2017). Site A mean Hg concentration was 0.026 µg/L ± 0.0012 with maximum of 0.04 µg/L in December 2019. The maximum concentration of Hg at Site A is attributed AMD from mining water seepage and precipitation into the river. Once Hg is released to the environment, it can be converted to a biologically toxic form of methylmercury (MeHg) by microorganisms found in soil and in the aquatic environment (Lebepe *et al.,* 2016; Le Roux *et al.,* 2016). Walters *et al.* (2017) has learned that mercury in surface water can occur through the accumulation of sediments that contain methylation and demethylation residuals.

The harmful methylmercury form of mercury readily crosses biological membranes and can accumulate to harmful concentrations in the exposed organism and become increasingly concentrated up the food chain (Naidoo, 2013; Self *et al.,* 2013; Walters *et al.,* 2017). The Hg concentration in all sites were within the recommended for drinking water 6 µg/L (SANS , 2015). Other studies have found out that coal-combustion for electrical power generation and industrial waste disposal are other sources of Hg in the environment (Malema *et al.,* 2014; Lebepe *et al.,* 2016; Ebenebe *et al.,* 2017; Edokpayi *et al.,* 2017). Power generation industries also deposit a significant amount of fly ash materials that are disposed of in landfill sites (Ayanda *et al.,* 2012; Maya *et al.,* 2015). Fly ash contains high amounts of radioactive metals and, when not properly removed, ash may be a source of heavy metals such as Hg in water (Schutte, 2018). According to López-Antón (2009), carbonaceous particles present in fly ashes are capable of retaining mercury species in different proportions depending on their characteristics and the process conditions. Various studies on fly ashes suggest that retention capacity of Hg concentrations depends not only on their unburned content, but also on their surface area, morphology and petrographic characteristics (López-Antón *et al.,* 2009; Le Roux *et al.,* 2016; Schutte, 2018).

In the Westbank area, Western Cape (South Africa), mercury concentrations value ranged between 0.0005 - 0.0020 µg/L which was within recommended limit of drinking (SANS, 2015) and exceeded the 0.04 µg/L for aquatic ecosystem (Le Roux *et al.,* 2016). Numerous studies carried out in South Africa rivers shared the same sentiment about mercury levels, they discovered that mercury concentrations of above 0.0017 µg/L were attributable to acid mine drainage, coal power stations and can have effects on the water downstream. High level of Hg have effects on the incidence of low-white blood cells in fish and other aquatic species, thereby affecting the aquatic ecosystem (DWAF, 1996a; DWS, 2017).

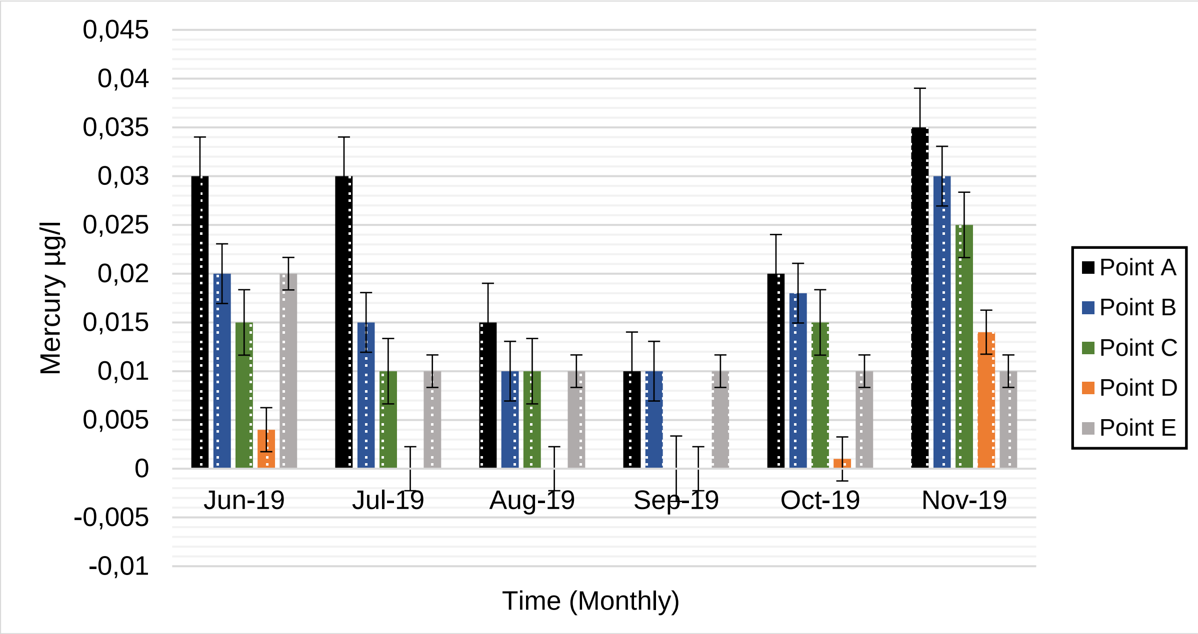

Figure 11. Mercury concentrations during the study period (June – December 2019) for the Naauwpoortspruit River.

4.2.1.9. Copper (Cu)

The concentrations of Cu varied from 0.001 – 0.0035 mg/L in all the sites (Figure 12). The other sites complied with the water quality guidelines with the exception of the DWS Aquatic Ecosystems guideline (DWAF,1996a), which recommends a concentration of 0.0012 mg/L, 0.0024 mg/L as the chronic effect value in drinking water and 0.0075 mg/L as the acute effect value (SANS, 2015).

Site A mean Cu concentration was 0.023 mg/L ± 0.00067 with elevated concentration in June 2019 (0.0023 mg/L), which decreased in July 2019 (0.0017 mg/L) and August 2019 (0.0015 mg/L). In December, Site A recorded maximum Cu concentration of 0.0035 mg/L which is attributable to mining and industrial discharge, especially coal mine and steel industry which is prevalent in the upper stream of Naauwpoortspruit River. According to Mathebula (2015), concentration of Cu can be result from AMD seepage from mining activities into waterways, and tributaries ending in the river system. Waste rock from mining activities can pollute surface water when they produce acidic runoff mobilizing Cu, Fe, Pb etc (Sims *et al.,* 2013).

Site E had a mean of 0.017 mg/L ± 0.0006 with maximum Cu concentration of 0.0025 mg/L in December 2019, which was within 0.0024 mg/L drinking water guideline (SANS, 2015). Even though Cu concentration was within South Africa water guidelines, accumulation of Cu is toxic to humans and aquatic ecosystem. From the study area, coal sediments from mining activities can pollute surface water when it produces acidic runoff and flying ash from the power station.

Wind can also play part in the transportation of small rocks and other debris that have elevated concentration of Cu, and they can be deposited in surface water. This process it's called weathering and takes time to have effects unless there are winds high speed and can speed the process (Masindi and Muedi, 2018). Recently, Lebepe *et al.* (2016) found a high level of alkaline pH and unacceptable limit of Cu concentration in the water column at Loskop and Boshielo Dams. The study found out that Cu residual from active and abandoned mines finds its way into surface water through AMD (DWS, 2016; Lebepe et al., 2016). Another study carried out in South Africa rivers, Orange and Olifant's River catchment revealed that high levels of copper are attributable to AMD, active mining deposits, weathering, wastewater treatment works, industries smelting copper (DWS, 2016; Olujimi *et al.,* 2015; Verlicchi et al., 2020). In Tai Lake, China, Cu concentrations in surface water (0.0024 – 0.0171 mg/L) were higher than the set limit and higher when comparing it to the present study with 89% non-compliance (Le Roux *et al.,* 2012; Soleimani *et al.,* 2018; Agoro *et al.,* 2020). High concentrations of copper have effects on the health of humans and aquatic ecosystems (Mwangi, 2014).

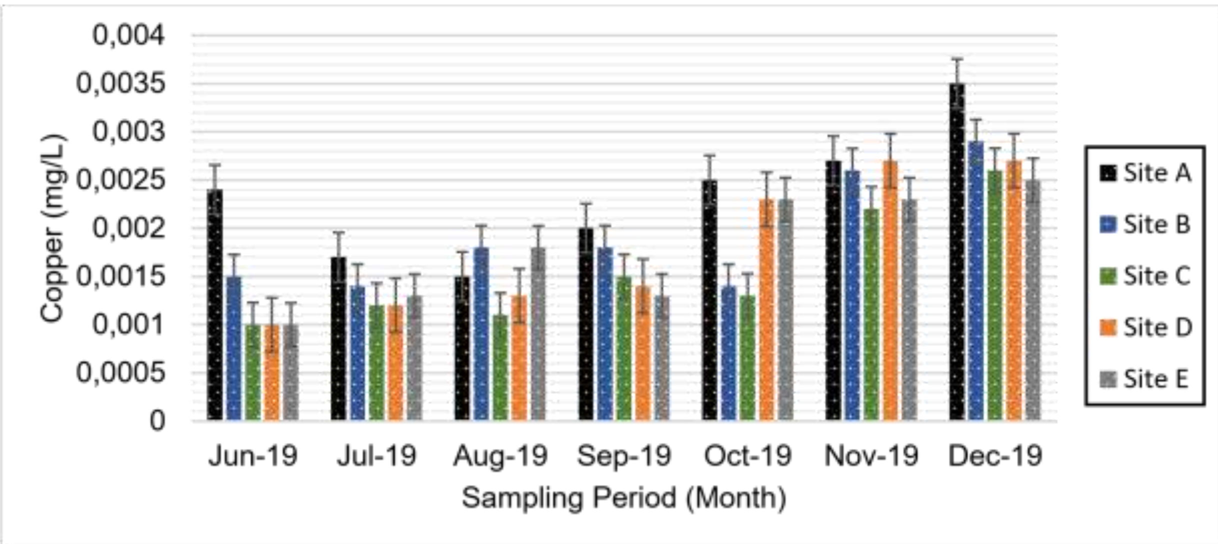

Figure 12. Copper concentration during the study period (June – December 2019) for the Naauwpoortspruit River.

4.2.1.10. Zinc (Zn)

The concentration of Zn was high at all selected sites of Naauwpoortspruit River in November and December 2019 (Figure 13). To protect the aquatic ecosystem, DWS recommended that a limit of 0.002 mg/L and 5 mg/L for drinking water (DWAF, 1996a; SANS, 2015; DWS, 2007). Site A mean Zn concentration measured a 0.045 mg/L ± 0.04 with a maximum of 0.098 mg/L in December 2019. The elevated Zn concentration at Site A could be attributed to metal production processes from steel industry and mining residuals with mining and steel industry are found in the upper stream of Naauwpoortspruit river. These anthropogenic activities discharges effluent containing heavy metals such as Zn, Cu etc that are acidic into the environment (Dabrowski and Klerk, 2013; Musingwini 2014). Upper Olifants River , where Naauwpoortspruit River is found has been comprised of coal mining, power generation industries which are discharging AMD into the environment (DWS, 2018; Verlicchi *et al.,* 2020).

Site B mean Zn concentration of 0.041 mg/L ± 0.03 was measured, with a maximum concentration of 0.089 mg/L in December 2019. The maximum Zn concentration at Site B was above recommended aquatic ecosystem limit of 0.002 mg/L and is attributed to agricultural activities (e.g., use of pesticides and insecticides) and domestic waste such as worn rubber tyres of vehicles (Olujimi *et al.,* 2015; Pollard *et al.,* 2017; Soleimani *et al.,* 2018). High temperatures are also suggested to be factors that can increase Zn in water (Change, 2016; Soleimani *et al.,* 2018). In the Stellenbosch River, Cape Town, Olujimi *et al.* (2015) found that the Zn concentration ranged between 0.172 to 0.722 mg/L which exceeded the acceptable limit and was attributed to industrial and wastewater effluent. A study by Verlicchi et al. (2020) found that high concentrations of metals (e.g., Zn and Cu) are polluting surface and groundwater in South Africa rivers due to the percolation of AMD which is not monitored especially in the abandoned mining area. High level of Zn can have lethal effect of zinc on fish is thought to be from the formation of insoluble compounds in the mucus covering the gills (DWAF, 1996a; Obersholster *et al.,* 2017).

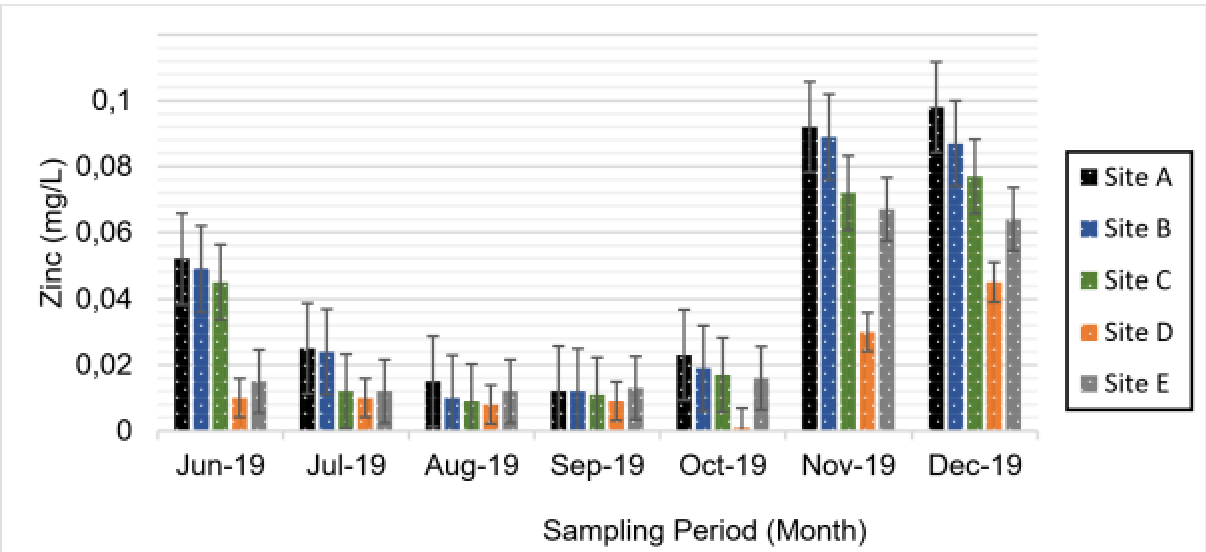

Figure 13. Zinc concentrations during the entire study period (June – December 2019) for the Naauwpoortspruit River.

4.2.1.11. Iron (Fe)

As shown on the Figure 14, the concentration of Fe in the Naauwpoortspruit River was below the 5 mg/L of DWS Agricultural Irrigation guidelines (DWAF, 1996b) throughout the study. The Fe concentration showed variation from June to December 2019 ranging from 0.06 - 0.71 mg/L. The maximum Fe concentration of 0.71 mg/L was recorded at Site B in December 2019, it was compliant to 2mg/L of drinking water and exceeding the 0.3 mg/L for aesthetic concerns (SANS, 241 (2015). (DWAF, 1996a; SANS, 2015; DWS, 2017). The maximum concentrations of Fe into the environment can be due to mining acid mine drainage, mineral processing, landfill leachates and the corrosion of iron and steel which are found closer to Site A and Site B of Naauwpoortspruit River (Gonah, 2014; Donoso *et al.,* 2017). Elevated dissolved metals are primarily from AMD, originating from abandoned and active coal mines. This AMD is a significant transporter of a non-point source of heavy metals to surface water sources (Gonah, 2014).

The Fe concentration showed variation at Site A with the mean of 0.214 mg/L ± 0.085 in June to December 2019. The maximum Fe concentration at Site was 0.39 mg/L in November 2019 was complaint to 2 mg/L of SANS 241 of drinking water and exceed aesthetic of 0.3 mg/L (SANS, 2015). Site C had a mean of 0.166 mg/L ± 0.15 with the maximum of 0.45 mg/L recorded in December 2019. Fe concentration at Site C from June was 0.09 mg/L, showed decline to 0.07 mg/L in July and begun to increase during November reaching 0.31 mg/L and December reaching 0.45 mg/L. the variation of Fe at sampling point can be due to many different factors, such as geology of the area and other residue mining and industries into the surface water (Dallas, 2004; Mathebula, 2015). November and December in South Africa is summer, particular at the study area, where the weather is mostly associated with hot and rainfall. Rainfall during November and December can coincide with elevated Fe concentration within Site A and Site B.

Eskom power grid. According to the Eskom Integrated report for 2020, these power plants are expected to emit more than 25 million tons of fly ash per year (Eskom, 2020). A significant amount of fly ash material from coal power stations can be disposed of in landfill sites and about 5 percent of the generated ash is usually used as backfill material (Ayanda *et al.,* 2012; Maya *et al.,* 2015). Fly ash contains significant quantities of radioactive metals and, when not properly removed, ash may be a source of metals (Schutte, 2018). Metals and other dissolve solids are leached from the ash heaps by the wastewater derived from the ash slurry and by subsequent infiltration by rain (Ayanda *et al.,* 2012). This subsequently imposes a risk on surface and groundwater. This makes the Naauwpoortspruit River turbid and nutrient enriched as it is closer to the power stations and mines. Elevated Fe concentration and other trace metals such as Al and Mn may cause pansteatitis to fish and other animals such as crocodile (Dabrowski and Klerk, 2013). According to Dabrowski (2013), a crocodile was found dead in 2011 near the Kranspoortspruit River and it was diagnosed with pansteatitis.

In a study of rivers feeding into Katse Dam, Lesotho, Fe levels were higher (13.60 mg/L) at Bokong River when comparing to the current study Site A (0.39 mg/L) and Site B (0.71 mg/L) and non-compliant to the DWS Agricultural Irrigation (5 mg/L) (Mathebula, 2015; DWS, 2017). Elevated concentration of Fe in Katse Dam was attributed to mining operations in the catchment area (Mathebula, 2015). A study of Daguija River (Zhu *et al.,* 2015) found high-level Fe of 10 mg/L when comparing to SANS: 241 (SANS, 2015) of 2 mg/L.

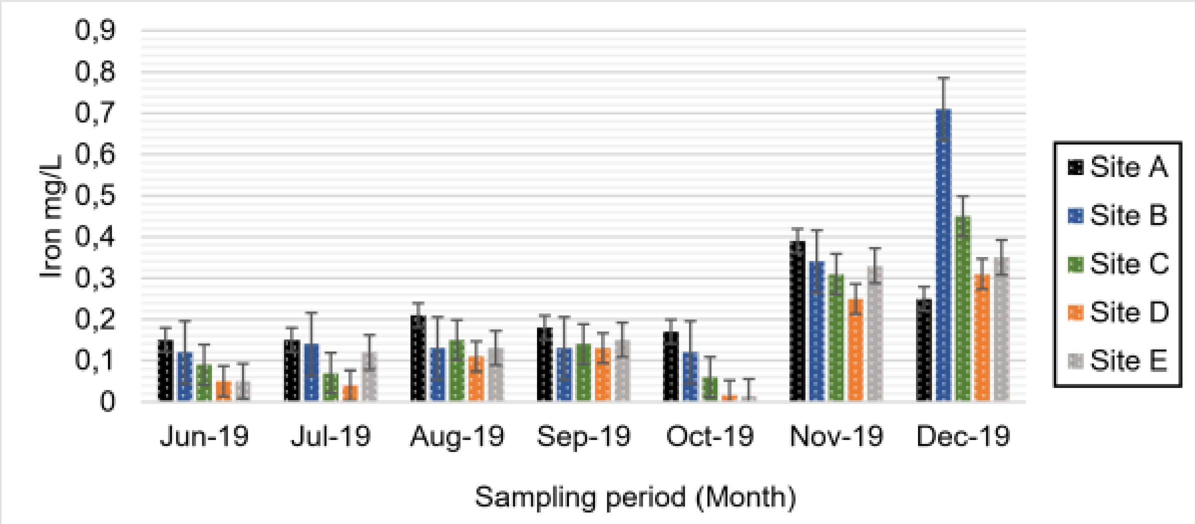

Figure 14. Iron concentrations during the entire study period (June – December 2019) for the Naauwpoortspruit River.

4.2.1.12. Manganese (Mn)

Manganese sources into surface water can be from natural and anthropogenic activities and can be toxic over time (Mathebula, 2015; Retief *et al.,* 2020). The primary uses of manganese are in metal alloys, dry cell batteries, micro-nutrient fertilizer additives, organic compounds used in paint driers, and chemical reagents (Rodrigues *et al.,* 2017). Manganese can reach both surface water and groundwater as a consequence of agricultural activity and urban runoff. As shown in Figure 15, the concentration of Mn showed maximum at Site A in December 2019. This Mn concentration of 0.19 mg/L was recorded at Site A was non-compliant to DWS Aquatic Ecosystem (0.18 mg/L) and Agricultural Irrigation (0.02 mg/L) (DWAF, 1996a; 1996b). The Mn concentration from June to October 2019 was within the acceptable guideline in all the sites.

The trend of Mn concentration at Site A showed that November (0.13 mg/L) and December (0.19 mg/L), with a mean of 0.0812 mg/L ± 0.057. Site A is characterized by industries (coal mining, steel, and iron smelters which are known sources for releasing manganese into the atmosphere) that result in high Mn into the surface water (Musilova *et al.,* 2016; Retief *et al.,* 2020). During the dry period in Naauwpoortspruit River, stagnant water induces soil saturation and mobilization of Fe and Mn oxides, resulting in increased concentration depending on the depth of the soil profile (Mariame *et al.,* 2013; Donoso *et al.,* 2017). During the rain period, runoff erodes the resulting Mn and Fe from the soil profile into the surface water. In a study of Mvudi River, Thohoyandou, Edokpayi *et al.* (2016) recorded Mn concentrations in the range of 0.081 – 0.52 mg/L at the upstream which was above the mean of Site A range of 0.041 – 0.19 mg/L and may be attributed to mining activities and geological structure of the rock in the catchment.

Site B mean Mn concentration was 0.075 mg/L ± 0.054 with the maximum of 0.179 mg/L recorded in December. Site B Mn concentration was compliant to DWS Aquatic Ecosystem and drinking water (0.4 mg/L) (DWAF, 1996a; SANS, 2015). Manganese is an essential micronutrient for plants and animals and is a functional component of nitrate assimilation and an essential catalyst of numerous enzyme systems in animals, plants and bacteria (DWAF, 1996a; Retief *et al.,* 2020; Mathebula, 2015). However, high concentration of Mn above the required drinking water guideline can cause undesirable taste (SANS, 2015) and can be toxic, which may lead to disturbances in various metabolic pathways, in particular disturbances of the central nervous system caused by the inhibition of the formation of dopamine (a neurotransmitter) and laundry problems (DWAF, 1996a).

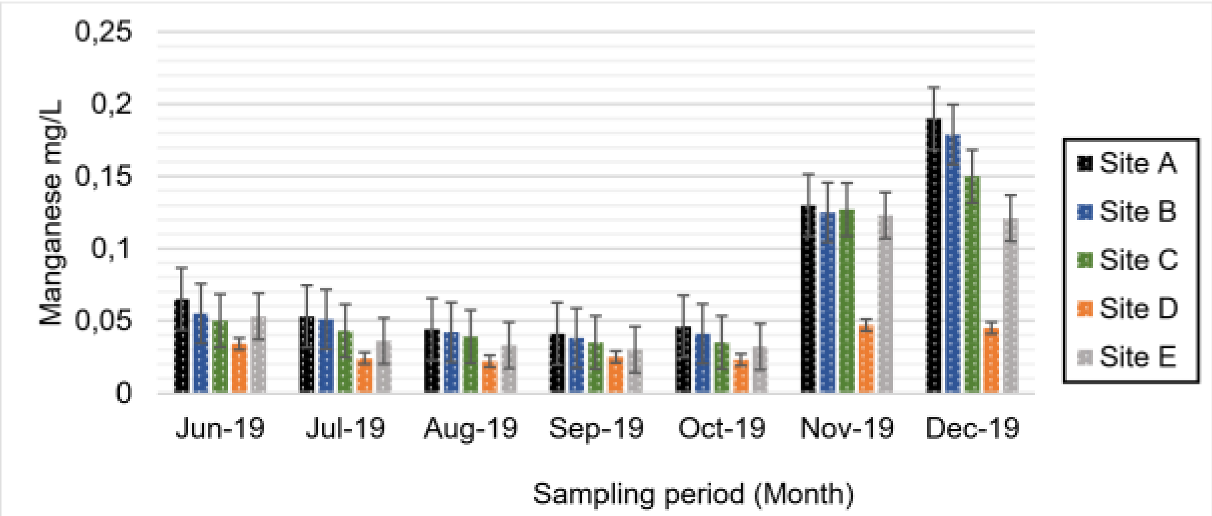

Figure 15. Manganese concentration during the study period (June – December 2019) for the Naauwpoortspruit River.

4.2.2. Microbiological analysis

The Naauwpoortspruit River was analysed for microbial indicators and the monthly results showed variations between sites and are presented in figures 16 to 19. Diluted water samples at 10^3 and 10^6, were used as suitable dilutions to permit the easy enumeration of colonies growing on the plates. Total coliforms, faecal coliforms, total heterotrophic bacteria, and *E. coli* are microbial indicators used for this study.

4.2.2.1. Total and Faecal coliforms

Figure 16 highlights the concentrations of total coliforms bacteria in Naauwpoortspruit River over the study period and for the different sampling sites. Site A mean total coliforms concentration was 0.61 x 10^3 cfu/ 100 mL ± 0.35 x10^3 with a maximum concentrations of 1.2 x 10^3 cfu/ 100 ml. Site B showed a mean total coliforms concentration of 1.007 x10^3 cfu/ 100 mL ± 0.92 x 10^3 with a maximum concentration of 2.35 x 10^3 cfu/ 100 ml. Site C showed a mean concentration of 1.07 x 10^3 cfu/ 100 mL ± 0.83 x 10^3 with a maximum concentration of 2.4 x 10^3 cfu/ 100 mL. Site D and Site E measured a mean concentration 1.54 x10^3 cfu/ 100 mL ± 1.4 x 10^3 and 1.65 x 10^3 cfu/ 100 mL ±1.38 x 10^3, with maximum concentrations of 4.15 x 10^3 cfu/ 100 mL and 4.2 x 10^3 cfu/ 100 mL, respectively.

Faecal coliforms at Site A showed a mean concentration of 0.57 x 10^3 ± 0.24 x 10^3 with a maximum of 0.9 x 10^3. Site B showed a mean total coliforms concentration of 1.24 x 103 cfu/ 100 mL ± 0.76 x10^3 with a maximum concentration of 2.2 x 10^3 cfu/ 100 mL. Site C showed a mean concentration of 1.18 x 10^3 cfu/ 100 mL ± 0.78 x 10^3 with a maximum concentration of 2.5 x 10^3 cfu/ 100 mL. Site D and Site E measured a mean concentration 1.47 x 10^3 cfu/ 100 mL ± 1.33 x 10^3 and 1.99 x 10^3 CFU/ 100 mL ±1.15 x 10^3, with maximum concentrations of 3.4 x 10^3 cfu/ 100 mL and 5.4 x 10^3 cfu/ 100 mL, respectively. The trend shows the variation of total and faecal coliforms per site, which all sites were not complying with recommended drinking water guidelines of 0 cfu/ 100mL, 10 cfu/ 100mL in operational and complying with DWS Irrigation guideline of 10 000 cfu/100 mL (DWAF, 1996b; SANS, 2015; DWS, 2017).

The high prevalence of these bacteria in water indicates that the surface water was polluted with faecal matter, most likely due to the faecal matter accumulated by WWTP, the leakage of waste, and the feeding of livestock near the river (Omoregbe *et al.,* 2017; Islam *et al.,* 2018). When it rains, faecal matter flows into the surface water contaminating the river. Microbes including *Salmonella spp., Shigella spp.,* and *E. coli* are members of the faecal coliform bacterial community. These pose a health risk e.g., diarrhoea, on occasional fever and cholera), to downstream users of water, either for drinking or agriculture without pre-treatment

(Morokong *et al.,* 2016; Matjuda *et al.,* 2019; Messina *et al.,* 2019; WHO, 2019). Mulamattahil *et al.* (2014) reported that water from Modimola Dam, Mafikeng, South Africa had high faecal bacteria above 100 CFU/mL. The study revealed that Modimola Dam is a source of drinking water to water treatment and nearby communities and high faecal contamination could be attributed to wastewater treatment effluent upstream of Modimola Dam (Mulamattahil *et al.,* 2014).

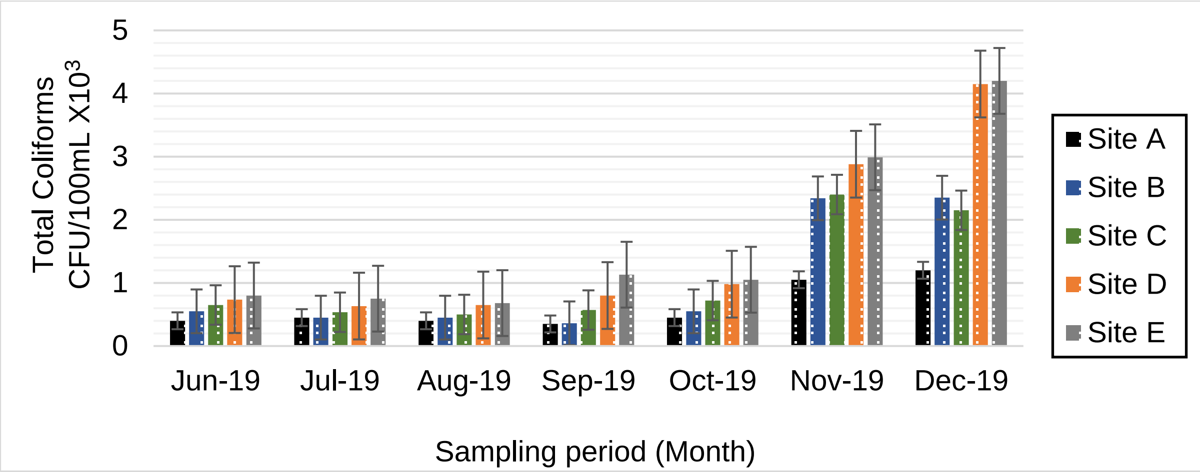

Figure 16. Total Coliforms counts per month during the study period (June – December 2019) for the Naauwpoortspruit River.

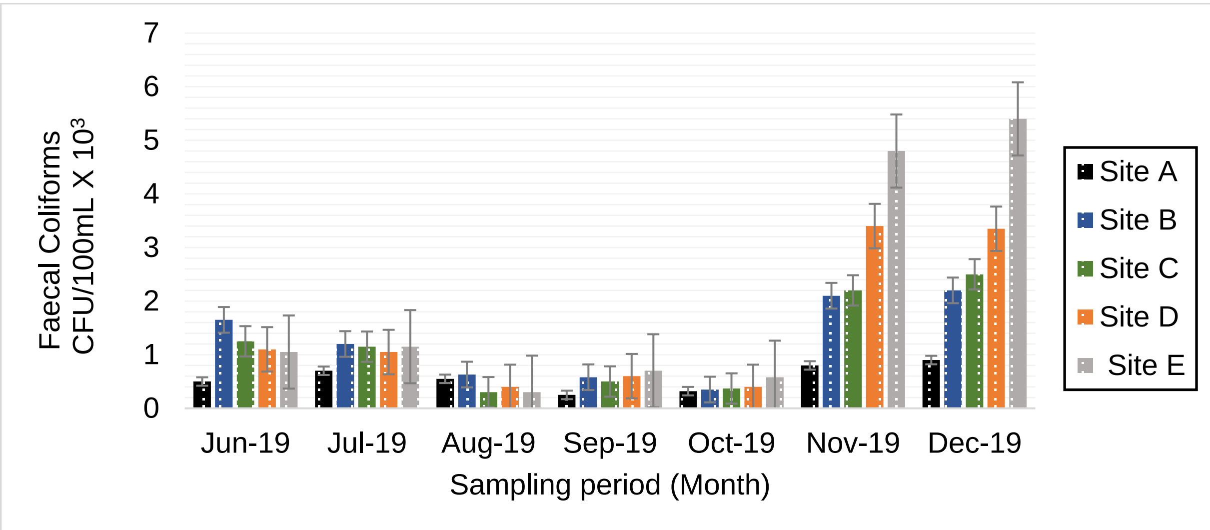

Figure 17. Faecal Coliforms counts per month during the entire study period (June – December 2019) for the Naauwpoortspruit River.

4.2.2.2. Heterotrophic Bacteria (HPC)

In all selected sites of the Naauwpoortspruit River, elevated levels of HPC (9.9 x 10^6 cfu/100 mL) were found in November 2019 at Site E (Figure 18). The trend for the study showed HPC levels in all sites not within acceptable DWS domestic use guideline of 1/10 cfu/100mL, SANS: 241 of 100/100 cfu/100mL (DWS, 1996c; SANS, 2015). Site A mean HPC concentration was 2.212 x10^6 ± 2.06 x$10^{6,}$ with a maximum of 5.3 x 10^6 cfu/mL was in December 2019. High concentration HPC in November and December 2019 could be attributed to the rain period over the Naauwpoortspruit River, where rain was measured above 300 mm at eMalahleni Town. HPC bacteria pose health risks for consumers of water (Augustyn *et al.,* 2016; Marie *et al.,* 2018; Herbig *et al.,* 2019). Waterborne diseases caused by inadequate hygiene and water supplies lead to a global health danger (WHO, 2019).

During December, HPC variation, with a high level of 9.2 x10^6 cfu/100mL, was also observed which could be due to municipal WWTP effluent near the river. According to Elbossaty (2017), the regrowth of heterotrophic bacteria in water pipelines or chambers can be influenced by high temperature, disposal of nutrients to bacteria, and lack of residual disinfectant. Municipal waste treatment, agriculture, and household effluents can be a source of heterotrophic bacteria in surface water (UNICEF, 2015; Rodrigues *et al.,* 2017). In a study of the uMhlangane River, South Africa, a high HPC count of 14.9 X 10^6 cfu/100 mL was found to be from industrial and WWTP effluent (Marie et al., 2018). At Site E on the Naauwpoortspruit River downstream, agricultural operations are ongoing including livestock grazing, fishing, and livelihood farming. During fishing, people using the bush to relieve themselves as toilet result in faecal waste which can be washed into river during precipitation, thereby increasing faecal pollution of the surface water (Chigor *et al.,* 2013; Mathebula, 2015).

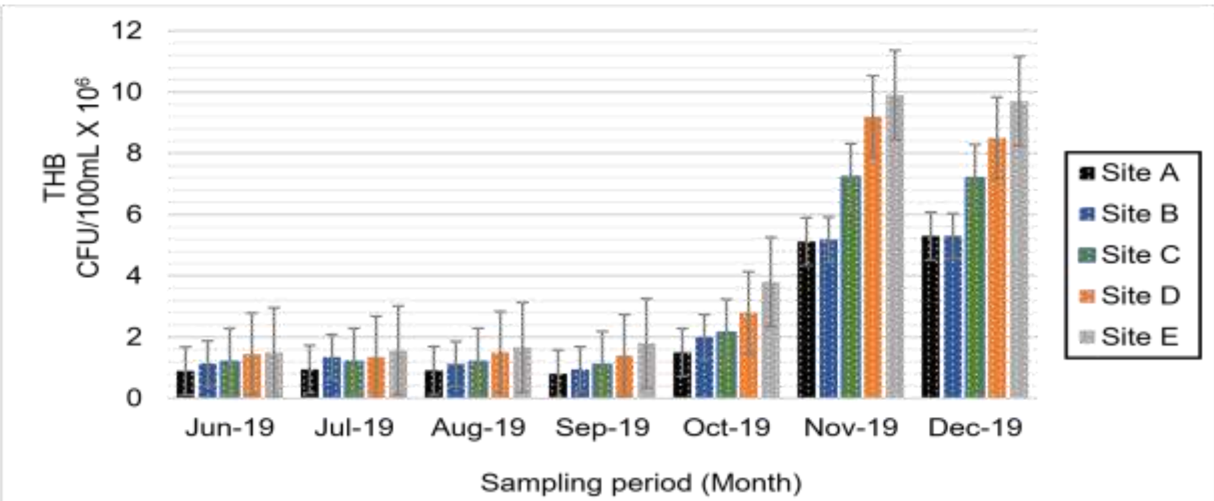

Figure 18. Total heterotrophic bacteria per month during the study period, June – December 2019 for the Naauwpoortspruit River.

4.2.2.3. *E. coli*

According to SANS: 241 (2015), the acceptable *E. c*oli limit in drinking water is 0 and DWS aquatic ecosystem is 0 – 1000 mg/L (DWS,1996a; SANS, 2015; DWS, 2017). From the study, *E. coli* concentrations from all selected sites of Naauwpoortspruit River ranged from 0.1 x 10^3 – 2.7 x 10^3 CFU/100mL (Figure 19). The high concentration of *E. coli* of 2.7 X10^3 CFU/mL was detected at Site E in November 2019, which is non-complaint to DWS aquatic ecosystem guideline and WHO (2011) drinking water limit of 0 mg/L (WHO, 2011; SAN, 2015). This high level of *E. coli* may be from WWTP effluent (Frieden, 2015; Olujimi et al., 2015). Various studies have shown that WWTPs are not successful in South Africa and that their effluents are not complying with wastewater guidelines (Le roux, 2014; Harmony Gold, 2014; Naidoo, 2013; DWS, 2017).

E. coli variance has demonstrated that during rainfall, faecal matter is streaming into the river and contaminates surface waters in November and December 2019. High *E. coli* detected per site, Site A (1.2 x 10^3 CFU/100mL), Site B (1.6 x 10^3 CFU/100mL), Site C (1.65 x10^3 CFU/100mL), Site D (2.1 x 10^3 CFU/100mL) and Site E (2.7 x 10^3 CFU/100mL). Industrial activities, municipal WWTPs, sewerage, and animal grazing in the vicinity of the study area also contribute to this accumulation of *E. coli* levels in the surface water (Edokpayi et al., 2018; WHO, 2018; Wen *et al.,* 2020). Major concern can be different *E. coli* isolates from a different source, that can pose a health danger to downstream water users, for example, *E. coli* O157:H7 can cause haemorrhagic enteritis and haemolytic uraemic syndrome in humans (Nguyen *et al.,* 2016; Ridanovic *et al.,* 2017). *E. coli* from this study were further tested for antibiotic resistance patterns within the Naauwpoortspruit River to determine antibiotic resistance trends.

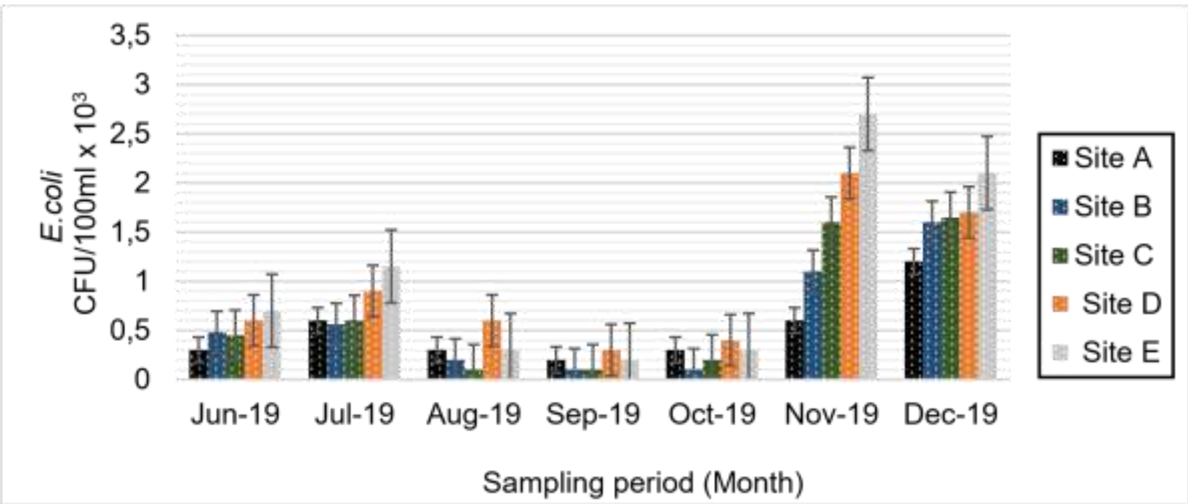

Figure 19. *E. coli* counts per month during the entire study period (June – December 2019) for the Naauwpoortspruit River.

4.3. Statistical analysis of the physicochemical and microbiological parameter's relationships.

A dendrogram was developed to determine the association between physicochemical and microbiological parameters for each sampling run. Figure 20 represents all the five selected sites of Naaauwpoortspruit from June – December 2019. The trend observed from the dendrogram reveals that TDS and pH in June 2019 were strongly associated with levels of HPC isolates. pH also plays an important role in the survival and growth of microbiological parameters in the water while TDS is associated with salinity and faecal contamination from sewerage leakage and WWTP effluent (Edokpayi *et al.,* 2017). Microbiological activities are sensitive to pH changes (Bester, 2015; Nguyen *et al.,* 2016).

In another case, water sampled in December revealed *E. coli* and total coliforms also associated with physical parameters. Site D is associated with many of the listed parameters (phosphates, *E. coli*, and total coliforms) due to WWTP effluent. The pattern in dendrograms also revealed that sampled water from site A and site B shared similar characteristics such as Hg, Cu and Zn concentrations due to mining and industrial discharge. The water quality of these sites is impacted by trace metals and other natural sediments which can seepage as AMD into the surface water. These physiochemical parameters were strongly correlated to *E. coli* and faecal coliforms levels.

In September 2019, sampled water from Site C and Site D formed a cluster with site E. This indicates that various sources were very similar, and this was observed during sampling as the sites are close to each other. COD and nitrates were the physicochemical parameters that had the most profound effect on these water samples. Antibiotic resistance patterns from the sample of Point E show correlation to physicochemical parameters due to the association between sampling sites which has impurities from WWTP effluent.

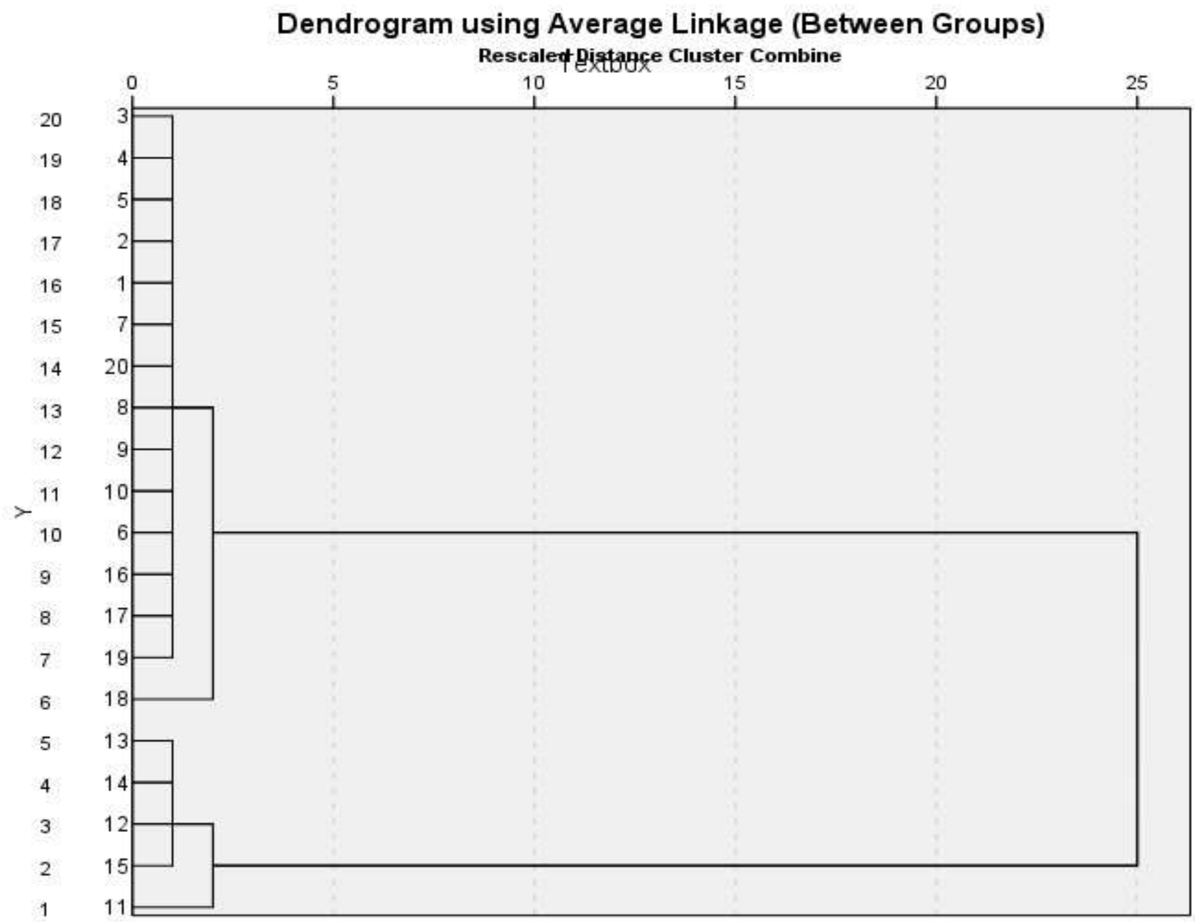

Figure 20. Correlation between physicochemical parameters and microbiological parameters at Naauwpoortspruit River.

4.3. Antibiotic resistance studies for faecal coliforms and *E. coli* bacteria.

4.3.1. Antibiotic resistance patterns

The antibiotic resistance pattern of each isolate was evaluated, and to determine the percentage of faecal coliforms and *E. coli* that were resistant to each antibiotic (Figure 21 and Figure 22).

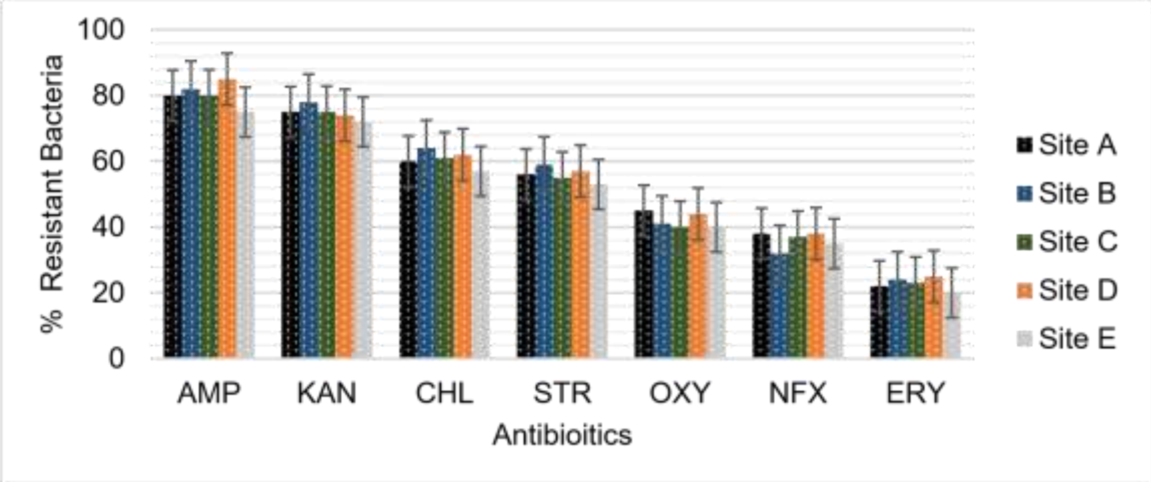

Figure 21. Results for antibiotic resistance patterns amongst faecal coliform bacteria from the Naauwpoortspruit River.

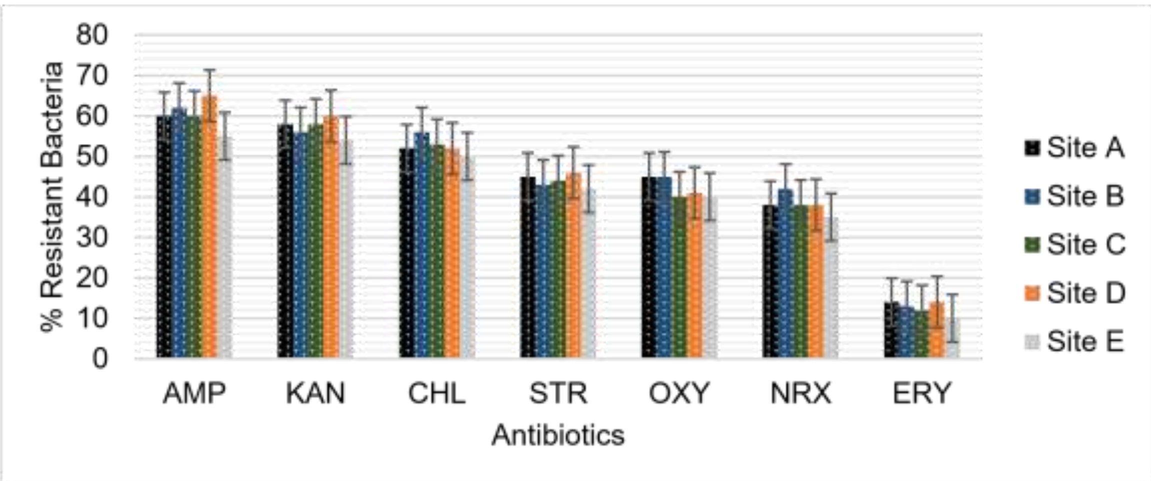

Figure 22. Results for antibiotic resistance patterns amongst *E. coli* isolates from the Naauwpoortspruit River.

Antibiotic resistant trends for isolates are shown in Figure 21 and Figure 22. Variation in the percentage resistance between isolates at the various sites shows a widespread prevalence of antimicrobial resistance in the vicinity of Naauwpoortspruit River. The prevalence of resistance of faecal and *E. coli* in the environment may be caused by their concomitant co-selection regulatory factors. There were greater numbers (60%) of bacterial resistant to *β*-lactam antibiotics than to other antibiotics. This was in line with the observation made by Chihomvu *et al.* (2014) where isolates were 94% effective to tetracycline. Tetracycline is effective against gram-positive bacteria such as *Streptococci, Corynebacteria, Clostridia, Bacillus* and active also against Gram-negative bacteria especially *Pseudomonas spp, Enterobacteriaceae,* and *Acinetobacter spp* (Jones *et al.,* 2014; Mendes *et al.,* 2015). According to Overbey *et al.* (2015) and Sandhu *et al.* (2016), exposure to zinc and manganese levels can increase the resistance of bacteria to ox-tetracycline and ciprofloxacin antibiotics as they share a mobile genetic factor in the water. Tetracycline resistance is typically due to one or more of these: the acquisition of traveling genetic components bearing tetracycline specific resistance genes, ribosomal binding site mutations as well as chromosome mutations resulting in enhanced intra-resistance system expression (Pal *et al.,* 2015; Benmalek *et al.,* 2016; Grossman *et al.,* 2016). Results from the study on surface water microbial communities showed that FC ranged from 0.35 x 10^3 – 4.2 x10^3 CFU/100mL and *E. coli* ranged from 0 – 1.7 x 10^3 CFU/100mL in Naauwpoortspruit River. Over 60% of faecal coliforms were resistant to ampicillin, kanamycin and between 40 – 60% were resistant to chloramphenicol, oxytetracycline, streptomycin, and 20 – 40% to norfloxacin and erythromycin. Between 60 – 80% of *E. coli* were resistant to ampicillin, between 40 – 60% were kanamycin, chloramphenicol, streptomycin, ox-tetracycline, and 20 – 40% resistant to norfloxacin and erythromycin.

4.3.2. Prevalent multiple antibiotic resistant (MAR) phenotypes

MAR means the resistance of bacteria to more than 2 antimicrobials was tested (Molale, 2012; Alonso *et al.,* 2017). The *E. coli* tested for MAR showed resistance to ampicillin (10 µg) and kanamycin (30 µg), oxytetracycline (30 µg), chloramphenicol (30 µg), streptomycin (30 µg), and norfloxacin (10 µg). To establish minimum inhibitory concentration (MICs) the concentrations of antibiotics needed to be dissolved before a stock solution has been achieved and then diluted to reach an adequate starting concentration (Kowalska-Krochmal *et al.,* 2021). Therefore, the quantities in each antibiotic are specified for testing depending on the antibiotics concerned in surface water. Bacteria endowing high resistance levels were detected at Site D, downstream of Naauwpoortspruit River when comparing it to other sites along the river. Antibiotic resistant *E. coli* phenotype indicating resistance to six antibiotics AMP-KAN-CHL-STR-OXY-NFX was observed (Table 6). Site D is situated downstream of Naauwpoortspruit River, which receives wastewater treatment effluent, agriculture, and industries effluent. The MAR levels were however predicted to be high. This is because significant antibiotic resistance causes such as heavy metals from factories and WWTP are available. Exposure to heavy metals such as Zn, Cu, and Hg and antibiotic ampicillin appears to enhance the MAR of the bacterial community (Benmalek *et al.,* 2015).

Site A in the Naauwpoortspruit River also found susceptibility to bacterial isolates to three or four antibiotics. Amp-KAN-CHL was the dominant MAR isolate phenotype. In the upstream site, there is inflow from mining and steel industrial activities, and this could explain the similar antibiotic resistance pattern. It is possible that AMP, KAN, CHL, and heavy metals in the surface water co-select genes responsible for the dissemination and proliferation of antibiotic resistance bacteria (Czekalski *et al.,* 2014; Wen *et al.,* 2017).

4.3.3. Analysis of multiple antibiotic resistance index

The MAR indexes were calculated for the chosen sampling sites and the findings are shown in Figure 23. The MAR index is a good tool for health risk assessment which identifies if isolates are from a region of high or low antibiotic use (Davis *et al.,* 2016). The MAR index greater than 0.2 suggests a 'high risk' source of pollution. The highest MAR index came from Site D at 0.38, followed by Site B at 0.08, followed by Site A and Site E at 0.06. The least was obtained from Site C with a value of 0.02.

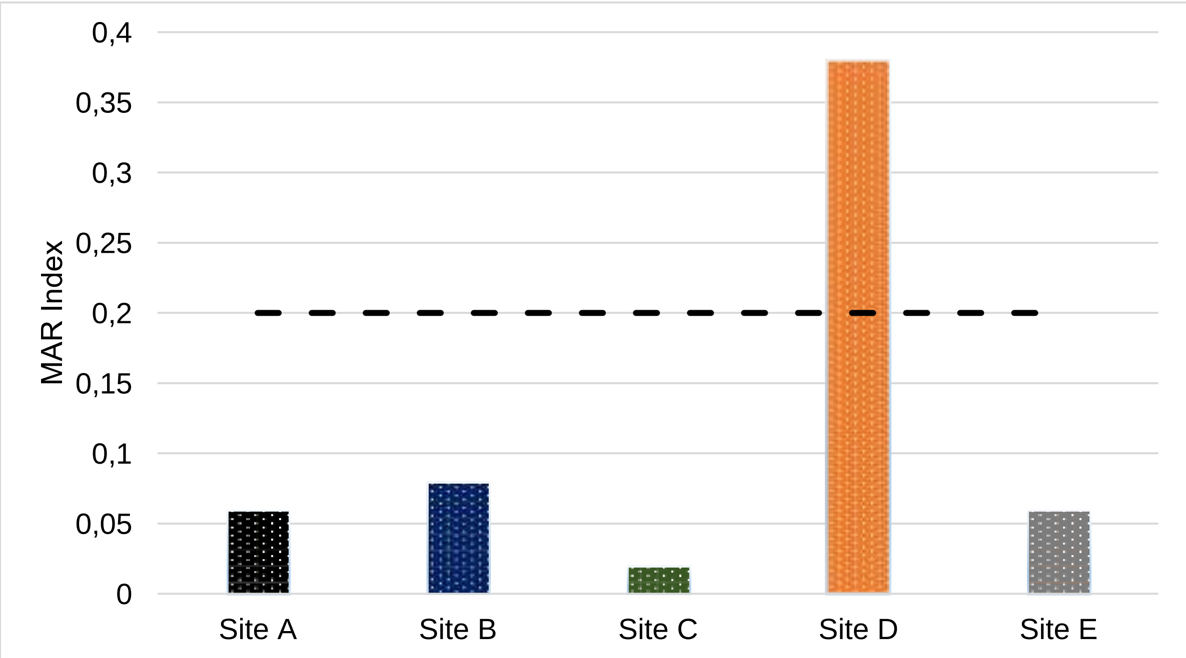

Figure 23. MAR index of *E. coli* resistant bacteria at different sites within the Naauwpoortspruit River. Dash line represents MAR threshold value (0.2) to differentiate the low and high risk.

4.4. Summary of results

The study aimed to assess water quality and prevalence of antibiotic-resistant bacteria in Naauwpoortspruit River, Mpumalanga, South Africa. In a bid to achieve the research objectives, selected physicochemical parameters, and levels of microbial indicator bacteria were determined in selected sampling sites. Results show that water quality in the river was impacted by pollution with significant anthropogenic activities (agriculture, mining, and industrial) occurring in the environment. The results of the study showed variation throughout the study period, and different factors affecting specific site per month. This was evident with pH at Site A being acidic in June, Site E recording a high phosphate of 2.2 mg/L in December., high ammonia concentration of 33.4 mg/L in June 2019. High levels of microbial pollution (FC, HPC, *and E. coli*) were also observed at sites D and E during November and December. Variation of trace metals trends e.g., Hg (0 - 0.04 µg/L) was complying with Aquatic Ecosystem guideline while concentration of Zn (0.098 mg/L), and Cu (0.0035 mg/L) were not complying with the recommended limits. The levels of Zn and Cu in Naauwpoortspruit River were attributed to AMD from mining and industrial activities. High antibiotic resistance levels for β-lactams were found downstream. Different factors, including exposure to antibiotic drugs, heavy metals, and other anthropological factors, may affect the tolerance of bacteria to antibiotics.

Other research studies have discovered that levels of Hg and Zn in surface water give microbial strains some level of tolerance (Di Cesare *et al.,* 2015; Pal *et al.,* 2015; BengtssonPalme *et al.,* 2018). This can be noted with a strong correlation between Hg and Zn at Site A to the detection of microbiological agents such as *E. coli* and total coliforms (Benmalek *et al.*, 2016; Li *et al*., 2017). According to Benmalek *et al.* (2016) the presence of significant concentrations of trace metals in the environment ecosystems leads to both contamination of soil and water and causing deleterious impact on environment life. Accumulation of metal in surface water of Naauwpoortsruit River can be from mining and atmospheric deposition (Drabwoski and De Klerk, 2013). The accumulation of trace metal elements above threshold levels have a detrimental effect on the microbial communities and their vital activities (Ahemad and Malik, 2012). Thus, microbial populations exposed to heavy metals present in the environment contain bacteria which have acquired a variety of mechanism for adaptation and resistance to these toxic elements, and, among them, bioaccumulation which involves complexation of the metal ions inside and outside the cell with biosorption (Srivasta & Kowshik, 2013), mineralisation and precipitation, enzymatic oxidation or reduction of the toxic metals, and the efflux systems of metal ions outside the cell (Czekalski *et al.,* 2014; Benmalek *et al.,* 2015; Chen *et al.,* 2019). Then through a process of co-selection, the resistance of bacteria to metals and antibiotics can overlap genetic mechanisms including co-resistance, cross-resistance (single genetic elements that control antibiotic and metal resistant genes), and co-regulation (which provide a common regulatory framework for antibiotic and metal resistant genes) (Jan *et al.,* 2015; Rahman *et al.,* 2016). Heavy metal tolerant microorganisms with resistance to contaminants will act as possible bioremediation agents for heavy metal polluted sites (Wen *et al.*, 2017). Further studies on the Naauwpoortspruit River heavy metal resistant bacteria could be required to detect HMRBs as bioremediation agents. Naauwpoortspruit River is anthropogenically polluted and there is a significant occurrence of antibiotic-resistant bacteria.

CHAPTER FIVE

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

5.1. Study Summary and Conclusions

Water pollution is a global issue and the protection of water resources from pollutants is of paramount importance for sustainable development. Overall results of physicochemical and level of microbial indicator bacteria indicated poor water conditions and presence of water stressors in the Naauwpoortspruit River. The stressors are anthropogenic activities (e.g., mining, wastewater treatment, agriculture, and domestic waste) within the vicinity of the study area. Mining and industrial activities are the sources of AMD and nutrient discharge that can cause a threat to natural biodiversity and downstream users of water (Musilova *et al.,* 2016; Walters *et al.,* 2017; Pollard *et al.,* 2017).

Results from the study provide insight into the condition of water in the Naauwpoortspruit River. Based on the comparison with the guidelines and standards for the aquatic ecosystem and for the various intended uses (drinking water, agriculture, irrigation & watering), the water quality can be deemed to be good quality, even though there were non-compliances with some of the guidelines and standards (DWAF, 1996a; DWS, 2017). The concentration of phosphates, ammonia and other trace metals were high at the different sites and months of the river perhaps due to natural activities (e.g. weathering, atmospheric deposition, ecological composition) and anthropogenic activities (e.g. mining and steel industries, sewerage and agriculture). Trace metals from AMD can cause noxious effect on the organisms and needs to be monitored throughout the study area.

The level of microbial contamination by faecal coliforms and *E. coli* in the Naauwpoortspruit River coupled with antibiotic-resistant bacteria is of high concern. Undoubtedly these levels of microbial contamination are mostly from sewerage pipe leakage, industries, and untreated wastewater effluent flowing into the Naauwpoortspruit River. High levels of heavy metals can be toxic, and most organisms cannot survive heavy metals contaminations (Griffins *et al.,* 2014; Bourceret *et al.,* 2016). However, bacteria can evolve different resistance mechanisms in heavy metal pollution, and thus persist in contaminated environments (Pal *et al.,* 2015; Di Cesare *et al.,* 2017). Elevated levels of antibiotics resistance pattern were observed downstream compared to upstream of Naauwpoortspruit River. These findings are endorsed by many studies regarding antibiotic resistance in surface water (Czekalski *et al*., 2014; Frieden, 2015; Bengtsson-Palme *et al.,* 2018; Wen *et al.,* 2020). The results indicate that exposure to heavy metals may play a key role in the prevalence and dissemination of antibiotic resistance bacteria. Statistics correlation showed that the development of microbiological parameters loads has a strong correlation with physicochemical parameters due to the association of sampling sites in the river environment. This urges the need for continuous surveillance within the research area as surface water is impacted by anthropogenic activities accumulating downstream due to runoff.

5.2. Recommendations

Recommendations to enhance the water quality of the Naauwpoortspruit River include:

a. Critical intervention to reduce nutrient input from residential and wastewater treatment as there are the most important sources of bacteria and nutrients in the river.
   Recommendations include the following:
   i. There should be an improvement in the maintenance and operation of wastewater treatment as it can reduce eutrophication and microbiological contamination in the river.
   ii. There should be continuous control interventions and testing of phosphates to reduce the loads that would improve the trophic status of Naauwpoortspruit River.
b. Water quality trends shows that phosphates and other trace metals were above the recommended limits at different sites and months exceeding DWS Aquatic Ecosystem and drinking water limit. High phosphate and trace metals comes from industrial and mining activities within the Naauwpoortspruit River. Recommendations include:
   i. Continuous improvement in policing and monitoring of activities along the sampling to reduce illegal discharges.
c. Heavy metals from mining and industrial activities have increased levels reaching the Naauwpoortspruit River. There should be a set priority to treat AMD and maintain good quality water within surface water. Recommendations include:
   i. Suitable mine closure plans need to be well prepared to avoid the proliferation of acid mine drainage and salinity problems.
   ii. There is a need to do rehabilitation of abandoned mines and treatment of acid mine drainage to mitigate current polluted water quality in Naauwpoortspruit River.
d. There is a need to further investigate the specific sources, and occurrence of antibiotic resistance bacteria within the study, using methods such as PCR to identify resistant genes of isolates. The PCR can also be able to assess the level of gene expression of the identified genes.

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APPENDIX A: Study area enlarged satellite and site images used for references.

Appendix Figure 1A. Location of Site A (Point A) sampling site in Naauwpoortspruit River

Appendix Figure 1B. Location of Site B (Point B) sampling site in Naauwpoortspruit River

Appendix Figure 1C. Location of Site C (Point C) sampling site in Naauwpoortspruit River

Appendix Figure 1D. Location of Site D (Point D) sampling site in Naauwpoortspruit River

Appendix Figure 1E. Location of Site E (Point E) sampling site in Naauwpoortspruit River

Appendix B: Numerical data and statistical analysis

Appendix Table 1. Prevalent multiple antibiotic resistance patterns for *E. coli* resistant in the Naauwpoortspruit River.

Antibiotic resistant profile						**Number of resistant isolates**
AMP	KAN					(7.4%)
AMP	KAN	CHL				(4.2%)
AMP	KAN	CHL	STR			(1.58%)
AMP	KAN	CHL	STR	OXY		(1.05%)
AMP	KAN	CHL	STR	OXY	NFX	(1.58%)

Appendix Table 2. Different sites showing resistance to different antibiotics

Antibiotics	**AMP**	**KAN**	**CHL**	**STR**	**OXY**	**NFX**	**ERY**
Site A	R	R	R	R	R	R	S
Site B	R	R	R	R	R	R	S
Site C	R	R	R	R	R	R	S
Site D	R	R	R	R	R	R	S
Site E	R	R	R	R	R	R	S

R= Resistance S= Sensitive I= Intermediate

Appendix Table 3. Multiple antibiotic resistance (MAR) indices for *E. coli* isolates at various sampling sites of Naauwpoortspruit River.

Sampling site	**Total numbers of the test (isolates)**	**No of the resistant test (resistant isolates)**	**MAR *p***
Site A	42	3	0.06
Site B	26	4	0.08
Site C	20	1	0.02
Site D	64	18	0.38
Site E	37	3	0.068

Appendix Table 4. List of significant positive and negative correlated water quality parameters

Level of Significance	**Positively Correlated Parameters**	**Negatively Correlated Parameters**

α= 0.05	EC – pH (r=0.001) EC – TDS (r= 0.01) EC – COD (r=0.397) pH - TDS (r=0.018 pH – COD (r=0.00008) pH – BOD (r=0.0008) Sulphate – Nitrate (r= 0.523) Nitrate – Phosphate (r=0.981) HPC – *E. coli* (r = 0.039) FC- TC (r = 0.42)	EC – BOD (r=-0.703) Ammonia – Nitrate (r=-0.816) Sulphate – Ammonia (r=-0.444) Ammonia – Phosphate (r=- 0.850) pH - Total coliforms (r=-0.11) pH- *E. coli* (r=-0.10)

Appendix Table 4. pH concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	Site A	Site B	Site C	Site D	Site E
Jun-19	4.45	5.01	6.45	7.05	7.15
Jul-19	6.01	6.24	6.56	6.34	6.56
Aug-19	7.33	7.33	7.47	7.89	7.2
Sep-19	7.25	7.45	7.63	7.78	7.86
Oct-19	7.25	7.45	7.46	7.25	7.44
Nov-19	6.15	6.67	7.86	7.76	7.53
Dec-19	6.23	6.5	7.03	7.9	7.83
Min	5.45	6.01	6.45	6.34	6.56
Max	7.33	7.45	7.86	7.9	7.86
Average	6.52	6.80	7.20	7.42	7.38
Standard Deviation	0.17	0.6	0.66	0.65	0.45

Appendix Table 5. Shows EC concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	Site A	Site B	Site C	Site D	Site E
19-Jun	58.63	64.8	78.5	78.6	80.1
19-Jul	63.72	71.4	74.4	76.5	80.5
19-Aug	62.5	70.5	81.3	84.2	87.5

19-Sep	76.56	84.9	85.5	88.5	84.2
19-Oct	66.35	84.75	88.9	88.6	85.2
19-Nov	81.3	93.35	92.1	98.5	112.5
19-Dec	87.4	100.4	101.3	99.5	113.3
Min	58.63	64.8	74.4	76.5	80.1
Max	87.4	100.4	101.3	99.5	113.3
Average	70.92	81.4	86	87.77	91.9
Standard Deviation	10.84	16.27	9.06	8.92	14.57

Appendix Table 6. TDS concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	**Site A**	**Site B**	**Site C**	**Site D**	**Site E**
Jun-19	381.1	421.5	510.25	510.9	520.65
Jul-19	414.18	464.1	483.6	497.25	523.25
Aug-19	406.25	458.25	528.45	547.3	568.75
Sep-19	497.25	551.85	555.75	575.25	547.3
Oct-19	431.3	550.88	577.85	574.9	533.8
Nov-19	528.45	606.76	598.65	640.25	731.25
Dec-19	568.1	652.6	658.45	646.75	736.45
Min	381.1	421.5	483.6	497.25	520.65
Max	568.1	652.6	658.45	646.75	736.45
Average	460.94	529.42	559	570.37	594.49
Standard Deviation	70.46	84.75	58.91	57.98	96.52

Appendix Table 7. COD concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	**Site A**	**Site B**	**Site C**	**Site D**	**Site E**
Jun-19	44	45	179	119.3	43
Jul-19	35	63	149	75.3	74
Aug-19	127.1	175	132.3	123	84.3
Sep-19	133.4	133	250.3	120	110.8
Oct-19	78	141	171.5	144	102.5
Nov-19	40	29	188.5	162	93.5

Dec-19	45.7	65	162	86	68
Min	35	29	132.3	75.3	43
Max	133.4	175	250.3	162	110.8
Average	71.88	93	176.08	118.5	82.3
Standard Deviation	41.58	34.2	27.62	18.48	19.98

Appendix Table 8. BOD concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	Site A	Site B	Site C	Site D	Site E
Jun-19	358.86	320.6	250	180	82.2
Jul-19	390.45	354.65	171.5	144	88.5
Aug-19	310.45	234.3	188.5	162	90.5
Sep-19	485.4	476.5	313.5	105.5	109
Oct-19	540.9	534.87	372	189	112.5
Nov-19	403.5	395	449	129	104.5
Dec-19	410.	380.75	330.5	110	102.3
Min	310.45	234.3	171.5	105.5	82.2
Max	540.9	534.87	449	189	112.5
Average	414.29	385.2	296.43	145.64	98.5
Standard Deviation	65.87	72.79	68.25	93.66	84.91

Appendix Table 9. Nitrate concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	Site A	Site B	Site C	Site D	Site E
Jun-19	4.5	4.4	4.8	5.4	5.85
Jul-19	3.45	3.8	4.45	5.4	5.5
Aug-19	3.39	4.01	4.43	4.93	5.33
Sep-19	4.2	4.5	4.2	4.15	5.3
Oct-19	6.2	7.32	6.1	6.4	7.5
Nov-19	7.1	7.43	7.4	8.3	8.9
Dec-19	7.2	7.3	7.7	8.3	8.5
Min	3.39	3.8	4.2	4.15	5.3
Max	7.2	7.43	7.7	8.3	8.9

Average	5.14	5.53	5.58	6.12	6.69
Standard Deviation	1.65	1.71	1.48	1.62	1.56

Appendix Table 10. Sulphate concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	Site A	Site B	Site C	Site D	Site E
Jun-19	114.3	102.4	102	104	96
Jul-19	112.5	114.4	117	87	95
Aug-19	113.5	81.8	73	84	81
Sep-19	104.5	102.8	95	101.5	99.1
Oct-19	106	114.2	104.63	93.66	83
Nov-19	114	112.54	103.3	102	99.3
Dec-19	124.3	113.1	103.5	102.5	91.2
Min	104.5	81.8	73	84	81
Max	124.3	114.4	117	104	99.3
Average	112.72	105.89	99.77	96.38	92.08
Standard Deviation	6.47	11.83	13.48	8.18	7.43

Appendix Table 11. Phosphate concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	Site A	Site B	Site C	Site D	Site E
Jun-19	0.3	0.2	0.4	0.87	0.71
Jul-19	0.52	0.59	0.64	0.7	0.6
Aug-19	0.12	0.6	0.5	0.6	0.55
Sep-19	0.4	0.34	0.57	0.75	0.47
Oct-19	0.45	0.82	1.3	1.6	1.4
Nov-19	1.3	1.4	1.5	2.1	1.75
Dec-19	1.4	1.3	1.6	2.2	2.1
Min	0.12	0.2	0.4	0.6	0.47
Max	1.4	1.4	1.6	2.2	2.1
Average	0.64	0.75	0.93	1.26	1.08
Standard Deviation	0.5	0.45	0.51	0.69	0.65

Appendix Table 12. Ammonia concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	Site A	Site B	Site C	Site D	Site E
Jun-19	33.4	16.2	13.2	12	13.4
Jul-19	17.2	15.1	15.4	15.1	10
Aug-19	26.3	22.3	15.4	11.3	7.1
Sep-19	25.3	23.3	16.7	18.4	11
Oct-19	24.5	12.1	15.9	12.4	9.7
Nov-19	14.9	9.4	10.5	8.4	6.4
Dec-19	15.3	11.3	13	14.6	10.5
Min	14.9	9.4	10.5	8.4	6.4
Max	33.4	23.3	16.7	18.4	13.4
Average	22.41	15.67	14.3	13.17	9.72
Standard Deviation	6.86	5.38	2.16	3.19	2.37

Appendix Table 13. Mercury concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	Point A	Point B	Point C	Point D	Point E
Jun-19	0.03	0.02	0.015	0.004	0.02
Jul-19	0.03	0.015	0.01	0	0.01
Aug-19	0.015	0.01	0.01	0	0.01
Sep-19	0.01	0.01	0	0	0.01
Oct-19	0.02	0.018	0.015	0.001	0.01
Nov-19	0.035	0.03	0.025	0.014	0.01
Dec-19	0.04	0.025	0.015	0.01	0.15
Min	0.01	0.01	0	0	0.01
Max	0.04	0.03	0.025	0.014	0.15
Average	0.025714	0.018	0.012	0.004	0.031
Standard Deviation	0.000045	0.000064	0.000035	0.000053	0.0000064

Appendix Table 14. Copper concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	Site A	Site B	Site C	Site D	Site E
Jun-19	0.0024	0.0015	0.001	0.001	0.001

Jul-19	0.0017	0.0014	0.0012	0.0012	0.0013
Aug-19	0.0015	0.0018	0.0011	0.0013	0.0018
Sep-19	0.002	0.0018	0.0015	0.0014	0.0013
Oct-19	0.0025	0.0014	0.0013	0.0023	0.0023
Nov-19	0.0027	0.0026	0.0022	0.0027	0.0023
Dec-19	0.0035	0.0029	0.0026	0.0027	0.0025
Min	0.015	0.014	0.01	0.01	0.01
Max	0.035	0.029	0.026	0.027	0.025
Average	0.023	0.019	0.015	0.018	0.017
Standard Deviation	0.00067	0.0006	0.0006	0.0007	0.0006

Appendix Table 15. Zinc concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	**Site A**	**Site B**	**Site C**	**Site D**	**Site E**
Jun-19	0.052	0.049	0.045	0.01	0.015
Jul-19	0.025	0.024	0.012	0.01	0.012
Aug-19	0.015	0.01	0.009	0.008	0.012
Sep-19	0.012	0.012	0.011	0.009	0.013
Oct-19	0.023	0.019	0.017	0.001	0.016
Nov-19	0.092	0.089	0.072	0.001	0.067
Dec-19	0.098	0.087	0.077	0.1	0.054
Min	0.012	0.01	0.009	0.001	0.012
Max	0.098	0.089	0.077	0.1	0.067
Average	0.045	0.041	0.034	0.019	0.027
Standard Deviation	0.04	0.03	0.03	0.35	0.023

Appendix Table 16. Iron concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	**Site A**	**Site B**	**Site C**	**Site D**	**Site E**
Jun-19	0.15	0.12	0.09	0.05	0.05
Jul-19	0.15	0.14	0.07	0.04	0.12
Aug-19	0.21	0.13	0.15	0.11	0.13

Sep-19	0.18	0.13	0.14	0.13	0.15
Oct-19	0.17	0.12	0.06	0.016	0.014
Nov-19	0.39	0.34	0.31	0.25	0.33
Dec-19	0.25	0.71	0.45	0.31	0.35
Min	0.15	0.12	0.06	0.016	0.014
Max	0.39	0.71	0.45	0.31	0.35
Average	0.214	0.226	0.166	0.115	0.144
Standard deviation	0.085	0.22	0.15	0.11	0.13

Appendix Table 17. Manganese concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	Site A	Site B	Site C	Site D	Site E
Jun-19	0.065	0.055	0.05	0.034	0.053
Jul-19	0.053	0.051	0.043	0.024	0.036
Aug-19	0.044	0.042	0.039	0.022	0.033
Sep-19	0.041	0.038	0.035	0.025	0.03
Oct-19	0.046	0.041	0.035	0.023	0.032
Nov-19	0.13	0.125	0.127	0.047	0.123
Dec-19	0.19	0.179	0.15	0.05	0.121
Min	0.041	0.038	0.035	0.022	0.03
Max	0.19	0.179	0.15	0.047	0.123
Average	0.0812	0.075	0.068	0.031	0.061
Standard deviation	0.057	0.054	0.048	0.01	0.042

Appendix Table 18. Total Coliforms counts concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	Site A	Site B	Site C	Site D	Site E
Jun-19	0.4×10^3	0.55×10^3	0.65×10^3	0.735×10^3	0.8×10^3
Jul-19	0.45×10^3	0.45×10^3	0.535×10^3	0.633×10^3	0.75×10^3
Aug-19	0.4×10^3	0.45×10^3	0.5×10^3	0.65×10^3	0.68×10^3
Sep-19	0.35×10^3	0.36×10^3	0.57×10^3	0.8×10^3	1.13×10^3

Oct-19	0.45×10^3	0.55×10^3	0.72×10^3	0.98×10^3	1.05×10^3
Nov-19	1.05×10^3	2.34×10^3	2.4×10^3	2.88×10^3	2.99×10^3
Dec-19	1.2×10^3	2.35×10^3	2.15×10^3	4.15×10^3	4.2×10^3
Min	0.35×10^3	$0,36 \times 10^3$	0.5×10^3	0.63×10^3	0.68×10^3
Max	1.2×10^3	2.35×10^3	2.4×10^3	4.15×10^3	$4,2 \times 10^3$
Average	0.61×10^3	1.007×10^3	1.07×10^3	1.54×10^3	1.65×10^3
Standard Deviation	$0.35 X10^3$	0.92×10^3	0.83×10^3	1.4×10^3	1.38×10^3

Appendix Table 19. Faecal Coliforms counts concentration data and standard deviation during the study period (June - December 2019) for the Naauwpoortspruit River.

Date	Site A	Site B	Site C	Site D	Site E
Jun-19	0.5×10^3	1.65×10^3	1.25×10^3	1.10×10^3	1.05×10^3
Jul-19	0.7×10^3	1.20×10^3	1.15×10^3	1.05×10^3	1.15×10^3
Aug-19	0.55×10^3	0.63×10^3	0.3×10^3	0.4×10^3	0.3×10^3
Sep-19	0.25×10^3	0.58×10^3	0.5×10^3	0.6×10^3	0.7×10^3
Oct-19	0.32×10^3	0.35×10^3	0.37×10^3	0.4×10^3	0.58×10^3
Nov-19	0.8×10^3	2.1×10^3	2.2×10^3	3.4×10^3	4.8×10^3
Dec-19	0.9×10^3	2.2×10^3	2.5×10^3	3.35×10^3	5.4×10^3
Min	0.25×10^3	0.35×10^3	0.3×10^3	0.4×10^3	0.3×10^3
Max	0.9×10^3	2.2×10^3	2.5×10^3	3.4×10^3	5.4×10^3
Average	0.57×10^3	1.24×10^3	1.18×10^3	1.47×10^3	1.99×10^3
Standard Deviation	$0.24 X10^3$	$0.76 X10^3$	0.78×10^3	1.33×10^3	1.15×10^3

Appendix Table 20. Heterotrophic plate counts concentration data and standard deviation during the study period (June- December 2019) for the Naauwpoortspruit River.

Date	Site A	Site B	Site C	Site D	Site E

Jun-19	0.9 $x10^6$	1.14 $x10^6$	1.2 $x10^6$	1.45 $x10^6$	1.5 $x10^6$
Jul-19	0.95 $x10^6$	1.35 $x10^6$	1.2 $x10^6$	1.35 $x10^6$	1.56 $x10^6$
Aug-19	0.915 $x10^6$	1.12 $x10^6$	1.2 $x10^6$	1.5 $x10^6$	1.67 $x10^6$
Sep-19	0.8 $x10^6$	0.95 $x10^6$	1.1 $x10^6$	1.4 $x10^6$	1.8 $x10^6$
Oct-19	1.5 $x10^6$	2 $x10^6$	2.15 $x10^6$	2.8 $x10^6$	3.8 $x10^6$
Nov-19	5.12 $x10^6$	5.18 $x10^6$	7.23 $x10^6$	9.2 $x10^6$	9.9 $x10^6$
Dec-19	5.3 $x10^6$	5.3$x10^6$	7.2 $x10^6$	8.5 $x10^6$	9.7 $x10^6$
Min	0.8 $x10^6$	0.95 $x10^6$	1.1 $x10^6$	1.35 $x10^6$	1.5 $x10^6$
Max	5.3 $x10^6$	5.3 $x10^6$	7.23 $x10^6$	9.2 $x10^6$	9.9 $x10^6$
Average	2.212 $x10^6$	2.434 $x10^6$	3.04 $x10^6$	3.742 $x10^6$	4.275 $x10^6$
Standard Deviation	2.06 $x10^6$	1.95 $x10^6$	2.87 $x10^6$	3.53$x10^6$	3.86 $x10^6$

Appendix Table 21. *E. coli* counts concentration data and standard deviation during the study period (June- December 2019) for the Naauwpoortspruit River.

Date	**Site A**	**Site B**	**Site C**	**Site D**	**Site E**
Jun-19	0.3 x 10^3	0.48 x 10^3	0.45 x 10^3	0.6 x 10^3	0.7 x 10^3
Jul-19	0.6 x 10^3	0.56 x 10^3	0.6 x 10^3	0.9 x 10^3	1.15 x 10^3
Aug-19	0.3 x 10^3	0.2 x 10^3	0.1 x 10^3	0.6 x 10^3	0.3 x 10^3
Sep-19	0.2 x 10^3	0.1 x 10^3	0.1 x 10^3	0.3 x 10^3	0.2 x 10^3
Oct-19	0.3 x 10^3	0.1 x 10^3	0.2 x 10^3	0.4 x 10^3	0.3 x 10^3
Nov-19	0.6 x 10^3	1.1 x 10^3	1.6 x 10^3	2.1 x 10^3	2.7 x 10^3
Dec-19	1.2 x 10^3	1.6 x 10^3	1.65 $x10^3$	1.7 x 10^3	2.1 x 10^3
Min	0.2 x 10^3	0.1 x 10^3	0.1 $x10^3$	0.3 x 10^3	0.2 x 10^3
Max	1.2 x 10^3	1.6 x 10^3	1.65 $x10^3$	2.1 x 10^3	2.7 x 10^3
Average	0.5 x 10^3	0.591 $x10^3$	0.657$x10^3$	0.942$x10^3$	1.064$x10^3$
Standard Deviation	0.35 x 10^3	0.57 x 10^3	0.69 x 10^3	0.69 x 10^3	0.98 x 10^3

Appendix Table 22. Antibiotic resistance profile of Faecal Coliforms from the different sampling sites to the individual antibiotics.

Antibiotic Resistance Index (ARI)	**Site**	**Site B**	**Site C**	**Site D**	**Site E**

% Resistance to;					
kanamycin	80	82	80	85	75
streptomycin	75	78	75	74	72
chloramphenicol	60	64	61	62	57
ampicillin	56	59	55	57	53
erythromycin	45	41	40	44	40
norfloxacin	38	32	37	38	35
Ox tetracycline	32	34	33	35	30

Appendix Table 23. Antibiotic resistance profile of *E. coli* from the different sampling sites to the individual antibiotics.

Antibiotic Resistance Index (ARI)	**Site**	**Site B**	**Site C**	**Site D**	**Site E**
% Resistance to;					
kanamycin	60	62	60	65	55
streptomycin	58	56	58	60	54
chloramphenicol	52	56	53	52	50
ampicillin	45	43	44	46	42
erythromycin	45	45	40	41	40
norfloxacin	38	42	38	38	35
Ox tetracycline	32	31	29	34	30

Appendix Table 24. Correlation pattern of physical characteristics for the Naauwpoortspruit River.

Physicochemical Parameters (t-test)	**pH**	**EC**	**TDS**	**COD**	**BOD**
pH	1				
EC	0.001	1			
TDS	0. 018	0.11	1		
COD	0.00008	0.397	0.459	1	
BOD	0.0008	-0.703	0.90	0.525	1

Appendix Table 25. Correlation pattern of chemical characteristics for the Naauwpoortspruit River.

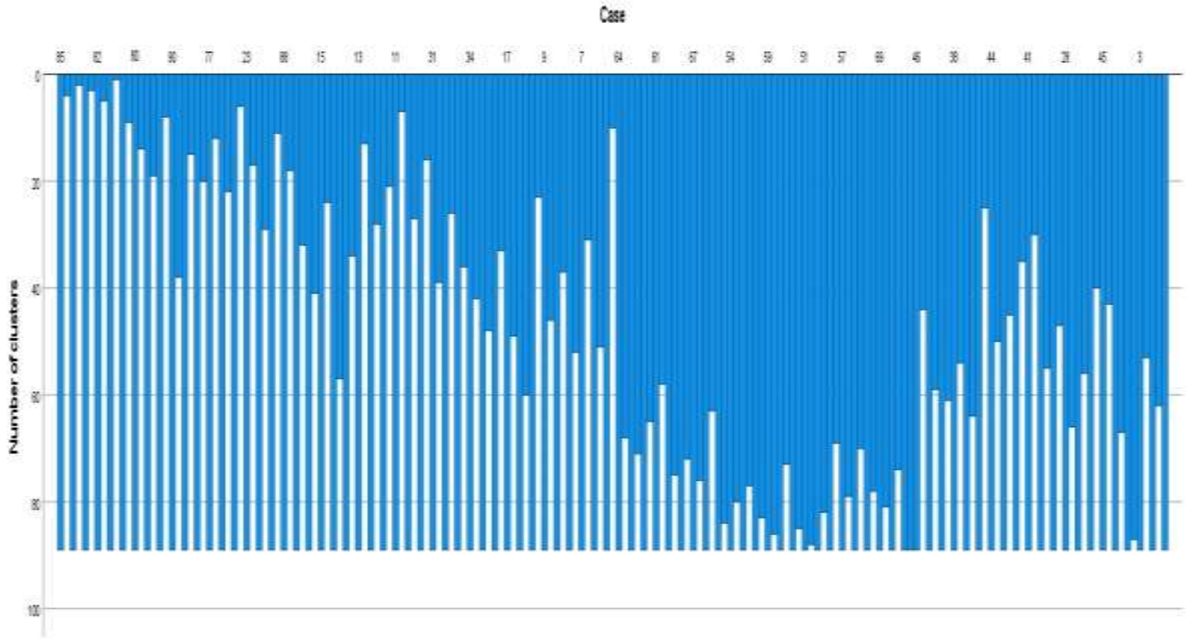
Chemical Parameters	Nitrate	Sulphate	Phosphates	Ammonia
Nitrate	1			
Sulphate	0.523	1		
Phosphate	0.981	0,535	1	
Ammonia	-0.816	-0.444	-0.850	1

Appendix Table 26. Correlation pattern of heavy metals characteristics for the Naauwpoortspruit River.

Heavy metals Parameters	Mercury	Copper	Zinc	Iron	Manganese
Mercury	1				
Copper	0.68	1			
Zinc	0.035	0,038	1		
Iron	0.015	0.015	0.533	1	
Manganese	0.019	0.018	0.416	0.957	1

Appendix Table 27. Correlation pattern of microbial characteristics for the Naauwpoortspruit River.

Microbial Parameters	Faecal Coliforms	Total Coliforms	HPC	***E. coli***
Faecal Coliforms	1			
Total Coliforms	0.69	1		
HPC	0.079	0.42	1	
E. coli	0.049	0.04	0.039	1



Appendix Figure 1. Shows cases of hierarchical Cluster analysis cases from different sites of Naauwpoortspruit River from June – December 2019).

Appendix C : Ethics Clearance

UNISA university of south africa

CAES HEALTH RESEARCH ETHICS COMMITTEE

Date: 03/12/2018

Dear Mr Mudau

NHREC Registration # : REC-170616-051
REC Reference # : 2018/CAES/156
Name : Mr KL Mudau
Student #: 50173065

Decision: Ethics Approval from 01/12/2018 to 30/11/2019

Researcher(s): Mr KL Mudau
khuthadzom152@gmail.com

Supervisor (s): Prof M Tekere
tekerm@unisa.ac.za; 011-471-2270

Working title of research:

An assessment of water quality and occurrence of antibiotic resistant bacteria at Naauwpoortspruit River, Mpumalanga Province, South Africa

Qualification: MSc Environmental Management

Thank you for the application for research ethics clearance by the CAES Health Research Ethics Committee for the above mentioned research. Ethics approval is granted for a one-year period. After one year the researcher is required to submit a progress report, upon which the ethics clearance may be renewed for another year.

Due date for progress report: 30 November 2019

Please note the points below for further action:

1. How many times will samples be collected at each point?
2. Are any of the sampling points situated on privately owned land? If so, permission must be obtained to access these and submitted to the committee before sample collection may commence.
3. Which laboratory facility will be used? If it is not the Unisa laboratories, permission must be obtained and submitted to the committee before laboratory analysis may commence.

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*The **low risk application** was **reviewed** by the CAES Health Research Ethics Committee on 29 November 2018 in compliance with the Unisa Policy on Research Ethics and the Standard Operating Procedure on Research Ethics Risk Assessment.*

The proposed research may now commence with the provisions that:

1. The researcher(s) will ensure that the research project adheres to the values and principles expressed in the UNISA Policy on Research Ethics.
2. Any adverse circumstance arising in the undertaking of the research project that is relevant to the ethicality of the study should be communicated in writing to the Committee.
3. The researcher(s) will conduct the study according to the methods and procedures set out in the approved application.
4. Any changes that can affect the study-related risks for the research participants, particularly in terms of assurances made with regards to the protection of participants' privacy and the confidentiality of the data, should be reported to the Committee in writing, accompanied by a progress report.
5. The researcher will ensure that the research project adheres to any applicable national legislation, professional codes of conduct, institutional guidelines and scientific standards relevant to the specific field of study. Adherence to the following South African legislation is important, if applicable: Protection of Personal Information Act, no 4 of 2013; Children's act no 38 of 2005 and the National Health Act, no 61 of 2003.
6. Only de-identified research data may be used for secondary research purposes in future on condition that the research objectives are similar to those of the original research. Secondary use of identifiable human research data require additional ethics clearance.
7. No field work activities may continue after the expiry date. Submission of a completed research ethics progress report will constitute an application for renewal of Ethics Research Committee approval.

Note:

*The reference number **2018/CAES/156** should be clearly indicated on all forms of communication with the intended research participants, as well as with the Committee.*

Yours sincerely,

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