Effects of organic and inorganic pollutants on the quality of river water and evaluation of possible negative effects on human health in the Eastern Cape Province

A thesis submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE (PHARMACY)

RHODES UNIVERSITY

Where leaders learn

By

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Dedication

To God, Jesus and the holy spirit

Declaration

I declare that this thesis is my work and I have prepared it alone. This thesis is being submitted in fulfillment of the Degree of Master of Science in Pharmaceutical Chemistry to Rhodes University, Grahamstown, South Africa. This thesis has never been submitted before for any degree in any other institution.

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Abstract

The quality of river water has been gradually decreasing for the past years due to pollution by faecal pathogens, organic and inorganic contaminants. This has caused environmental concern which led to the creation of a large area of research in many countries. In this study, the quality of river water was monitored by measuring physicochemical properties, bacteriological quality, screening of pharmaceuticals and biomonitoring water quality using aquatic macroinvertebrates. The physical parameters indicated the following; chemical oxygen demand ranged from 6 to 45 mg/L, turbidity ranged from 0.00 to 718 NTU, pH ranged from 5.89 to 9.77 and electric conductivity ranged from 0.00 to 2.95 mS. The concentrations of chemical parameters included phosphate ranged from 0.00 to 16 mg/L, chloride ranged from 80 to 518 mg/L, ammonium ranged from 5 to 279 mg/L, nitrate ranged from 0 to 50 mg/L, nitrite ranged from negative to positive, iron concentration was 0 mg/L in all the rivers throughout the sampling seasons while sulphate ranged from 5 to 103 mg/L. The bacteriological quality of river water ranged as follows; total coliform ranged from 0.20 to 255682 cfu/100 mL, faecal coliform ranged from 0.02 to 1091 cfu/100 mL and heterotrophic bacteria ranged from 213 to 1153543 cfu/mL. Several bacterial species were identified with analytical profile index kit with *Klebsiella oxytoca*, *Vibrio alginolyticus*, *Providencia stuartii*, *Ewingella Americana*, *Providencia alcalifaciens/ rustigianii*, *Enterobacter cloacae*, *Leclercia adecarboxylata*, *Proteus penneri*, *Aeromonas salmonicida ssp salmonicida* and *Escherichia coli* being the most dominant pathogenic bacteria present in the rivers. Pharmaceuticals screening was conducted using enzyme-linked immunoassay kits with fluoroquinolones detected in a concentration range of lower than detectable limits (LDL) to 0.4 ppb while sulfamethoxazole was in a concentration range of LDL to 1.4 ppb. Pre-concentration and extraction of the target pharmaceuticals in water samples were conducted by lyophilization followed by solid-phase extraction on Water Oasis HLB cartridge and ultra-performance liquid chromatography- electron spray ionization tandem mass spectrometry was used for detection of the compounds. The antibiotics detected were sulfamethoxazole at a concentration range of LDL to 3484 ng/0.5 L, clarithromycin at 2.4 to 1640.2 ng/0.5 L and erythromycin at LDL to 372.1 µg/0.5 L and anti-epilepsy drug detected was carbamazepine at a concentration of 40.9 to 18288.1 ng/0.5 L in river water samples. Ibuprofen and ciprofloxacin were not detected. The biological quality of river water was assessed using aquatic macroinvertebrates. The presence of highly sensitive families such as Heptageniidae, Crambidae and Glossosomatidae was a confirmation that the quality of river water was in a good state on upper stream sites, while middle stream and lower stream sites were impacted.

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List of abbreviations

WHO	World health organization
DWAF	Department of Water Affairs
DEAT	Department of Environmental Affairs and Tourism
MDB	Municipal Demarcation Board
UNICEF	United Nation International Children's Emergency Fund
WWTP	Wastewater Treatment Plant
STP	Sewage Treatment Plant
CSIR	Council for Scientific and Industrial Research
ND	Not detected
NS	Not sampled
SPE	Solid phase extraction
GWRC	Global Water Research Coalition
SANS	South Africa National Standards
LDL	Lower than Detectable Limits
HLB	Hydrophilic-Lipophilic-Balance
ELISA	Enzyme-Linked Immunoassay
UPLC	Ultra-Performance Liquid Chromatography
ESI	Electron Spray Ionization
MS	Mass Spectrometry
RHP	River Health Program
FEPA	Federal Environment Protection Agency
USEPA	United State Environmental Protection Agency
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
LC	Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
UPLC-TOF-MS	Ultra-Performance Liquid Chromatography Time of Flight Mass Spectrometry
CE	Capillary Electrophoresis
QqQ-MS	Triple quadrupole Mass Spectrometry
SRM	Selected Reaction Monitoring

COD	Chemical oxygen demand
EC	Electric conductivity

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1. Biomonitoring of the freshwater quality using aquatic macroinvertebrates South African Journal of Aquatic Sciences

2. Detection of pharmaceutical residues in surface waters of the Eastern Cape Province International Journal of Environmental Research and Public Health

This thesis is structured as follows: for each chapter there is an introduction, literature review, aims and objectives, methods and materials, results and discussion, conclusion and references.

Chapter 1

1.0. Introduction

Water is a fundamental natural resource which humans, animals and plants need to have access to in order to survive (World Health Organisation (WHO), 2008; Khadse *et al*. 2012). Water plays a fundamental role in the survival, growth and maintenance of a health life in all living organisms as it is used to transport nutrients, carry out biochemical processes and facilitate excretion of waste products (Laskey, 2015). Therefore, access to water of good quality in humans, plants and animals is essential to life (WHO, 2010). In addition, water is used for domestic purposes by humans, such as cooking, cleaning and sanitation, as well as playing a crucial in agricultural and industrial processes (Abia *et al*. 2016). In developing countries such as Pakistan, Kenya, India and South Africa, rivers, dams, underground water and rainwater has been serving as a source of drinking water for many years and monitoring of the quality of water in these resources is important for protection of public health (Department of Water Affairs (DWAF), 1996b; Fenwick, 2006; Cabral, 2010).

South Africa is regarded as a water scare country due to minimal average rainfall and high evaporation rate (Mulamattathil *et al*. 2014; Abia *et al*. 2016). South Africa has an annual rainfall of 450 mm which is lower than the world average rainfall of 860 mm. The annual average rainfall in South Africa varies in each season with maximum rainfall attained in summer season. Nevertheless, the maximum average rainfall in south-western regions of the country is obtained in winter season (Botai *et al*. 2018). The minimal rainfalls in conjunction with high evaporation rate and rising water demand due to increasing human population, industrialisation, and agricultural activities are the main causes that the water levels in water resources are depleting (Liang *et al*. 2013; Abia *et al*. 2016). In addition, the decrease in the quality of water in water resources due to contamination is one of the main factors which lead to limited water supply (Olaniran *et al*. 2013).

It is estimated people who lack access to safe drinking water worldwide are 768 million (UNICEF/WHO, 2013). South Africa has an estimated population of 57.7 million people (Stats South Africa, 2018). Approximately 5 million people lack access to safe drinking water in South Africa (Momba *et al*. 2013; Wanda *et al*. 2016). This high percentage of population without access to safe drinking water causes people to rely on untreated river water, underground water and rainwater as their source of drinking water (DWAF, 1996b; Edokpayi *et al*. 2015; Abia *et al*. 2016). The challenge with these water resources lies on the quality of water and absence of sufficient treatment before use, thus putting the health of individuals at risk (Cabral, 2010; Wanda *et al*. 2016). The most affected individuals are those who live in rural areas or informal settlements (Olaniran *et al*. 2013; Wanda *et*

al. 2016). Eastern Cape Province is the third largest Province in the country with population size of approximately 7 million people (Stats South Africa, 2016). The population of each district is presented in table 1.

Table 1. 1: Human population in the Eastern Cape Province categorised by each district. (Stats South Africa, 2016)

Province	District	Population size
Eastern Cape Province	Buffalo City	368 520
	OR Tambo	313 889
	Nelson Mandela bay	247 759
	Amatole	222 415
	Alfred Nzo	195 979
	Chris Hani	191 356
	Sarah Baartman	138 182
	Joe Gqabi	95 294

In 2016 it was estimated that approximately 75% of the households residing in the Eastern Cape Province had access to piped water supply with population of about 928 332 having access to adequate sanitation (Stats South Africa, 2016). The Eastern Cape Province is surrounded by many rural areas and access to adequate quality water supply in these rural areas is a problem. According to Department of Environmental Affairs and Tourism (DEAT), the small percentage of South Africans with lack of access to tap water supply was observed in rural areas of the Eastern Cape Province (Municipal Demarcation Board (MDB) 2010). This forces the individuals who live in those rural areas to use untreated river water and underground water as their source of drinking water resulting in illness and deaths (DWAF, 1996b; Schaefer, 2008; UNICEF/ WHO, 2009).

For more than three decades aquatic environments have been polluted by pharmaceutically active compounds (Gros *et al*. 2006; López-Roldán et al. 2010; Afonso-Olivares *et al*. 2013; Madikizela *et al*. 2017). A variety of pharmaceutical residues have been detected in surface water, underground water and drinking water in concentration range of ng/L to µg/L (Dinha *et al.* 2011; González *et al*. 2010; Sim *et al*. 2010; Yiruhan *et al*. 2010; Li *et al*. 2009; Tamtam *et al*. 2008). Pharmaceuticals are important chemicals used for maintaining public health and improve lives of individuals having communicable and chronic illnesses. Various pharmaceuticals are used to prevent or treat diseases in both humans and animals. Amongst pharmaceuticals used, antibiotics are the most concerning since they are used in large quantities and are continuously present in water and soil. Antimicrobials are group of drugs

(antibiotics, antivirals, antifungals and antiprotozoals) used to eliminate or prevent the growth of microorganisms (such as bacteria, viruses, fungi, archaea, protozoa and microalgae) (Brandt *et al*. 2015; Grenni *et al*. 2018).

The global use of antibiotics exceeds 90 718 474 kg every year and the concern of the consequence of these drugs in the environment has increased for past years. Most of these drugs are stable and are therefore are able to bypass the Wastewater Treatment Plant (WWTP) processes and get discharged into aquatic environments (Kümmerer, 2009; Danner *et al*. 2019). Large application of antibiotics in the treatment of pathogenic infections and improvement production yield in agriculture causes antibiotics to be omnipresent in the environment. Although antibiotic use benefits human and animal health its continuous presence in the environment pose undesirable effects in non-targeted organisms following exposure. In the environment antibiotics disturbs the microbial communities and results in the alteration of nutrient cycling and/or degradation of pollutants (Flaherty and Dodson, 2005; Kaushal, 2009; Danner *et al*. 2019) and have potential of causing changes in the microbial genetics thus leading to the development of antibiotic resistant genes (Singer *et al*. 2016; Danner *et al*. 2019). Antibiotic resistance poses risk in human and animal health (Bruhn, 2003; Kümmerer, 2009; Pawlowski *et al*. 2016; Grenni *et al*. 2018).

Approximately 50-80% of the ingested antibiotics are excreted by the body via faeces and urine as parent compounds as well as metabolites. High excretion rate has been reported for some antibiotics such as tetracycline (80-90%) and ciprofloxacin (50-80%) while for erythromycin (5-10%), sulfamethoxazole (15-30%) and clarithromycin (25%) low excretion rate has been observed (Mompelat *et al*. 2009, Danner *et al*. 2019). In WWTPs antibiotics are eliminated through adsorption by sludge, biological and non-biological degradation processes. The challenge lies with the degradation processes being ineffective for other antibiotics since WWTPs were not designed for removal of pharmaceuticals and therefore are released with the treated effluents into environmental waters such as rivers and streams and thus contaminating surface waters (Monteiro and Boxall, 2015; Grenni *et al*. 2018).

Antibiotics are present in noticeable concentrations in aquatic environments for example ofloxacin and ciprofloxacin has been reported in hospital effluents close to the Ter River in Spain with concentration above 13 µg/L (Rodriguez-Mozaz *et al*. 2015). In Sweden, ciprofloxacin and erythromycin were detected in concentrations of 1.41 µg/L and 0.47 µg/L in sewage influents while in treated effluents 0.06 µg/L and 0.35 µg/L were observed (Östman *et al*. 2017). While erythromycin concentrations of 260 ng/L have been detected in Umgeni River in South Africa (Matongo *et al.* 2015). Ampicillin and

ciprofloxacin were detected in concentration of 9 µg/L and 27 µg/L respectively in WWTP effluents of Msunduzi River in South Africa (Agunbiade and Moodley, 2016). Ciprofloxacin and nalidixic acid have been detected in South Africa in concentrations of 23 µg/L and 14 µg/L respectively (Agunbiade and Moodley, 2016). Approximately 10 µg/L of sulfamethoxazole and sulfapyridine were detected in surface water in Spain (Díaz-Cruz *et al*. 2008; Ginebreda *et al*. 2010) while in Umgeni River in South Africa concentration range of up to 1240 ng/L were detected (Matongo *et al*. 2015).

The detection of organic and inorganic contaminants in aquatic environments have been documented (Gros *et al*. 2006; Afonso-Olivares *et al*. 2013; Olaniran *et al*. 2013; López-Roldán *et al*. 2010; da Silva and Oliveira, 2018). Their presence in aquatic environments alters the physicochemical properties of water and causes decrease in the quality of water (Olaniran *et al*. 2013). Changes in physicochemical properties of water can negatively affect the aquatic biodiversity and human health. For instance, the presence of nitrates causes development of algae blooms in aquatic environments and results in methaemoglobin in humans thus decreasing oxygen levels (Davie, 2003; Nas and Berktay, 2006; Gupta *et al*. 2017). While the changes in pH, temperature, dissolved oxygen and salinity in aquatic environments affects the biochemical processes such as photosynthesis, cycling of nutrients and respiration (Olaniran *et al*. 2013). The presence of pharmaceuticals such as carbamazepine, ibuprofen, diclofenac and estrogen have toxicological effects on human and animal health respectively (Triebskorn *et al*. 2007; López-Roldán *et al*. 2010; Ramaswamya *et al*. 2011). In addition, heavy metals in aquatic environments pose toxic effects in aquatic life and humans following exposure (Olaniran *et al*. 2013).

According to the toxicology studies on eukaryotic algae, amoxicillin was found to be highly sensitive on *Synechococcus leopolensis* while clarithromycin was highly sensitive on *Pseudokirchneriella subcapitata* as they were found toxic at a concentration of 2 µg/L (Andreozzi *et al*. 2004; Isidori *et al*. 2005). Ciprofloxacin was reported to inhibit the growth of cynobacteria *Mycrocystis aeruginosa* at 5 µg/L and 17 µg/L (Halling-Sørensen *et al*. 2000; Robinson *et al*. 2005). In surface waters ciprofloxacin within the same concentration range was found in South Africa, 14.3 µg/L (Agunbiade and Moodley, 2016) and 9.66 µg/L in France (Feitosa-Felizzola and Chiron, 2009). Ofloxacin was reported to pose ability of inhibiting the growth of bacterium *Vibrio fischeri* at a concentration of 0.9 µg/L and algae *Pseudokirchnella subcapitata* at a concentration of 4.74 µg/L (aus der Beek *et al*. 2016). Ofloxacin have been reported in concentration of 17.7 µg/L in Asia (aus der Beek *et al*. 2016) while in Spain concentration of 8.7 µg/L was reported (Ginebreda *et al*. 2010). Antibiotics such as erythromycin, norfloxacin, chloramphenicol, oxytetracycline, streptomycin, and tylosin are usually present in surface

waters at concentrations higher than 1 µg/L and are regarded one of the antibiotics that are very toxic to aquatic life (European Commission, 1996; Petrie *et al*. 2014).

Pathogenic microorganisms have been detected in aquatic environments and their presence has negative effects on human health as they lead to waterborne diseases (Paulse *et al*. 2012; Abia *et al*. 2016). The main waterborne diseases include cholera, gastroenteritis, typhoid fever, other serious salmonellosis, bacillary dysentery or shigellosis, sever diarrhoeas and gastroenteritis. The causative organisms of waterborne disease are *Vibrio cholerae, Vibrio parahaemolyticus, Salmonella enterica sp*. *Shigella dysenteriae, Shigella flexneri, Shigella bodydii* and *Shigella sonnei*, and *Escherichia coli* (Cabral, 2010).

The danger of microbial infection is linked to consumption of water polluted by human or/and animal faeces. The discharge of wastewater by WWTPs are the main sources of faecal coliforms together with pathogens in aquatic systems. The people who are affected by microbial diarrheal diseases are usually those that are financially disadvantaged with lack of adequate sanitation services. This is one of the major contributing factors to waterborne diseases for children under the age of 5 in developing countries such as Asia and African countries (UNICEF/WHO, 2009; Cabral, 2010; Edokpayi *et al*. 2015). The Council for Scientific and Industrial Research (CSIR) reported that diarrhoea is a third killing disease in South Africa and does not only affect children but also adults who are between the age of 45 to 65 years (CSIR, 2015; Edokpayi *et al*. 2015). Inadequate sanitation and shortage of access to clean and safe drinking water is one of the major causes of waterborne diseases and deaths (Prüss *et al*. 2002; WHO, 2009; Chigor *et al*. 2013). Nevertheless, people from developed countries such as USA also suffer from microbial water transmitted illnesses (Cabral, 2010). An outbreak of waterborne diseases in USA reported between 2013 and 2014 resulted in 289 cases of sicknesses with 108 cases of hospitalization and 17 deaths (Hawthorne, 2018)

Continuous pollution of the aquatic systems by pathogens, pharmaceuticals, inorganic contaminants, pesticides and heavy metals have been well documented by several authors (Fatta-Kassinos *et al*. 2011; Afonso-Olivares *et al*. 2013; Olaniran *et al*. 2013; Mulamattathil *et al*. 2014; Wanda *et al*. 2016). The sources of these pollutants into the aquatic environments includes acid rains, weathering of rocks, surface runoff in agricultural lands, discharge of treated hospital and community wastewater by the Sewage Treatment Plants (STP) into the rivers, industrialisation, increase in human population and improper disposal of waste (Suthar *et al*. 2010; Olaniran *et al*. 2013; Chigor *et al*. 2013). The presence of these pollutants in aquatic environments decreases the quality of water, affects the aquatic biodiversity and human health (Naz and Berktay, 2006; Gupta *et al*. 2017).

Numerous techniques are used for monitoring the water quality in water resources such as: biomonitoring, physicochemical testing, microbial testing, pharmaceutical screening and ecotoxicology testing (Dickens and Graham, 2002; Momba *et al*. 2013; Paulse *et al*. 2012; Abia *et al*. 2016; Wanda *et al*. 2016; Ramaswamya *et al*. 2011; Fent *et al*. 2006). Aquatic macroinvertebrates have been used in monitoring the quality of water in aquatic systems. The biomonitoring technique is based on the sensitivity of the aquatic macroinvertebrate families to water pollution. The difference in the sensitivity of macroinvertebrate families to pollution is the reason they have been used as water quality indicators (Dickens and Grams, 2002). Aquatic macroinvertebrates are important also in toxicological studies as their high sensitivity makes them useful in assessment of the quality of water and toxicity of organic and inorganic contaminants (Dickens and Grams, 2002).

Contamination of aquatic systems is a serious environmental issue that needs to be addressed for the protection of public health as these contaminants can be life-threating in both humans and animals (Mulamattathil *et al*. 2014; Liang *et al*. 2013; López-Roldán *et al*. 2010; Gros *et al*. 2006; da Silva and Oliveira, 2018; Valcárcel *et al*. 2011). Several studies reporting contamination of water in those four selected rivers have been documented and indicated that the health of people living near those rivers may be affected since river water is sometimes used as source of water for domestic purposes (Chigor *et al*. 2013; Sibanda *et al*. 2013, Odume, 2011; Odume and Mgaba, 2016). This indicates that contamination of river water is a serious environmental issue that needs to be addressed for the protection of public health. This study aims to assess the quality of river water in four selected rivers of the Eastern Cape Province by measuring the physicochemical properties of water, biological quality of water, screening for the presence of pharmaceuticals and biomonitoring of the quality of river water using aquatic macroinvertebrates. This study also aims to determine the impact of human activities towards pollution of aquatic environments during autumn, winter and spring season and assess the possible health effects that might arise by using inadequately treated river water. This study was motivated by the scarcity of water in South Africa and that rivers in rural areas are sometimes used as a source of water due to limited reliable and accessible clean and safe drinking water. Also, the limited information on the presence of antibiotics in aquatic environments in Eastern Cape Province rivers contributed on the initiation of this study. This study reports the occurrence of pharmaceutical residues (antibiotics and anti-epilepsy drug) in South African surface waters. The effectiveness of using enzyme-linked immunoassay (ELISA) kit versus ultra-performance liquid chromatography electron spray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) for screening of the presence of antibiotics in river water. The efficiency of using lyophilisation technique as part of sample preparation method during pre-concentration of pharmaceuticals in river water samples prior solid phase extraction

(SPE) process was also reported. The use of freshwater macroinvertebrates for determining the ecological state of the river and quality of water was also documented in this study.

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Chapter 2

Effects of seasonal variation on the quality of river water in Eastern Cape Province during dry season

2.0. Introduction

Water is an important natural resource which needs to be properly managed and protected to ensure the health of both humans and animals is maintained. Section 24 of the South African constitution states, "Every human has the right to an environment that in not harmful to human health or well-being". Thus, it is important that humans and animals have access to clean and safe drinking water to maintain a healthy lifestyle (Hendrick and Pool, 2012). Monitoring the quality of surface water by measuring the physicochemical properties of water is fundamental for the protection of both the environment and public health (Okoh *et al*. 2007; Chigor *et al*. 2013) since river water is used for domestic purposes, irrigation and livestock watering (Ramakrishnaiah *et al*. 2009; Jindal and Sharma, 2011; Olaniran *et al*. 2013; Gupta *et al*. 2017). South Africa is water scarce and one of the major causes to limited drinking water supply is the decrease in the quality of water due to contamination caused by inorganic compounds and heavy metals, thus making the available water in water resources unfit for consumption (Olaniran *et al*. 2013).

Organic and inorganic pollution in aquatic systems alters the physicochemical properties of water and results in the decrease in water quality. The biochemical processes in the aquatic environments have been reported to be affected by the physicochemical parameters such as temperature, pH, dissolved oxygen, salinity, and nutrient loading. The changes in these parameters in aquatic environments demonstrates that the water has been disrupted in a manner that will decrease its quality thus affecting the people which uses the water for domestic purposes (Hacioglu and Dulger, 2009; Olaniran *et al*. 2013). In aquatic environments, the chemical constituents of water are influenced by the several aspects and that comprises of weathering of rock, the type of rain and average rainfall in that particular area, the chemical processes that occur between the soil and sediments and amount of pollution caused by different pollution sources (da Silva and Sacomani, 2001; Olaniran *et al*. 2013).

2.1. Factors triggering decrease in water quality

Previous studies have indicated that the quality of surface water is influenced by several factors which includes natural and anthropogenic factors such as weathering and erosion of rocks, rainfall, surface runoff, increase in human population and industrialization (Olaniran *et al*. 2013). Nevertheless, in South Africa the major source of contamination in surface waters are discharge from STPs (Suthar *et*

al. 2010; Chigor *et al.* 2013). The STPs in South Africa more especially in the Eastern Cape Province have been reported to be inefficient in removing pollutants thus they have high impact on the physicochemical quality of the surface waters (Chigor *et al.* 2013). The effluent discharge from STPs are recognized to contribute to nutrient loading in water systems and have potential of introducing pathogens that are harmful to human health (Suthar *et al*. 2010; Olaniran *et al*. 2013; Chigor *et al*. 2013). Increase in human population, industrialization, farming and effluent discharge from STPs are main causes of pollution in aquatic environments and this causes river water to be unfit for domestic, agricultural, recreational and aesthetic purposes (Olaniran *et al*. 2013).

2.2. Effects of human pollution on the quality of river water

The discharge of wastewater from STPs stimulates the growth of toxic algal blooms and that affects the aquatic life and limits food harvesting areas (Nas and Berktay, 2006; Gaw *et al*. 2014; Gupta *et al*. 2017). Human pollution in freshwater systems results in acidification which is caused by the presence of sulphur and nitrogen compounds, movement of organic compounds from soil, soil erosion and sedimentation. In addition, high levels of nitrogen and phosphorus compounds in aquatic environments results in the modification of the river flow, disrupts the habitats, introduce foreign or invasive species and can selectively remove other species (Anderson *et al*. 2008; Olaniran *et al*. 2013).

The presence of chemical contaminants from manufacturing industries and hospitals more specially the persistent chemicals in freshwater systems have been reported to affect the quality of water and has potential of affecting the public health even at minimal concentrations (McMichael *et al*. 2001; Yassi *et al*. 2001; Olaniran *et al*. 2013). The detection of toxic heavy metals (lead and cadmium) in the environment has caused great concern in environmental scientists, government agencies and health specialist since these chemicals are not vital metals that the human body can use and can be harmful to human health upon exposure (Fatoki *et al*. 2002; Olaniran *et al*. 2013). In aquatic environments the presence of lead and cadmium from weathering and abrasion of rocks and industrial pollutions has direct negative impact on the biota whereas in humans the impact is indirect. These metals have been reported to cause dermal, lung and nasal sinus cancer in humans (Olaniran *et al*. 2013).

2.3. Seasonal variation on the physicochemical parameters of river water

Seasonal variations in the concentration of inorganic substances have been reported by previous studies (Mason, 2002; Environments Canada, 2003; Ayman and Işik, 2015). High nitrate and phosphorus concentrations in drinking water have been reported in winter season as compared to the summer season. This was due to thermal stratification and during winter season water density becomes uniform,

allowing nitrate and phosphate to accumulate in the surface of the water from the deep body of the water (Ayman and Işik, 2015). Seasonal variation in the concentration of nitrates have been observed in Canada when during winter and spring season high concentration of nitrates was reported while during summer and autumn season a decrease in the concentration as a result of biological productivity and nitrate uptake by plants was reported (Environments Canada, 2003). The concentration of nitrates remains low in aquatic environments during summer season even if fertilizers are introduced due to nitrogen being used by growing plants and elevated levels of evaporation and transpiration. During winter season the decrease in transpiration and evaporation occur as nitrate is filtered from the soil as concentrations increases (Mason, 2002).

2.4. The physicochemical parameters

2.4.1.Chlorine

Naturally chlorine does not exist, however, in the environment chlorine occurs in the form of chloride which is an anion of the element chlorine. Salts consists of chlorides includes sodium chloride, potassium chloride, calcium chloride and magnesium chloride and are very soluble in water. Chloride is usually present in water at concentrations that range from few to hundreds mg/L in freshwater and about 19 800 mg/L in sea water. Sources of chlorine in surface water include sewage effluent discharge, irrigation return flows and several industrial processes (Department of Water Affairs and Forestry, 1996).

High levels of chloride in surface water used for domestic purposes can be problematic as it increases the saltiness of water, elevates corrosion rate of metals and can be lethal and affect reproduction of other sensitive aquatic plants and freshwater macroinvertebrates. High chloride concentrations have also been reported to affect microbial community assembly (DWAF, 1996b; Kaushal, 2009). The South African water quality standard for chloride is 0 – 100 mg/L where there are no health effects. The concentrations which are in a range of 100 – 600 mg/L has no health effects, however, corrosion in domestic appliances may occurs. Concentrations which are higher than 600 mg/L tend to have salty taste and cannot satisfy thirst. The health effects for chlorine are observed in concentration that are higher than 1 200 mg/L were nausea and disturbance of the electrolyte balance may arise, especially in children, where fatalities due to dehydration may occur (DWAF, 1996b; Kaushal, 2009). The federal agencies in the United States and Canada recommends that chloride should not exceed 250 mg/L in surface waters, and this is for protection of both aquatic organisms and human health (Kaushal, 2009). In surface waters, chloride concentration of 1000 mg/L has been reported to have lethal effects on aquatic plants and freshwater macroinvertebrates (Kaushal, 2009).

Chlorination is one of the traditional methods commonly used in WWTP to treat wastewater as it can reduce levels of microorganisms in wastewater before discharge. However, the presence of chloride in aquatic environments can cause development of toxic by-products that can have negative impact on sensitive aquatic organisms such as mayflies and stone flies at concentration of 1000 mg/L (Brungs, 1973; Kaushal, 2009; Hendrick and Pool 2012).

2.4.2. Ammonium

Ammonium occurs in form of ammonium ions at neutral and low pH solutions whereas at high pH solutions it occurs in the form of a gas, which can be emitted into the atmosphere. Ammonium can be converted to nitrite by ammonification process and nitrite pose toxicological effects in infants (DWAF, 1996b). In the body nitrite can combine with haemoglobin, and results in the formation of methaemoglobin, which is not able to carry oxygen (Mason, 2002). This is dangerous in infants under three months and it may become critical when intake of vitamin C is not sufficient (DWAF, 1996b). Concentrations which are lower than 0.2 mg/L of ammonium nitrogen are usually reported in surface waters that are not contaminated by organic wastes. Nevertheless, concentration which are higher than 10 mg/L have been reported in raw untreated sewage. Under anaerobic conditions where organic decomposition occurs, higher levels of ammonium have also been reported. Ammonia and nitrites are sources of nitrogen in plants and stimulates growth. Ammonium salts are commonly used as fertilizers in agriculture and therefore their presence in agricultural runoff has been reported (DWAF, 1996b).

According to South African Water Quality guidelines, the concentrations of ammonia that has been reported in drinking water pose no toxicological effects in humans. However, ammonia tends to have adverse effects on water at higher concentrations as can affect the disinfection of water and promotes the development of nitrite in distribution systems which can result in the alteration in the odor and taste of water when concentration is higher than 1.5 mg/L. The toxicity of ammonia is associated with pH as it tends to be more toxic at alkaline conditions as compared to neutral conditions whereas no toxicity has been reported under acidic conditions. For domestic use the target water quality range for ammonia is 0 to 1.0 mg/L where ammonia poses not health or aesthetic concern. Concentrations higher than 10.0 mg/L are hazardous in domestic waters as can induce the formation of nitrites and critically affects disinfection by chlorination (DWAF, 1996b).

2.4.3. Nitrate

Nitrate comes from the end product of ammonium or nitrite. Nitrate (NO_3^-) and nitrite (NO_2^-) are the oxoanions of nitrogen which is found in +V and +III oxidation state (Department of Water Affairs and Forestry, 1996). In the environment, nitrates and nitrites co-exists and can be interconverted. In the

oxidising conditions, nitrite can be converted to nitrate which is the most stable positive oxidation state of nitrogen. Reduction of the nitrate concentration in the environment is achieved by denitrification process carried out by bacteria such as *Pseudomonas, Micrococcus* and *Bacillus* where nitrate is converted to nitrite then molecular nitrogen before it is released as nitrogen gas into the atmosphere. The temperature has effect on denitrification process as the rate of denitrification in the environment increases with increasing temperature (Cavari and Phelps, 1977; Holmes *et al.* 1996).

The presence of nitrate in drinking water causes health risk as it can be converted to nitrite due to bacterial reduction in gastrointestinal tract. In aquatic environments nitrate is present in concentration less than 5 mg/L of nitrate-nitrogen or 22 mg/L of nitrate. The sources of nitrate in water resources includes oxidation of vegetable and animal debris, human and animal wastes. High levels of nitrates are reported in treated sewage waste. High nitrate concentration is usually detected in groundwater sources due to agricultural and urban runoff. The presence of nitrate and phosphate in the environment stimulates plant growth whereas in aquatic systems it stimulates the growth of algae and results in the presence of algae blooms, it also decreases the concentration of dissolved oxygen thus affecting aquatic life (DWAF, 1996b; Davie, 2003; Nas and Berktay, 2006; Gupta *et al*. 2017). In addition, nitrates can react with secondary and tertiary amines and amides which comes from food to form nitrosamines which are carcinogens. The target water quality range of nitrate/nitrite is 0 to 6 mg/L. In concentration of 6 to 20 mg/L of nitrate/nitrite results in health complication in children were as in adults, mucous membrane irritation occurs in concentrations higher than 20 mg/L (DWAF, 1996b; Nyamangara *et al*. 2013; Gupta *et al*. 2017).

Nitrate have less toxicological effect in aquatic organisms compared to ammonia and nitrite as have limited uptake and no main physiological effects (Camargo *et al*. 2005). Nevertheless, nitrates can cause harmful effects on aquatic organisms such as death, reduction of growth, fertility and hatching, behavioural changes as signs of stress, bent spines and other physical abnormalities (Environment Canada, 2003).

2.4.4. Sulfate

Naturally, sulfate as a component of water comes from complete breakdown of the chemical components of rock and soil such as calcium sulfate and other sulfate containing minerals which are moderately soluble in water. Sulfate salts are soluble in water while on the other hand calcium sulfate is partially soluble. Hence, addition of other sulfates in water can greatly increase the concentration of sulfates in water. According to the South African water quality standards concentration of sulfate in surface water is approximately 5 mg/L, however, in water contaminated by sulfates concentrations

higher than 100 mg/L have been reported. This is due to dissolution of sulfate mineral, acid rain and release of sulfate containing effluents in surface waters. In sea water concentration which are higher than 900 mg/L have been reported (DWAF, 1996b).

In drinking water, higher concentration of sulfate has been linked with diarrhea (which is thought to be cause by magnesium) and results in unpleasant taste of water (DWAF, 1996b; WHO, 2004). This normally occurs when sulfate concentrations are approximately or higher than 600 mg/L and the likelihoods of individuals adapting to those concentrations are higher if they are constantly exposed. The target water quality range is 0 to 200 mg/L where there is no health concern, however, in concentrations higher than 200 mg/L water tends to be salty and sensitive individuals may start experiencing diarrhea. Whereas in concentrations higher than 1 000 mg/L may be harmful to all individuals (DWAF, 1996b).

2.4.5. Phosphorus

Phosphorus occurs in the form of orthophosphate (PO_4^{3-}), metaphosphate and limited number of phosphate salts in aquatic systems. Phosphorus is essential in plant and animal growth and development (Karels and Petnkeu, 2010). Phosphorus is an important nutrient as it is involved in the formation of primary energy (adenosine triphosphate) transporter for living organisms and is involved in the formation of DNA backbone (Chapman, 1996; Davies and Day, 1998). The sources of phosphate in the environment are sediments, burst sewage pipes, wastewater discharge from STPs, surface runoff from agricultural fields, weathering of rocks, decomposition of organic material and atmospheric deposition (Karels and Petnkeu, 2010). Elevated levels of phosphate due to human activities are introduced by domestic wastewater as detergents, industrial effluents and fertilizers runoff in surface waters (Ahuja, 2009). Domestic sewage has high phosphate concentration due to detergent washing powder containing high levels of phosphate (Abel, 2002). The phosphorus that is linked with the organic and mineral elements of sediments in aquatic environments can be moved by bacteria into water column thus raising the levels (Dallas and Day, 2004). Low phosphorus level can result in decreased plant production in aquatic environments while high levels increases plant production but also have indirect detrimental effects as it stimulates the growth of toxic algae blooms. The presence of toxic algae blooms in the environment results in an anoxic condition which critically affects survival of fish and other aquatic animals (Karels and Petnkeu, 2010).

2.4.6. Iron

Iron is present in many minerals, is the most abundant element which forms 5% of the earth's crust. Iron (Fe) usually is found in three oxidation states which are Fe, ferrous iron (Fe^{2+}) and ferric iron

(Fe^{3+}), however, in the environment iron commonly occurs in the form of Fe^{3+}. In aquatic environments iron occur as dissolved Fe^{2+}, Fe^{3+} or suspended iron hydroxides (Knepper, 1981; Elinder, 1986; WHO, 2003). Iron is an essential micronutrient needed by all living organisms as it is important in various metabolic processes (National research council, 1979; WHO, 2003). Iron is found in liver, kidney, fish and green vegetables at concentration of 20 to 150 mg/kg while in red meat and egg yolk is present at concentration of 10 to 20 mg/kg. Rice, many fruits and vegetables have low concentration of iron 1 to 10 mg/kg (WHO, 2003). According to South African standards iron is usually present at concentration range of 0.001 to 0.5mg/L in uncontaminated surface waters and in sea water concentration of 0.002 mg/L are usually found (DWAF, 1996b).

The concentration of iron in aquatic environments is influenced by pH, where at neutral, alkaline pH and oxidising conditions, the dissolved iron is usually in a concentration range of µg/L but at reducing conditions ferrous iron maybe formed and high concentration can be detected in mg/L range. In acidic environments (pH 3.5) the dissolved iron can be in hundreds mg/L and this is usually reported in acid mine drainage (DWAF, 1996b).

Exposure to high iron concentration causes haemochromatosis which results in tissue damage due to accumulation of iron (WHO, 2003). Naturally in aquatic environments iron is usually present in low concentrations therefore the risk of food poisoning is rare. The target water quality range is 0 to 0.1 mg/L, health effects may occur in children and sensitive individuals at concentration range of 1 to 20 mg/L. However, at concentration higher than 30 mg/L chronic health effects such as liver damage, liver cirrhosis, pancreatic islet cell damage, diabetes, hypothyroidism, and hypogonadism may occur (DWAF, 1996b; McDowell and Sticco, 2020).

2.4.7. pH

In natural waters pH is a result of complex acid-base equilibria of different dissolved compounds such as carbon dioxide-bicarbonate-carbonate equilibrium system which is influenced by temperature (DWAF, 1996b). According to DWAF, no health effects have been linked to the pH of water. Nevertheless, harmful effects are reported at very low (acidic) and very high (alkaline) pH conditions, where solubilization of toxic heavy metals and protonation or deprotonation of other ions have been reported. Usually the pH of natural waters remains in a range of 6.5 to 8.5. Water tends to taste sour at pH lower than 6.0 and can have toxicological effects to humans due to the presence of toxic metals while at pH higher than 9.0 it tastes bitter and results in deprotonation of species such as ammonium. In addition, pH which is in a range of 3.5 to 4.5 influences the aquatic life whereas pH higher than 11 results in eye irritation and skin condition. According to the South African Water Quality guidelines

the target water quality range is 6.0 to 9.0 where it poses not harmful effects to health because of the presence of toxic metals or protonated species (DWAF, 1996b; Leo and Dekkar, 2000; Adarsh and Mahantesh, 2006; Edokpayi *et al*. 2015; Gupta *et al*. 2017). In certain catchment areas, the pH of water can be affected by the constituents of rocks and soil. Several other factors affecting the pH of water includes nutrient cycling, and release of industrial effluents, variation in temperature, acid rain, microbial activity and decomposition process (DWAF, 1996b). Slightly high average pH values have been reported in Mvudi River during dry season as compared to wet season (Edokpayi *et al*. 2015).

2.4.8.Turbidity

Turbidity is defined as the measure of the capacity of water to scatter light and an indication of the amount of suspended materials in water (DWAF, 1996b). In water turbidity occurs because of the suspension of a mixture of inorganic materials such as clay, soil particles and organic materials in water (Momba *et al*. 2006; Olaniran *et al*. 2013). The turbidity of natural waters usually ranges from 1 nephelometric turbidity unit (NTU) to 1 000 NTU in very turbid water (DWAF, 1996b).

Turbid water has no effects health; however, microbial contamination is commonly in turbid water and disinfection process usually requires higher concentration of chloride. Due to this, drinking water which are very turbid and have been chlorinated might be harmful for human health. High turbid water may be problematic for water treatment processes like flocculation and filtration resulting in high treatment expenses (Igbinosa and Okoh, 2009; Olaniran *et al*. 2013). Therefore, the target water quality range for turbidity is 0 to 1 where the water is clear. Levels which are above 5 NTU can put an individual at risk of being sick while at levels higher than 10 NTU may increase the risk of diseases and infections (DWAF, 1996b).

2.4.9. Temperature

In aquatic environments temperature is an important parameter as it influences the quality of water. Changes in water temperature can be detrimental to aquatic life when it lies outside the ordinary range. Changes in water temperature is usually induced by thermal pollution (resulting from industrial pollution), removal of surrounding trees along aquatic environments (allowing more solar rays to penetrate water), surrounding air temperature, and increase in turbidity due to increased suspended solid (Mandal, 2014). The effect of air temperature to the water temperature is depended on water depth, since shallow water are prone to temperature changes compared to deep waters. In aquatic environments changed in water temperature affects the solubility of dissolved oxygen since more gases tend to dissolve more in cold water compared to warm water, hence aquatic organisms that require high amounts of dissolved oxygen inhabit cold water. Increase in water temperature stimulate plant

growth due to increase in the rate of photosynthesis while it alters the ecosystems, the rate of metabolism in organisms and resistance of organisms to pollutants and diseases (Olaniran *et al*. 2013; Mandal 2014).

2.5. Aim

To monitor water quality of four selected rivers (Buffalo River, Bloukrans River, Swartkops River and Tyhume River) in the Eastern Cape Province.

2.6. Objectives

To monitor the seasonal changes of the physico-chemical from the selected rivers.

2.7. Methodology

2.7.1. Description of the study area

Nkonkobe Local Municipality is located in the Amatole district in the Eastern Cape Province in South Africa. According to Statistic South Africa consensus in 2011 the Nkonkobe Local Municipality has a population of approximately 127 115. Eastern Cape Province is one of the regions in South Africa that consist of rural and poor people, with lacking water supply systems. This Province is surrounded by rural communities of rural discrete villages and subsistence farmers and towns serving those villages and farmers (Momba *et al*. 2006). The Tyhume River originates from the Amatole Mountains in Hogsback and passes through several rural areas down to Alice and further down rural areas until it joins the Keiskamma River at Manqulweni location. This river serves as a domestic water source in close by communities where there is lack of tap water supply (Sibanda *et al*. 2013). The Tyhume River catchment and sampling sites are shown in figure 2.1.

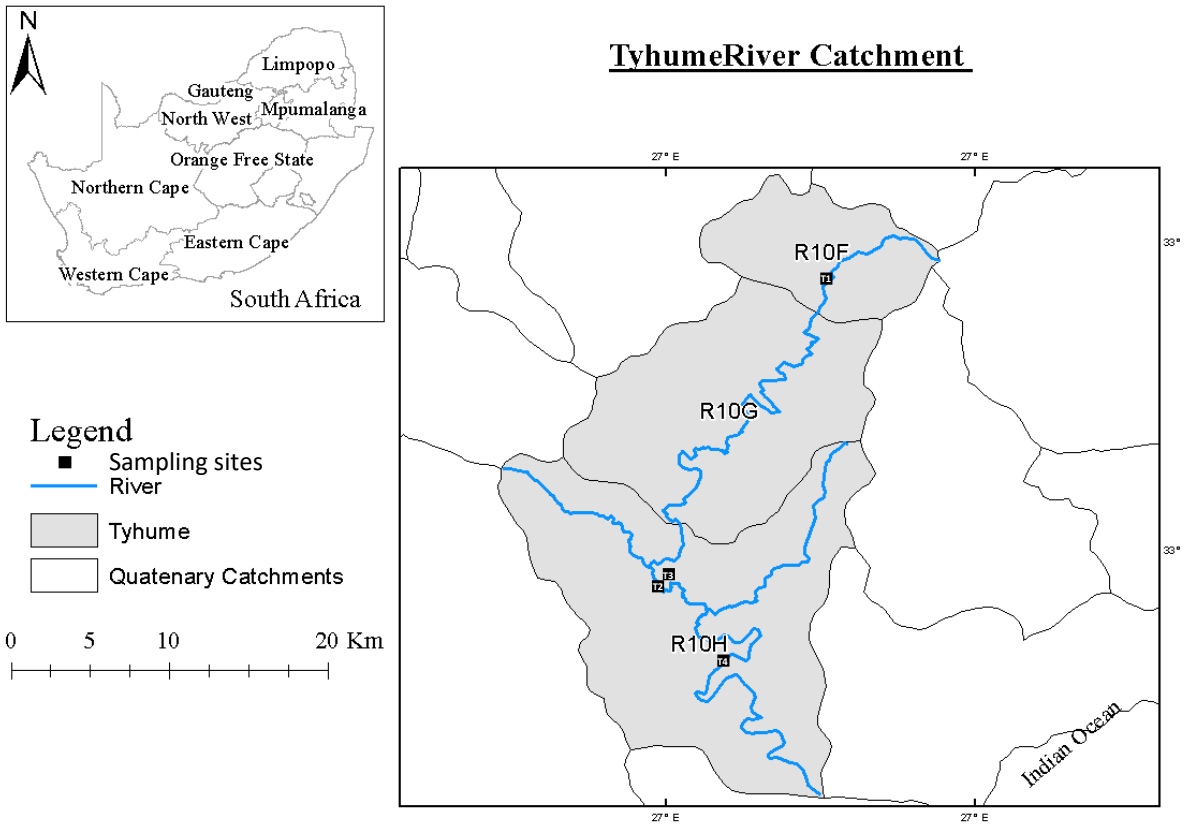

Figure 2. 1: The Tyhume River catchment with four sampling sites.

Buffalo River is situated in the Amatole district under Buffalo City Metropolitan Municipality in the Eastern Cape Province in South Africa. Buffalo River rises to height of 1 200 m in the Amatole Mountains found Eastern Cape Province and flows south-eastwards for approximately 126 km before draining into the Indian Ocean at East London harbour. The main tributaries of the Buffalo River are Cwengcwe, Izele, Mgqakwebe, Ngqokweni and Yellowwoods River. Approximately 570 000 people are subsidized by this catchment within an area of 1.287 km^2 and moderate warm climate. In the coastal zone the temperature is ranging from 8 to 39 °C with the annual average temperature of 21 °C while in the inland regions the temperature ranges between -2 to 42 °C with the annual average temperature of 18 °C. Buffalo River has and average rainfall of 400 mm to more than 1000 mm in summer season with an average annual rainfall of 700 mm (River Health Programme (RHP), 2004). Buffalo River has about nine WWTPs that discharge treated effluent directly or indirectly to it. Nevertheless, there are four dams along the Buffalo River that are used for water supply in urban areas of Buffalo City Municipality those areas include King Williams Town and neighbouring areas such as Zwelitsha, Mdantsane and East London (Chigor *et al*. 2012; Chigor *et al*. 2013). The Buffalo River catchment and sampling sites are shown in figure 2.2.

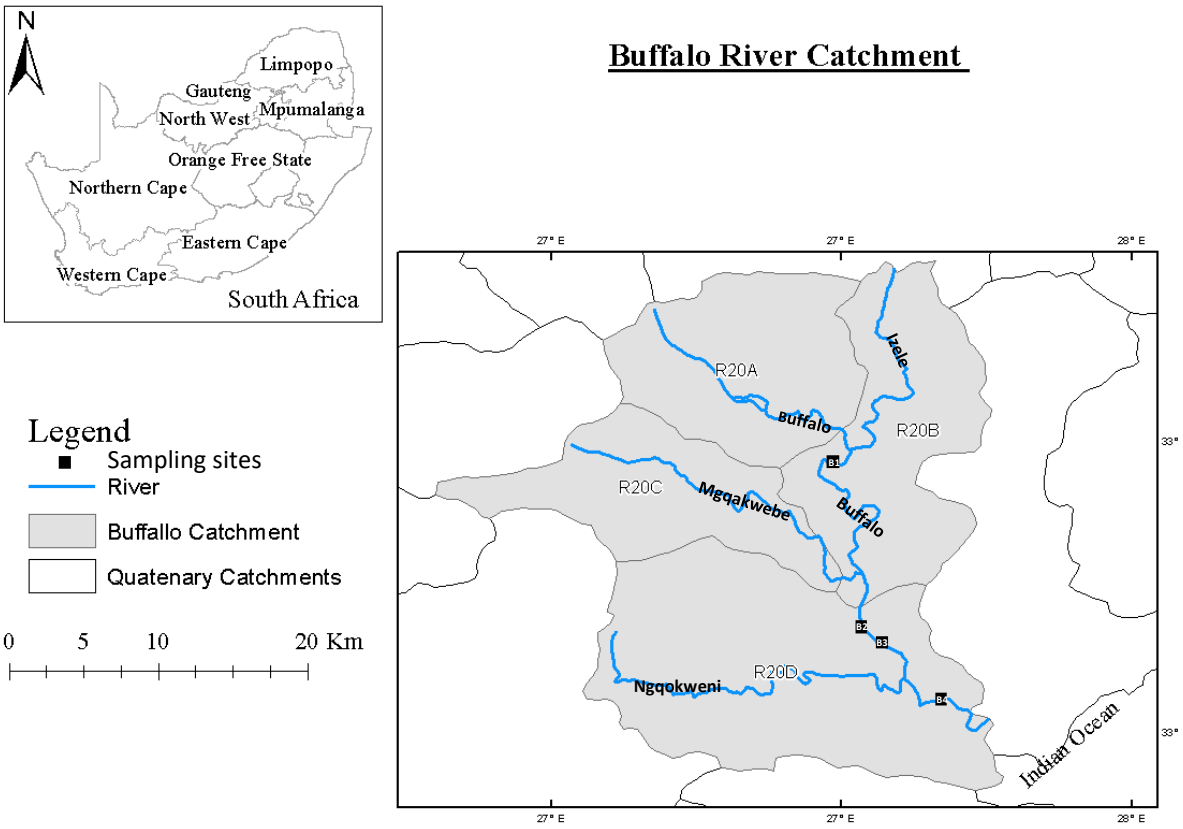

Figure 2. 2: The Buffalo River catchment with four sampling sites.

The Swartkops River is located in the north of Port Elizabeth in the Eastern Cape Province in South Africa. Swartkops River originates from the two rivers, KwaZunga River and Elands River which both originates from the Groot Winterhoek Mountain. The KwaZunga River flows from the north and the Elands River flows from the southwest and connects in an area called Kruisriveir which is before the town called Uitenhage to form Swartkops River. Swartkops River flows and passes Uitenhage, Kwanobuhle, dispatch and Swartkops village before it empties into the Indian Ocean at Algoa Bay in Port Elizabeth (DWAF, 1996a, Odume, 2011). The KwaZungu River and Elands River are the major tributaries of Swartkops River. Swartkops River also has small tributaries which are Brak and Chatty River and both originates from the north of Port Elizabeth. The Brak River joins Swartkops River at Uitenhage whereas Chatty joins Swartkops River before the Swartkops village. Swartkops River serves as a valuable ecological resource as it supports an estuary that offers vital breeding habitats for water birds and fish (Taljaard *et al.* 1998, Odume, 2011). However, because of its location, this river suffers from pollution due to human activities such as industrial discharge, deforestation, agricultural activities (DWAF, 1996a; DWAF, 2003; Odume, 2011) and sewage effluent discharge from Uitenhage, Dispatch and Kwanobuhle municipalities (Binning and Baird, 2001; Odume, 2011). The Swartkops

River catchment has a length of about 120 km and width of 42 km and the total river length of 155 km with a total catchment area of 1360 km^2 and has an average runoff of 84.2 x 10^6 m3 (Bate *et al*. 2004; Odume, 2011). The catchment has a climate that is warm and moderate with an average temperature of 6 °C in winter season and 27 °C in summer season (Nelson Mandela Bay Municipality, 2006). Although the catchment get rain all year round, the highest rainfall occurs in June and October (Haigh, 2002; Odume, 2011). Rainfall pattern in the catchment vary significantly with a minimum monthly mean of 60 mm (Nelson Mandela Bay Municipality, 2006; Odume, 2011). The river has persistent tidal limit of 16 km from the mouth of the ocean where the causeway cease disturbance by saltwater further upstream (Swartkops River Water Resources Management Plan, 1999). The Swartkops River catchment and sampling sites are shown in figure 2.3.

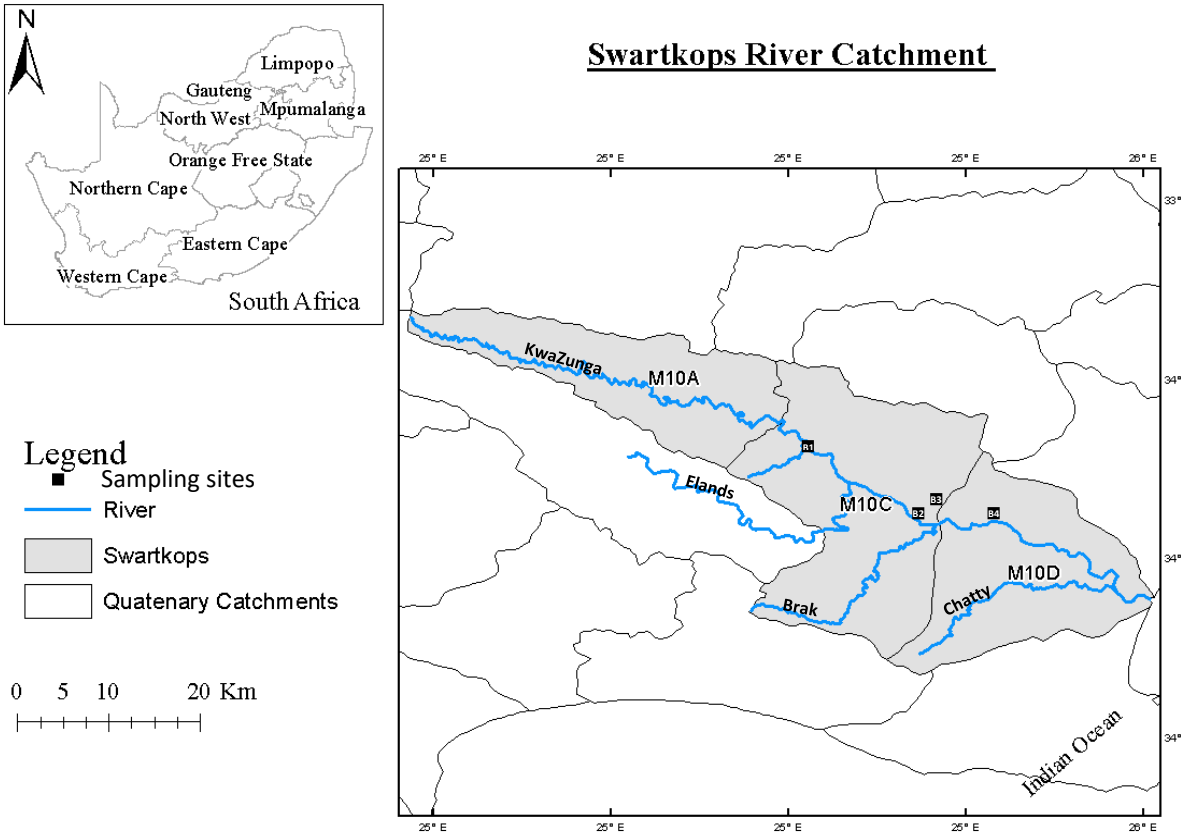

Figure 2. 3: The Swartkops River catchment with four sampling sites.

The Bloukrans River is situated in the Makana Municipality in Sarah Baartman District which is located in the western part of the Eastern Cape Province in South Africa. Grahamstown is a small town located in Makana Municipality. This town is surrounded by informal settlements and affordable housing. The municipality consists of a number of game reserves and private game farm which are Shamwari, Kwandwe, Thomas Baines Nature Reserve, Bayethe and the Andries Vosloo Nature Reserve. The rivers which are situated within the Municipality are Bushmans, Kariega, Palmiet/Berg,

Bloukrans, Kowie and Great Fish River. The Bloukrans River originate near Grahamstown and flows to south-easterly direction where is joins the Kowie River which continues to flow south-eastwards and it drains into the Indian Ocean at Port Alfred. The Grahamstown WWTP situated in Makana Municipality region discharges its wastewater into the Bloukrans River and is the major pollution point source as it consists of municipal and industrial effluent (from two tanneries). Bloukrans River is used as a source of water for irrigation by the surrounding farms and for spiritual rituals. The Palmiet River was used as upper-stream site (reference site) for Bloukrans River samples due to the challenge of obtaining the sampling permit for the upper-stream site of Bloukrans River since the site was within a private property (Von Der Meden *et al*. 2004). Palmiet River is situated within the same eco-region as Bloukrans River therefore the natural macroinvertebrate assemblage would be the same. The Bloukrans River catchment and sampling sites are shown in figure 2.4.

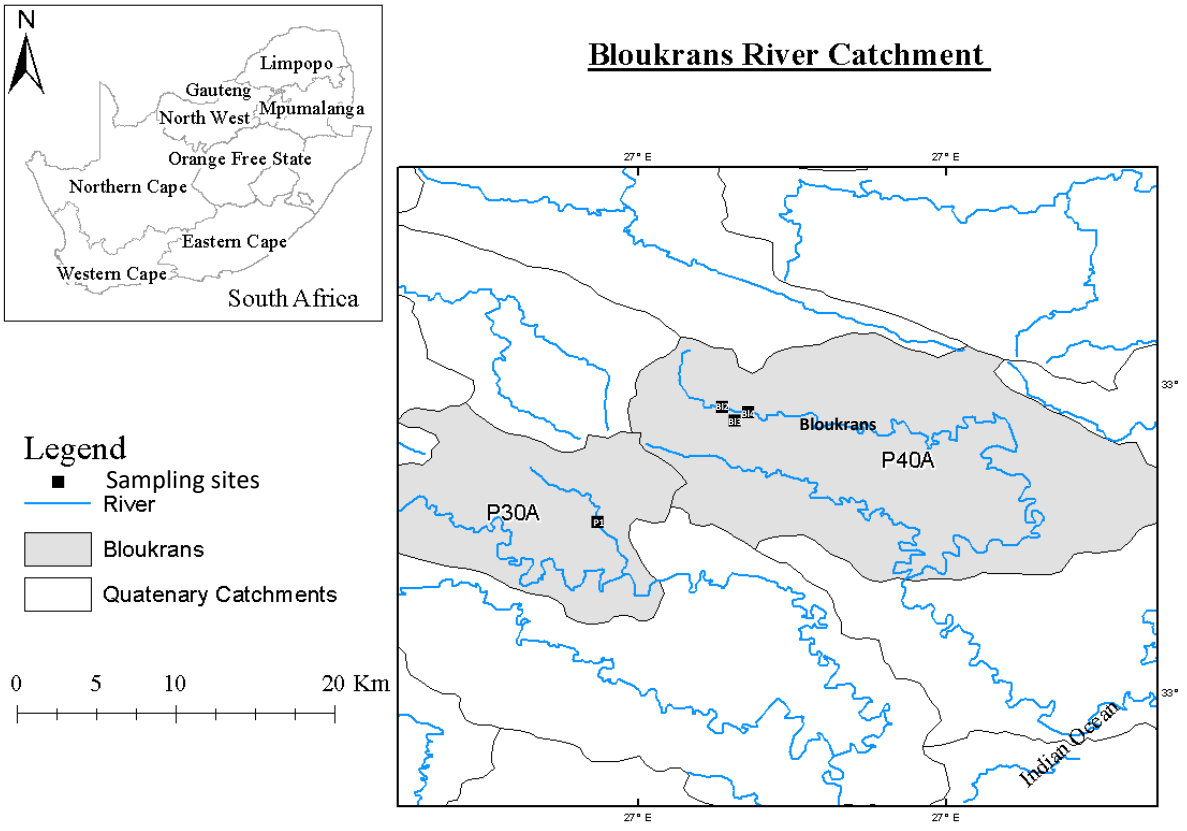

Figure 2. 4: The Bloukrans River catchment with four sampling sites.

The sampling sites co-ordinates of the Palmiet River, Bloukrans River, Buffalo River, Tyhume River and Swartkops River are shown in table 2.1

Table 2. 1: Co-ordinates of the sampling sites in all the rivers

River	Site no.	Site name	Latitude	Longitude
Palmiet	P1	Upper stream	33.369625	26.476542
Bloukrans	Bl2	Middle-stream	33.314295	26.551907
	Bl3	Wastewater treatment plant	33.316896	26.559300
	Bl4	Lower stream	33.317766	26.568247
Buffalo	B1	Upper stream	32.789741	27.369707
	B2	Middle-stream	32.896940	27.392820
	B3	Wastewater treatment plant	32.900088	27.404174
	B4	Lower stream	32.934406	27.440321
Tyhume	T1	Upper stream	32.610883	26.909413
	T2	Middle-stream	32.797323	26.847497
	T3	Wastewater treatment plant	32.792744	26.849658
	T4	Lower stream	32.827368	26.888672
Swartkops	S1	Upper stream	33.716609	25.288034
	S2	Middle-stream	33.791756	25.407598
	S3	Wastewater treatment plant	33.784194	25.426816
	S4	Lower stream	33.792082	25.490763

The sampling permit for South African Scoring System version 5 was obtained from the Department of Economic Development, Environmental Affairs and Tourism in Eastern Cape Province in South Africa. The sampling permit for WWTPs was attained from Buffalo City Metropolitan Municipality, Makana Municipality, University of Fort Hare which is located in the Nkonkobe Local Municipality and Nelson Mandela Bay Metropolitan Municipality.

2.7.2. Chemicals and reagents

The phosphate test kit (1.00798.0001), ammonium test kit (1.00683.0001), nitrate test kit (1.10020.0002), sulphate test kit (1.14548.0001), chlorine test kit (114801.0001), iron (1.10004.0001), chemical oxygen demand (COD) test kit (1.14538.0065 and 1.14539.0495) and potassium hydrogen phthalate was purchased from Merck Millipore (Darmstadt, Germany). The electric conductivity (EC) calibration solution (HIL 7030/500) for Hanna Instruments was purchased from (Sigma-Aldrich, Germany). The buffers solutions were purchased from Merck Millipore (Gauteng, South Africa). The ammonium chloride and di-potassium sulphate were purchased from Merck Millipore (Gauteng, South Africa). The di-potassium hydrogen phosphate and potassium chloride were manufactured by Minema

chemicals (Johannesburg, South Africa) and were supplied by Spellbound laboratory solutions (Port Elizabeth, South Africa).

2.7.3. Sample collection

Sample collection was conducted in three seasons: autumn, winter and spring season of 2018. In each of the four selected rivers, four sites were targeted for sample collection (Figure: 2.1 to 2.4). In each of the four selected rivers one sample was collected in each site twice a season therefore making a total of 96 samples collected. The samples were collected using a sterile scotch bottle. The bottle was rinsed twice with the river water prior to sample collection and 1 L of water sample was collected in each site by holding the container on the handle and dipping the container approximately 0.5 m below water surface facing away from the water current. All samples were protected from direct sunlight, stored on ice and transported to the laboratory for analysis. Samples were filtered through 0.45 µm pore-sized membrane filters purchased from Merck Millipore (Gauteng, South Africa), stored at 4°C and analysed within 48 hours of collection.

2.7.4. Procedure

2.7.4.1. Preparation of standard curves for testing chemical property of water

Ammonium chloride, di-potassium sulphate, di-potassium hydrogen phosphate, potassium chloride and potassium hydrogen phthalate were used as standards for water chemical parameter testing. A standard solution of 500 mg/L was prepared by dissolving the weighed powder into distilled water. The standard (stock) solutions were then used to prepare seven diluted standard solutions. The concentrations of the diluted standards varied depending on detection range of each test used. The absorbance of these standards was read three times using UV-vis spectrophotometer, model UVmini-1240 at 420 nm (sulphate), 450 nm (chlorine) , 610 nm (COD) and 650 nm (phosphate and ammonium) respectively. The standard curve was then drawn using concentrations and absorbance and was used to determine the concentrations of the target chemicals. Standard curves are listed on appendix 1, figure 1 to 6.

2.7.4.2. Physicochemical properties of water

The physical parameters such as pH, EC, temperature and turbidity were measured at the site. The turbidity meter and the multi-parameter meter were pre-calibrated using the standard solutions and buffers respectively before use. Three measurements were taken for each parameter in each site. The pH, EC and temperature were measured using a calibrated multi-parameter meter, Hanna Instruments,

model HI 98130 (Sigma-Aldrich, Germany). The electrode of the instrument was rinsed with distilled water before each sample was tested to prevent cross contamination. Turbidity was measured using a calibrated turbidity meter, model TU-2016 (Lutron electronic enterprise, Taiwan). The measurements were taken three times and the turbidity meter vial was rinsed with distilled water before each test. The physical parameter such COD and chemical parameters such as iron, nitrates, ammonium, phosphate, chlorine and sulphate were measured in the laboratory using the test kits. In all the samples the physicochemical parameters of river water were measured in triplicates.

The COD of the samples was measured using the COD test kit purchased from Merck Millipore (Darmstadt, Germany) and this was done following the manufactures protocol. Approximately 3 mL of river water sample was mixed with 0.3 mL of COD solution A (mercury(II) sulphate) and 2.30 mL of solution B (sulphuric acid, potassium dichromate). The solution was then incubated at 148 °C for 2 hours. Following incubation, the samples were cooled at room temperature for 10 minutes and the absorbance was read at 610 nm using a UV-vis spectrophotometer, model UVmini-1240 (Shimadzu Corporation, Japan). The absorbance readings were the recorded, and the concentration was determined using the COD standard curve. The ammonium concentration of the samples was measured using the ammonium test kit and this was done following the manufactures protocol. About 5 mL of reagent NH_4-1 was added into 0.10 mL of the sample and the solution was mixed. Following that, 1 level microspoon of reagent NH_4-2 powder was added and the solution was mixed properly. The solution was incubated at room temperature for 15 minutes and the absorbance was measured at 650 nm using UV-vis spectrophotometer. The absorbance readings were recorded, and ammonium concentration was determined using ammonium standard curve. This method for ammonium test is analogous to EPA 350.1, APHA 4500-NH_3 F, ISO 7150-1, and DIN 38406-5.

The concentration of sulphate on the samples was measured using the sulphate test kit following the manufactures protocol. About 0.50 mL of reagent SO_4-1 was added into 5 mL sample and mixed properly. Following that, 1 level of microspoon of reagent SO_4-2 powder was dissolved into the solution and the solution was incubated at room temperature for 2 minutes. After the incubation, the absorbance was read at 420 nm using UV-vis spectrophotometer and the results were recorded. Sulphate concentration was then determined using sulphate standard curve. This method for sulphate test is analogous to EPA 375.4, APHA 4500-SO_4^{2-} E, and ASTM D516-11.

The chlorine concentration was measured using the chlorine test kit and this was done following the manufactures protocol. About 1 mL of the sample was mixed with 2.5 mL of reagent Cl-1 and 0.5 mL of reagent Cl-2. The solution was incubated at room temperature for 1 minute and the absorbance was

read at 450 nm using UV-vis spectrophotometer. Absorbance results were recorded, and the concentration of chlorine was determined using the equation obtained from chlorine standard curve. This method for chlorine test is analogous to EPA 325.1 and APHA 4500-Cl^- E. The concentration of phosphate was measured from the samples using the phosphate test kit following the manufactures protocol. About 5 mL of sample was mixed with 5 drops of PO_4-1 reagent, followed by the addition of 1 level microspoon of PO_4-2 powder. The solution was mixed properly and incubated at room temperature for 5 minutes. The absorbance was read at 650 nm using UV-vis spectrophotometer and the results were recorded. The concentration of the phosphate was determined using the phosphate standard curve.

The nitrate concentration was measured using the nitrate test kit following the manufactures protocol. The two reaction zones of nitrate test strips were immersed into the sample for 1 second, the excess liquid was removed, and the strips were incubated for 1 minute at room temperature. Following incubation, the colour of the strip was compared to the NO_2^- and NO_3^- reaction zone on the manual. The corresponding concentration to the colour of the strip was then recorded. The concentration of iron on the samples was measured using the iron test kit, and this was done following the manufactures protocol. The reaction zone of the iron test strip was immersed into the sample for 1 second, the excess liquid was removed from the strip and the strip was incubated for 10 seconds at room temperature. Following incubation, the colour of the strip was compared to the colour on the manual and the corresponding concentration to the colour of the strip was recorded. The data was statistically analysed using Microsoft excel and ANOVA.

2.8. Results and discussion

This study monitors the physicochemical characteristics of river water during autumn, winter and spring season and the results are presented on table 2.2 to 2.5. Seasonal variations on some of the physicochemical parameters of river water were observed in this study. The phosphate concentrations throughout the sampling seasons ranged from 0.08 mg/L (B1-autumn) to 8 mg/L (B3-winter) in Buffalo River samples; 0.00 mg/L (S1-spring) to 16 mg/L (S3-winter) in Swartkops River samples; 0.13 mg/L (T1-winter) to 8 mg/L (T3-winter) in Tyhume River samples and 0.03 mg/L (Bl1-spring) to 13 mg/L (Bl3-winter) in Bloukrans River samples. High phosphate concentration was observed in spring season at Buffalo River (0.24 to 4.48 mg/L) and Tyhume River (0.19 to 6.84 mg/L), while in Bloukrans River and Swartkops River high phosphate concentrations (0.05 to 13 mg/L and 0.15 to 16 mg/L) were observed in winter season. The chloride concentrations in Buffalo River ranged from 121 (B1-autumn) to 372 mg/L (B4-spring); 80 mg/L (Bl1-spring) to 518 mg/L (Bl2-spring) in Bloukrans River; 205 mg/L (S1-autumn) to 450 mg/L (S2-winter) in Swartkops River and 130 mg/L (T1-winter)

to 276 mg/L (T4-winter). High chloride concentration was observed in spring at Buffalo River (267 to 370 mg/L), Bloukrans River (80 to 518 mg/L) and Tyhume River (149 to 263 mg/L), however, at Swartkops River there was no much variation in chloride concentration observed during autumn, spring and winter season.

The ammonium concentration throughout the sampling seasons ranged from 13 mg/L (B1-spring) to 104 mg/L (B4-winter) in Buffalo River samples; 5 mg/L (Bl1- autumn) to 273 mg/L (Bl2-winter) in Bloukrans River samples; 8 mg/L (S2-autumn) to 210 mg/L (S3-winter) in Swartkops River samples and 7 mg/L (T1-autumn) to 279 mg/L (T4-winter) in Tyhume River samples. In all the rivers, high concentration of ammonium was observed in winter. In seven out of 11 samples obtained from Buffalo River nitrite tested negative, however, samples obtained in WWTPs during winter and spring season and lower-stream sample collected in spring season tested positive for nitrite. Nitrite was negative in all the samples obtained in Bloukrans River except for the sample obtained from WWTP in autumn season. In Swartkops River samples nitrite tested negative in all the samples except for the middle-stream sample in winter and lower-stream sample in spring season. In Tyhume River samples nitrite tested negative in all the samples obtained in autumn and winter season except for the sample obtained from the WWTP during winter season, during spring season nitrite tested positive in all the samples obtained at Tyhume River. The nitrate concentration ranged from 0 mg/L (B1-autumn) to 25 mg/L in Buffalo River; 0 mg/L (Bl1-autumn) to 50 mg/L (Bl3-autumn) in Bloukrans River samples; 0 mg/L (S1-autumn) to 13 mg/L (S3-winter) in Swartkops River samples and 0 mg/L (T1-autumn) to 25 mg/L (T3-winter). High nitrate concentration was observed in winter at Buffalo River, while at Bloukrans River high nitrate concentration was observed in autumn; at Swartkops River and Tyhume River there were no significant variations observed between the seasons. Throughout the sampling seasons iron concentration was 0 mg/L in all the rivers. The sulphate concentration ranged from 9 mg/L (B1-autumn) to 43 mg/L (B4-spring) in Buffalo river samples; 11 mg/L (Bl1-winter) to 98 mg/L (Bl2-autumn) in Bloukrans River; 5 mg/L (S1-autumn) to 103 mg/L (S4-spring) in Swartkops River and 7 mg/L (S1-autumn) to 42 mg/L (S2-spring) in Tyhume River samples. In all the rivers, high sulphate concentrations were observed in spring.

The COD of river water samples ranged from 8 mg/L (B1-winter) to 21 mg/L (B1-spring) in Buffalo River samples; 10 mg/L (Bl1-winter) to 45 mg/L (Bl2-autumn) in Bloukrans River; 6 mg/L (S1-autumn) to 17 mg/L (S3-winter) in Swartkops River and 7 mg/L (T1-autumn) to 14 mg/L (T3-winter) in Tyhume River. The COD was high during spring season at Buffalo River; high in winter season at Swartkops River and Tyhume River while it was high in autumn season at Bloukrans River. The turbidity of river samples ranged from 11.46 NTU (B1-winter) to 577 NTU (B3-spring) in Buffalo

River; 0.00 NTU (Bl1-winter) to 415 NTU (Bl2-winter) in Bloukrans River; 0.00 NTU (S1-autumn) to 718 NTU (S3-spring) in Swartkops River and 0.00 NTU (T1-winter) to 238 NTU (T2-spring) in Tyhume River samples. In all the rivers the turbidity was high during spring season. The temperature of the river water ranged from 10.90 °C (B1-winter) to 22.78 °C (B1-spring) in Buffalo River; 11.87 °C (Bl1-winter) to 22.10 °C (Bl3-autumn) in Bloukrans River; 12.75°C (S1-winter) to 23.00°C (S2-autumn) in Swartkops River and 8.48°C (T1-winter) to 18.07°C (T4-spring) in Tyhume River. High temperatures were observed in autumn season at Bloukrans River whereas at Tyhume River, Bloukrans River, and Swartkops River high water temperature were observed in autumn and spring season. The pH of the samples ranged from 7.79 (B1-winter) to 9.58 (B3-spring) in Buffalo River; 5.89 (Bl1-autumn) to 9.14 (Bl2-spring) in Bloukrans River; 7.67 (S1-autumn) to 9.77 (S3-spring) in Swartkops River and 7.55 (T1-winter) to 9.09 (T4-winter) in Tyhume River. High pH values were observed in spring at Buffalo River, Bloukrans River and Swartkops River, whereas at Tyhume River high pH values were observed in winter. The EC of the samples ranged from 0.08 mS (B1-autumn) to 0.61 mS (B1-spring) in Buffalo River; 0.00 mS (Bl1-autumn) to 1.64 mS (Bl2-1.64) in Bloukrans River; 0.13 mS (S1-winter) to 2.95 (S2-spring) in Swartkops River and 0.03 mS (T1-autumn) to 0.40 mS (T4-winter) in Tyhume River. High EC values were observed in spring season at Buffalo River, in Tyhume River high EC values were observed in winter season, at Swartkops River high EC values were observed in winter and spring season while at Bloukrans River, high EC values were observed in autumn season.

Table 2. 2: The average values of the physicochemical properties of Buffalo River collected in autumn, winter and spring season.

		Chemical properties							Physical properties				
	Site no.	**Phosphate (mg/L)**	**Chlorine (mg/L)**	**Ammonium (mg/L)**	**Nitrite (mg/L)**	**Nitrate (mg/L)**	**Iron (mg/L)**	**Sulphate (mg/L)**	**COD (mg/L)**	**Turbidity (NTU)**	**Temperature (°c)**	**pH**	**Electric conductivity (mS/cm)**
Autumn	B1	0.08 ± 0.00	121 ± 0.01	14 ± 0.02	(-)	0 ± 0.00	0 ± 0.00	9 ± 0.00	10 ± 0.01	25.63 ± 0.53	19.02 ± 0.13	8.21 ± 0.04	0.08 ± 0.00
	B2	0.43 ± 0.00	193 ± 0.03	17 ± 0.01	(-)	0 ± 0.00	0 ± 0.00	16 ± 0.00	9 ± 0.01	83.83 ± 1.08	20.34 ± 0.15	8.09 ± 0.03	0.21 ± 0.01
	B3	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	B4	0.69 ± 0.00	187 ± 0.27	16 ± 0.02	(-)	22 ± 0.00	0 ± 0.00	19 ± 0.02	11 ± 0.01	119.5 ± 3.34	21.03 ± 0.00	8.13 ± 0.01	0.26 ± 0.00
Winter	B1	0.15 ± 0.02	261 ± 0.09	36 ± 0.16	(-)	0 ± 0.00	0 ± 0.00	11 ± 0.01	8 ± 0.01	11.46 ± 0.40	10.90 ± 0.00	7.79 ± 0.02	0.30 ± 0.01
	B2	0.34 ± 0.05	301 ± 0.32	96 ± 0.18	(-)	10 ± 0.00	0 ± 0.00	20 ± 0.01	9 ± 0.01	21.00 ± 0.84	12.00 ± 0.00	8.17 ± 0.03	0.31± 0.00
	B3	8 ± 1.09	280 ± 0.16	63 ± 0.00	(+)	25 ± 0.00	0 ± 0.00	29 ± 0.01	11 ± 0.00	33.39 ± 1.01	16.00 ± 0.00	8.26 ± 0.01	0.37 ± 0.00
	B4	3 ± 0.15	313 ± 0.31	104 ± 0.22	(-)	18 ± 0.00	0 ± 0.00	26 ± 0.02	21 ± 0.00	12.87 ± 3.67	12.82 ± 0.06	8.33 ± 0.02	0.44 ± 0.01
Spring	B1	0.24 ± 0.00	370 ± 0.09	13 ± 0.02	(-)	0 ± 0.00	0 ± 0.00	13 ± 0.01	21 ± 0.05	29.78 ± 0.00	22.78 ± 0.00	9.15 ± 0.01	0.61 ± 0.01
	B2	0.35 ± 0.01	314 ± 0.07	13 ± 0.01	(-)	5 ± 0.00	0 ± 0.00	29 ± 0.01	18 ± 0.04	35.20 ± 0.40	21.72 ± 0.08	8.76 ± 0.01	0.50 ± 0.01
	B3	5 ± 0.01	267 ± 0.10	16 ± 0.02	(+)	13 ± 0.00	0 ± 0.00	40 ± 0.02	16 ± 0.03	577 ± 9.24	22.53 ± 0.03	9.58 ± 0.04	0.41 ± 0.01
	B4	4.48 ± 0.01	372 ± 0.08	15 ± 0.01	(+)	10 ± 0.00	0 ± 0.00	43 ± 0.03	18 ± 0.01	31.40 ± 8.10	21.45 ± 0.10	8.65 ± 0.13	0.55 ± 0.04

NS: Not sampled

Table 2. 3: The average values of the physicochemical properties of Bloukrans River collected in autumn, winter and spring season.

		Chemical properties							Physical properties				
Season	**Site no.**	**Phosphate (mg/L)**	**Chlorine (mg/L)**	**Ammonium (mg/L)**	**Nitrite (mg/L)**	**Nitrate (mg/L)**	**Iron (mg/L)**	**Sulphate (mg/L)**	**COD (mg/L)**	**Turbidity (NTU)**	**Temperature (°c)**	**pH**	**Electric conductivity (mS/cm)**
Autumn	P1	0.35 ± 0.01	214 ± 0.07	5 ± 0.01	(-)	0 ± 0.00	0 ± 0.00	14 ± 0.01	45 ± 0.01	23 ± 1.20	19 ± 0.29	5.89 ± 0.02	0.00 ± 0.04
	Bl2	3 ± 0.03	475 ± 0.01	25 ± 0.02	(-)	0 ± 0.00	0 ± 0.00	62 ± 0.05	26 ± 0.01	118 ± 4.16	20 ± 0.06	7.72 ± 0.14	1.64 ± 27.07
	Bl3	9 ± 0.03	436 ± 0.07	32 ± 0.11	(+)	49 ± 0.00	0 ± 0.00	54 ± 0.06	22 ± 0.02	28 ± 1.47	22 ± 0.10	7.77 ± 0.01	0.95 ± 5.00
	Bl4	4± 0.03	448 ± 0.00	24 ± 0.10	(-)	0 ± 0.00	0 ± 0.00	49 ± 0.03	19 ± 0.01	27 ± 0.46	17 ± 0.12	7.68 ± 0.03	1.36 ± 76.69
Winter	Bl1	0.05 ± 0.00	215 ± 0.03	123 ± 0.06	(-)	0 ± 0.00	0 ± 0.00	11 ± 0.00	10 ± 0.00	0.00 ± 0.00	11.87 ± 0.06	7.55 ± 0.01	0.12 ± 0.00
	Bl2	7 ± 0.04	437 ± 0.03	273 ± 0.11	(-)	0 ± 0.00	0 ± 0.00	56 ± 0.08	32 ± 0.00	232 ± 7.02	13.17 ± 0.03	9.11 ± 0.12	1.17 ± 0.01
	Bl3	13 ± 0.05	374 ± 0.05	224 ± 0.14	(-)	0 ± 0.00	0 ± 0.00	53 ± 0.05	24 ± 0.00	48.96 ± 2.13	15.47 ± 0.06	8.25 ± 0.02	0.71 ± 0.00
	Bl4	6 ± 0.02	436 ± 0.03	217 ± 0.06	(-)	0 ± 0.00	0 ± 0.00	57 ± 0.06	26 ± 0.01	83.50 ± 4.09	13.28 ± 0.12	8.79 ± 0.03	1.09 ± 0.00
Spring	Bl1	0.03 ± 0.00	80 ± 0.06	6 ± 0.05	(-)	0 ± 0.00	0 ± 0.00	14 ± 0.01	11 ± 0.01	0.00 ± 0.00	13.27 ± 0.06	8.75 ± 0.03	0.12 ± 0.00
	Bl2	111 ± 001	518 ± 0.00	149 ± 0.69	(-)	0 ± 0.00	0 ± 0.00	98 ± 0.03	42 ± 0.00	415 ± 2.65	15.93 ± 0.06	9.14 ± 0.01	1.37 ± 0.01
	Bl3	10 ± 0.02	467 ± 0.10	93 ± 0.89	(-)	10 ± 0.00	0 ± 0.00	68 ± 0.02	23 ± 0.01	28.60 ± 1.04	17.20 ± 0.00	8.68 ± 0.01	0.78 ± 0.01
	Bl4	5 ± 0.01	481 ± 0.24	68 ± 0.23	(-)	0 ± 0.00	0 ± 0.00	82 ± 0.02	24 ± 0.00	47.72 ± 0.94	13.87 ± 0.06	8.44 ± 0.02	1.15 ± 0.01

NS: Not sampled

Table 2. 4: The average values of the physicochemical properties of Swartkops River collected in autumn, winter and spring season.

		Chemical properties							Physical properties				
	Site no.	**Phosphate (mg/L)**	**Chlorine (mg/L)**	**Ammonium (mg/L)**	**Nitrite (mg/L)**	**Nitrate (mg/L)**	**Iron (mg/L)**	**Sulphate (mg/L)**	**COD (mg/L)**	**Turbidity (NTU)**	**Temperature (°c)**	**pH**	**Electric conductivity (mS/cm)**
Autumn	S1	0.04 ± 0.00	205 ± 0.07	11 ± 0.02	(-)	0 ± 0.00	0 ± 0.00	5 ± 0.00	6 ± 0.01	0.00 ± 0.00	22.0 ± 0.06	7.7 ± 0.04	0.23 ± 11.27
	S2	2 ± 0.01	426 ± 0.00	8 ± 0.00	(-)	10 ± 0.00	0 ± 0.00	48 ± 0.00	12 ± 0.02	22.0 ± 0.53	23.0 ± 0.00	8.7 ± 0.12	0.40 ± 0.00
	S3	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	S4	6 ± 0.02	421 ± 0.06	10 ± 0.02	(-)	10 ± 0.00	0 ± 0.00	70 ± 0.08	16 ± 0.02	22.9 ± 1.67	20.9 ± 0.15	9.0 ± 0.01	0.03 ± 0.16
Winter	S1	0.15 ± 0.02	245 ± 0.15	153 ± 0.03	(-)	0 ± 0.00	0 ± 0.00	11 ± 0.00	10 ± 0.01	0.00 ± 0.00	12.75 ± 0.00	7.87 ± 0.02	0.13 ± 0.00
	S2	2 ± 0.01	450 ± 0.00	186 ± 0.04	(+)	10 ± 0.00	0 ± 0.00	62 ± 0.21	11 ± 0.00	11.41 ± 0.45	15.22 ± 0.03	8.29 ± 0.01	2.95 ± 0.00
	S3	16 ± 0.23	435 ± 0.04	210 ± 0.06	(-)	13 ± 0.00	0 ± 0.00	57 ± 0.04	17 ± 0.01	268 ± 12.62	18.20 ± 0.00	8.26 ± 0.02	1.49 ± 0.00
	S4	10 ± 0.02	447 ± 0.04	186 ± 0.08	(-)	10 ± 0.00	0 ± 0.00	62 ± 0.03	11 ± 0.01	0.00 ± 0.00	14.85 ± 0.09	8.53 ± 0.03	2.26 ± 0.02
Spring	S1	0.00 ± 0.00	255 ± 0.02	10 ± 0.02	(-)	0 ± 0.00	0 ± 0.00	13 ± 0.01	7 ± 0.00	0.00 ± 0.00	17.17 ± 0.15	9.03 ± 0.01	0.15 ± 0.01
	S2	1 ± 0.00	431 ± 0.01	12 ± 0.01	(-)	0 ± 0.00	0 ± 0.00	92 ± 0.10	9 ± 0.00	0.00 ± 0.00	22.47 ± 0.21	9.55 ± 0.07	2.95 ± 0.00
	S3	8 ± 0.02	448± 0.00	28 ± 0.04	(-)	10 ± 0.00	0 ± 0.00	99 ± 0.03	10 ± 0.00	718 ± 52.37	20.57 ± 0.12	9.77 ± 0.03	1.5 ± 0.01
	S4	3 ± 0.01	442± 0.07	10 ± 0.01	(+)	10 ± 0.00	0 ± 0.00	103 ± 0.11	13 ± 0.01	0.00 ± 0.00	16.70 ± 0.17	9.22 ± 0.02	2.38 ± 0.01

NS: Not Sampled

Table 2. 5: The average values of the physicochemical properties of Tyhume River collected in autumn, winter and spring season.

		Chemical properties							Physical properties				
Season	**Site no.**	**Phosphate (mg/L)**	**Chlorine (mg/L)**	**Ammonium (mg/L)**	**Nitrite (mg/L)**	**Nitrate (mg/L)**	**Iron (mg/L)**	**Sulphate (mg/L)**	**COD (mg/L)**	**Turbidity (NTU)**	**Temperature (°c)**	**pH**	**Electric conductivity (mS/cm)**
Autumn	T1	0.83 ± 0.01	133 ± 0.20	7 ± 0.01	(-)	0 ± 0.00	0 ± 0.00	7 ± 0.01	7 ± 0.00	31.49 ± 1.33	13.50 ± 0.00	7.74 ± 0.00	0.03 ± 0.00
	T2	0.32 ± 0.06	153 ± 0.06	12 ± 0.03	(-)	0 ± 0.00	0 ± 0.00	12 ± 0.00	9 ± 0.00	56.67 ± 0.58	16.63 ± 0.51	8.04 ± 0.01	0.12 ± 0.00
	T3	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	T4	0.33 ± 0.09	175 ± 0.10	12 ± 0.05	(-)	0 ± 0.00	0 ± 0.00	19 ± 0.04	11 ± 0.02	83.67 ± 1.53	17.50 ± 0.00	8.10 ± 0.02	0.15 ± 0.00
Winter	T1	0.13 ± 0.01	130 ± 0.09	275 ± 0.06	(-)	0 ± 0.00	0 ± 0.00	7 ± 0.00	10 ± 0.01	0.00 ± 0.00	8.48 ± 0.14	7.55 ± 0.05	0.03 ± 0.00
	T2	0.53 ± 0.01	207 ± 0.07	270 ± 0.16	(-)	5 ± 0.00	0 ± 0.00	14 ± 0.01	12 ± 0.02	14.42 ± 0.45	11.50 ± 0.00	8.29 ± 0.01	0.26 ± 0.00
	T3	8.16 ± 0.04	224 ± 0.08	274 ± 0.21	(+)	25 ± 0.00	0 ± 0.00	27 ± 0.02	14 ± 0.00	28.57 ± 1.30	14.93 ± 0.12	8.81 ± 0.01	0.36 ± 0.01
	T4	0.84 ± 0.02	276 ± 0.07	279 ± 0.13	(-)	18 ± 0.00	0 ± 0.00	17 ± 0.02	11 ± 0.01	11.93 ± 2.30	6.53 ± 0.12	4.42 ± 0.01	0.22 ± 0.01
Spring	T1	0.19 ± 0.01	149 ± 0.08	11 ± 0.01	(+)	10 ± 0.00	0 ± 0.00	11 ± 0.01	9 ± 0.01	39.24 ± 0.43	12.07 ± 0.17	8.51 ± 0.02	0.03 ± 0.01
	T2	1.19 ± 0.07	243 ± 0.09	17 ± 0.04	(+)	10 ± 0.00	0 ± 0.00	42 ± 0.01	13 ± 0.00	238 ± 2.51	15.37 ± 0.03	8.18 ± 0.01	0.26 ± 0.01
	T3	6.84 ± 0.03	247 ± 0.15	30 ± 0.01	(+)	25 ± 0.00	0 ± 0.00	39 ± 0.15	12 ± 0.00	27.35 ± 2.08	17.25 ± 0.00	8.29 ± 0.03	0.37 ± 0.03
	T4	1.58 ± 0.02	263 ± 0.08	16 ± 0.10	(+)	10 ± 0.00	0 ± 0.00	36 ± 0.01	11 ± 0.00	166 ± 0.76	18.07 ± 0.32	8.67 ± 0.02	0.33 ± 0.00

NS: Not Sampled

2.9.1. Chemical properties

During dry season water level in water resources are low due to limited average rain fall and high water demands. This results in shortage of water supply and causes people to use different water resources to meet their daily domestic water needs. Therefore, constant monitoring of the water quality in water resources is important for protection of public health and development of strategies for proper management of water resources (Kannel *et al*. 2007; Alexakis, 2011). In this study the physicochemical properties of river water in the Eastern Cape Province were studied.

Throughout the sampling period, high phosphate concentrations were detected in samples near the urban areas (0.32 to 111 mg/L), WWTP (5 to 16 mg/L) and lower stream sites (0.33 to 10 mg/L) in all the rivers. This was an indication that human activities and WWTP are the major source of phosphate in surface waters (Table: 2. 2-2. 5). In addition, the seasonal variation on phosphate concentration were observed as high phosphate concentrations were detected in winter and spring season from the effluent samples of WWTPs and lower-stream sites in all the rivers expect for Bloukrans River where high phosphate concentrations were also observed from middle-stream site (Bl2). High phosphate concentration at Bl2 may be as a result of contaminating by sewage waste due to blocked sewage pipes from the town. The observed trend in phosphate concentration may be due to phosphate being used as a mineral fertilizer in agriculture therefore during winter season concentrations in water tends to be higher whereas in spring season high phosphate concentration may result from surface runoff (Shabalala *et al*. 2013).

Dilution due to rainfall was observed on the upper-stream site of Bloukrans River where phosphate concentration of 0.03 mg/L of was detected during spring season. Similar results have been reported in several studies (Radhika and Gangaderr 2004; Ololade and Ajayi, 2009; Olaniran *et al*. 2013). There are no guidelines in South Africa for the targeted phosphate concentration for domestic water use. Nevertheless, according to WHO phosphate concentration must be 0.5 mg/L in domestic water (Olaniran *et al*. 2013). The detected phosphate concentration ranged from 0.08 to 0.69 mg/L in Buffalo River samples during autumn season and it complied with WHO standards. However, during winter and spring season only site B1 and B2 (0.15 to 0.35 mg/L) complied with targeted limit while B3 and B4 samples exceeded the targeted limit (>5 mg/L) (Table 2.2). The phosphate concentration (0.00 to 0.35 mg/L) complied with the targeted limit range only at the upper-stream sites of Bloukrans River (Bl1) and Swartkops River (S1) throughout the sampling season. In Tyhume River samples, phosphate concentration (0.13 to 0.83 mg/L) was within the target range during autumn and winter season except for T3 (8.16 mg/L) in winter season. While only site T1 was within the target range (0.19 mg/L) in spring season. This indicates that the phosphate concentration from the middle-stream site to lower-

stream site in Bloukrans River and Swartkops River, and some sites in Buffalo River and Tyhume River was not suitable for domestic water use, aquatic life, irrigation, livestock watering and recreational purposes (Federal Environmental Protection Agency (FEPA), 1991; United State Environmental Protection Agency (USEPA), 2004; Olaniran *et al*. 2013).

High levels of phosphate in human triggers muscle damage, breathing difficulties and kidney failure (Nyamangara *et al*. 2013; Gupta *et al*. 2017). In aquatic environments high phosphate concentration promotes the growth of microorganisms, causes eutrophication and decreases the concentration of dissolved oxygen (Davie, 2003; Gupta *et al*. 2017). The main source of phosphate into the aquatic environments includes industrial and sewage waste (DWAF, 1996b).

The chloride concentration observed in this study was higher than the South African water quality standards (0 to 100 mg/L) in all the rivers except for P1 site (80 mg/L) in spring season. The observed chloride concentration was within the range of 100 to 600 mg/L and according to DWAF there are no health effects reported for chloride at this concentration at this concentration (DWAF, 1996b). High chloride concentrations on the upper-stream sites may be due to weathering of rocks and ion exchange in soils (Kaushal, 2009). High chloride concentration in samples obtained from the WWTPs may be due to the use of chloride gas or tablets as final disinfection step during wastewater treatment. Chlorine is used to treat drinking water in water supply systems. The presence of chloride in aquatic environments lowers the concentration of microorganisms, however higher concentrations may have negative effect on the on both human and animal health (Brungs, 1973; Department of Water and Affairs, 1996; Hendrick and Pool 2012).

During water quality testing measuring ammonia concentrations in water is important as ammonia can indicate sewage, animal waste and microbial pollution (WHO, 2003). In all the samples the ammonium concentration was above the acceptable limit (0 to 1 mg/L). This indicates that all the sites were contaminated either by ammonium containing compounds from the middle-stream site to lower-stream site due to entry of agricultural runoff and sewage discharge from WWTPs. In all the rivers high ammonium concentration were observed in winter season as compared to other sampled seasons and this may be due to decrease in ammonification process and plant uptake (DWAF, 1996b).

In all the rivers the concentration of nitrate and nitrite (0 to 6 mg/L) on the samples obtained from the upper-stream site was within the acceptable target range for domestic water use. However, on the middle stream site concentrations above the acceptable target water quality range for nitrite and nitrate was observed in samples obtained from Buffalo River in winter season, Tyhume River in spring season and Swartkops River in autumn and winter season. High nitrite and nitrate concentration in samples

obtained from the WWTPs and lower stream site in all the rivers was observed. This was due to nitrate as being the end product of ammonium or nitrite during ammonification process and therefore the presence in sewage samples has been reported. In addition, high nitrate concentration in samples obtained from the middle-stream sites may be due to high ammonium concentration as ammonium salts are used as fertilizers in agriculture (DWAF, 1996b). No variation was observed in nitrate concentration between the sampled seasons. According to WHO the recommended nitrate limit for domestic water is 50 mg/L (WHO, 1996) and all the samples fell within the acceptable limit for nitrates. The sites with the nitrate concentration of less than 10 mg/L were suitable for livestock watering and recreational purposes (FEPA, 1991; USEPA, 2004; Olaniran *et al*. 2013).

In all the rivers throughout the sampling season iron was within the water quality target range (0 to 0.1 mg/L) for domestic water. This may be because of the effect of pH in iron concentration, as at neutral and alkaline pH conditions iron is usually present in concentration of µg/L. In all the water samples the pH was either neutral or alkaline, expect for upper-stream site at Bloukrans River where slightly acidic pH was detected. The pH conditions observed in water samples maybe the cause that iron was lower than the detection limit (3 mg/L) of the iron kit used.

In all the rivers sulphate concentration was high in samples obtained from middle-stream, WWTPs and lower-stream sites. This observation indicates that human activities and WWTPs have significant contribution in contamination of surface waters. Sulphate concentration complied with water quality target range of 0 to 200 mg/L in all the rivers throughout the sampling season. The sulphate concentration observed in this study was similar to the one that was reported at Boesmanspruit River and Witrandspruit River (Tate and Husted, 2015). Significant correlation between sulphate concentration and pH was observed at Buffalo River, $r(12) = 0.631$, $p < 0.05$, Bloukrans River, $r(12) = 0.595$, $p < 0.05$ and Swartkops River, $r(12) = 0.591$, $p < 0.05$ while at Tyhume River significant correlation was observed between sulphate concentration and temperature, $r(12) = 0.668$, $p < 0.05$.

2.9.2. Physical properties

The temperature of water affects the rate of chemical and biological reactions in water environments (Olaniran *et al*. 2013). Studies have reported that an increase in temperature (30 to 35 °C) in aquatic environments increase the toxicity of several substances like heavy metals and pesticides and it also escalates the sensitivity of organisms towards toxic constituents in water bodies (Momba *et al*. 2006; Olaniran *et al*. 2013; Li *et al*. 2013). In aquatic environments water temperature is affected by the turbidity of water, and this is due to the ability of the suspended solids to absorb and scatter sunlight (Mandal, 2014). There was no significant correlation (p-value ranged from 0.20 to 0.50) observed

between water temperature and water turbidity in all the rivers. According to South African water quality guidelines for domestic water use the temperature of water must be below 25 °C (DWAF, 1996b; Olaniran *et al*. 2013). Water temperature complied with the target water quality range in all the rivers. The water temperature recorded at Buffalo River and Bloukrans River corresponded to the temperature that was reported by Olaniran and co-workers (2013) during autumn season at Umgeni River and Umdloti River located in Durban, KwaZulu-Natal Province in South Africa. Seasonal variation on the temperature of river water has been observed in this study as the lowest temperature (8.48 °C) was observed during winter season while the highest temperature (23.00 °C) was reported in autumn season.

In definition "electric conductivity is the measure of the ability of water to conduct an electric current" and this results from dissolved ions in water which have a potential to carry an electric charge. The EC of water is determined by the concentration of ions present in water and the nutrient load (DWAF, 1996b; Wanda *et al*. 2016). Significant correlation between electric conductivity and other parameters such as temperature, pH, chemical oxygen demand, chloride and iron concentration of water has been previously reported in a study conducted by Shweta et al. (2014). In this study, no significant correlation was observed between EC and temperature in all the studied rivers, while significant moderate correlation was observed between EC and pH, r (12) = 0.658, $p < 0.05$ only at Buffalo River. Significant positive correlation was observed between EC and COD at Bloukrans River, r (12) = 0.776, $p < 0.05$ and Tyhume River, r (12) = 0.717, $p < 0.05$. While significant moderate correlation between EC value and Cl concentration was observed at Bloukrans River, r (12) = 0.569, $p = 0.053$ and Tyhume River, r (12) = 0.829, $p < 0.05$.

High EC values were observed during spring season at Buffalo River, whereas at Bloukrans River there was slight variations on the EC values throughout the sampling season and at Swartkops River and Bloukrans River high EC values were observed in winter and spring season. High EC values during winter season has been previously reported in Mvudi River (Edokpayi *et al*. 2015). High EC values observed during spring season may be due to surface runoff. In all the rivers high EC values were observed in the middle-stream, WWTPs and lower-stream site samples whereas on the upper-stream sites low EC reading were observed. High EC values are cause by the presence of dissolved inorganic compounds in water due to pollution by humans and WWTPs. According to South Africa National Standards (SANS) for drinking water, the water quality guidelines for EC is 170 mS/m. The water EC was within the permissible limit except for the samples obtained at site S2 during winter and spring season and S4 during spring season at Swartkops River (SANS, 2011; Wanda *et al*. 2016). High values

of EC observed in those sites may be caused by industrial discharge since those sites are nearby manufacturing industries.

The pH of the water is essential in determining the corrosive nature of water since lower pH value indicates increased corrosiveness of water. Positive correlation has been reported between the pH and EC of the water (Gupta *et al.* 2009; Shweta *et al.* 2014). Significant correlation between pH and other water parameters such as temperature, Cl and SO_4^{2-} concentration was observed in this study. Significant correlation was observed between pH and temperature at Buffalo River, $r(12) = 0.831$, $p < 0.001$ and Swartkops River, $r(12) = 0.871$, $p < 0.001$ and Tyhume River, $r(12) = 0.875$, $p < 0.001$. Significant correlation was observed between pH and Cl concentration in Buffalo River, $r(12) = 0.768$, $p < 0.001$, Swartkops River, $r(12) = 0.831$, $p < 0.001$ and Tyhume River, $r(12) = 0.588$, $p < 0.05$. Significant correlation was also observed between COD and pH at Buffalo River, $r(12) = 0.733$, $p < 0.05$, Swartkops River, $r(12) = 0.705$, $p < 0.05$ and Tyhume River, $r(12) = 0.810$, $p < 0.05$. In most of the samples the pH of river water was within the target water quality range. The pH of the sample obtained from upper-stream site of Bloukrans River in autumn was slightly below the target water quality range whereas in middle-stream sites in winter and spring the pH was slightly above the target water quality range. This may be due to photosynthetic assimilation of dissolved inorganic carbon by planktons or caused by water evaporation through the loss of half-bound CO_2 and precipitation of mono-carbonate (Iqbal *et al.* 2004; Wang *et al.* 2007; Olaniran *et al.* 2013). The pH values observed in this study were similar to the pH values that were observed in Umgeni River and Umdloti River that is in Durban, KwaZulu-Natal Province, South Africa (Olaniran *et al.* 2013).

The turbidity was above the target water quality range in all the samples obtained from Buffalo River. In all the samples obtained from Bloukrans River turbidity was above the target water quality range except for samples obtained from the upper-stream site in winter and spring which were within the acceptable target water quality range. Turbidity was above the acceptable water quality range in all the samples obtained from Tyhume River except for the upper-stream site sample obtained in winter. All the samples obtained from upper-stream site in Swartkops River were within the target water quality range together with the samples obtained from lower-stream site in winter and middle-stream and lower stream site in spring. In most of the rivers, low turbidity was observed in samples obtained from winter as compared to other seasons as expected. This was caused minimal disturbance due to low average rainfall in winter as compared to other seasons causing velocity of the water to decrease. This allows the suspended materials to settle on the bottom of the water (Momba *et al.* 2006; Olaniran *et al.* 2013; Edokpayi *et al.* 2015). High turbidity values observed in other seasons (rainy seasons) may be due to rainfall which causes surface runoff, transporting materials into the river. High turbidity

values are also linked with the presence of micro-organisms, clay, silts and other suspended materials in water which causes the water to appear cloudy and lose its natural beauty (Edokpayi *et al.* 2014; Edokpayi *et al.* 2015). High turbidity decreases the number of light rays that penetrates water and that has a negative effect on benthic organisms (Anhwange *et al.* 2012; Edokpayi *et al.* 2015). High turbidity in samples obtained from the middle-stream site are a result of human contamination. Throughout the sampling season, high turbidity was observed in all the samples obtained from the WWTPs and this indicates that WWTPs has significant contribution in the turbidity of river water. High turbidity values observed in autumn and spring in river samples may be because of disturbance due to surface runoff and erosion (Momba *et al.* 2006; Olaniran *et al.* 2013). Surface waters which are affected by different pollution sources and various category of contaminants, turbidity is a measure of water pollution. In contaminated water bodies, high turbid values show the presence of suspended organic constituents which may lead to microbial growth (Momba *et al.* 2006; Olaniran *et al.* 2013). High turbidity values have been reported to affect the aquatic life and results in decrease of water quality (Verma *et al.* 1984; Gupta *et al.* 2017).

COD is one of the water quality parameters used to measure the amount of oxygen needed to carry biochemical reactions and chemical oxidation in water. It also measures the amount of organic pollution in water in mg/L concentration range (Shweta *et al.* 2014). Significant correlation was observed between COD and temperature at Buffalo River, $r(12) = 0.858$, $p < 0.001$, Swartkops River, $r(12) = 0.582$, $p < 0.05$ and Tyhume River, $r(12) = 0.698$, $p < 0.05$. Significant correlation was also observed between COD and pH at Buffalo River, $r(12) = 0.733$, $p < 0.05$, Swartkops River, $r(12) = 0.705$, $p < 0.05$ and Tyhume River, $r(12) = 0.810$, $p < 0.05$. Significant correlation was also observed between EC and COD at Bloukrans River, $r(12) = 0.776$, $p < 0.05$ and Tyhume River, $r(12) = 0.717$, $p < 0.01$. In this study high COD values were observed in all the samples and no much variations were observed between the seasons except for Buffalo River samples were high COD values were observed in spring season. In South Africa there are no guidelines for COD limits in surface water. These high COD values observed in this study indicates that rivers are contaminated by organic and inorganic substances, organic contaminants from WWTPs, industries and agriculture. The presence of dissolved oxygen in aquatic environments results in the oxidation of organic substances thus the decrease in the level of organic substance in water and high oxygen demand. The concentration of dissolved oxygen in water bodies is influence by the temperature (Akan *et al.* 2008; Olaniran *et al.* 2013). Dissolved oxygen is important in aquatic environments as low levels of dissolved oxygen may affect aquatic organisms (Cox, 2003, Gupta *et al.* 2017).

2.9. Conclusion

According to the results obtained in this study, the sampled rivers were not suitable as sources of drinking water as most of the parameters did not comply with the South African Water Quality guidelines for domestic water use more special in the middle-stream and lower-stream sites. The health of individuals may be at risk following exposure to such contaminated water. However, the river water was suitable for agricultural purposes as it was nutrient rich and would probably stimulate plant growth. High levels of COD observed in this study also indicated the level of pollution in those rivers and the negative effect it may have on water invertebrates as high COD levels causes low dissolves oxygen concentration in water. Only iron and sulphate complied with the South African water Quality guidelines in all seasons, while phosphate, nitrates, nitrite, turbidity, temperature, pH and electric conductivity were above the acceptable target in some of the sites during the sampling seasons. High turbidity levels are usually associated with microbial contamination and water that are turbid may not be safe for human consumption. The results obtained in this study has shown that people who use these rivers as their source of drinking water in those areas may have negative health effects. Although no significant seasonal variation on the physicochemical properties of river water was observed in this study, constant monitoring of surface waters is important to determine the quality of water for the protection of public health.

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Chapter 3

Detection of pharmaceutical residues in surface waters of the Eastern Cape Province using ELISA and LC-ESI-MS/MS (triple quadrupole).

3.0. Introduction

The presence of pharmaceuticals in environmental samples has been well documented (Gros *et al.* 2006; López-Roldán *et al.* 2010; Afonso-Olivares *et al.* 2013; Madikizela *et al.* 2017) for over three decades and they are being referred to as emerging contaminants (Afonso-Olivares *et al.* 2013; da Silva and Oliveira, 2018). Pharmaceuticals are chemicals used in the diagnosis, treatment, prevention of diseases and healthy functioning of both human and animal bodies (Jones *et al.* 2001). Owing to its broad application in human and veterinary medicine, large amounts of pharmaceuticals are produced yearly (Madikizela *et al.* 2017; da Silva and Oliveira, 2018). The increase in human population worldwide (Wasentanda *et al.* 2016) is one of the major reasons for the increase in human pharmaceuticals application (Vieno *et al.* 2005). For instance, the consumption of antibiotics globally is estimated to range from 100 000 to 200 000 tons per year (Wise, 2002; Senta *et al.* 2008; Dinha *et al.* 2011; Hendrick and Pool, 2012).

Pharmaceuticals undergo metabolism in the body, resulting in some completely degraded and other metabolites excreted in body wastes. Approximately 5 to 90% of the ingested antibiotic dosage is excreted as the metabolite or parental compound depending on the chemical properties of the compound (Halling-Sørensen, 2001; Jjemba, 2002; Sarmah *et al.* 2006; Kümmerer, 2009; da Silva and Oliveira, 2018; Dinha *et al.* 2011). These pharmaceuticals end up in the sewage systems and eventually enter the environment through sewage leakages, discharge of wastewater from STPs which deposit into the aquatic systems or through the waste disposal of unused or unfinished medication (Ayman and Isik, 2015; Madikizela *et al.* 2017). In addition, the use of sludge and animal manure in agriculture as a fertilizer may cause contamination of the agricultural soils and results in the entry of antibiotics into the aquatic systems by leaching to aquatic systems or penetration into the groundwater (Hirsch *et al.* 1999; Dinha *et al.* 2011).

Worldwide pharmaceutically active compounds (PhACs) have been identified from environmental samples such as surface water, groundwater, seawater, sediments and drinking water at low concentrations usually ng/L to µg/L (Tamtam *et al.* 2008; Li *et al.* 2009; González *et al.* 2010; Sim *et al.* 2010; Yiruhan *et al.* 2010; Dinha *et al.* 2011). Some of the pharmaceuticals have been reported to persist when present in the environment and can make their way to humans via food-chain or drinking water (Khetan and Collins 2007; Fatta-Kassinos *et al.* 2011).

Over the past years, the presence of pharmaceuticals has been reported by several authors (Jones *et al.* 2001; Vieno *et al.* 2005; Gros *et al.* 2006; López-Roldán *et al.* 2010; Afonso-Olivares *et al.* 2013; Madikizela *et al.* 2017; da Silva and Oliveira 2018) in drinking water thus raising concern on the quality of drinking water (Fatta-Kassinos *et al.* 2011). The inadequate information on the effect of pharmaceuticals detected in drinking water to humans (Fatta-Kassinos *et al.* 2011) has indicated the necessity for continual testing for water quality in water resources and regulatory agencies (Trenholm *et al.* 2009). Water is essential to life in both plants and animals (Edokpayi *et al.* 2015). It is therefore important that humans and animals have access to clean and safe drinking water to maintain a healthy lifestyle. Rivers, dams and underground water has been serving as a source for drinking water for many years (Cabral, 2010) and monitoring of water quality in these water resources is crucial for the protection of public health (Gros *et al.* 2006; López-Roldán *et al.* 2010; da Silva and Oliveira, 2018).

3.1. Sources of pharmaceuticals into the environment

Pollution of aquatic systems by pharmaceuticals have been documented worldwide (Gros *et al*. 2006; López-Roldán *et al.* 2010; Afonso-Olivares *et al*. 2013; Madikizela *et al*. 2017; da Silva and Oliveira 2018). The source of pharmaceuticals into the aquatic environments involves improper disposal of unused or unfinished medication, agricultural runoff, effluent discharge from industries, hospitals and WWTPs which are considered as the main source of pharmaceuticals into the aquatic environments (Afonso-Olivares *et al.* 2013; Baranowska and Kowalski, 2010). Several studies on WWTPs have indicated WWTPs to be inefficient in removing pharmaceuticals. Therefore, those PhACs pass the WWTP without being properly removed and are released into the rivers or marine system, thus contaminating surface water and groundwater which are the main source of drinking water for humans and animals (Jones *et al.* 2001; Paíga and Delerue-Matos, 2016).

3.2. Removal of pharmaceutical in WWTPs and surface water

The WWTPs are not designed for removal of pharmaceuticals in wastewater therefore, pharmaceuticals pass WWTPs without being properly removed and are released into the aquatic environments (Gros *et al.* 2006; Vieno *et al.* 2005; Petrovic *et al.* 2006). Nevertheless, some WWTPs are using advanced methods such as ozonation and UV advanced oxidation treatment processes and those treatment processes have been reported to considerably lower the concentration of pharmaceuticals before effluents are discharged into the environment (Ternes *et al.* 2002; Trenholm *et al.* 2009; López-Roldán *et al.* 2010). The traditional methods such as chlorination and filtration or flocculation have been reported to remove low percentages of pharmaceuticals in water (Petrovic *et al.* 2006; López-Roldán *et al.* 2010) while processes such as nanofiltration and reverse osmosis have

also been reported to be effective in the removal of pharmaceuticals from water (Zwiener and Frimmel, 2000; Ternes *et al.* 2002; López-Roldán *et al.* 2010).

Hendrick and Pool (2012) reported three STPs to be inadequate in the removal of fluoroquinolones as there was no much difference in concentration between the influent and effluent samples. The influent and effluent concentration which ranges from 300 to 500 ng/L has been reported in countries such as France, Greece, and Italy and from 30 to 1100 ng/L in Switzerland. The release of fluoroquinolones into aquatic bodies can negatively impact fish species as well as consumers of fish (Miranda *et al.* 2001; Pathak and Gopal, 2005; Hendricks and Pool, 2012). Pharmaceuticals such as ibuprofen, naproxen, ketoprofen, diclofenac, and bezafibrate have been reported to be removed by biodegradation and sorption on STPs (Ternes *et al.* 2004; Vieno *et al.* 2005, Urase 2005). While in surface waters they are removed by biodegradation, sorption, and photodegradation. Ibuprofen has been reported to be removed by biodegradation (Buser *et al.* 1999; Winkler *et al.* 2001; Vieno *et al.* 2005) and its relatively high sorption coefficient also allows it to be removed by sedimentation (Tixier *et al.* 2003; Vieno *et al.* 2005). Photodegradation has been reported to readily degrade diclofenac whereas poor absorption by the sediments has been reported (Buser *et al.* 1998; Tixier *et al.* 2003; Andreozzi *et al.* 2003; Vieno *et al.* 2005).

The type of the sediment influences sorption of ibuprofen and diclofenac, therefore removal of pharmaceuticals in surface waters can be site-specific (Vieno *et al.* 2005). Sorption of the pharmaceuticals through the soil is achieved by various mechanisms which include ion exchange, surface adsorption of minerals, and a creation of complexes with metals, hydrogen bonding, and association with the organic substance. The ionization of majority pharmaceuticals makes pH to be an important factor in sorption of the pharmaceuticals into the soil. Basic pharmaceuticals demonstrate great sorption in soil, and this is due to soil organic matter being negatively charged (Fatta-Kassinos *et al.* 2011).

The removal of the pharmaceuticals in treatment plants has been reported to be achieved by biodegradation and sorption with biodegradation being the main process. Both biodegradation and sorption processes are temperature dependent, with low temperatures reported to favour sorption while biodegradation is reduced. This has been reported in the winter season (7 °C) where low elimination of pharmaceuticals has been reported as compared to high elimination rate which was reported in spring and summer season when water temperature ranged from 13 to 21 °C (Vieno *et al.* 2005). The absence of the nitrification process in the treatment plant during the winter season at Aura STP was reported to be due to low water temperature thus, resulting in inadequate removal of the nitrogen. The

absence of nitrifying microorganisms in Aura STP during winter season was reported to be the cause of poor elimination of pharmaceuticals (Vieno *et al.* 2005).

3.3. Seasonal effect on the concentration of pharmaceuticals

Higher concentrations of pharmaceuticals in surface waters during rainy seasons can be expected due to agricultural runoff. Thus, making agriculture one of the main sources of pharmaceuticals in surface waters. The elevated levels of pharmaceuticals reported from river samples in Aksaray, Turkey was assumed to be influenced by season. A study done by Ayman and Işik (2015) demonstrated that samples obtained in December 2010 (winter season) and June 2011 (summer season) had high concentrations of pharmaceuticals as compared to samples that were obtained in April 2011 (spring season) and this was due to surface runoff. The concentration of the pharmaceuticals in the effluent samples of the STP has been reported to be high during the winter season as compared to spring and summer season (Vieno *et al.* 2005).

According to the study conducted by Tauxe-Wuersch et al. (2005) the degradation of ibuprofen and ketoprofen during winter season was decreased and this was due to lower biodegradation in the STP as a result of heavy rainfall. However, Vieno et al. (2005) reported lower degradation of pharmaceuticals to be caused by low water temperature (7 °C) resulting in decreased biodegradation. Thus, high contamination of river water by pharmaceuticals during the winter season is influenced by a decrease in water temperature (Vieno *et al.* 2005). High concentration of pharmaceuticals was observed in dry season in Yangtze River and Pearl River Estuary in China as compared to the wet season (Liang *et al.* 2013). In contrast, high concentrations of antibiotics were reported during wet season in China and this was thought to be due to increased concentrations in runoff from the veterinary medicines (Gaw *et al.* 2014). Seasonal changes in the concentration of antibiotics has been reported as higher concentration of antibiotics has been reported in winter season as compared to the summer season. This was explained by the fact that majority of people get sick in winter season as compared to summer season (Castiglioni *et al.* 2004; Hendrick and Pool, 2012).

3.4. Antibiotics in the environment

Detection of antibiotics in environmental samples have been documented by several authors (Yang *et al.* 2010; Dinha *et al.* 2011; Mulamattathil *et al.* 2014). Their presence in the environment may lead to the development of antibiotic resistance even at low concentration, therefore, posing a health concern for both humans and animals. In addition, some of the antibiotics persist in the environment lasting up to months (Brij Verma John, 2007; Shelver *et al.* 2008; Dinha *et al.* 2011). Antibiotics may also be a threat to aquatic life as their effect is still unknown (Hendricks and Pool, 2012). The presence of

antibiotics such as ciprofloxacin and sulfamethoxazole in untreated river water has been reported while antibiotic resistance has been reported for trimethoprim (Mulamattathil *et al.* 2014). Although the Sulfonamide are extremely lipophilic, their presence in the environment can be problematic since they are persistent and resilient to biodegradation (Shelver *et al.* 2008). According to Shelver et al. (2008) elimination of some antibiotics such as sulfamethoxazole metabolite by WWTP leads to the formation of the parent compound that is biologically active. Therefore, resulting in increased concentration in the effluent samples which are discharged into the rivers. Inadequate removal (33 to 75%) of the sulfamethoxazole by the STP have been reported, therefore sulfamethoxazole is released into the environment with wastewater effluents (Shelver *et al.* 2008). The extensive application of sulfamethoxazole antibiotic in humans and animals (Peng *et al.* 2006; Hendricks and Pool, 2012) as well as its persistence to degradation makes it to be omnipresent in the environment (Hong *et al.* 2008; Hendricks and Pool, 2012). Sulfamethoxazole has been detected in concentration range of 0.22 to 0.68 µg/L in WWTP effluent samples and similar concentration range has been reported for sulfapyridine (Shelver *et al.* 2008).

Sulfonamides have an important application as veterinary medicine for both prevention and treatment of diseases and are also used as growth promoters. In the body, these compounds are subjected to metabolism and are then excreted by the body through urine and faeces partially or unchanged at all (Hendricks and Pool, 2012). In humans, sulfonamides have important application in the treatment of bacterial infections. Being the most antibiotic used by humans they contribute about 16 -21% of the antibiotics used annually. Therefore, this explains why sulfonamides are usually present in environmental samples (Shelver *et al.* 2008).

Quinolones, fluoroquinolones and quinolone carboxylic acids have an important application as antibiotics and veterinary medicine in humans and animals respectively (Hendricks and Pool 2012; Peng 2006). Fluoroquinolones have wide application in aquaculture and are commonly the main reason that they detected in high concentrations in the environment more especially in the areas with fishponds (Afonso-Olivares *et al.* 2013; Zou *et al.* 2011). The presence of fluoroquinolones and sulfamethoxazole have been reported in the influent and effluent sewage samples (Hendricks and Pool 2012). Fluoroquinolones have been reported in concentration which ranges from 89 to 92 ng/L in influent samples of the STPs. This was explained by the fact that fluoroquinolones are the most commonly used antibiotics in South African (Hendricks and Pool, 2012).

The presence of carbamazepine in surface waters have been reported worldwide. Carbamazepine is extremely persistent in the environment with the removal rate to 10% by the STPs (Zhang *et al.* 2008).

Carbamazepine was detected in all the samples obtained in the Llobregat River in Spain in a concentration range of 8 to 179 ng/L. Although its concentration was not too high, its presence in all the samples indicated that this drug was among the commonly consumed drugs and this may be problematic as the WWTPs are not efficient in removing it. Therefore, it passes the WWTPs partially or not removed at all and gets released with the effluent discharge (López-Roldán *et al.* 2010).

According to the Global Water Research Coalition (GWRC) carbamazepine, sulfamethoxazole, diclofenac, ibuprofen, naproxen, bezafibrate, atenolol, erythromycin and gemfibrozil are high priority pharmaceuticals (class 1) needs to be regularly monitored in the environment (GWRC 2004), since high concentrations of these pharmaceuticals are introduced into the environment following human use (López-Roldán et al. 2010).

3.5. Effect of pharmaceuticals in aquatic organisms

There is inadequate information on the long-term effect of the wide range of pharmaceuticals detected in environmental samples at low concentrations on both humans and animals (López-Roldán *et al.* 2010). Nevertheless, their presence in aquatic environments has the potential of affecting the quality of water which in turn may affect the aquatic biodiversity and human health (Baranowska and Kowalski, 2010; Maldaner *et al.* 2012; da Silva and Oliveira, 2018). The constant exposure of pharmaceuticals to aquatic environments can lead to chronic effects such as alterations in the metabolic or/and reproductive systems in non-targeted organisms (Cooper *et al.* 2008; López-Roldán *et al.* 2010). In addition, the presence of pharmaceuticals in the environment such as antibiotics can lead to the development of antimicrobial resistance as some microorganisms have potential to alter their genetic material and may have toxicological effects to both human and animal upon long-term exposure (Gros *et al.* 2006; Maldaner *et al.* 2012; da Silva and Oliveira, 2018). The transfer of resistant bacteria to humans can occur through water or food if plants are irrigated with water, wastewater or sludge containing resistant bacteria (Baquero *et al*. 2008; Fatta-Kassinos *et al.* 2011).

Carbamazepine has been reported to affect the circulating thyroid hormones at a concentration of 179 ng/L while sulfamethoxazole has been reported to change the thyroid function at a concentration of 119 ng/L (López-Roldán *et al.* 2010). Carbamazepine has been reported to have effects on the ultrastructure of the fish kidney, liver, and gills at a concentration of 1000 ng/L (Triebskom *et al.* 2007; Ramaswamya *et al.* 2011). According to Fent et al. (2006) diclofenac was associated with the disappearance of the Orient White-backed Vulture in India and Pakistan, while in mammals, diclofenac was reported to affect kidneys and liver. Propranolol in north-eastern Spain surface water was reported to have toxic effects on zooplankton and benthic organisms. In addition, exposure of estrogens to the

marine environment has been associated with the feminization of male fish resulting in a lack of mating fish (Valcárcel *et al.* 2011; Agunbiade *et al.* 2014).

It has been observed that some beta-blockers target specific receptors (e.g. atenolol target β1) while others are not that specific e.g. propranolol which targets β2. Beta-blockers bind to the beta-adrenergic receptors which are found in many tissues in mammals including the heart and inhibit its activation and this leads to a decrease in heart rate and contraction (Fent *et al.* 2006). In addition to that beta-blockers were considered to lower the heartbeat, fertility, and size of *D. magna* at a concentration of 0.11 mg/L. Long-term exposure of *C. dubia* at 250g/L and in *H. Azteca* at 100 µg/L to propranolol was associated with a reduction in reproduction (Fent *et al.* 2006).

The uptake of pharmaceuticals by plants in the environment when treated wastewater is used for irrigation has the potential of affecting plant growth. The effect, however, was unclear whether it is caused by pharmaceuticals itself or results from the presence of antibiotics which change the microbiota of the soil, therefore, affecting plant-microorganism symbiosis (Chander *et al.* 2005; Fatta-Kassinos *et al.* 2011). It has been later reported that the presence of antibiotics in soil has an indirect effect on the plant growth, by disturbing the soil communities. The decrease in soil bacteria results in lack of food supply for soil fauna and affects the soil function as plant residues decomposition becomes slow, denitrification process becomes reduced, and nutrients are recycled slowly (Migliore *et al.* 1998; Kaushal, 2009; Fatta-Kassinos *et al.* 2011; Danner *et al*. 2019).

3.6. Instrumental analysis

Detection of pharmaceuticals and pesticides from the environmental samples have been achieved by use of gas chromatography coupled with mass spectrometry (GC-MS), high-resolution liquid chromatography-tandem combined with mass spectrometry (LC-MS/MS), ultra-performance liquid chromatography time-of-flight mass spectrometry (UPLC-TOF-MS) and capillary electrophoresis (CE) combined with detectors such as MS, UV-Vis, FID, ECD. The GC-MS and LC-MS methods in conjunction with extraction, derivatization and clean-up technique offers means of detecting pharmaceuticals up to a concentration of ng/L. Capillary electrophoresis has been used for identification of pharmaceuticals in wastewater samples due to it being less complex and cost-effective as compared to GC and LC. However, CE is less sensitive than GC and LC detecting concentrations up to µg/L, thus making the technique only suitable for highly contaminated waters (wastewater) rather than surface water (López-Roldán *et al.* 2010; Baranowska and Kowalski 2010; Ahmed *et al.* 2010, Fatta-Kassinos *et al.* 2011; Afonso-Olivares *et al.* 2013).

Both GC-MS and LC-MS are suitable for analysis of pharmaceuticals in the environmental samples. Nevertheless, GC-MS is more suitable for analysis of non-polar and volatile compounds, although analysis of the pharmaceuticals in low concentrations can be obtained by addition of the derivatization process. Gas chromatography provides advantages of being highly sensitive with high resolution, excellent accuracy, and precision with wide dynamic range (Hada *et al.* 2000; Cochran 2002; Fatta-Kassinos *et al.* 2011). The derivatization of polar pharmaceuticals is essential in GC and optimization techniques have been developed as this process can affect the accuracy of the technique due to loss of analytes. The efficiency of the derivatization process relies on the type of compound in the study as well as the derivatization agent used. Usually, acid anhydrides, alkyl chloroformates, benzyl halides, and diazomethane are used for derivatization, although diazomethane is not usually used due to its toxicity and carcinogenicity. Derivatization may be incomplete at times, therefore, affecting the results of the analytes or totally hinder analysis of some compounds, e.g. GC-MS is not suitable for atenolol and sotalol analysis due to this reason. In addition, thermolabile compounds degrade during GC-MS analysis e.g. carbamazepine produces iminostilbene as degradation product (Petrovic *et al.* 2003; Fatta-Kassinos *et al.* 2011).

Liquid chromatography is the most preferred method for analysis of polar organic compounds and offers an advantage of rapid analysis of pharmaceuticals in environmental samples. The major disadvantage of HPLC method for analysis of organic pollutants in environmental samples is the matrix effect (the ion-suppression) resulting in the decrease in sensitivity, linearity, accuracy, and precision of the technique (Petrovic and Barcelo, 2007; Fatta-Kassinos *et al.* 2011). The most preferred technique for analysis of pharmaceutical residues in water samples is LC-MS/MS (López-Roldán *et al.* 2010; Baranowska and Kowalski, 2010; Ahmed *et al.* 2010; Fatta-Kassinos *et al.* 2011; Afonso-Olivares *et al.* 2013). This is due to its high sensitivity (Afonso-Olivares *et al.* 2013), selectivity and robustness (Petrovic *et al*. 2006; López-Roldán et al. 2010; Masiá *et al.* 2013). The high sensitivity of LC-MS/MS with triple quadrupole (QqQ) analyser is the reason this method is convenient for the detection of pharmaceuticals in surface waters (up to ppb) through target analysis in multiple reaction modes (López-Roldán *et al.* 2010; Gros *et al.* 2006; López-Serna, 2011; Baena-Nogueras *et al.* 2016).

The ionization methods commonly used in LC-MS/MS analysis is electrospray ionization (ESI) and this is due to its high sensitivity, reliability, and robustness. Analysis using the multiple reaction monitoring modes allows for identification, confirmation, quantification and caters for low detection limits this is a result of an increase in signal to noise ratio (Maldaner *et al.* 2012; Afonso-Olivares *et al.* 2013). Detection of pharmaceuticals using LC-MS/MS with QqQ allows for both identification and confirmation of the target compound. This is a result of three identification point (IP) needed for

verification of the presence of compounds of interest. The QqQ analysers have been demonstrated to be extra sensitive and precise for quantification (Gros *et al.* 2006; López-Roldán *et al.* 2010). However, the disadvantage of this method is that the analytes which are not demonstrating two selected reaction monitoring (SRM) transitions, meaning one product ion can be produced from the precursor ion cannot be confirmed. This is the problem that makes this technique not suitable for analysis of compounds such as ibuprofen and gemfibrozil as they have poor fragmentation (López-Roldán *et al.* 2010).

The UPLC-TOF-MS technique has been applied for detection of pharmaceuticals in wastewater (Bueno *et al.* 2007) and in river water (López-Roldán *et al.* 2010) and was reported to be effective. The high sensitivity of TOF-MS allows it to measure the correct mass of the compounds thus providing confirmation needed for obtaining reliable results (López-Roldán *et al.* 2010). However, TOF-MS is ineffective for detection of the pharmaceuticals in minimal polluted waters such as river water, groundwater, and drinking water, and QqQ in SRM is usually applied (Petrovic *et al.* 2006; López-Roldán *et al.* 2010).

3.7. Sample preparation methods

Low concentrations of pharmaceuticals in environmental samples makes a direct analysis of pharmaceuticals by chromatographic techniques to be challenging thus, pre-concentration of the sample is fundamental. The pre-concentration step is not only essential only for providing means for the detection of pharmaceuticals in low concentrations but also reduces the matrix effect during LC-MS analysis (Baena-Nogueras *et al.* 2016). Sample extraction methods for concentration of pharmaceuticals in environmental samples are liquid-liquid extraction, ultrasonic-assisted extraction/centrifugation, SPE, liquid-phase microextraction and freeze-drying (Kostopoulou and Nikolaou, 2008; Fatta-Kassinos *et al.* 2011). Traditionally extraction of pharmaceuticals from aquatic environments has been achieved by liquid-liquid extraction technique for the past years. However, the disadvantage of this method lies in utilizing large volumes of organic solvents such as chloroform which are hazardous to human health, time consuming and produces large amounts of chemical wastes (Togola and Budzinski, 2008; Trenholm *et al.* 2009; Mastroianni *et al.* 2010; da Silva and Oliveira, 2018) and only effective for extraction of compounds that are soluble to organic solvents and has low recovery for highly polar compounds (Savjani *et al.* 2012). The SPE method is the preferred sample preparation technique due to small organic solvent required for extraction and column clean-up, short sample preparation time and the ability to pre-concentrate the sample. Its ability to isolate target compounds and reproducibility, allows for optimization and sample clean-up in different sample matrix prior to chromatographic analysis (Maldaner *et al.* 2012; Afonso-Olivares *et al.* 2013; Aga *et al.* 2016).

3.8. The type of SPE used for analysis of pharmaceuticals

Different types of SPE cartridges suitable for analysis of acidic, neutral and basic compounds have been developed such as octadecyl silica (C18), Isolute ENV+, Lichrolut EN, and polymeric mixed-mode sorbents which are Oasis MXC, Oasis MAX, Strata-X-C, and Strata-X-A (Maldaner *et al.* 2012). The challenge with these cartridges lies on analysis of samples containing compounds with different polarities, chemical, and physical properties simultaneously. This challenge is overcome by advanced SPE column with polymeric hydrophilic-lipophilic balance, Oasis HLB (Baranowska and Kowalski 2010; Maldaner *et al.* 2012). Oasis HLB allows for simultaneous extraction of acidic, neutral and basic analytes over a broader pH range (Maldaner *et al.* 2012) with methanol, acetone and ethyl acetate as an elution solvent (Fatta-Kassinos *et al.* 2011).

3.9. Enzyme immunoassay for screening for the presence of antibiotics

Enzyme-linked immunosorbent assay (ELISA) technique is one of the traditional techniques used to screen for presence antibiotic residues in meat, milk, surface water, groundwater, wastewater, soil and manure (Aga *et al.* 2003; Kumar *et al.* 2004; Kandimalla *et al.* 2007; Černoch *et al.* 2012; Barber *et al.* 2009; Galarini *et al*. 2014; Bradley *et al.* 2014;). ELISA techniques are useful for the screening of structural similar antibiotic mixtures in a sample (Aga *et al.* 2016). The compounds with similar structures are difficult to differentiate with immunoassays, therefore, LC/MS or LC-MS/MS techniques are used for detection and quantification of structurally similar compounds. Nevertheless, due to high costs which are involved in LC/MS or LC-MS/MS methods, the technique cannot be used for routine analysis of the pharmaceuticals in environmental samples therefore, ELISA techniques are usually used (Shelver *et al.* 2008). The frequent monitoring of the antibiotics in environmental samples is essential as it provides information about the antibiotics present in the environment, their concentration and the potential of causing negative effects to the environment (Aga *et al.* 2016). The use of ELISA allows for simultaneous screening of various samples within a short period of time and at low costs. The antibodies used in ELISA are usually designed to target one analyte but because they are class specific, they display a high degree of cross-reactivity with other structurally similar compounds (O'kennedy *et al.* 1990; Huet *et al.* 2006; Wang *et al.* 2007; Černoch *et al.* 2012; Aga *et al.* 2016). In LC-MS/MS technique small changes in the structure of the analyte can hinder detection whereas in ELISA the wide-range of cross-reactivity can be beneficial in screening procedures as the assay can detect the presence of transformation products in the sample (Aga *et al.* 2016). Even though the ELISA methods are not a replacement for the quantitative instrumental techniques, they display advantage of low costs analysis and detection of compounds with similar structure as a result of the transformation of antibiotics in the environment (Aga *et al.* 2016).

The use of enzyme immunoassays provides the following advantages: the assays are highly sensitive, highly specific, and need small volume of reagents, the assays require less time for analysis and can screen various samples provide both qualitative and quantitative results, the detection is fairly straight forward as results may be detected visually or by the use of special readers, the results are reproducible, has automated and manual methods, has multi-purposes, not sensitive to radiation and can be disposed of with waste, some assays are portable and can be used on the field and can use monoclonal or polyclonal antibodies (O'kennedy *et al.* 1990; Shelver *et al.* 2008; Aga *et al*. 2016).

The disadvantages related to the use of enzyme immunoassays include: the specificity of an antibody to an antigen, unspecific binding of antibodies to solid phase, inadequate washing, the quality and pureness of the enzyme-labelled antibodies, achievability of labelling, impact of labelling on the binding ability of the antibody, the sensitivity of the assay, the discharge of the immobilized antigen or antibody from the solid phase, the potency of the antibody and enzyme, the polyclonal antibodies may have challenge of specificity of different groups (O'kennedy *et al.* 1990; Ivanov *et al*. 2008; Jaria *et al*. 2020). These drawbacks can be suppressed by purification and adequate control of the antibody before use for analytical purposes, and so far, the development of monoclonal antibodies has helped to solve such a challenge. The imprecise attachment of the antibodies can be solved by using sufficient blocking measures and these may differ in each assay. The monoclonal antibody technology has the benefit of producing sticky antibodies that bind to the surface of the plastic, glass etc. (O'kennedy *et al.* 1990; Shelver *et al.* 2008; Jaria *et al*. 2020).

3.10. Aims

1. Detection of targeted pharmaceutical residues in river water samples of Buffalo, Tyhume, Bloukrans and Swartkops River using UPLC-ESI-MS/MS.

2. Determine the impact of humans activities towards contamination of river water by comparing the lower-stream sites to the upper-stream site.

3.11. Objectives

1. Screen for the presence of pharmaceutical residues in river water using ELISA kit and confirm the results using LC-MS/MS.

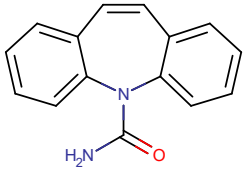
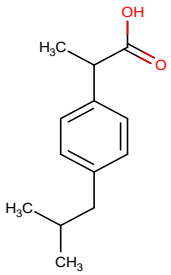
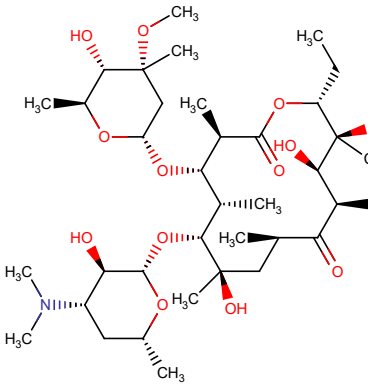
2. Measure the concentration of the targeted pharmaceutical residue in each site and determine level of pollution by comparing each site to the upper-stream site.

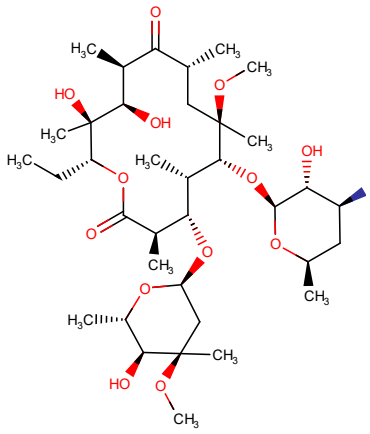
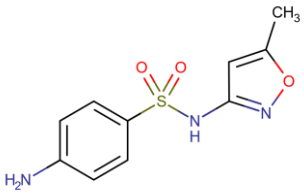
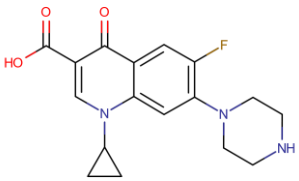
3.12. Methodology

The target pharmaceutical compounds in this study were carbamazepine, ibuprofen, ciprofloxacin, sulfamethoxazole, erythromycin and clarithromycin, the standards of target pharmaceuticals were

purchased from Merck Millipore (Gauteng, South Africa). The standard solutions (1000 µg/mL) of the target compounds were prepared by dissolving the weighed standard powder into methanol (HPLC gradient-grade) purchased from Merck Millipore (Gauteng, South Africa). The structure and molecular weight of the target pharmaceuticals are shown in table 3.1.

Table 3. 1: List of target pharmaceutical compounds and their structures (Drugbank, 2018).

Compound group	Target compound	Use	Molecular weight (g/mol)	Structure
Anticonvulsants	Carbamazepine	Anti-epileptic	236.274	
Non-steroidal anti-inflammatory drug	Ibuprofen	Anti-inflammatory	206.285	
Macrolide antibiotic	Erythromycin	Antibiotic	733.937	

Macrolide antibiotic	Clarithromycin	Antibiotic	747.953	
Sulfonamide	Sulfamethoxazole	Antibiotic	253.276	
Fluoroquinolone	Ciprofloxacin	Antibiotic	331.347	

3.12.1. Sample preparation for pharmaceutical analysis

The 96 (1 L) water samples were collected from the four selected rivers, stored in ice and transported into the laboratory for analysis (for detailed sample collection refer to chapter 2). The analysis of the samples was conducted within 48 hours. Prior to sample analysis water samples were filtered through 0.45 µm pore-sized membrane filters purchased from Merck Millipore (Gauteng, South Africa). For ELISA screening the filtered samples were transferred into sterile vials purchased from Merck Millipore (Gauteng, South Africa) and kept on ice. The screening was performed using the ELISA kits purchased from Abraxis LLC (Warminster, England) following the manufactures protocol. Pre-concentration of the samples was conducted by freeze drying method. About 500 mL of the filtered river water samples were freeze using liquid nitrogen on a round bottom flask connected to a Buchi Rotavapor, model R-215 (Labortechnik AG, Flawil Switzerland) at a speed of 160 rpm. The samples were frozen until they reached a temperature of approximately -40 °C and dried on a freeze dye machine VirTis BenchTop K, model #2KBTES-55 (SP Scientific, Europe). The freeze dyer condenser temperature was set at -50 °C and the vacuum was set at 300 mT. The samples were left on a freeze dryer machine until a powder sample was obtained. The product yield was weighed and transferred

into a sterile 10 ml vial and labelled properly. The lyophilised samples were sent to Stellenbosch University for LC-ESI-MS/MS analysis.

3.12.2. Pharmaceutical screening

3.12.2.1. Fluoroquinolones

Screening of fluoroquinolones was performed using the screening kit following the manufacturer's protocol. Approximately 50 µl of the standard solutions, samples, enzyme conjugate, and antibody solution were added to the ELISA microtiter plate pre-coated with secondary antibodies (goat anti-rabbit) specific to a unique antigenic site on the fluoroquinolone molecule. The solution was then mixed properly, and the plate was then incubated for 1 hour at room temperature. Following incubation, the wells were washed 3 times with 1X wash buffer solution and tapped dry on a stack of paper towel. Approximately, 100 µL of the substrate (colour) solution was then added to the wells and incubated at room temperature for 20-30 minutes. After incubation 100 µL of the stop, the solution was added to the wells to stop the enzyme reaction. The optical density was read at 450 nm with a microtiter plate reader. The zero standard (0 ppb) results in maximum binding of the enzyme conjugate. The results are reported in percentage of zero standards. Interpretation of the results was performed manually by plotting a standard curve using the results obtained for the standards and the concentrations of the samples were read from this curve (Appendix 1: figure 7 and 8).

3.12.2.2. Sulfamethoxazole

The screening for sulfamethoxazole was performed using the sulfamethoxazole ELISA kit following the manufacturer's protocol. The test was performed by adding 75 µl of the samples, control, and standards to the microtiter plate pre-coated with (goat anti-rabbit). Following that 50 µL of the anti-sulfamethoxazole antibody solution was added to each well. The solution was then mixed for 20–30 seconds and the plate were incubated at room temperature for 20 minutes. Following 20 minutes incubation, 50 µL of the sulfamethoxazole enzyme conjugate solution was then added to each well. The plate was then mixed properly for 20-30 seconds and incubated for 40 minutes at room temperature. After incubation, the wells were then washed 3 times with 1X wash buffer solution and tapped dry on a stack of paper towel. When the washing step was done, 150 µL of substrate (colour) solution was added to all wells and incubated for 30 minutes at room temperature. About 100 µL of the stop solution was added after incubation to stop the enzyme reaction. The optical density was then read at 450 nm with a microtiter plate reader. The zero standard (0 ppb) results in maximum binding of the enzyme conjugate. The results are reported in percentage of zero standards. Interpretation of the results was performed manually by plotting a standard curve using the results obtained for the

standards and the concentrations of the samples were read from this curve (Appendix 1: figure 9 and 10).

3.12.3. Extraction of targeted pharmaceutical residue

Prior to sample analysis, the lyophilised samples were reconstituted in a 9 mL of 10% methanol consisting of 1 ml of 50 g/L p-aminosalicylic acid used as an internal standard. Pharmaceutical residues were isolated from the sample by passing the 10 mL sample through SPE cartridge, packed with Oasis HLB 6 cc Vac cartridge, 200 mg sorbent purchased from Waters (Milford, USA). The cartridge was washed with water and the analytes were eluted off using 1mL methanol. The percentage recovery of the analytes after sample clean-up was 103 % ± 6.9.

3.12.4. Chromatographic separation

Chromatographic separation was carried out on in Acquity UPLC linked to a Xevo TQS triple quadrupole Mass Spectrometer (Waters, South Africa). The analytes were separated on 2.1 x 100 mm, 1.7 µm UPLC BEH reverse phase C19 analytical column (Waters, South Africa). The mobile phase consisted solvent A: 0.1% formic acid in water and solvent B: 0.1% formic acid in acetonitrile. The column was initially eluted 0.1 % formic acid in water for 0.5 minutes at a flow rate of 0.3 mL/minute then increased to 0.1% formic acid in acetonitrile for 9.5 minutes at a flow rate of 0.3 mL/minute then back to 0.1% formic acid in water for 1.5 minutes at a flow rate of 0.3 minutes. Before the next injection, the column was allowed to calibrate for 5 minutes. The analysis was carried out for 12 minutes and the retention times were between 4.59 to 5.70 minutes for the four detected analytes. Mass spectrometry was performed using Xevo TQS triple quadrupole Mass Spectrometer (Waters, South Africa) equipped with an electrospray ionization source. The mass spectrometer was carried out in a multiple reaction monitoring mode and the cone voltage and collision energy were optimized for each analyte in positive ionization mode. The spectrum of the target pharmaceuticals is presented on appendix 1, figure 11 to 14.

3.13. Results and discussion

Detection of pharmaceutical contaminants in surface waters have been reported worldwide (Gros *et al.* 2006; López-Roldán, *et al.* 2010; Afonso-Olivares *et al*. 2013; Olaniran *et al*. 2013; da Silva and Oliveira, 2018). Recent studies have reported that the concentrations present in surface water can pose toxicological effects on the environmental (Halling-Sørensen *et al.* 2000; Andreozzi *et al.* 2004; Isidori *et al.* 2005; Robinson *et al.* 2005; aus der Beek *et al.* 2016). In this study the presence of pharmaceutical residues in river water of the Eastern Cape Province Rivers was investigated. The screening of pharmaceutical was performed by ELISA and confirmation of the presence of

pharmaceuticals was conducted by LC-ESI-MS/MS. The concentration of sulfamethoxazole and fluoroquinolone screened by ELISA are presented on table 3. 2. Sulfamethoxazole was detected in 12 out of 13 samples with 92.3% detection frequency while fluoroquinolones were detected in 8 out of 13 samples with 61.54% detection frequency. Sulfamethoxazole was obtained in high concentrations ranging from not detected to 1.4 ppb while fluoroquinolones were obtained in a concentration ranging from not detected to 0.4 ppb.

Table 3. 2: Mean concentration of the detected antibiotics in Buffalo, Bloukrans, Swartkops and Tyhume River water samples by ELISA screening during autumn season.

River	Season	Site number	Sulfamethoxazole (ppb)	Fluoroquinolones (ppb)
Buffalo	**Autumn**	B1	ND	ND
		B2	0.7	ND
		B3	NS	NS
		B4	0.9	0.1
Bloukrans	**Autumn**	Bl1	0.2	ND
		Bl2	1.4	0.4
		Bl3	1.4	0.5
		Bl4	1.3	0.4
Swartkops	**Autumn**	S1	0.1	ND
		S2	1.1	ND
		S3	NS	NS
		S4	1.2	0.4
Tyhume	**Autumn**	T1	0.1	0.3
		T2	0.2	0.2
		T3	NS	NS
		T4	0.4	0.2

NS: not sampled; ND: not detected

The pharmaceuticals detected in river water samples collected from Buffalo, Bloukrans, Swartkops and Tyhume River are presented in Table 3.3. Carbamazepine, Erythromycin, sulfamethoxazole and clarithromycin were the psychiatric and antibiotic drugs detected respectively. However, two target compounds, ibuprofen and ciprofloxacin were not detected from all the samples. The instrumental parameters for UPLC-ESI-MS/MS method are shown in table 3. 4. Carbamazepine was the only pharmaceutical present in all the samples with 100% detection frequency. Similar results have been observed in Umgeni River, KwaZulu Natal, in South Africa (Matongo *et al*. 2015). Followed by clarithromycin present in all the samples except for site P1 at Palmiet River with 93.75% detection frequency. Erythromycin and sulfamethoxazole were detected in 11 and 12 of the 16 analysed samples with detection frequency of 68.75% and 75% respectively. Slightly higher detection frequency (83%) of sulfamethoxazole was obtained in Umgeni River, KwaZulu Natal, in South Africa, while lower detection frequency (42%) for erythromycin was obtained in the same river (Matongo *et al*. 2015). In

all the samples erythromycin was detected in high concentrations ranging from not detected (P1, B1, B2, S1 and T1) to 372.100 ng/0.5 L (Bl3). Higher erythromycin concentrations (260 ng/L) have been reported in Umgeni River samples compared to the concentrations reported in this study (Matongo *et al.* 2015). Carbamazepine was following with a concentration range of 40.9 (P1) to 18288.1 ng/0.5 L (S3). However, higher concentration range of carbamazepine (190 ng/L to 1650 ng/L) has been reported in Umgeni River. (Matongo *et al*., 2015). Sulfamethoxazole was detected with a concentration range of not detected (B2, S1 and T1) to 3484 ng/0.5 L (Bl3) while clarithromycin was observed at concentration range of 2.4 (B1) to 1640.2 ng/0.5 L (Bl2). In a study conducted in Umgeni River similar sulfamethoxazole concentration range (not detected to 1240 ng/L) was reported (Matongo *et al*. 2015). Significant correlation was observed between carbamazepine and pH at Buffalo River, $r(2) = 0.966$, $p < 0.05$, while at Bloukrans River significant correlation was observed between temperature and carbamazepine, $r(2) = 0.952$, $p < 0.05$ and between erythromycin and temperature $r(2) = 0.998$, $p < 0.01$. Positive correlation was observed between temperature and clarithromycin at Swartkops River, $r(2) = 0.954$, $p < 0.05$.

Table 3. 3: The pharmaceuticals detected with UPLC-ESI-MS/MS in river water samples collected from Buffalo, Bloukrans, Swartkops and Tyhume River during spring season.

		Class					
		Anti-epilepsy	**Macrolide antibiotic**	**Antibiotic**	**Macrolide antibiotic**	**Antibiotic**	**Anti-inflammatory**
River	**Site no.**	**Carbamazepine (ng/0.5 L)**	**Erythromycin (ng/0.5 L)**	**Sulfamethoxazole (ng/0.5 L)**	**Clarithromycin (ng/0.5 L)**	**Ciprofloxacin (ng/0.5 L)**	**Ibuprofen (ng/0.5 L)**
Palmiet	P1	40.9	ND	3.3	ND	ND	ND
Bloukrans	Bl2	7181.8	266800	2267.6	1640.2	ND	ND
	Bl3	13164.8	372100	3484	770.9	ND	ND
	Bl4	4858.9	81800	2987.2	239.1	ND	ND
Buffalo	B1	108	ND	10.1	2.4	ND	ND
	B2	168.9	ND	ND	4.2	ND	ND
	B3	7481.9	131500	1822.7	157.5	ND	ND
	B4	2379	41.1	652.7	33.7	ND	ND
Swartkops	S1	1130.7	ND	ND	72	ND	ND
	S2	2645.6	59000	1459.4	132.4	ND	ND
	S3	18288.1	17300	989.9	130.4	ND	ND
	S4	8672.6	30300	1333.2	49.1	ND	ND
Tyhume	T1	61.7	ND	ND	2.7	ND	ND
	T2	471.9	14200	167.2	41	ND	ND
	T3	2548.8	58900	456.6	220	ND	ND
	T4	1150.1	5600	ND	6.9	ND	ND

ND: not detected

Table 3. 4: The analytical parameters for UPLC-ESI-MS/MS method.

Pharmaceutical	Limit of detection (ng/L)	Limit of quantification (ng/L)	Parent compound (m/z)	Daughter compound (m/z)	Cone voltage	Collision voltage	Ion mode
Carbamazepine	0.1	0.1	273.0000	135.0000	20	10	ESI +
Ibuprofen	0.5	1.4	ND	ND	ND	ND	
Erythromycin	0.9	2.3	734.0000	158.0000	15	15	ESI +
Ciprofloxacin	1.0	3.4	ND	ND	ND	ND	
Sulfamethoxazole	0.3	0.9	254.0000	147.0000	20	25	ESI +
Clarithromycin	<0.1	<0.1	748.8000	590.6000	30	20	ESI +

ND: not detected

Sulfamethoxazole and fluoroquinolones concentration observed were lower than detection limit (0.015 ppb) of ELIZA test kit used at site B1 in Buffalo River. While at Bloukrans River and Swartkops River, concentration lower than the detection limits were observed only for fluoroquinolones at site BL1, BL2, S1 and S2 (Table 3.1). The results obtained corresponds to the results which were previously reported by Shelver et al. (2008) where concentrations lower than detection limits has been reported on upper-stream site samples. The sulfamethoxazole concentration observed at site S1 in Swartkops River can be explained by the fact that this site is situated in Groendal nature reserve. Since sulfamethoxazole is one of the commonly used antibiotics, it is possible that people who visit this nature reserve might have urinated near the river or improperly disposed this drug and through surface runoff it ended up in river water. In addition, sulfamethoxazole has been reported in aquatic systems in several studies and its presence in the environment maybe problematic since it is resilient to degradation enabling it to last in the aquatic environments (Holm *et al.* 1995; Kümmerer, 2001; Scheytt *et al.* 2005; Hendick and Pool, 2012).

The pharmaceuticals detected by UPLC-ESI-MS/MS from the analysed river samples were carbamazepine, erythromycin, sulfamethoxazole and clarithromycin. Ibuprofen and ciprofloxacin were the two target pharmaceuticals that were not detected by UPLC-ESI-MS/MS in all river water samples. Ibuprofen is a commonly used anti-inflammatory drug, therefore it was expected to be present in river water in high concentrations more specially in WWTP effluent samples (Matongo *et al*. 2015). However, it was not detected, and this may be due to its poor fragmentation since confirmation of pharmaceuticals on UPLC-ESI-MS/MS(QqQ) requires two SMR transition. Since one product ion can be produced from the precursor ion, ibuprofen could not be confirmed thus making it undetectable (López-Roldán *et al.* 2010). Similar results have been reported in a study that was conducted by López-

Roldán et al. (2010) where ibuprofen could not be detected by QqQ-MS but was detected by TOF-MS. Ciprofloxacin is one of the commonly used antibiotics to treat various bacterial infections and its wide use in aquaculture is the reason it found in environmental samples (Afonso-Olivares *et al.* 2013; Zou *et al.* 2011; Hendricks and Pool, 2012). Ciprofloxacin was not detected in all the samples and this may be due to UPLC-ESI-MS/MS machine used was unable to detect it since the standard solution was also not detected. This indicates that ciprofloxacin was probably present in the samples but not detected. Numerous studies have reported carbamazepine to be very persistent and stable in the environment (Clara *et al.* 2004; Zhang *et al.* 2008; Ramaswamya *et al.* 2011; Paiga and Delerue-Matos 2016). The presence of antibiotics such as sulfamethoxazole in river water can be problematic since it is persistent and resilient to biodegradation (Shelver *et al.* 2008).

Sulfamethoxazole was not detected at B2, S1, T1 and T4 sites since the concentrations were lower than the detection limits. Concentrations which were obtained at site P1 and B1 sites are probably due to human contamination since these sites are used to conduct spiritual ritual and camping. Sulfamethoxazole has been reported to change the thyroid function at a concentration equals to or higher than 119 ng/L. In all the sites the sulfamethoxazole concentration was higher than 119 ng/L indicating that the river water has a potential of affecting human and animal health following exposure (López-Roldán *et al.* 2010). The presence of carbamazepine in all river water samples was an indication that carbamazepine is one of the commonly used drugs in the Eastern Cape Province. Higher concentrations of carbamazepine were obtained from the effluent samples of WWTPs compared to other sites in all the studied rivers. This may be due to WWTPs being in sufficient in removing carbamazepine from wastewater and is therefore discharged with wastewater into surface waters (Zhang *et al.* 2008; Ramaswamya *et al.* 2011). This indicates that the municipal waste and hospital waste are the main source of carbamazepine in WWTPs.

Higher concentrations of carbamazepine were obtained from the Swartkops River samples as compared to other rivers indicating that carbamazepine is one the commonly used drug at Uitenhage with concentration ranging from 1130.7 ng/L (S1) to 18288.1 ng/0.5 L (S3). The lowest concentrations of carbamazepine were obtained from the upper-stream sites in all the rivers except for Swartkops River upper-stream site. No health effects have been reported for the concentration of carbamazepine obtained at P1, B1 and T1 sites. High concentration of carbamazepine observed at site S1 may be explained by the site being inside the Groendal nature reserve and that the people who visit this site may be using carbamazepine. The presence of carbamazepine in river water may be as a result of excretion via urine or improper disposal. Concentrations which were obtained at site B2 and T2 were higher than 179 ng/L and according to López-Roldán et al. 2010 such concentrations can lead to

disturbance in circulating thyroid hormones. In most of the sites the concentration of carbamazepine was above 1000 ng/L and these concentrations affects the ultrastructure of the fish kidney, liver, and gills (Zhang *et al.* 2008; Baquero *et al.* 2008).

In all the antibiotics that were detected in river water samples, erythromycin was the only antibiotic detected in higher concentrations. The absence of erythromycin on upper-stream sites of the rivers and B2 was an indication that these sites were not contaminated by erythromycin even though these sites were contaminated by clarithromycin and carbamazepine. High concentration of erythromycin was obtained in effluent samples of WWTPs in all the rivers except for site S2 in Swartkops River. High concentration at S2 can be explained by effluent discharge from the WWTP located upstream this site and that this site is near the houses so improper disposal, sewage leakage, and surface runoff might have contributed to high concentrations obtained. The high concentrations of erythromycin observed in effluent samples was an indication that the WWTP processes are inadequate in removing erythromycin and that WWTPs are the main source of erythromycin in surface waters. The high concentrations observed in middle-stream sites and WWTPs sites was an indication that erythromycin is one of the commonly used antibiotics since it is used to treat variety of bacterial infections. Lower concentrations observed in lower-stream sites was due to dilution by river water as the river is flowing further down. However, at Swartkops River high concentration of erythromycin was observed at lower-stream site, and this may be due to high contamination by the WWTP upper-stream as low concentration of erythromycin was observed from the WWTP effluent discharge near this site. The concentration of erythromycin obtained in this study were higher than the concentrations which were reported in Sweden influent and effluent samples (0.47 and 0.35 µg/L) of WWTP (Östman et al. 2017). The erythromycin concentration detected in this study was higher than 1 µg/L and this indicates possible toxic effects since concentrations higher than 1 µg/L have been reported to affect aquatic life (European Commission, 1996; Petrie *et al*. 2014).

Clarithromycin was detected in all the samples except for P1 site at Palmiet River and this was an indication that clarithromycin is one of the commonly used antibiotics since its used to treat variety of bacterial infections. Higher concentrations of clarithromycin were detected in effluent samples of WWTPs at Buffalo River and Tyhume River while at Swartkops River and Bloukrans River, higher concentrations were obtained on middle-stream sites (S2 and Bl2). High concentration at this site at Bloukrans River may be caused by sewage leakage while at Swartkops River it due to effluent discharge from the WWTP upstream. Low concentration of clarithromycin at lower-stream sites of the rivers was due to dilution by river water as the river flows further down. Clarithromycin concentration observed in this study was less than 2 µg/L in all the sites indicating that the chances toxicity to

eukaryotic algae (Andreozzi *et al.* 2004; Isidori *et al.* 2005) and other aquatic organisms was minimal. However, clarithromycin can have negative effects of microbial community and lead to antibiotic resistance even at these concentrations.

3.14. Conclusion

This study reports successful screening of the presence of fluoroquinolones and sulfamethoxazole in river water. The absence or low concentration of antibiotics detected from the samples obtained from the upper-stream sites was an indication that these sites received minimal anthropogenic impacts. The presence of antibiotics on the middle-stream sites was an indication that the communities have a contribution on the pollution of river water and the presence of antibiotics on effluent samples of the WWTPs and on the lower-stream site indicated that WWTPs are the main sources of pollution. Detection of carbamazepine in all the samples in high concentrations was an indication that this drug is one of the commonly used drugs. This is an indication of possible environmental effects since this drug has negative effects on aquatic organisms. High concentration of erythromycin in river water samples was an indication that this drug is one on the commonly used antibiotics. Its presence in high concentration in the environment pose a threat into aquatic species as this drug is considered on one of the priority drugs that can have health effects on aquatic organisms. In this study it was observed that upper-stream sites are less impacted compared to lower-stream sites thus indicating community, WWTP and surface runoff are source of contamination in surface waters. The detection pharmaceuticals in river water of the Eastern Cape Province was an indication that these rivers are polluted by pharmaceuticals. Therefore, constant monitoring of the concentration of pharmaceutical contaminants in essential to protect public health, aquatic biodiversity and the quality of river water.

3.15. References

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Chapter 4

Detection of life-threatening pathogens in environmental surface waters in the Eastern Cape Province

4.0. Introduction

South Africa is considered as a water scarce country due to minimal average rain fall and high evaporation rate which has resulted to limited access to freshwater in water resources (Eberhard and Robinson, 2003; Steyn *et al.* 2004; le Roux *et al.* 2012; Genthe *et al.* 2013; Mulamattathil *et al.* 2014; Abia *et al.* 2016). Water is fundamental to life in both humans and animals, hence access to uncontaminated drinking water for humans and animal is vital for the maintenance of a healthy life (WHO, 2008; Cabral 2010; WHO, 2010; Edokpayi *et al*. 2015). In developing countries such as South Africa, rivers, dams and underground water has been serving as a source of drinking water for decades (Fenwick, 2006; Cabral 2010). The Statistics South Africa documented that approximately 84.5% of South African population from 2002 had access to piped water or tap water in their dwellings or about 200 m away from their houses (Statistics South Africa, 2011; Luyt *et al.* 2012).

According to the Department of Water Affairs and Forestry, people in the cities have constant supply of treated domestic water, while people in rural areas get to use partially treated water and even untreated water in certain rural areas as their source of drinking water (DWAF, 1996b; Obi *et al.* 2002; WHO/UNICEF, 2006; Gwimbi, 2011; Edokpayi *et al.* 2015). Moreover, the quality of river water in most of the rivers in the urban communities is monitored whereas rivers in the rural area there is inadequate information on the quality of water and these rivers are susceptible to contamination (Edokpayi *et al.* 2015). It has been reported that worldwide people who lack access to safe drinking water are about 1.1 billion, with 85% of them live in rural areas. This shows that a large population of people who live in the rural areas to rely on polluted/untreated river water and underground water as a source of drinking water, thus resulting in illnesses and deaths (DWAF, 1996b; Schaefer, 2008; UNICEF/WHO, 2009; Mulamattathil *et al.* 2014; Edokpayi *et al.* 2015; Abia *et al.* 2016). Many children in those areas suffer from diarrhoeal diseases particularly those with compromised immune system, their mortality rate is 11 times higher (Adefisoye and Okoh, 2017). The Department of Water Affairs in 2010 reported that about 70% of children under 5 years of age in South Africa suffered from the diarrhoeal disease due to lack of access to safe drinking water (Wanda *et al.* 2016). The use of ground water sources and surface waters due to lack of access to safe drinking water has been reported in the Eastern Cape Province, North-West Province and Mpumalanga Province in South Africa (Chigor *et al.* 2013, Wanda *et al.* 2016).

Generally, drinking water is utilized for non-drinking purposes such as irrigation, sanitation, general cleaning and industrial purposes resulting in large volumes of water being utilized daily (Mulamattathil *et al.* 2014; Abia *et al.* 2016). This high consumption of water causes stress and depletion of water. In addition, the availability of fresh water is also greatly affected by human pollution resulting in the water that is present in water resources unfit for drinking purposes due to decrease in water quality (Liang *et al.* 2013; Abia *et al.* 2016). The ways of dealing with the challenge of high-water demand in South Africa includes protection of water systems and implementation wastewater reuse, however, advancement on wastewater treatment plant (WWTP) processes will be necessary to ensure that the quality of drinking water is not affected (Mulamattathil *et al.* 2014). One of the major strategies in water management practices include detection and minimization of sources of faecal contaminants (Liang *et al.* 2013).

Determination of the source of faecal pollutants in aquatic environments is fundamental in the development of water protection strategies and determination of the risk of health-related complications following exposure to contaminated waters (Liang *et al.* 2013). Traditionally, faecal indicator bacteria are employed in water quality monitoring programs, however, those faecal indicator bacteria have no ability in differentiating faecal coliforms that are of human or animal origin (Field and Samadpour, 2007; Liang *et al.* 2013). Thus, the bacteroidales DNA markers that are specific for human (HF183) and ruminant (CF128 and CF193) for determination of the source of faecal coliforms have been developed (Bernhard and Field, 2000; Liang *et al.* 2013). In addition, the enterococcal surface protein of *Enterococcus faecium* (HS-esp) is also used as a DNA marker for distinction of human and animal waste (Scott *et al.* 2005; Peed *et al.* 2011; Liang *et al.* 2013). These DNA markers are essential as they provide ways of detecting the source of contamination so that necessary strategies may be developed for the protection of aquatic environments (Liang *et al.* 2013).

The issue of faecal pollution in aquatic environments has been a challenge for the past 21st century (Liang *et al.* 2013). Frequent assessment of the microbial quality of water intended for domestic purposes is important for protection of public health (Momba *et al.* 2006). According to the National Water Act of 1998 (Act No. 36 of 1998) it is important that national's water resources are utilized, protected, developed, preserved, properly managed and controlled in a suitable manner for the benefit of everyone (Chigor *et al.* 2013).

Overcoming the challenge of a lack of access to clean and safe drinking water in South Africa more special in rural areas depends on the availability of a reliable data of water quality of the water resources. The data is used in water quality monitoring programs to assess or enhance the water quality

networks, thus making it possible to determine pollution sources and develop strategies to prevent and protect the water resources used as source of drinking water from contamination. Following assessment of the state of water quality, scientist can indicate whether immediate action is needed in those areas (Wanda *et al.* 2016).

4.1. Pathway of microorganisms in aquatic systems

Open water resources such as dams, rivers, lakes and underground water are subjected to microbial contamination. Microorganisms usually enter the aquatic environment through rainfall runoff of wild and domestic animals' faeces in land fields, agricultural runoff, effluent discharge from WWTPs and bust or leaking sewage pipes. When microorganisms are present in aquatic environments, they decrease the quality of water, thus the water will be unfit for human consumption (Paulse *et al.* 2012; Liang *et al.* 2013; Mulamattathil *et al.* 2014).

Coliforms are present in the intestinal microflora of all the warm-blooded animals including humans. These coliforms enter the environment through excretion of body waste materials. They are eliminated from the environment via sewage treatment plants (STPs) (Edberg *et al.* 2000; Hendrick and Pool, 2012). Nevertheless, several studies have reported inadequate removal of faecal coliforms by the STPs and these coliforms are discharged with the treated sewage effluents into the rivers, thus contaminating surface water. Their presence in the environment pose a threat to human health especially to those who are immunocompromised as they can cause diseases such as dysentery, typhoid and gastroenteritis following exposure to contaminated water (Hendrick and Pool, 2012; Rivera-Utrilla et al. 2013). Monitoring bacteriological quality of water using indicator bacteria such as *Escherichia coli* (*E. coli*), *Aeromonas* and *Pseudomonas aeruginosa* is significant to protect the health of both humans and animals (Mulamattathil *et al.* 2014).

The rapid increase in the world human population results in high water demands and generation of large volumes of sewage wastes thus putting more stress on WWTPs (Teklehaimanot *et al*. 2015; Abia *et al*. 2018). The WWTPs sometimes find it difficult to deal with the challenge of treating large volumes of sewage and end up releasing partially or untreated wastewater into the rivers. This is problematic because it introduces pathogens in the environment thus decreasing the quality of water. This affect the aquatic ecosystem and changes the biodiversity of macroinvertebrates (Abia *et al*. 2018). People who are affected by this are usually the communities downstream the WWTPs who are using river water as their source of drinking water in the absence of water supply (Durand, 2012; Lim *et al*. 2017; Abia *et al*. 2018). In addition, the discharged wastewater which contains pathogens or even

antibiotic resistant bacteria may find its way to the dams downstream which serves as a source of drinking water to other communities (Chigor *et al*. 2013).

4.2. Pathogens in aquatic environments

It has been documented that approximately 2.5 billion people worldwide lack access to adequate sanitation and that results in children suffering from diarrheal diseases and causing more than 1.5 million death each year. WHO has indicated that more than 5 million people per year die because of water linked illnesses where more than 50% are caused by microbial intestinal infections with cholera being the leading disease (Fenwick, 2006; Cabral, 2010). This high death rate will continue to be true more special in rural areas if the communities are not well informed about the quality of water in their water resources (Momba *et al.* 2006).

Escherichia coli is a gram-negative bacterium found in the intestinal flora of all warm-blooded animals. Although *E. coli* strains are part of the normal flora in humans, some strains have been implicated in infections and/or diseases. In the environment *E. coli* is introduced through excretion of faeces and present in different concentration. Thus, it is used as an indicator bacterium to evaluate the presence of faecal coliform and possible pathogens in the environment. In water resources *E. coli* last for 4 to 12 weeks depending on the environmental conditions, whereas in water distribution system it can last even longer. The presence of faecal coliforms in drinking water can lead to waterborne diseases such as cholera (Edberg et al 2000; van der Hoven et al. 2017).

In the Eastern Cape Province, South Africa, pathogens such as *Escherichia coli*, *Salmonella*, *Shigella*, *Vibrio cholerae* have been identified in effluent samples from WWTP and thus indicating inadequate removal of microorganisms before discharge of wastewater into the river. This results in the contamination of surface water, groundwater and freshwater which are the main source of drinking water, therefore causing concern in public health (Müller *et al.* 2001; Momba, 2006; Paulse *et al.* 2012). The presence of *Enterobacter sp*., *Citrobacter sp*., *Shewanella sp*., and *Aeromonas sp*., in the Berg and Plankenburg Rivers located in the Western Cape Province, South Africa is an indication of faecal contamination (Paulse *et al.* 2012). *Citrobacter sp*. is classified as an opportunistic pathogen which may cause illness in an individual's having a compromised immune system. Identification of *Bacillus cereus* in Berg and Plankenburg Rivers indicated a health risk since the presence of this bacteria results in acute nausea, vomiting and diarrhoea in infected individuals (Paulse *et al.* 2012).

Among the pathogens which are commonly isolated in aquatic environments, enteric pathogens are the most prevalent pathogens, and these include specie such as *E. coli*, *V. cholerae* and S. *typhimurium* which are mostly transferred to humans by consumption of microbial polluted foods and water

(Momba *et al.* 2006). The presence of pathogenic organisms in groundwater, surface water and freshwater have also been reported by Pandey and co-workers (2014) in India indicating that contamination by faecal coliform is a global serious environmental issue that needs to be addressed so that humans can have access to clean and safe drinking water (Mulamattathil *et al.* 2014).

Pseudomonas species are gram-negative, aerobic motile bacilli that contain single polar flagellum in their cells (Barbara and Iglewski, 1996; Çiçek *et al*. 2016) or more polar flagella in case of *P. fluorescens, P. putida, and P. luteola* (Wisplinghoff, 2017). They are characterized by their ability to produce catalase enzyme, indophenol and oxidase positive, inability to ferment glucose and production of fluorescent pigments (Burn, 2018). These bacteria have been reported to cause bacteraemia, sepsis, septic arthritis, meningitis, endocarditis and peritonitis in infected individuals and can be life threatening in patients with immunocompromised system or underlying diseases like cancer or burns (Barbara and Iglewski, 1996). The high motility rate associated with *Pseudomonas* is caused by weakened immune system, antibiotic resistance and production of extracellular bacterial enzymes and toxins (Barbara and Iglewski, 1996)

4.3. Seasonal variation of the bacterial quality of water

Season variation on the concentration of *E. coli* in river water has been reported, and according to Edokpayi et al. it would be difficult to predict when the faecal contamination will probably be high (Edokpayi *et al*. 2015). This may be due to their observations of high faecal count in dry seasons instead of wet season. High *E. coli* concentration is expected during wet season due to increased average rainfalls, surface runoff and high turbidity values however, excessive ultraviolet radiation from the sunlight during wet season was thought to decrease *E. coli* concentration in water (Edokpayi *et al*. 2015). High concentration of the *Enterococci* has been reported in wet season as compared to high *E. coli* concentration that was observed in dry season. Several studies have also reported seasonal variation on the faecal indicator bacteria in South African rivers. The study that was conducted by Chigor et al. 2013 and Lin et al. 2015 reported that there were no significant variations on the concentration of indicator bacteria between wet and dry season in Buffalo River and Umgeni River in South Africa (Edokpayi *et al*. 2015). High concentration of indicator bacteria during dry season have also been reported by Fatoki et al 2001 in Umtata River, whereas Sibanda et al. 2013 reported high concentration of faecal indicator bacteria during dry season compared to wet season in studies on Tyhume River in the Eastern Cape Province, South Africa (Edokpayi *et al*. 2015).

4.4. Occurrence of antibiotic resistance

There is growing concern on the appearance of microbial antibiotic resistance which is associated with an exposure of antibiotics to the environment (Valcárcel *et al*. 2011; Mulamattathil *et al*. 2014). The use of antibiotics in aquaculture industry in developed and developing countries has been reported to increase the appearance bacteria resistant to antibiotics in the environment. The increase in antibiotic resistance has also been demonstrated to result from the exchange of resistant gene among bacteria of animal and human pathogens in aquacultures. For instance *Aeromonas*, which are fish pathogens pose the ability to transfer a resistant gene to *E. coli* isolated from humans (Cabello 2006).

The presence of *Salmonella spp*. in the effluent samples from the WWTP in Durban, South Africa has been documented (Odjadjare *et al*. 2015). *Salmonella spp*. are gram-negative bacilli that can cause diseases such as gastroenteritis, typhoid fever, osteomyelitis, septicaemia and meningitis in both humans and animals when present in the environment (Soyer *et al*. 2009; Fookes *et al*. 2011; Odjadjare *et al*. 2015). Approximately 93.8 million outbreaks of gastroenteritis that are reported worldwide each year are linked to *Salmonella spp*. leading up to 155 000 deaths. The high number of infections was associated with the failure of antibiotics in treating the disease due to increase in antimicrobial resistance (Majowicz *et al*. 2010; Odjadjare *et al*. 2015).

The increase in therapeutic use of antibiotics in human and veterinary medicine have been documented and the presence of antibiotics in the environment is associated with development of antibiotic resistance thus causing difficulties in treating infections. In addition, the use of biological processes in WWTPs have been associated with the selective increase in antimicrobial resistance and can also result in multiple antibiotic resistant strains (Mulamattathil *et al*. 2014; Schafhauser *et al*. 2018).

A study conducted by Malema and co-workers 2018 reported *E. coli* strains isolated from harvested rainwater to be resistant antibiotics such as Cephalothin, Tetracyclnes, Colistin sulfate, Ampicillin and Streptomycin and further suggested the possibility of the transfer of gene of resistance to similar strains (Malema et al. 2018). Moreover, the presence of pathogenic *E. coli* strains in water can cause diarrheal diseases, urinary tract infections, sepsis and wound infections (Zamxaka and Muyima 2004; Malema *et al*. 2018). Hence monitoring for the presence of *E. coli* in drinking water is important for the protection of public health (Edberg *et al*. 2000; Wanda *et al.* 2016).

4.5. Natural purification system

Bacteria are the most prevalent group of microorganisms present in the aquatic environments particulary in bed sediments and are essential in the cycling of nutrients and breaking down of contaminants that enter the water bodies (Wang *et al*. 2013; Abia *et al*. 2018). These chemical and

biological contaminants that enter water bodies apply stress on the aquatic ecosystems resulting in change of the biodiversity in aquatic environments. For instance, the presence of chemical contaminants such as antibiotics and heavy metals in aquatic environments may change the microbial diversity thus posing a negative effect that may put the health of individuals at risk. The presence of biological contaminants such as pathogens may cause infections and illness in humans and animals using river water as their source of drinking water. Microbial pathogens such as *Vibrio cholerae, Salmonella spp*. *Shigella spp*. and pathogenic strains of *E. coli* have been detected by several authors in aquatic environments. These microbial strains may cause serious life-threatening diseases upon human exposure in such contaminated waters (Paulse *et al*. 2012; Abia *et al*. 2016). In addition, the pollutants that enter the water bodies may tend to accumulate in sediments and can be undetected during water quality testing. These pollutants usually get re-suspended when the water is disturbed thus affecting water quality (Abia *et al*. 2016; Abia *et al*. 2018).

4.6. Aim

- To determine microbial quality of water in Buffalo River, Tyhume River, Bloukrans River and Swartkops River located in the Eastern Cape Province.

4.7. Objectives

1. To monitor the presence of faecal coliforms, total coliforms and heterotrophic bacteria using m-Fc agar, m-Endo agar and R2A agar.
2. To identify possible pathogens in the rivers using the analytical profile index (API 20E kit).
3. Determine the season effects on the bacterial quality of river water.

4.8. Methodology

4.8.1. Materials and reagents

The R2A agar, nutrient broth and glycerol were purchased from Sigma Aldrich (Johannesburg, South Africa). The m-Fc agar, m-Endo agar, the membrane filters 0.45 µm pore-sized and parafilm were purchased from Merck Millipore (Gauteng, South Africa). The 90 mm sterile petri dish and 1.5 mL Eppendorf tubes were purchased from Lasec (Port Elizabeth, South Africa). The API 20E kit was purchased from bioMérieux (Midrand, South Africa).

4.8.2. Water collection

The 96 water samples were collected from the Bloukrans River, Buffalo River, Tyhume River and Swartkops River. Four sampling sites which were sparsely distributed along the river identified. These were the upper-stream, middle-stream, WWTP and lower-stream. Prior to sample collection, clean

schott bottles were autoclaved at 121 °C for 15 minutes and rinsed with river water. This was done to remove the chemicals which were used to clean the bottle as may affect with the sample. One litre of water sample was collected from each of the four different sites of each river. The samples were properly labelled, stored in ice and transported into the laboratory for analysis. Samples were kept at 4 °C until analysis was completed. In this study sample collection was conducted in three seasons which are autumn, winter and spring. In all four rivers water samples were collected twice a season.

4.8.3.Isolation and identification of microorganisms

The faecal coliforms, total coliforms and heterotrophic bacteria were isolated from water samples. The m-Fc agar was used as a selective medium for isolation of faecal coliforms, the m-Endo was used for selection of total coliforms and R2A agar for selection of heterotrophic bacteria. Isolation of faecal coliforms and total coliforms was performed by membrane filtration technique using the m-Fc agar plates and m-Endo agar plates respectively. The m-fc agar plates and m-Endo agar plates and were prepared according to the manufactures protocol and stored at 4 °C until use. On a sterile environment, approximately 100 ml of each sample was filtered through 0.45 µm pore-sized filter using a water pump (Vacuubrand GMBH + CO KG from Lasec, Germany). The membrane filters were carefully placed on the m-Fc agar plates and m-Endo agar plates respectively without trapping any air bubbles. For each sample 100 mL of water sample was filtered and samples were analysed in triplicates. The control plates were prepared for each sample by filtering 100 mL of sterile saline solution through 0.45 µm pore-size filter. The m-Fc agar plates were then incubated at 44.5 °C and the m-Endo agar plates were incubated at 37 °C for 24 hours. Following incubation, colony counting was performed, and results were recorded as colony forming units (CFU) per 100 mL. The plates were then sealed with parafilm and stored in the fridge until colony isolation was conducted (Mulamattathil *et al*. 2014).

The R2A agar plates were prepare according to the manufactures protocol and stored at 4 °C until use. Isolation of heterotrophic bacteria was performed by spread plating method on the R2A agar plates following Mulamattathil *et al*. 2014 method. Approximately 100 µL of the sample was placed on the R2A agar plate and spread using a sterilized hockey stick. The control plate for each sample was prepared by spread plating 100 µL sterile saline water. All the samples were analysed in triplicates and incubated at 35 °C for 72 hours. Following incubation, colony counting was performed, and the results were recorded as cfu/mL. The plates were then sealed with parafilm and stores in the fridge until colony isolation was conducted.

4.8.4. Isolation of pure colonies

On a sterile environment, about 4 colonies were picked randomly from each plate using a sterile inoculation loop and sub-cultured on the m-Fc agar, m-Endo agar and R2A agar plates using a streaking method (Mulamattathil *et al*. 2014). The plates were then incubated at 44.5 °C for isolation of thermotolerant coliforms, 37 °C and 35 °C for 24 hours and 72 hours respectively. Pure colonies were obtained by sub-culturing twice using the streaking method, and the plates were stored at 4 °C until the glycerol stocks were prepared (Mulamattathil *et al*. 2014).

4.8.5. 60% Glycerol stock.

The glycerol stocks were prepared by mixing liquid culture with glycerol. Approximately 60% of the pure glycerol was mixed with 40% of distilled water and autoclaved at 121 °C for 15 minutes. The nutrient broth was prepared following the manufactures protocol. The pure colonies were then inoculated in the liquid broth and incubated at 44.5 °C, 37 °C and 35 °C for 24 hours. Following incubation, the 500 µl of the liquid culture and sterile 60% glycerol were added into a sterile Eppendorf tubes mixed properly and store at -80 °C.

4.8.6. Identification using API 20E

Identification of microorganisms in the samples was performed using a commercial analytical profile index (API) 20E test kit which is a standardized biochemical test for identification and differentiation of members of the family Enterobacteriaceae and other Gram-negative rods. This was conducted following the manufacture's protocol and the results were analysed using API catalogue.

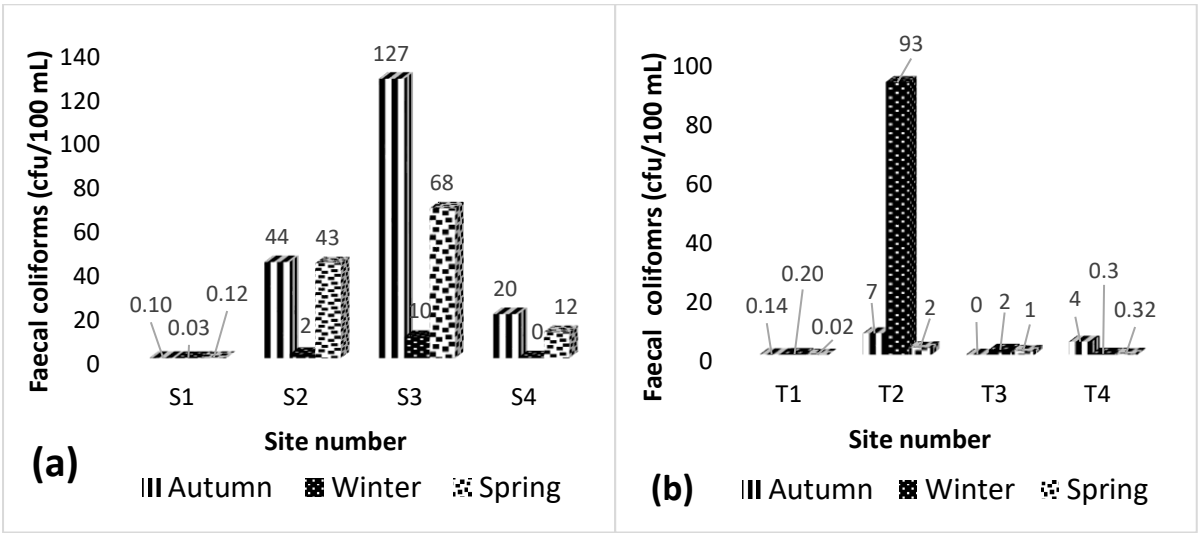
4.9. Results and discussion

The aim of this study was to determine the bacterial quality of river water. This study was motivated by the scarcity of water in South Africa and use of river water as a source of domestic water during this period. The use of river water is more common in rural areas and this is caused by a lack of reliable and accessible clean and safe drinking water (Chigor *et al*. 2013, Wanda *et al*. 2016; Beshiru *et al*. 2018). Thus, monitoring of the quality of river water used for domestic purposes is vital for protection of public health. One of the objectives of this study was to screen for the presence of faecal contaminating microorganisms. This was motivated by the fact that faecal indicator bacteria such as *E. coli* in water is an indication of faecal contamination and possible presence of faecal pathogens which may severe impact human health more specially the immunocompromised individuals (Mulamattathil *et al*. 2014).

Faecal coliforms, total coliforms and heterotrophic bacteria were isolated in the all samples obtained during autumn, winter and spring season. In all the samples the concentration of heterotrophic bacteria

was high as compared to total coliforms and faecal coliforms throughout the sampling seasons (Table 3.1). The trend of these results was similarly to the results reported by Mulamattathil et al. (2014). Nevertheless, the concentration of heterotrophic bacteria in this study was much higher than the concentration reported in their study. High concentration of heterotrophic bacteria may be as result of wide diversity of heterotrophic bacteria present in aquatic environments (Mulamattathil *et al*. 2014). In addition to this, heterotrophic bacteria were isolated with non-selective agar therefore allowing the growth of many microorganisms that are of faecal and non-faecal origin. The abundance of the presence of faecal coliforms in sampled rivers is shown in figure 4.1 (a, b, c and d) with Bloukrans River being the most faecal contaminated river.

Throughout the sampling season, the concentration of faecal coliforms on the upper-stream site in Swartkops and Tyhume River compiled with South African water quality guidelines (0 cfu/100 mL) for domestic water use. However, the concentration of faecal coliforms in Bloukrans River upper-stream site was slightly above the acceptable water quality target range during autumn and winter season while higher faecal coliform concentration was observed during winter season. High concentration of *E. coli* during dry season as compared to wet season has been previously reported in Mvudi River in Limpopo Province in South Africa (Edokpayi *et al*. 2015). In Buffalo River, the concentration of faecal coliforms at upper-stream site was above the acceptable water quality target range during autumn and spring season. Higher concentration of faecal coliforms at this site maybe due to surface runoff of wild and domestic animal faeces. Similar observation has been reported in a study conducted by Abia and co-workers (2016) in Apies River where high faecal coliform concentration have been observed in sample obtained from the upper-stream site.



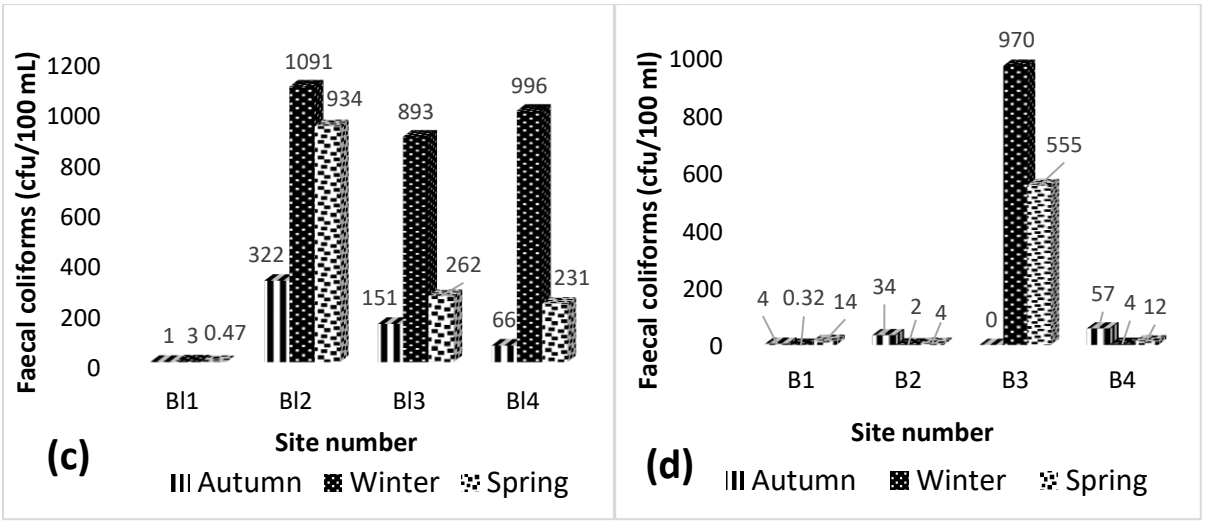


Figure 4. 1: Concentration of faecal coliforms in Swartkops River (a), Tyhume River (b), Bloukrans River (c) and Buffalo River (d) during autumn, winter and spring season at four sampling sites.

Higher concentration of faecal coliforms was observed in samples obtained near urban areas at Bloukrans River, Buffalo River and Tyhume River. Similar trend was reported by Abia and co-workers (2016) when during the rainy season the concentration of faecal indicator bacteria in surface waters was higher near urban areas as compared to other sites. Among all the studied rivers, the Bloukrans River middle-stream site (Bl2) was the most faecal polluted site. This was probably due to blocked sewage pipes which caused the sewage from the town to be released directly into the river and lesser sewage inflow to the WWTP. The direct release of sewage into the Bloukrans River may probably be the main reason that this river was the most polluted river as compared to other studied rivers.

In all the rivers expect for the Tyhume River, higher concentration of faecal coliform was observed from the effluent samples obtained from the WWTPs thus, indicating that WWTPs are the major source of faecal coliforms and possible pathogens into the aquatic environments. The decrease in faecal coliform concentration was observed in samples obtained from the downstream sites in all the rivers. This may result from dilution by river water as the river flows down-stream. High concentration of faecal coliforms in the middle-stream sites of all the rivers indicates that town/communities has considerable contribution in pollution of surface waters. Due to their waste ending up into the river through surface runoff and surface drainage. Higher concentration of *E. coli* in downstream sites as compared to upper-stream sites have also been documented in a study that was conducted in Mvudi River in Limpopo Province (Edokpayi *et al*. 2015).

The physicochemical parameters of water have major influence on the survival and growth of bacteria in aquatic systems. The presence of inorganic contaminants such as nitrate, phosphate, ammonium,

sulfate, and iron in river water promotes the growth of bacteria since these inorganic compounds are vital for formation of RNA, proteins and metabolism. Equally higher temperature and neutral pH in aquatic environments provides conducive environments for biological reactions and bacterial growth. Hence, the favourable conditions in these rivers may be one of the reasons higher bacterial concentrations were observed. Positive correlation between phosphate concentration, faecal coliform and heterotrophic bacteria concentration was observed at Buffalo River while at Bloukrans River positive correlation was observed between phosphate concentration, total coliform and heterotrophic bacteria. Moderate significant correlation between chloride concentration and heterotrophic bacteria concentration was observed at Bloukrans River $r(12) = 0.641$, $p < 0.05$. The moderate correlation between chloride and heterotrophic bacteria was an indication that the chloride present in aquatic environments was not effective in reducing the concentration of heterotrophic bacteria. Although significant decrease was observed at on WWTP of Tyhume River catchment.

Table 4. 1: Average colony forming unit of total coliforms, faecal coliforms and heterotrophic bacteria of sampled rivers in each season.

	South African Water Quality Guidelines for domestic use		Autumn				Winter				Spring			
		Site no.	Buffalo River	Tyhume River	Bloukrans River	Swartkops River	Buffalo River	Tyhume River	Bloukrans River	Swartkops River	Buffalo River	Tyhume River	Bloukrans River	Swartkops River
Total coliform (cfu/100 mL)	0 - 100 cfu/100 mL	1	90	20	267	0.20	43	15	20	3,37	7912	888	0.30	217
		2	637	273	112560	88667	1300	2717	134537	11880	24750	162153	325333	65567
		3	NS	NS	8710	254333	3433	10	8877	3850	502	58097	4047	255682
		4	423	393	2010	48240	237	67	2757	13487	3667	143673	148000	12267
Faecal coliform (cfu/100 mL)	0 cfu/100 mL	1	4	0.14	1	0,10	0.32	0.20	3	0,03	14	0.02	0.47	0.12
		2	34	7	322	44	2	93	1091	2	4	2	934	43
		3	NS	NS	151	127	970	2	893	10	555	1	262	68
		4	57	4	66	20	4	0.33	996	0.02	12	0.32	231	12
Heterotrophic bacteria (cfu/mL)	0 - 5 cfu/ mL	1	2600	213	1167	250	983	567	500	300	420	3300	593	375
		2	12833	227	243880	21000	25167	28143	578210	7167	15867	2600	1153543	21000
		3	NS	NS	347060	12667	113333	17000	549400	25500	4033	4900	245220	12667
		4	28500	373	494460	14333	3500	8347	288667	12667	3240	1900	192290	14333

NS: not sampled

The bacterial species which were identified in the rivers in four sites during autumn, winter and spring are shown in table 4.2 to 4.5.

Table 4. 2: Identified bacteria from Tyhume River water samples obtained at four sites during autumn, winter and spring season.

Culture media	Season	Site	Organism	% identity	T value	Comment
m-Endo agar	Autumn	T1	*Klebsiella oxytoca*	89.7	75.8	Acceptable identification
			Klebsiella pneumoniae ssp pneumoniae 2	76.9	0.67	Doubtful identification
		T2	*Escherichia coli 1*	96.3	0.87	Very good identification
			Enterobacter aerogenes	98.9	1	Good identification to the genus
		T3	*Klebsiella oxytoca*	99.0	0.84	Very good identification
			Enterobacter aerogenes	98.9	1	Good identification to the genus
			Yersinia frederiksenii/intermedia	99.7	0.94	Excellent identification
		T4	*Escherichia coli 1*	99.9	0.94	Excellent identification
			Kluyvera spp	80.1	0.67	Acceptable identification
	Winter	T1	*Yersinia frederiksenii/intermedia*	99.7	0.84	Good identification to the genus
		T2	*Escherichia coli 1*	99.9	1	Excellent identification
		T3	*Citrobacter freundii*	86.3	0.67	Acceptable identification
		T4	*Aeromonas hydrophila/caviae/sobria 1*	56.2	0.43	Doubtful identification
			Rahnella aquatilis	73.2	0.42	Doubtful identification
	Spring	T1	*Yersinia frederiksenii/intermedia*	99.7	0.94	Very good identification to the genus
			Serratia fonticola	91.8	0.37	Doubtful identification
		T2	*Enterobacter aerogenes*	98.9	1	Good identification to the genus
		T3	*Escherichia coli 1*	96.3	0.87	Very good identification
			Klebsiella oxytoca	99.0	0.84	Very good identification
		T4	*Escherichia coli 1*	99.9	0.94	Excellent identification
			Pantoea spp 4	62.8	0.91	Acceptable identification
R2A agar	Autumn	T1	*Proteus penneri*	58.2	0.38	Doubtful identification
		T2	*Proteus mirabilis*	99.9	0.64	Very good identification
		T3	*Providencia stuartii*	98.4	0.68	Good identification
		T4	*Pseudomonas fluorescens/putida*	82.4	0.83	Good identification
	Winter	T1	*Proteus penneri*	58.2	0.38	Doubtful identification
			Vibrio alginolyticus	95.3	0.72	Very good identification
		T2	*Aeromonas salmonicida ssp salmonicida*	75.0	0.64	Doubtful identification
			Proteus penneri	58.2	0.38	Doubtful identification
			Proteus mirabilis	56.5	0.36	Doubtful identification
		T3	*Proteus penneri*	58.2	0.38	Doubtful identification
			Salmonella ser.Typhi	99.9	1	Excellent identification
			Proteus mirabilis	56.5	0.36	Doubtful identification
		T4	*Proteus penneri*	58.2	0.38	Doubtful identification
			Proteus mirabilis	99.9	0.64	Very good identification
	Spring	T1	*Providencia stuartii*	79.0	0.74	Good identification to the genus
		T2	*Stenotrophomonas maltophilia*	99.3	0.86	Very good identification
			Shigella spp.	81.5	0.96	Very good identification
		T3	*Pseudomonas aeruginosa*	98.7	0.92	Good identification
			Vibrio fluvialis	71.7	1	Good identification
		T4	*Pseudomonas luteola*	99.9	0.66	Very good identification
			Escherichia coli 2	99.4	0.85	Very good identification

The identified bacteria at upper-stream site, T1 of Tyhume River were *Klebsiella oxytoca* (winter season); *Yersinia frederiksenii/intermedia* and *Providencia stuartii* (autumn season) and *Yersinia frederiksenii/intermedia* and *Vibrio alginolyticus* (spring season). Wild animal faeces are possible sources of these bacteria into the river water and are directly released into the water or transported by rain through surface runoff. *Yersinia frederiksenii/intermedia* is considered as one of the non-pathogenic bacteria in the genus *Yersinia* hence its presence in river water does not rise concern to human health (Springer *et al*. 2018). *Yersinia frederiksenii/intermedia* has been isolated from water, soil, food, domestic, wild animals and humans and no clinical infections has been reported from it (Springer *et al*. 2018). The presence of *Klebsiella oxytoca* during winter season at this site can lead to intestinal, urinary and respiratory tract following exposure (Cabral, 2010; Lavan *et al*. 2016). *Providencia stuartii* is a gram-negative opportunistic pathogen which usually causes urinary tract infections in hospitalized patients with catheter. Multiple drug resistance in *Providencia stuartii* species are usually the cause of mortality in infected patients (Aubert *et al*. 2005; De Vecchi *et al*. 2013; Wie, 2015). *Providencia stuartii* is known to be able to migrate from the urinary tract to other organs thus causing diseases such as endocarditis, pericarditis, peritonitis and meningitis (Krake and Tandon, 2004; Simon *et al*. 2010; Sipahi *et al*. 2010; Unverdi *et al*. 2011). The presence of this pathogenic bacteria in water during autumn season maybe concerning since it may lead to health complications more specially to immunocompromised individuals. The presence of *Vibrio alginolyticus* at this site can be threat to human health as this bacterium can be infectious causing variety of infections and inflammation such as otitis, ocular, intracranial, peritonitis, osteomyelitis and wound infections in both humans and animals. *Vibrio alginolyticus* usually is isolated in marine, estuaries and aquatic environments and is the most dominant in the in the genus *Vibrio* (Fu *et al*. 2016). People living near this site use this river water as source of domestic water in the absence of alternative water safe and clean water supply. The presence of these pathogenic bacteria in water indicated that this water is not fit for domestic purpose and people using this water are in danger of infection. Seasonal variations on the abundance of pathogenic bacteria at T1 site was not observed as only one pathogen was identified in each season.

Bacteria detected at middle-stream site, T2 were *Escherichia coli 1*, *Enterobacter aerogenes* and *Proteus mirabilis* during autumn season, *Stenotrophomonas maltophilia*, *Enterobacter aerogenes* and *Shigella spp.* during and spring season and *Escherichia coli 1* during winter season. The possible sources of these pathogens into the river is surface runoff from agricultural fields as these bacteria were identified during autumn and spring season rains. The presence of bacteria such as *E. coli* and *Shigella* at this site can result in diarrhoea disease and gastroenteritis (Cabral, 2010) following

exposure of an individual to this water. *Stenotrophomonas maltophilia* is a multi-drug resistant gram-negative aerobic bacillus and is commonly found in aquatic environments (Mahmood *et al*. 2018). This pathogen is not common to humans, however, cases of *S. maltophilia* infections have been increasingly reported in humans. This low virulent pathogen needs to bypass human immune defence system to cause infection hence immunocompromised patients are usually the ones at risk and infections can be life threatening to exposed individuals (Mahmood *et al*. 2018). The presence of *Proteus mirabilis* in river water can lead to *Proteus* infections such as urinary tract infections. *Proteus mirabilis* is a gram-negative rod-shaped facultative anaerobe that belongs to the family of Enterobacteriaceae. It is characterized by its capability to ferment maltose and inability to ferment lactose. Its swarming motility and ability to self-elongate helps it with colonization to a host. *Proteus mirabilis* inhabits the gastrointestinal tract of humans and forms part of normal flora. *Proteus* had been isolated from soil and water samples and is usually present in abundance (Foris and Snowden, 2018). The genus Enterobacter is a non- spore forming gram-negative facultative anaerobe that belongs to the family Enterobacteriaceae. *Enterobacter aerogenes* has been reported to cause respiratory, urinary, and wound infection (Davin-Regli and Pages, 2015). The presence of these pathogens in water can be problematic as have serious implications to human health.

The identified bacteria at site T3 during autumn season were *Klebsiella oxytoca, Enterobacter aerogenes, Yersinia frederiksenii/intermedia* and *Providencia stuartii* while during winter season were *Escherichia coli 1*, *Klebsiella oxytoca* and *Salmonella ser.Typhi* and during spring season were *Escherichia coli 1*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa* and *Vibrio fluvialis.* The presence of these pathogenic bacteria from effluent samples of the WWTP is an indication that WWTPs are the sources of these pathogens into surface water.

On site T4 bacteria identified were *Pseudomonas fluorescens/putida, Pseudomonas luteola, Pantoea spp. 4* and *Escherichia coli* 2 during spring season while during winter season only *Proteus mirabilis* was identified and during autumn season identified bacteria were *Kluyvera spp* and *Escherichia coli 1*. *Pseudomonas aeruginosa* and *Pseudomonas luteola* are opportunistic pathogen commonly found in water, soil and moist environments (Çiçek *et al*. 2016). The presence of these bacteria in water can lead to bacteraemia, sepsis, septic arthritis, meningitis, endocarditis and peritonitis following exposure of an individual (Barbara and Iglewski, 1996). This is concerning since river water at this site is used for irrigation and sometimes used for domestic purposes in the absence of tap water supply. Indicating that the health of people living near this area maybe at risk.

Table 4. 3: identified bacterial species in Buffalo River at four sites during autumn, winter and spring season.

Culture media	Season	Sites	Organism	% identity	T value	Comment
m-Endo agar	Autumn	B1	*Pantoea spp 2*	63.7	0.41	Doubtful identification
		B2	*Kluyvera intermedia*	92	0.74	Good identification
		B3	*Escherichia coli 1*	99.5	0.96	Very good identification
			Aeromonas hydrophila/caviae/sobria 1	76	1	Good identification
		B4	*Aeromonas hydrophila/caviae/sobria 1*	67	0.17	Doubtful identification
	Winter	B1	*Proteus mirabilis*	56.5	0.36	Doubtful identification
			Enterobacter cloacae	94	0.18	Doubtful identification
		B2	*Proteus mirabilis*	56.5	0.36	Doubtful identification
		B3	*Proteus penneri*	58.2	0.38	Doubtful identification
			Escherichia coli 1	99.5	0.96	Very good identification
			Citrobacter freundii	99.4	0.91	Good identification
			Klebsiella oxytoca	98.2	0.73	Good identification
		B4	*Serratia ficaria*	67.7	0.38	Doubtful identification
	Spring	B1	*Ewingella americana*	98.6	0.92	Excellent identification
			Enterobacter cloacae	94	0.18	Doubtful identification
		B2	*Vibrio fluvialis*	71.7	0.71	good identification
		B3	*Aeromonas hydrophila/caviae/sobria 1*	97	0.78	Good identification
			Citrobacter freundii	99.4	0.91	Good identification
			Escherichia coli 1	99.5	0.96	Very good identification
			Klebsiella oxytoca	98.2	0.73	Good identification
		B4	*Raoultella ornithinolytica*	80.8	0.68	Doubtful identification
			Pasteurella multocida 2	94.4	0.34	Doubtful identification
R2A agar	Autumn	B1	*Providencia alcalifaciens/rustigianii*	75.6	0.62	Good identification to the genus
		B2	*Ewingella americana*	98.6	0.92	Excellent identification
		B3	*Citrobacter freundii*	99.4	0.91	Good identification
		B4	*Pasteurella multocida 2*	94.4	0.34	Doubtful identification
	Winter	B1	*Proteus mirabilis*	56.5	0.36	Doubtful identification
			Providencia stuartii	98.4	0.68	Good identification
		B2	*Proteus mirabilis*	56.5	0.36	Doubtful identification
			Enterobacter aerogenes	98.9	1	Good identification to the genus
		B3	*Escherichia coli 2*	93.2	0.92	Very good identification
			Salmonella ser.Typhi	98.3	0.92	Good identification
		B4	*Providencia alcalifaciens/rustigianii*	75.6	0.62	Good identification to the genus
	Spring	B1	*Providencia alcalifaciens/rustigianii*	75.6	0.62	Good identification to the genus
		B2	*Pseudomonas luteola*	93.9	0.67	Good identification
			Shigella spp.	81.5	0.96	Good identification
		B3	*Providencia alcalifaciens/rustigianii*	75.6	0.62	Good identification to the genus
			Shigella spp.	81.5	0.96	Good identification
		B4	*Proteus vulgaris group*	99.9	1	Excellent identification
			Proteus penneri	73.3	0.17	Doubtful identification

Identified bacteria at site B1 were *Providencia alcalifaciens/rustigianii* during autumn season, *Providencia stuartii* during winter season, *Providencia alcalifaciens/rustigianii* and *Ewingella Americana* during spring season. *Providencia alcalifaciens/rustigianii* have been associated with

diarrhoea and gastroenteritis disease in both children and adults (Haynes and Hawkey, 1989; Albert *et al*. 1992; Guth and Perrella, 1996; Vieira *et al*. 2003; Choi *et al*. 2015). *Kluyvera intermedia, Vibrio fluvialis, Ewingella Americana, Enterobacter aerogenes, Pseudomonas luteola* and *Shigella spp.* were identified at site B2. The bacteria which were identified at site B3 were *Escherichia coli 1, Aeromonas hydrophila/caviae/sobria 1, Escherichia coli 1, Citrobacter freundii, Klebsiella oxytoca, Aeromonas hydrophila/caviae/sobria 1, Citrobacter freundii, Escherichia coli 1, Klebsiella oxytoca, Citrobacter freundii, Escherichia coli 2, Salmonella ser.Typhi, Providencia alcalifaciens/rustigianii* and *Shigella spp.* At site B4 bacteria identified were *Providencia alcalifaciens/rustigianii* and *Proteus vulgaris group.*

The presence of pathogenic bacteria rises a concern in human health since river water are directed into the dams which serves as the source of drinking water. Some of these pathogenic bacteria are able to bypass the water treatment process by chlorination and enter water distribution systems (Mulamattathil *et al*. 2014). This results in the presence of pathogenic bacteria in drinking water which has been reported in previous studies (Mulamattathil *et al*. 2014; Obi *et al*. 2002). The presence of opportunistic pathogens such as *Pseudomonas* and *Aeromonas spp*. in drinking water in Mafikeng, South Africa has been reported in a study that was conducted by (Mulamattathil *et al*. 2014) and can results in illnesses on exposed individuals.

Table 4. 4: Identified bacterial species in Bloukrans River at four different sites during autumn, winter and spring season.

Culture media	Season	Sites	Organism	% identity	T value	Comment
m-Endo agar	Autumn	Bl1	*Enterobacter cloacae*	94	0.81	Good identification
		Bl2	*Vibrio fluvialis*	71.7	0.71	Good identification
			Escherichia coli 1	99.5	0.96	Excellent identification
		Bl3	*Kluyvera spp*	90.4	0.73	Good identification
			Klebsiella oxytoca	98.2	0.83	Very good identification
			Raoultella ornithinolytica	80.8	0.68	Doubtful identification
		Bl4	*Klebsiella pneumoniae ssp pneumoniae 2*	95.8	0.8	Good identification
	Winter	Bl1	*Leclercia adecarboxylata*	81.8	1	Very good identification to the genus
		Bl2	*Raoultella ornithinolytica*	92	0.38	Doubtful identification
			Kluyvera intermedia	92	0.74	Good identification
			Ewingella americana	99.2	0.9	Very good identification
		Bl3	*Proteus penneri*	58.2	0.38	Doubtful identification
			Aeromonas hydrophila/caviae/sobria 1	76.0	1	Good identification
			Escherichia coli 2	93.2	0.92	Very good identification
		Bl4	*Vibrio fluvialis*	70.4	0.59	Acceptable identification
			Pasteurella multocida 2	95.1	0.34	Doubtful identification
			Klebsiella pneumoniae ssp pneumoniae 1	97.3	1	Good identification

		Bl1	*Proteus penneri*	58.2	0.38	Doubtful identification
		Bl2	*Pantoea spp 2*	63.7	0.41	Doubtful identification
			Kluyvera intermedia	90.6	0.91	Very good identification
		Bl3	*Providencia alcalifaciens/rustigianii*	75.6	0.62	Good identification to the genus
	Spring		*Vibrio fluvialis*	85.3	0.85	Good identification
			Aeromonas hydrophila/caviae/sobria 1	67	0.17	Doubtful identification
			Pantoea spp 2	66.3	0.46	Doubtful identification
		Bl4	*Klebsiella pneumoniae ssp pneumoniae 1*	97.3	1	Good identification
		Bl1	*Proteus penneri*	99.2	1	Very good identification
		Bl2	*Aeromonas hydrophila/caviae/sobria 1*	85.9	0.76	Good identification
	Autumn	Bl3	*Salmonella ser.Typhi*	99.9	1	Excellent identification
		Bl4	*Providencia stuartii*	98.4	0.68	Good identification
		Bl1	*Aeromonas salmonicida ssp salmonicida*	99.6	0.97	Very good identification
			Proteus penneri	58.2	0.38	Doubtful identification
		Bl2	*Aeromonas hydrophila/caviae/sobria 1*	64.2	0.92	Doubtful identification
			Escherichia coli 2	99.6	0.9	Very good identification
R2A agar	**Winter**	Bl3	*Aeromonas hydrophila/caviae/sobria 1*	85.9	0.76	Good identification to the genus
			Providencia alcalifaciens/rustigianii	75.6	0.62	Good identification to the genus
			Ewingella americana	99.2	0.6	Very good identification
		Bl4	*Pantoea spp 3*	99.7	0.95	Very good identification
			Providencia stuartii	98.4	0.68	Good identification
		Bl1	*Escherichia coli 2*	99.4	0.85	Very good identification
		Bl2	*Vibrio cholerae*	93.0	0.86	Very good identification
			Salmonella ser.Typhi	98.3	0.92	Good identification
	Spring	Bl3	*Vibrio fluvialis*	91.1	0.78	Good identification
			Shigella spp	81.7	0.96	Very good identification
		Bl4	*Pseudomonas luteola*	96.0	0.77	Good identification
			Pseudomonas fluorescens/putida	88.4	0.83	Good identification

Identified bacteria at Bl1 site were *Enterobacter cloacae, Leclercia adecarboxylata, Proteus penneri, Aeromonas salmonicida ssp salmonicida and Escherichia coli 2.* At Bl2 site bacteria identified were *Vibrio fluvialis, Escherichia coli 1, Kluyvera intermedia, Ewingella Americana, Aeromonas hydrophila/caviae/sobria 1, Escherichia coli* 2, *Vibrio cholerae* and *Salmonella ser.Typhi. At site Bl3 bacteria identified were Kluyvera spp., Klebsiella oxytoca, Aeromonas hydrophila/caviae/sobria 1, Escherichia coli 2, Providencia alcalifaciens/rustigianii, Vibrio fluvialis, Salmonella ser.Typhi, Ewingella Americana* and *Shigella spp.* While at site Bl4 bacteria identified were *Klebsiella pneumoniae ssp pneumoniae 2, Vibrio fluvialis, Klebsiella pneumoniae ssp pneumoniae 1, Providencia stuartii, Pantoea spp 3*, *Pseudomonas luteola* and *Pseudomonas fluorescens/putida.*

Table 4. 5: identified bacterial species in Swartkops River at four sites during autumn, winter and spring season.

Culture media	Season	Sites	Organism	% identity	T value	Comment
m-Endo agar	Autumn	S1	*Providencia stuartii*	98.4	0.68	Good identification
		S2	*Enterobacter aerogenes*	98.9	1	Good identification to the genus
		S3	*Salmonella ser.Typhi*	99.9	1	Excellent identification
		S4	*Shigella spp*	81.5	0.96	Very good identification
	Winter	S1	*Providencia stuartii*	79	0.74	Good identification to the genus
		S2	*Serratia odorifera 1*	64.5	0.22	Doubtful identification
		S3	*Pseudomonas luteola*	99.9	0.66	Very good identification
		S4	*Escherichia coli 2*	99.4	0.85	Very good identification
			Pseudomonas fluorescens/putida	82.4	0.83	Good identification
	Spring	S1	*Escherichia coli 1*	96.3	0.87	Very good identification
		S2	*Stenotrophomonas maltophilia*	99.3	0.86	Very good identification
			Klebsiella oxytoca	97.8	0.73	Good identification
		S3	*Shigella spp*	81.5	0.96	Very good identification
			Klebsiella oxytoca	99.0	0.84	Very good identification
			Escherichia coli 1	96.3	0.87	Very good identification
		S4	*Enterobacter aerogenes*	98.9	1.0	Good identification to the genus
			Vibrio fluvialis	71.7	1	Good identification
			Pseudomonas aeruginosa	99.2	0.92	Very good identification
R2A agar	Autumn	S1	*Providencia stuartii*	98.4	0.68	Good identification
		S2	*Enterobacter aerogenes*	98.9	1	Good identification to the genus
		S3	*Salmonella ser.Typhi*	99.9	1	Excellent identification
		S4	*Shigella spp*	81.5	0.96	Very good identification
	Winter	S1	*Proteus penneri*	99.2	1	Very good identification
		S2	*Providencia alcalifaciens/rustigianii*	75.6	0.62	Good identification to the genus
		S3	*Aeromonas salmonicida ssp salmonicida*	99.6	0.97	Very good identification
		S4	*Pantoea spp 3*	99.7	0.59	Very good identification
			Proteus penneri	58.2	0.38	Doubtful identification
	Spring	S1	*Aeromonas salmonicida ssp salmonicida*	99.6	0.97	Very good identification to the genus
		S2	*Providencia alcalifaciens/rustigianii*	75.6	0.62	Good identification to the genus
			Pseudomonas aeruginosa	99.2	0.92	Very good identification
			Pseudomonas luteola	99.9	0.66	Good identification
		S3	*Pseudomonas aeruginosa*	98.7	0.92	Good identification
			Escherichia coli 2	99.4	0.85	Very good identification
		S4	*Pseudomonas luteola*	99.9	0.66	Very good identification
			Escherichia coli 2	99.4	0.85	Very good identification

The identified bacteria at S1 site were *Klebsiella pneumoniae ssp pneumoniae 2, Vibrio fluvialis, Klebsiella pneumoniae ssp pneumoniae 1, Providencia stuartii, Pantoea spp 3, Pseudomonas luteola* and *Pseudomonas fluorescens/putida.* At site S2 identified bacteria were *Enterobacter aerogenes, Stenotrophomonas maltophilia, Klebsiella oxytoca, Enterobacter aerogenes, Providencia alcalifaciens/rustigianii, Pseudomonas aeruginosa* and *Pseudomonas luteola.* At site S3 identified bacteria were *Salmonella ser.Typhi, Pseudomonas luteola, Shigella spp., Klebsiella oxytoca, Escherichia coli 1, Salmonella ser.Typhi, Aeromonas salmonicida ssp salmonicida, Pseudomonas aeruginosa, Escherichia coli 2.* While at S4 site the identified bacteria were *Shigella spp., Escherichia coli 2, Pseudomonas fluorescens/putida, Enterobacter aerogenes, Vibrio fluvialis, Pseudomonas aeruginosa, Shigella spp, Pantoea spp 3, Pseudomonas luteola* and *Escherichia coli 2.*

4.10. Bacterial identification

The presence of faecal coliforms and heterotrophic bacteria in river water samples was confirmed using apiweb identification database up to species level. Detection of bacteria of faecal origin such as *Escherichia coli*, *Shigella spp*., *Vibrio cholerae* and *Salmonella spp*. in river water samples was a confirmation of faecal pollution. The presence of these pathogens in river water can result in sicknesses and death following exposure, as these pathogens causes diseases such as gastroenteritis, diarrhoea, salmonellosis, dysentery, cholera and typhoid fever . Waterborne diseases are one of the most important diseases in South Africa being the cause of about 20% death in children under the age of 5 years with lack of adequate sanitation and assess to clean and safe drinking water (Momba *et al*. 2006).

The presence of *Vibrio cholerae* has been also previously reported in Tyhume River water by Momba et al. (2006). This in an indication that the people who resides in rural areas around Alice are in a risk of suffering from cholera as some use river water for domestic purposes during shortage of tape water supply. The genus of *Vibrio* is classified as small curved-shaped rods that contain single polar flagellum. Vibrio's are gram-negative facultative anaerobes with the ability to use both fermentative and respiratory metabolism. The growth of all the bacterial species is stimulated by sodium and is recognised as crucial requirement for the majority of bacteria. Nearly all the vibrio species are oxidase-positive and can convert nitrate to nitrite. This is problematic as nitrite is toxic to human health causing methaemoglobin, which is not able to carry oxygen, and this is dangerous to children (Mason, 2002). Some of the *Vibrio* species such as *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* have pili in their cell structures made of protein TcpA. The formation of the protein TcpA is co-regulated with expression of cholera toxin and is the main determining factor for vivo colonization.

The *Vibrio* species are known to be infectious to humans with *V. cholerae* being the most common species isolated from human specimens. *V. cholerae* is the cause of disease cholera. *V. fluvialis*, *V. hollisae* and *V. mimicus* species are known to cause diseases such as diarrhoea or gastrointestinal tract infections. The species *V. parahaemolyticus* is recognized as the cause of severe food-borne gastroenteritis especially in countries such as Japan and South East Asia. The food-borne infections are linked with consumption of raw or partially cooked shellfish like oysters, shrimp, crab and lobster. *V. vulnificus* is the main cause of septicaemia and wound infections (Farmer *et al*. 2003; Farmer *et al*. 2005; Cabral, 2010). The genus *Vibrio* are aquatic bacteria and their species dispersal depends on sodium rich environment and favourable water temperature. The species which require low sodium concentration are commonly present in freshwater environments. *V. cholerae* are able to grow at a temperature of 40 °C with pH of 9 to 10, their growth is induced by the presence of sodium chloride (Farmer *et al*. *et al*. 2004; Farmer *et al*. 2005; WHO, 2008; Cabral, 2010).

The genus Salmonella belongs to the Enterobacteriaceae family. The morphology of Salmonella is characterized as gram-negative motile straight rods. The Salmonella species are oxidase negative and catalase-positive, in the presence of D-glucose they form gas and use citrate as their sole source of carbon. In mammals Salmonella inhabits in the intestinal tract and is introduced into the environment through excretion of body wastes (Dworkin *et al*. 2003; Cabral 2010). Hence the Salmonella is commonly detected from wastewater samples and is introduced in aquatic environments via effluent discharge from WWTPs. The sources of Salmonella into the environmental waters involves, municipal sewage, agricultural runoff and surface runoff (WHO, 2008; Arvanitidou *et al*. 2005). The removal of Salmonella during WWTP processes requires the use of germicides and failure to use germicides results in Salmonella strains released with the effluent discharge (Popoff *et al*. 2005; Cabral 2010). Salmonella causes two types of salmonellosis in infected individuals, the typhoid or paratyphoid fever and gastroenteritis (Dworkin *et al*. 2003; Popoff *et al*. 2005; Tindall *et al*. 2005). The presence of Salmonella species in Aliakmon River and Axios River water situated in Grace was reported in a study that was conducted by Arvanitidou et al. (2005). Salmonella has been isolated in environmental samples such as poultry, red meat, milk and dairy products (Ben Aissa *et al*. 2007; Cabral, 2010).

The genus *Shigella* belong to the family Enterobacteriaceae and is classified by being immotile gram-negative straight rods. Shigella species do not produce gas when fermenting sugars and have no ability to ferment salicin, adonitol and myo-inositol. Citrate, malonate and acetate do not serve as source of carbon and there is no production of H_2S. The cells are oxidase-negative, catalase-positive and have no ability to decarboxylate lysine (Kapperud et al. 1995; WHO, 2008; Strockbine *et al*. 2005). World-wide people suffering from Shigellosis are estimated to be 164.7 million leading up to 1.1 million of deaths each year where 61% of death are reported for children under the age of 5 years (Germani *et al*. 2003; Emch *et al*. 2008). Shigella lives in the intestinal tract of humans and primates (Kapperud et al. 1995; Strockbine *et al*. 2005; Germani *et al*. 2003; Tetteh *et al*. 2003; WHO, 2008). In the environment Shigella is introduced through excretion of body wastes and spreads by consumption of faecal contaminated water or food. In aquatic environments Shigella lives up to 6 months at a temperature of ±25 °C and this great survival favours spread via water, hence shigella species were detected in all the rivers at middle-stream, WWTP or lower-stream sites resprctively (Germani *et al*. 2003; Chompook *et al*. 2006).

Aeromonas spp. Kluyvera spp. Klebsiella pneumoniae ssp pneumoniae, Citrobacter freundii, Klebsiella oxytoca, Raoultella ornithinolytica were detected in river water samples and their presence in water samples can be problematic they can cause sicknesses and deaths in exposed individuals.

Their presence in river water puts public health at risk as rivers serves as a source of water in dams supplying drinking water.

The genus *Citrobacter* belongs to the family of Enterobacteriaceae. Citrobacter are facultative gram-negative anaerobes and are motile straight rods. Citrobacter species test negative for oxidase, while for Methyl-Red test and catalase they test positive. These species use citrate as their source of carbon, they are negative for Voges-Proskauer test and do not decarboxylate lysine (Holt *et al*. 1994). The study that was conducted by Gordon and FitzGibbon (2015) reported the presence of *C. amalonaticus*, *C. freundii* and *C. koseri* (*C. diversus*) in Australian mammal faeces. In humans, *C. freundii* resides in the intestines and can have or attain the ability to produce an enterotoxin and therefore becomes an intestinal pathogen. Citrobacter has been isolated from the environmental samples such as water, sewage, soil and food (Frederiksen *et al*. 2003; Grimont *et al*. 2003; Cabral, 2010). The presence of these bacterial pathogens in river water samples is an indication that the people who use these rivers as their source of domestic water are in great danger of infections.

Klebsiella and *Raoultella* are members of the family Enterobacteriaceae, they are non-motile straight rods covered by the capsule. *Klebsiella* and *Raoultella* species are characterised by being oxidase-negative, catalase-positive, possessing ability to decarboxylate lysine and inability to decarboxylate ornithine and arginine. Their cells are able to ferment nearly all the carbohydrates. *K. pneumoniae* is part of the microbial flora in humans and is commonly present in nasopharynx and the intestinal tract. Infectious *Klebsiella spp*. causes illnesses such as intestinal, urinary and respiratory tract. *Klesiella spp*. have been detected in environmental samples such as soil, vegetation and water and have been reported to influence biochemical and geochemical processes (Frederiksen *et al*. 2003; Grimont *et al*. 2003; Gordon and FitzGibbon, 2015).

4.11. Conclusion

This study reports the presence of faecal coliforms in surface waters of the Eastern Cape Province. The prevalence of indicator bacteria (*E. coli*) in all the samples during three sampled seasons indicates that faecal pollution is a serious environmental issue that need to be addressed for the protection of public health. The presence of pathogens such as *Aeromonas spp. Kluyvera spp. Klebsiella pneumoniae ssp pneumoniae, Citrobacter freundii, Klebsiella oxytoca, Raoultella ornithinolytica, Escherichia coli*, *Shigella spp*., *Vibrio cholerae* and *Salmonella spp*. in river water was an indication that the river water was faecal polluted and that could result in illnesses and deaths in exposed individuals. This indicates that the river water is not safe for domestic use. Since these pathogens are found in river water which also feed into dams that serves as source of drinking water, it is essential to monitor the presence of

these pathogens in these resources. According to the DWAF and WHO the acceptable standards for domestic water and drinking water, the faecal coliform and total coliform concentration in water must be 0 cfu/100 mL. The water resources which are used as source of drinking water must not contain contaminating pathogens as these may cause serious health complications in affected individuals. The detection of pathogenic bacteria in all the sites indicated that all the sites were not suitable to be used as a source of drinking water or domestic water that may put the health of individuals at risk.

4.12. References

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Chapter 5

Biomonitoring of the freshwater quality using aquatic macroinvertebrates

5.0. Introduction

Rivers are constantly degrading due to contamination caused by the endless release of domestic and industrial wastes (Benetti and Garrido, 2010), agricultural runoff of fertilizers and pesticides, which results in eutrophication and pollution of aquatic systems (García-Criado *et al*. 1999; Paz, 1993; Benetti *et al*. 2012). In addition, rivers are also affected by the building of dams and reservoirs thereby changing the ecological characteristics of their basins (Richter *et al*. 1997; Benetti *et al*. 2012). Pollution is the major cause of aquatic environments deterioration and results in the temporal or permanent modification, disturbance and harmful destruction of the aquatic habitat (Hellawell, 1986; RebouÇas *et al*. 1999; Uherek and Gouveia, 2014). Pollution changes the physicochemical properties of water and disturbs macroinvertebrates communities and their habitats (Oller and Goitia, 2005; Smolders *et al*. 1999, Smolders *et al*. 2003; Benetti *et al*. 2012). Previous studies (Dang *et al*. 2002; Bell *et al*. 2001; Gordan *et al*. 2014; Shimba and Jonah, 2016) have reported that during wet season contamination in aquatic environments increases due to surface runoff. The surface runoff from agricultural areas and mining industries can lead to temporal degradation of the nearby aquatic environment (Tate and Husted, 2015).

The freshwater systems are of immense importance in the environment with a great biodiversity of faunas that comprises of communities that have a complex structure and essential biological value. Nevertheless, their unique classification is the reason they are susceptible to environmental changes more especially when induced by anthropogenic activity, which usually results in permanent degradation of their biota (Baesley and Kneale, 2003; Dahl *et al*. 2004). The susceptibility of macroinvertebrate habitats is also linked to the potential influence caused by climate changes of which rivers and streams are more likely to be affected. The effect of climate change could lead to aquatic systems changed from permanent to seasonal whereas in some cases could even disappear. This affects the aquatic biodiversity and can lead to alteration in the biogeochemical cycles (Jenkins *et al*. 1993; Vörösmarty and Sahagian, 2000; Krajenbrink *et al*. 2019).

Majority of the aquatic macroinvertebrate families have limited ecological requirements and are extensively utilized as bio-indicators for evaluation of the quality of aquatic systems (Fernández-Díaz *et al*. 2008; Pérez-Bilbao and Garrido, 2009; Benetti and Garrido, 2010) to pinpoint certain parts of the contaminated streams and rivers that lack self-purification of organic contaminants (Chatzinikolaou and Lazaridou, 2007; Benetti *et al*. 2012). Therefore, reservation of good water

quality in water resources is fundamental since each taxon of macroinvertebrates have varied tolerance to each of the water quality parameters (Dallas and Day, 1993; Malan and Day, 1993; Fernández-Díaz, 2003; Benetti *et al*. 2012; Wolmarans *et al*. 2014).

5.1. Biomonitoring of water quality

In the past decades the quality of water was evaluated by monitoring of the physical and chemical properties of water. However, the technique was only effective in reflecting the contamination of water, without indicating the ecological state of the river (Alba-Tercedor, 1996; Benetti *et al*. 2012; Odume *et al*. 2012). The biological indicators such as aquatic macroinvertebrates are more useful in the assessment of the long-term changes on the quality of water since various macroinvertebrates have definite environmental conditions preference. Hence, alterations of environmental conditions can result in the elimination of sensitive species and replacement by tolerant species (Alba-Tercedor, 1996; Benetti *et al*. 2012).

The South African Scoring system (SASS) is a macroinvertebrate biomonitoring system which was developed to assess organic pollution (Dickens and Grahams, 2002; Wolmarans *et al*. 2014). SASS was developed by Chutter in 1998, it is biotic index created on the occurrence of families of freshwater macroinvertebrates and their marked sensitivity to changes in water quality (Chutter, 1998; Dickens and Graham, 2002). Several modifications were applied on SASS resulting in the development of SASS version 5 (Dickens and Graham, 2002). The freshwater macroinvertebrate families in SASS5 are given scores which are relative to marked sensitivity from score 1 to15 based on their sensitivity to the decrease in water quality and the results are represented both as the index score (SASS5 score and as the average score per recorded taxa (ASPT score). The SASS5 score is the sum of all the recorded taxa while ASPT value determined by the division of the total SASS5 score with the total number of taxa (Dickens and Graham, 2002).

Hence, SASS is widely used in water quality assessment due to the method being highly sensitive, easy to apply and interpret, less time consuming and cost effective (Dallas, 1997; Gordan *et al*. 2014). Macroinvertebrates are widely used as water quality assessors due to their differences in sensitivity to water contamination and habitat modification. The sensitivity of each taxon of macroinvertebrates is categorized based on SASS5 sensitivity scores ranging from 1 (tolerant) to 15 (highly sensitive) (Dickens and Graham, 2002). The highly sensitive families belong to the order, Plecoptera, Ephemeroptera, Lepidoptera and Trichoptera while the moderate families belong to the order Hydracarina, Coleoptera, and Odonata and the tolerant families belong to the order Turbellaria, Annelida, Hemiptera, Diptera, Gastropoda and Pelecypoda.

5.2. Human impacts in aquatic environments

Anthropogenic influences are the main cause of freshwater systems to be at risk of degradation (Bredenhand and Samways, 2009; Benetti *et al*. 2012). The biodiversity of freshwater ecosystems worldwide is gradually declining due to human activities (Dahl *et al*. 2004; Benetti *et al*. 2012). The five main types of threats to freshwater biodiversity are overexploitation, water contamination, modification of water flow, destruction or degradation of habitat and invasion by exotic species (Dudgeon et al, 2006; Benetti *et al*. 2012).

It is most likely that the human impact in freshwater ecosystems would threaten the aquatic biodiversity (Strayer, 2006; Benetti *et al*. 2012). Overexploitation of the rivers and aquifers for irrigation purposes is a serious issue in various places, in European countries over 60% of water is used annually for irrigation purposes (Abellán *et al*. 2006; Benetti *et al*. 2012). This could be problematic in a long run as can lead to drought and the disappearance of inland aquatic environments (Abellán *et al*. 2006; Belmar *et al*. 2010) or result in the alteration of the physicochemical properties of water (Velasco *et al*. 2006; Benetti *et al*. 2012).

Pollution of aquatic environments by organic, inorganic compounds and heavy metals has emerged as a public concern worldwide. The human and industrial growth causes different non-point sources of pollution thus decreasing the quality of freshwater (Beasley and Kneale, 2003, Benetti *et al*. 2012). Pollution causes a decrease in water quality and biodiversity of aquatic organisms, the negative effects of pollution are well documented in several studies (Garrido *et al*. 1998; Lytle and Peckarsky, 2001; Hirst *et al*. 2002; Beasley and Kneale, 2003; Beasley and Kneale, 2004; Harper and Peckarsky, 2005; Smolders *et al*. 2003; Fernández-Díaz *et al*. 2008; Song *et al*. 2009; Benetti and Garrido, 2010).

The building of dams and the creation of reservoirs is among the main factors that lead to alteration of the rivers, since the normal flow of the rivers is modified to be firm with decreased flows (Petts, 1984; Baeza *et al*. 2003; Benejam *et al*. 2010; Belmar *et al*. 2010; Zarfl *et al*. 2015). Modification in the marginal vegetation and water velocity can lead to changes in the biodiversity of aquatic organisms, with substitution of some species by others due to the destruction of habits and creation of new ones (Lessard and Hayes, 2003; Fulan *et al*. 2010; Sarr, 2011). The modification of river flow to provide water for humans is one of the major causes that lead to stream and river degradation (Gordon *et al*. 2004; Bredenhand and Samways, 2009; Tonkin *et al*. 2018; Krajenbrink *et al*. 2019) and one of the factors that lead to alteration in the stream community (Rosenberg *et al*. 2000; Krajenbrink *et al*. 2019).

Introduction of foreign invasive species in aquatic environments results in the extinction of some native species due to competition, predation and biotic homogenization. Example of exotic species

invasions includes Nile perch, the American indicator crayfish or the zebra mussel (Raehl, 2002; Benetti *et al*. 2012). In addition, the introduction of exotic plant species such as water hyacinths in streams as a result of pollution decreases dissolved oxygen levels and affects survival of sensitive aquatic macroinvertebrates (Aquatic insects, 2018).

5.3. The influence of habitat in macroinvertebrates

Aquatic macroinvertebrates have different habitat preferences, therefore conducive habitats have a vital role in survival and abundance of species. For instance, the family of Thiaridae prefers habitat with a warmer climate, slow current speed (< 0.1 m/s), poor water quality, nutrient-rich and aquatic vegetation (Thirion, 2006; Wolmarans *et al*. 2014). Although Thiaridae prefers a warmer climate it can survive in temperatures between 0 to 47 °C (Miranda *et al*. 2002, Wolmarans *et al*. 2014). The family of Chironomidae prefers nutrient-rich environments with slightly salty water (Thirion, 2006; Arimoro *et al*. 2011; Wolmarans *et al*. 2014). The family of Psychodidae prefers warmer climates, therefore, they are commonly found during spring season. During their larvae stage, they feed on algae, fungi, and bacteria in sewage polluted environments while during their adult stage they feed on polluted water. The family of Syrphidae prefers habitat with stagnant waters and highly polluted with sewage waste (College of letters and science field station, 2010; Bejarano and Estrada 2016).

The impact of suspended sediments on macroinvertebrates can either be direct or indirect. Direct impact results in the blockage and tearing of gills thus affecting the life of an organism and filter-feeding organisms causes reduced feeding ability (Bilotta and Brazier, 2008; Jones *et al*. 2011; Gordon *et al*. 2014). In addition, the settled residues can result in benthic macroinvertebrates and eggs being suffocated leading to deaths (Jones *et al*. 2012, Gordon *et al*. 2014). Indirect impact can lead to changes in the habitat (Wood *et al*. 1997; Gordon *et al*. 2014), food availability (Peeters *et al*. 2006), increased turbidity and results in altered predator-prey feeding mechanisms therefore causing reduced growth (Jones *et al*. 2012; Gordon *et al*. 2014) and overall change in the environment (Bo *et al*. 2007; Gordon et al 2014). This indicates that the concentration of instream residue must be regulated (Gordon *et al*. 2014). This study was motivated by the decreasing quality of river water due to human activities. The upper stream sites are usually in a good ecological state as compared to the middle stream sites and lower stream sites. This is due to less anthropogenic effects that upper stream sites experience.

Aims

1.To assess the quality of river water using benthic macroinvertebrates such as South African Scoring System version 5.

2. To determine the human impact on the quality of river water.

Objectives

1.To determine the ecological state of each site in all the sampled rivers by measuring SASS score.

2. To compare the middle stream site and lower stream site ecological state to the upper stream site of the river.

5.4. Methods and materials

The macroinvertebrates sampling was conducted in two seasons in four rivers. The selected seasons were winter season (June to August) and spring season (September to November). This was done to assess the quality of water when there is minimum average rainfall since South Africa is water scarce. In all the rivers three sampling sites were chosen, upper-stream site, middle-stream site, and lower-stream site. These sites were chosen based on the surrounding impacts, where the upper-stream sites were used as reference sites, the middle-stream sites were used to represent the impact of the community/urban areas towards the river pollution and the lower-stream sites were used as the representatives of the effects of urban areas and wastewater treatment plants pollution on the rivers. Sample collection was conducted following the South African Scoring Systems version 5 (SASS5) procedure (Dickens and Grahams, 2002).

The sampling procedure requires sampling to be conducted in three different biotopes which are stones (stones in-current (SIC) and stone out-current (SOC)), vegetation (marginal and aquatic vegetation (MAV)) and sediments (gravel, sand and mud, (GSM)). Sampling was conducted for 2 minutes in SIC and 1 minute for SOC, 2 meters of MAV and 1 minute for GSM. In each of the biotopes, sampling was conducted once in each site. The sampling of macroinvertebrates was conducted using a SASS5 net (frame 30 cm X 30 cm with a pore size of 1000 µm). The samples were transferred into white SASS5 trays which were half filled with river water/ distilled water in the case of turbid sites. The macroinvertebrates present in the samples were identified using SASS5 identification book and identification was conducted up to the family level for 15 minutes per sampled biotope by SASS accredited person and results were recorded on SASS5 scoring sheet. The macroinvertebrates were categorized based on the SASS5 score into three groups: tolerant (score range 1 to 5), moderately sensitive (score range 6 to 10) and extremely sensitive (score range 11 to 15) (Dickens and Grahams, 2002).

5.4.1. Water quality valuation

The South African Scoring System (SASS) was developed by Chutter in 1998, it is biotic index created on the occurrence of families of freshwater macroinvertebrates and their marked sensitivity to changes in water quality (Chutter, 1998; Dickens and Graham, 2002). Several modifications were applied on

SASS resulting in the development of SASS version 5 (Dickens and Graham, 2002). The freshwater macroinvertebrate families in SASS5 are given scores which are relative to marked sensitivity from score 1- 15 based on their sensitivity to the decrease in water quality and the results are represented both as the index score (SASS5 score and as the average score per recorded taxa (ASPT score). The SASS5 score is the sum of all the recorded taxa while ASPT value determined by the division of the total SASS5 score with the total number of taxa (Dickens and Graham, 2002).

The SASS5 score describes the state of water quality of polluted rivers while the ASPT value is more reliable and describes the biological water quality of a river precisely than SASS5 score (Dickens and Graham, 2002). The possibility of occurrent of spatial variations in freshwater macroinvertebrate groupings between the rivers of different ecoregions (Kleynhans *et al*. 2005), has led to the development of guidelines which includes geographical and longitudinal differences in the interpretation of SASS5 data (Dallas, 2007). Therefore, each level of the ecoregion which is divided into upper and lower zones has specific biological bands used for the interpretation of SASS5 data (Dallas, 2007). The Bloukrans River is situated in the Southern folded mountain lower zone (Table 5.1), the Tyhume River and Buffalo River upper-stream site is situated in the Western Bankveld upper zone (Table 5.2) while the middle-stream and the lower-stream site are in the Soutpansburg lower zone (Table 5.3) and the Swartkops River is in the Southern Eastern Coastal belt lower zone, table 5.4 (Dallas, 2007).

Table 5. 1: Biological band/ Ecological categories for interpreting SASS data for the Southern folded mountain lower zone, Bloukrans River (Dallas, 2007).

Biological band/ Ecological Category	SASS score	ASPT score	Ecological Category name	Description
E/F	0 – 42	0 – 4.2	Seriously modified	Seriously modified
D	>42 – 4.5	>4.2 – 4.5	Poor	Largely modified
C	>53 – 71	>4.5 – 4.8	Fair	Moderately modified
B	>71 – 104	>4.8 – 5.8	Good	Largely natural with few modifications
A	>104 – 180	>5.8 – 8	Natural	Unmodified natural

Table 5. 2: Biological band/ Ecological categories for interpreting SASS data for the Western Bankveld upper zone, Tyhume River and Buffalo River upper-stream site (Dallas, 2007).

Biological band/ Ecological Category	SASS score	ASPT score	Ecological Category name	Description
E/F	0 – 83	0 – 4.5	Seriously modified	Seriously modified
D	>83 – 115	>4.5 – 5.4	Poor	Largely modified
C	>115 – 151	>5.4 – 5.9	Fair	Moderately modified
B	>151 – 244	>5.9 – 6.4	Good	Largely natural with few modifications
A	>244 – 320	>6.4 – 8	Natural	Unmodified natural

Table 5. 3: Biological band/ Ecological categories for interpreting SASS data for Soutpansburg lower zone, Tyhume River and Buffalo River middle-stream and lower-stream site (Dallas, 2007).

Biological band/ Ecological Category	SASS score	ASPT score	Ecological Category name	Description
E/F	0 – 125	0 – 5.9	Seriously modified	Seriously modified
D	>125 – 144	>5.9 – 6.3	Poor	Largely modified
C	>144 – 165	>6.3 – 6.5	Fair	Moderately modified
B	>165 – 183	>6.5 – 7.3	Good	Largely natural with few modifications
A	>183 – 240	>7.3 - 8	Natural	Unmodified natural

Table 5. 4: Biological band/ Ecological categories for interpreting SASS data for Southern Eastern Costal belt lower zone, Swartkops River (Dallas, 2007).

Biological band/ Ecological Category	SASS score	ASPT score	Ecological Category name	Description
E/F	0 – 63	0 – 5.1	Seriously modified	Seriously modified
D	>63 – 82	>5.1 – 5.4	Poor	Largely modified
C	>82 – 100	>5.4 – 6	Fair	Moderately modified

B	>100 – 149	>6 – 7.1	Good	Largely natural with few modifications
A	>149 – 180	>7.1 – 8	Natural	Unmodified natural

5.4.2. Integrated Habitat Assessment Systems

The physical environmental structure of the streams and rivers is assessed using the Integrated Habitat Assessment System (IHAS) index which aims to provide information on the quantity, quality, and diversity of habitats available to macroinvertebrates (Ollis *et al.* 2006). IHAS was formed to be used with SASS in the biomonitoring programmes (McMillan, 1998). The IHAS is not part of the water quality assessment index, however, it provides sufficiency assessment and diversity of habitats for the interpretation of SASS5 results. IHAS is the scoring system formed by two sections: the sampling habitat which has 55 points and the stream condition which has 45 points, thus forming a total of 100 points. The sampling habitat consists of three sections: SIC (20 points), vegetation (15 points) and other habitats/general such as GSM, bedrock, algal presence and tray identification (20 points). The stream condition gives a description of the stream such as river makeup, the width, and depth of the stream, the velocity and the colour of the water as well as resent disturbance to the stream. In all the sites throughout the sampling period, the IHAS was conducted by completing the IHAS score sheet.

5.5. Results and discussion

The health and quality of the Buffalo River, Bloukrans River, Swartkops River, and Tyhume River water was evaluated during winter and spring season using SASS5 method. The results are summarised in table 5.5. The SASS5 scores at Buffalo River sites ranged from 49 (B2) to 110 (B1) with ASPT value increasing from 4.1 (B2) to 5 (B1) during winter season. The IHAS values ranged from 48% (B1) to 66% (B4) during the same period. While the SASS5 scores ranged from 35 (B2) to 105 (B1) with ASPT value ranging from 3.2 (B2) to 5.3 (B1) during spring season. The IHAS value ranged from 63% (B1) to 71% (B4) during the same period. The SASS5 at Bloukrans River sites ranged from 3 (Bl4) to 95 (Bl1) with ASPT value raising from 1.3 (Bl2) to 5.6 (Bl1) during winter season. The IHAS value ranged from 42% (Bl4) to 56% (Bl1) during the same period. While the SASS5 scores ranged from 4 (Bl4) to 196 (Bl1) with ASPT value ranging from 1.3 (Bl4) to 6.8 (Bl1) during spring season. The IHAS value ranged from 57% (Bl4) to 65% (Bl1) during the same period. The SASS5 scores at Swartkops River sites ranged from 53 (S4) to 116 (S1) with ASPT value increasing from 4.4 (S4) to 4.9 (S2) during winter season. The IHAS value ranged from 57% (S1) to 74% (S2) during the same period. While the SASS5 scores ranged from 61 (S2) to 173 (S1) with ASPT value increasing from 4.1 (S2) to 5.7 (S1) during spring season. The IHAS value ranged from 51% (S2) to 73% (S1) during the same period. The SASS5 scores at Tyhume River sites ranged from 121 (T2) to 163 (T1) with

ASPT value increasing from 5.3 (T4) to 6.6 (T1) during winter season. The IHAS values ranged from 52% (T4) to 65% (T1) during the same period. While the SASS5 scores ranged from 52 (T2) to 96 (T1) with ASPT values increasing from 3.9 (T4) to 6 (T1). The IHAS values ranged from 56% (T2) to 64 (T4) during the same period.

Table 5. 5: The SASS5 scores, number of taxa, ASPT values and IHAS values of Buffalo River, Bloukrans River, Swartkops River and Tyhume River sites during winter and spring season of 2018.

		Buffalo River			**Bloukrans River**			**Swartkops River**			**Tyhume River**		
	Site no.	**B1**	**B2**	**B4**	**Bl1**	**Bl2**	**Bl4**	**S1**	**S2**	**S4**	**T1**	**T2**	**T4**
Winter	SASS5 score	110	49	67	95	4	3	116	69	53	163	121	143
	No. of Taxa	22	12	14	17	3	2	24	14	12	25	22	27
	ASPT value	5	4.1	4.8	5.6	1.3	1.5	4.8	4.9	4.4	6.6	5.5	5.3
Spring	SASS5 score	105	35	61	196	9	4	173	61	70	96	52	66
	No. of Taxa	20	11	14	29	4	3	30	15	14	16	10	17
	ASPT value	5.3	3.2	4.4	6.8	2.3	1.3	5.7	4.1	4.6	6	5.2	3.9
Winter	IHAS value	48%	58%	66%	56%	46%	42%	68%	74%	57%	65%	54%	52%
Spring	IHAS value	63%	61%	71%	65%	65%	57%	73%	51%	55%	63%	56%	64%

According to the biological band of the Southern Eastern Coastal belt lower zone (Table 5.4), the SASS5 score of the upper-stream site (S1) in Swartkops River indicated that the quality of river water was in a good state (natural with few modifications) while the ASPT value indicated that the stream was seriously modified during winter season (Table 5.6). The SASS score reflects more on water quality that has been affected whereas the ASPT value is more advantageous than SASS score it considers both the SASS score and the number of taxa, hence it more accurate in reflecting the biological quality of water (Dickens and Grams, 2002; Tate and Husted, 2015). Due to this reason, the ASPT value was mostly considered in making a conclusion about the biological quality of water/ecological state of the river while SASS5 score was mostly considered in making a conclusion about the quality of water that has been affected. The ASPT value results observed at this site may have been affected by the fire which occurred around the stream and fallen trees into the stream (Figure 5.1: a and b), thus causing the stream to be negatively affected. The low water levels in conjunction with a slow flow rate at S1 may have also contributed to the macroinvertebrate disturbance.

As discussed in chapter 2, the physicochemical parameters of this site were within target water quality range except for the chloride and ammonium which were in high concentration. This further explains the reason that the SASS5 score was indicating that the quality of water was in a natural state with few modifications at this site. However, the presence of high chloride concentration may have a negative effect on aquatic macroinvertebrates as can result in the formation of toxic substances which can affect sensitive aquatic macroinvertebrate families such as mayflies and stoneflies (Brungs, 1973; Hendrick and Pool 2012). The SASS score results at this site were further supported by the presence of Heptageniidae a highly sensitive macroinvertebrate family, thus confirming the state of the quality of water. Nevertheless, only one species was observed from the marginal vegetation biotope. The absence of other highly sensitive families may be linked to high chloride concentration at this site. In addition, the presence of three fish species in the vegetation biotope and one fish species in the GSM biotope may be the reason few highly sensitive species were observed at this site since macroinvertebrates are food source for fish (Macroinvertebrates, 2019). The moderate sensitive macroinvertebrate families found at this site were Hydropsychidae 2 sp., Lepidostomatidae, Elmidae, and Anthericidae. Elmidae and Anthericidae were the dominant moderate sensitive families observed at this site during the winter season (Table 5.7). The SASS results are usually interpreted in conjunction with IHAS to provide a full insight into the state of the stream (Tate and Husted, 2015). The analysis of the habitat biotope scores indicated that the habitat diversity was sufficient to support macroinvertebrate biodiversity as IHAS value of 68% was obtained. There were about four different aquatic plant species observed at this site and the presence of algae on rocks and on aquatic vegetation was also noticed (Figure 5.1: a). Both the plants and algae are used as a food source by macroinvertebrates such as mayflies, stoneflies, caddisflies (Macroinvertebrates, 2019).

During the spring season, the SASS score was in a natural state while the ASPT value was in fair state (moderately modified) at site S1. These observations were supported by the physicochemical properties which were within the acceptable target range except for chloride. The IHAS value was 78% thus indicating that the habitat diversity for macroinvertebrate communities was in a good state and provided good living habitats for the macroinvertebrates. Abundant marginal vegetation was observed during the spring season at this site as compared to marginal vegetation observed during the winter season. The abundance in marginal vegetations may have contributed in increased sensitive species since there was more food for growth. The presence of algae on rocks and aquatic plants was also noticeable (Figure 5.1: b). In addition, target pharmaceuticals were detected in concentration of not detected for erythromycin, ibuprofen, ciprofloxacin and sulfamethoxazole, 79 ng/0.5 L for clarithromycin and 1130.7 ng/0.5 L for carbamazepine. Although the concentration of most target

pharmaceuticals was lower than the detection limits, detection of carbamazepine in high concentration may have had negative effects on the diversity of macroinvertebrates. These observations might have been influenced by the seasonal changes, the rise in water level and improved water quality which allows the stream condition to improve. The improved water quality and stream condition were supported by increased number of highly sensitive macroinvertebrate families observed during the spring season as Crambidae and Glossosomatidae were observed. The number of moderate sensitive families also increased during the spring season as Caenidae, Hydrometridae, Anthericidae and Dixidae were observed with Lestidae, Elmidae and Baetidae 2 sp. being in high abundance. In addition, the presence of two fish species in vegetation biotope and three fish species in GSM biotope might have influenced the abundance of macroinvertebrate species and presence of other macroinvertebrates.

Figure 5. 1: The aquatic vegetation observed at site S1 of Swartkops River during the winter season (a) and spring season (b).

Table 5. 6: The ecological status of Buffalo River, Bloukrans River, Swartkops River and Tyhume River in three sampling sites during winter and spring season of 2018.

		Buffalo River			Bloukrans River			Swartkops River			Tyhume River		
		B1	**B2**	**B4**	**Bl1**	**Bl2**	**Bl4**	**S1**	**S2**	**S4**	**T1**	**T2**	**T4**
Winter	Ecological Category for SASS5 score	Poor	Seriously modified	Seriously Modified	Good	Seriously modified	Seriously Modified	Good	Poor	Seriously Modified	Good	Seriously modified	Poor
	Ecological Category for ASPT value	Poor	Seriously modified	Seriously Modified	Good	Seriously modified	Seriously Modified	Seriously modified	Seriously modified	Seriously Modified	Natural	Seriously modified	Seriously modified
Spring	Ecological Category for SASS5 score	Poor	Seriously modified	Seriously modified	Natural	Seriously modified	Seriously modified	Natural	Seriously modified	Poor	Poor	Seriously modified	Seriously modified
	Ecological Category for ASPT value	Poor	Seriously modified	Seriously modified	Natural	Seriously modified	Seriously modified	Fair	Seriously modified	Seriously modified	Good	Seriously modified	Seriously modified

The SASS score indicated that the water quality was in a poor state while the ASPT value indicated that the stream was seriously modified at site S2 during the winter season. Both the SASS score and the ASPT value indicated that the stream was seriously modified at site S2 during the spring season. These results were supported by the absence of highly sensitive aquatic macroinvertebrates at site S2 during the winter season. The absence of highly sensitive macroinvertebrates may have been affected by pollution, dumping of rubbish and disturbance by livestock which recently occurred at this site. The target pharmaceuticals were also detected in high concentrations at this site except for ciprofloxacin and ibuprofen which were not detected. Although this site was seriously modified as a result of pollution it was still able to support moderate sensitive macroinvertebrate families such as Synlestidae, Lestidae, Aeshnidae, and Ancylidae. The highly sensitive family observed at site S2 during spring season was Crambidae with one species from the vegetation biotope. There were no moderate sensitive macroinvertebrate families observed at site S2 during the spring season and this may be due to pollution. The presence of invasive plant species such as water hyacinths (Figure 5.2: a, and b) at this site might be the main reason that the sensitive species were not observed at this site. The growth of water hyacinths was promoted by the presence of organic contaminants, therefore, indicating there was organic pollution at this site (Aquatic insects, 2018). The pollution of this site was further confirmed by the presence of tolerant families which survive well in water of poor quality (Dickens and Graham, 2002) such as Chironomidae and Oligochaeta with high abundance and Chironomidae observed at this site were bigger in size. The IHAS values indicated that the site was in a good and fair state during winter and spring season respectively and so the absence of sensitive families was due to pollution.

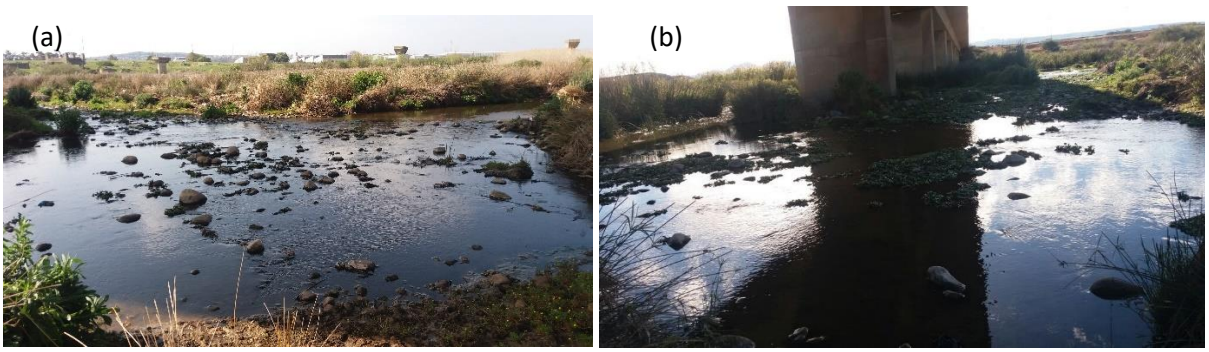

Figure 5. 2: The water hyacinth observed at site S2 during the winter season (a) and spring season (b) at Swartkops River.

Both the SASS score and the ASPT value indicated that the stream was seriously modified at site S4 during winter season. While the SASS score indicated that the water quality was in a poor state and the ASPT value indicated that the stream was seriously modified at site S4 during spring season. These

results indicated that the river water was polluted, and this was supported by the absence of highly sensitive aquatic macroinvertebrates. The presence of inorganic contaminants in high concentration at this site together with target pharmaceuticals were the main cause of decrease in water quality. The main source of these contaminants at this site is the Dispatch WWTP. The moderate sensitive macroinvertebrate family observed was Naucoridae in high abundance. However, the presence of fish in vegetation biotope might have contributed to the absence of highly sensitive families and few numbers of moderate sensitive families at this site. The habitat biotope scores indicated that the stream was in a fair state to support macroinvertebrates diversity. Therefore, the absence of sensitive families is due to pollution. The highly sensitive family observed at site S4 during spring season was Crambidae and indicated that the quality of water had improved although the state of the stream did not improve much. Lestidae was the only moderate sensitive macroinvertebrate observed during spring season at this site. The IHAS value was in a fair state during the spring season at this site indicating that the habitat biotope was able to support the macroinvertebrates and the presence of algae in stones at site S4 was the food source for caddisflies and mayflies. The pollution of these sites was further confirmed by the presence of tolerant families such as Chironomidae and Oligochaeta in high abundance. The presence of inorganic contaminants and target pharmaceuticals in high concentration at this site might have contributed to the contamination of this site and lead to negative effects of on aquatic biodiversity.

The growth of water hyacinths in site S2 and S4 was probably promoted by industrial and agricultural contaminants that are released into the Swartkops River near these sites. In addition, the Dispatch WWTP has no capacity to deal with huge amounts of sewage influents and therefore, the sewage ends up being discharged into the river (Aquatic insects, 2019). According to Knipe the elevated pollution levels in this river are the main cause of water hyacinth to be dominant and double in size every 14 days. If the growth of water hyacinths is uncontrolled, the water hyacinths can cover the whole river, absorb all the dissolved oxygen from water and that lead to the death of fish and plant life (figure 5.3: a and b). The decrease in the oxygen levels affects the sensitive macroinvertebrate survival, hence only few or no sensitive families were observed at sites S2 and S4.

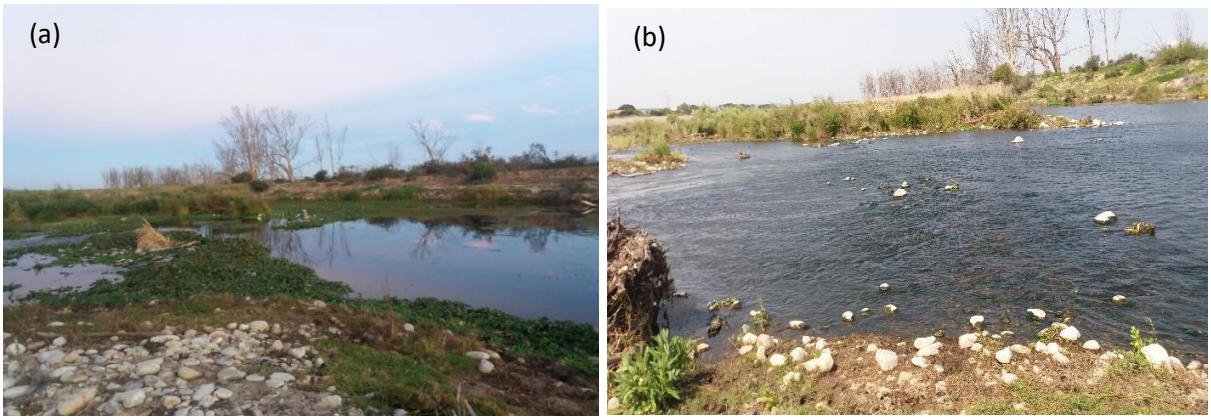

Figure 5. 3: The water hyacinths observed at site S4 of Swartkops River during the winter season (a) and spring season (b).

The SASS score and ASPT value of the upper-stream site/ reference site (P1) of Bloukrans River was in a good and natural state during winter and spring season respectively (Table 5.6). Similar results have been reported in a study that was conducted by Odume and Mgaba (2016) at this site. This may be due to less anthropogenic impacts which would lead to disturbance of the ecosystem, decrease in water quality and disturbance of aquatic biodiversity. In addition, the IHAS value of this site during winter and spring season ranged from 56% and 65% respectively, indicating that the habitat biotope was in a fair state for supporting the macroinvertebrate communities. These observations were supported by the observation of highly sensitive macroinvertebrate families in both winter and spring season. The Heptageniidae (flat-headed mayflies) was the only family observed during winter season whereas the number of highly sensitive families increased in the spring season as Crambidae, Helodidae, and Blephariceridae were observed in abundance. Thus, indicating that the stream conditions at this site allowed for macroinvertebrate recovery. The moderate sensitive macroinvertebrate families observed during winter season in high abundance were Leptophlebiidae, Aeshnidae, Gomphidae, and Ecnomidae. While during spring season the moderate sensitive macroinvertebrate families observed in high abundance were Hydracarina, Caenidae, Chlorolestidae, Synlestidae, Lestidae, Naucoridae, Lepidostomatidae, Leptoceridae, Anthericidae and Dixidae, Leptophlebiidae and Gomphidae. Dixidae is an air breather macroinvertebrate, therefore, it is not affected by depletion of dissolved oxygen in water were as other macroinvertebrates rely on dissolved oxygen for breathing. The low COD, temperature and EC readings observed at this site were indirectly indicating that the water was having enough oxygen levels. The number of sensitive families increased during the spring season as compared to the winter season and this may be due to improved water quality.

The SASS score and ASPT value of site Bl2 and Bl4 indicated that the stream was seriously modified during winter and spring season. Similar results have been reported in a study that was conducted by Odume and Mgaba (2016). These observations were further supported by the absence of highly sensitive families during winter and spring season. This may be due to the deterioration of the water quality caused by human pollution therefore causing the highly sensitive species unable to survive. The absence of moderate sensitive species in Bl2 and Bl4 sites during winter and spring season indicated that the water was critically polluted, and this was supported by the presence of tolerant families such as Oligochaeta and Chironomidae in high abundance. The presence of Syrphidae on-site Bl2 with an abundance of A indicated that this site was polluted by sewage waste thus further supporting the site observations which have been discussed in chapter 2 and 3 on this site. The presence of Chironomidae, Psychodidae, and Syrphidae in abundance at site Bl2 and Bl4 during spring season was an indication that the river was polluted with sewage waste and that the water was highly polluted. The sampling biotope for these sites was in a poor state as IHAS values of these sites was low during winter season. However, the sampling biotope improved during the spring season as obtained IHAS values were 65% and 57% respectively. Although the biotope habitat improved there was no much improvement in the quality of water and biological state of the river water, and this was confirmed by the absence of sensitive taxa.

The SASS score and ASPT value indicate that the upper-stream site (B1) of Buffalo River was in a poor state during winter and spring season. Although the water quality slightly decreased during the spring season, the poor state of the stream may possibly be due to low water levels and slow flow rate thus indicating long-term changes in environmental conditions. In addition to this, there was a recent disturbance by livestock at this site. The water parameters were still within the acceptable target range in this site although that was the case the biodiversity of macroinvertebrates could not recover. Similar results have been reported in other studies (Tate and Husted, 2015). The moderate sensitive families observed at this site were Leptophlebiidae, Gomphidae, Aeshnidae, Hydropsychidae 2 sp. and Philopotamidae in high abundance. The number of moderate sensitive species in spring increased as Caenidae, Leptophebiidae, Gomphidae, Aeshnidae, Naucoridae, Anthericidae, Lestidae and Platycnemidae were observed in high abundance. The middle-stream (B2) site and lower-stream (B4) site were seriously modified during winter and spring season. These observations may be caused by contamination of river water due to anthropogenic activities, agricultural runoff, sewage drainage and effluent discharge from WWTPs. High concentrations of inorganic compounds with relative high turbidity values detected in those sites may have also contributed to the decrease in water quality in those sites (García-Criado *et al*. 1999; Paz, 1993; Benetti *et al*. 2012). The deterioration of the river

ecological state in the middle-stream and lower-stream sites was confirmed by low SASS scores and ASPT value with few numbers of taxa observed. The fact that few or none of the taxa belonging to the order of Plecoptera, Ephemeroptera and Trichoptera observed from these sites was further indicating that the stream was critically affected, and the quality of water was compromised. Similar results have been reported in previous studies (Fjellheim and Raddum, 1992; Dallas, 2007; Tate and Husted, 2015). The biotope assessment of the middle-stream sites of the river indicated that the river was in a fair state in both the season as more than 50% IHAS values were observed.

There were no highly sensitive species observed during winter and spring season at Buffalo River in all the sites and this was indicating that the water quality was poor. The highly sensitive families are unable to cope with the changes in the aquatic environment conditions such as high-water temperature, high turbidity, high of deposits and low dissolved oxygen levels (environmental stressors) therefore only survive in in good water quality with good biotope habitats (Physical adaptation, 2019). Ancylidae was the only moderate sensitive family observed during the winter season at site B2 and there was only one species. The presence of sensitive families at this site might have also been influenced by the presence of fish as two species were observed from vegetation biotope. Sensitive families were replaced by tolerant families as Oligochaeta and Chironomidae were observed in high abundance at this site. However, during spring season Aeshidae was the only moderate sensitive species observed and only one species was observed at this site. The absence of sensitive families at this site might have been influenced by the presence of four fish species observed from the vegetation biotope while more than 10 fish species were observed from the GSM biotope. The families which were observed in high abundance were Oligochaeta and Chironomidae. The moderate sensitive families observed at site B4 during winter season were Leptophlebiidae, Elmidae, and Aeshnidae. While during the spring season, Caenidae, Leptophebiidae, Aeshidae were observed in high abundance. The most abundant families observed at this site were Oligochaeta and Chironomidae. The sampling habitat was in a fair state at this site and algae presence in rocks was observed.

The SASS score indicated that the stream was in a natural state while the ASPT value indicated that the stream was in a good state on the upper-stream site (T1) of Tyhume River during the winter season. Whereas during the spring season, the SASS score indicated that the stream was in a poor state while the ASPT value indicated that the stream was in a good state. The poor water quality at this site during spring season may be a due disturbance by the rain thus causing the substances which settle at the bottom to be re-suspended as the turbidity reading were high (Tate and Husted, 2015). The site T2 and T4 were seriously modified during winter and spring season. The highly sensitive families observed at T1 during winter season were Notonemouridae, Heptageniidae, Baetidae >2 sp. And

Hydropsychidae in abundance. Although the family of Baetidae is thought to be a moderate sensitive family it has few numbers of highly sensitive species (Tate and Husted, 2015) and those species were observed at this site. The moderate sensitive families observed during the winter season at this site were Hydracarina, Platycnemidae, Pisullidae, Tricorythidae, Lepidostomatidae, and Ancylidae in high abundance. The presence of highly sensitive families and moderate sensitive families were an indication that the stream was in a good state and further confirming the results for the physicochemical parameter. The highly sensitive families observed during spring season were Perlidae and Heptageniidae with high abundance. Highly sensitive families were observed in high numbers during the winter season as compared to the spring season and this may be due to the seasonal variation which has been reported in other studies (Dalas, 2004; Khoza *et al*. 2012; Wolmarans *et al*. 2014). The moderate sensitive families observed in high abundance at this site during spring season were Baetidae, Caenidae, Lestidae, and Ancylidae.

While there were no highly sensitive families observed from the middle-stream site (T2) during winter and spring season and the moderate sensitive families observed during winter season were Baetidae, Chlorocyphidae and Psychomyiidae, Gomphidae, Philopotamidae and Dixidae and Ancylidae with some of the families observed in high abundance. The moderate sensitive families observed during spring season were Baetidae and Caenidae. The pollution of this site was further confirmed by the abundance of tolerant families such as Oligochaeta and Chironomidae during the winter season and Oligochaeta, Chironomidae, and Syphidae during the spring season. The highly sensitive families observed from site T4 were Batidae >2 sp. and Hydropsychidae with moderately high abundance. The highly sensitive families observed in spring season was Prosopistomatidae at site T2 and was detected in high abundance. The moderate sensitive families observed at T4 during winter were Baetidae 2 sp., Synlestidae, Hydropsychidae, Dixidae and Ancylidae with high abundance. The abundance of macroinvertebrates at this site during winter and spring season might have been reduced by the presence of fish species in vegetation biotope. The presence of tolerant families in high abundance was evident as Oligochaeta and Chironomidae were observed. The moderate families in spring were Synlestidae and Ancylidae. The pollution in this site was further confirmed by the presence of tolerant families such as Oligochaeta, Chironomidae, and Psychodidae with high abundance. The aquatic macroinvertebrates families are presented in table 5.7.

Table 5. 7: The macroinvertebrates families identified in Buffalo River, Bloukrans River, Swartkops River and Tyhume River during winter season from the three sites. Estimate abundance: 1 = 1, A = 2-10, B = 10-100, C = 100-1000, D = >100.

Order	Family	Buffalo River			Bloukrans River			Swartkops River			Tyhume River		
		B1	B2	B4	Bl1	Bl2	Bl4	S1	S2	S4	T1	T2	T4
TURBELLARIA (Flatworms)		-	-	A	-	-	-	-	C	B	B	-	B
ANNELIDA	Oligochaeta (Earthworms)	B	B	A	B	B	D	B	A	D	B	B	B
	Hirudinea (Leeches)	-	A	-	-	-	-	-	-	-	B	-	-
CRUSTACEA	Potamonautidae (Crabs)	-	1	-	-	-	-	-	-	-	A	A	1
HYDRACARINA (Water mites)		-	-	-	-	-	-	-	-	-	A	-	-
PLECOPTERA (Stoneflies)	Notonemouridae	-	-	-	-	-	-	-	-	-	B	-	-
EPHEMEROPTERA (Mayflies)	Baetidae 1sp	A	B	B	B	-	-	B	-	A	B	B	B
	Baetidae 2sp	B	-	A	A	-	-	A	-	-	A	A	A
	Baetidae > 2sp	-	-	-	-	-	-	-	-	-	-	1	A
	Caenidae (Squaregills/Cainflies)	B	A	B	A	-	-	B	-	-	A	A	B
	Heptageniidae (Flatheaded mayflies)	-	-	-	B	-	-	1	-	-	B	-	-
	Leptophlebiidae (Prongills)	B	-	1	B	-	-	-	-	-	C	-	-
	Tricorythidae (Stout Crawlers)	-	-	-	-	-	-	-	-	-	B	-	-
	Chlorocyphidae	-	-	-	-	-	-	-	-	-	-	1	-
	Synlestidae (Chlorolestidae)(Sylphs)	-	-	-	-	-	-	-	A	-	-	-	A
	Coenagrionidae (Sprites and blues)	A	A	B	A	-	-	A	A	1	B	1	A
ODONATA (Dragonflies and Damselfies)	Lestidae (Emerald Damselflies)	-	-	-	-	-	-	-	A	-	-	-	-
	Platycnemidae (Brook Damselflies)	-	-	-	-	-	-	-	-	-	-	-	1
	Aeshnidae (Hawkers and Emperors)	A	-	A	B	-	-	-	1	-	-	-	-
	Gomphidae (Clubtails)	B	-	-	A	-	-	-	-	-	-	A	-
	Libellulidae (Darters)	-	-	-	A	-	-	A	-	-	-	-	-
LEPIDOPTERA (Moths)	Crambidae (Pyralidae)	-	-	-	-	-	-	-	-	-	-	-	1
HEMIPTERA (Bugs)	Belostomatidae (Giant water bugs)	A	-	-	-	-	-	-	A	-	-	-	-
	Corixidae (Water boatmen)	-	-	-	-	-	-	-	1	B	-	1	-
	Gerridae (Pond skaters/Water Striders)	-	-	-	-	-	-	A	-	-	-	B	-
	Naucoridae (Creeping water bugs)	-	-	-	-	-	-	-	-	B	-	-	-
	Nepidae (Water scorpions)	-	-	-	-	-	-	-	-	-	-	-	1
	Notonectidae (Backswimmers)	A	-	-	-	-	-	A	-	-	-	-	1

	Veliidae (Ripple bugs)	-	-	-	A	-	-	-	-	-	-	A	-
	Ecnomidae	-	-	-	A	-	-	-	-	-	-	-	-
	Hydropsychidae 1 sp	1	-	B	B	-	-	A	-	-	A	1	A
	Hydropsychidae 2 sp	-	-	-	-	-	-	1	-	-	A	-	A
	Hydropsychidae > 2 sp	-	-	-	-	-	-	-	-	-	A	-	A
TRICHOPTERA (Caddisflies)	Philopotamidae	1	-	-	-	-	-	-	-	-	-	A	-
	Psychomyiidae/Xiphocentronidae	-	-	-	-	-	-	-	-	-	-	1	-
	Cased caddis:	-	-	-	-	-	-	-	-	-	-	-	-
	Lepidostomatidae	-	-	-	-	-	-	1	-	-	B	-	-
	Leptoceridae	1	-	-	-	-	-	A	-	-	-	-	-
	Pisuliidae	-	-	-	-	-	-	-	-	-	A	-	-
	Dytiscidae/ Noteridae (Diving beetles)	A	1	-	-	-	-	A	A	B	A	-	A
COLEOPTERA (Beetles)	Elmidae/Dryopidae (Riffle bettles)	-	-	1	-	-	-	B	A	-	-	-	-
	Gyrinidae (Whirligig beetles)	1	1	-	A	-	-	A	-	-	-	-	-
	Hydrophilidae (Water scavenger beetles)	-	1	-	-	-	-	-	1	-	-	-	-
	Athericidae	-	-	-	-	-	-	A	-	-	B	-	-
	Ceratopogonidae (Biting midges)	A	-	-	-	-	-	C	1	1	A	A	D
	Chironomidae (Midges)	-	C	A	B	D	A	B	D	D	B	C	D
	Culicidae (Mosquitoes)	-	-	-	-	-	-	B	A	1	-	-	B
	Dixidae (Dixid midge)	-	-	-	-	-	-	-	-	-	-	A	A
DIPTERA (Flies)	Ephydridae (Shore flies)	-	-	-	-	-	-	-	-	-	-	-	1
	Muscidae (House flies, Stable flies)	-	-	-	-	-	-	-	-	-	-	-	B
	Simuliidae (Blackflies)	A	B	C	A	-	-	B	1	A	C	C	C
	Syrphidae (Rat tailed maggots)	-	-	-	-	A	-	-	-	-	-	-	-
	Tabanidae (Horse flies)	B	-	-	-	-	-	A	-	-	-	1	-
	Tipulidae (Crane flies)	A		-	A	-	-	-	-	-	A	-	-
GASTROPODA (Snails)	Ancylidae (Limpets)	-	1	-	-	-	-	A	-	-	B	B	B
	Lymnaeidae (Pond snails)	-	-	-	-	-	-	A	-	-	-	-	-
PELECYPODA (Bivalves)	Corbiculidae	1	-	B	-	-	-	-	-	-	-	-	-
	Sphaeriidae (Pills clams)	A	-	A	-	-	-	-	-	-	-	-	A

The aquatic macroinvertebrates families observed from four site of the rivers during spring season are presented in table 5.8.

Table 5. 8: The macroinvertebrates families identified in Buffalo River, Bloukrans River, Swartkops River and Tyhume River during spring season from three sites. Estimate abundance: 1 = 1, A = 2-10, B = 10-100, C = 100-1000, D = >100.

		Buffalo River			Bloukrans River			Swartkops River			Tyhume River		
Order	Family	B1	B2	B4	Bl1	Bl2	Bl4	S1	S2	S4	T1	T2	T4
ANNELIDA	Oligochaeta (Earthworms)	B	B	A	-	-	-	B	B	C	D	D	C
	Hirudinea (Leeches)	-	A	A	-	-	-	A	D	C	A	-	A
CRUSTACEA	Potamonautidae (Crabs)	A	A	B	A	-	-	A	-	-	B	A	B
HYDRACARINA (Water mites)		-	-	-	A	-	-	1	-	-	-	-	-
PLECOPTERA (Stoneflies)	Perlidae	-	-	-	-	-	-	-	-	-	A	-	-
EPHEMEROPTERA (Mayflies)	Baetidae 1 sp	A	1	B	B	-	-	B	-	A	C	D	A
	Baetidae 2 sp	-	-	-	-	-	-	A	-	-	B	A	-
	Caenidae (Squaregills/Cainflies)	B	-	A	A	-	-	1	-	-	B	A	A
	Heptageniidae (Flatheaded mayflies)	-	-	-	-	-	-	-	-	-	B	-	-
	Leptophlebiidae (Prongills)	B	-	A	B	-	-	-	-	-	A	-	-
	Prosopistomatidae (Water specs)	-	-	-	-	-	-	-	-	-	-	B	-
	Chlorocyphidae	-	-	-	A	-	-	-	-	-	-	-	-
	Synlestidae (Chlorolestidae)(Sylphs)	-	-	-	A	-	-	-	-	-	-	-	A
ODONATA (Dragonflies and Damselfies)	Coenagrionidae (Sprites and blues)	1	A	A	B	-	-	B	1	-	-	B	-
	Lestidae (Emerald Damselflies)	1	-	-	A	-	-	A	-	1	A	-	-
	Platycnemidae (Brook Damselflies)	1	-	-	-	-	-	-	-	-	-	-	-
	Aeshnidae (Hawkers and Emperors)	A	1	A	A	-	-	-	-	-	-	-	-
	Gomphidae (Clubtails)	B	-	-	B	-	-	-	-	-	-	A	-
	Libellulidae (Darters)	-	-	1	A	-	-	B	A	1	-	-	-
LEPIDOPTERA (Moths)	Crambidae (Pyralidae)	-	-	-	A	-	-	1	1	A	-	-	-
HEMIPTERA (Bugs)	Belostomatidae (Giant water bugs)	B	B	B	A	-	-	-	A	B	-	-	-
	Corixidae (Water boatmen)	B	B		A	-	-	-	C	1	-	-	A
	Gerridae (Pond skaters/Water Striders)	-	-	-	-	-	-	B	-	1	-	-	-
	Hydrometridae (Water measures)	-	-	-	-	-	-	1	-	-	-	-	-
	Naucoridae (Creeping water bugs)	A	-	-	A	-	-	-	-	-	-	-	-
	Nepidae (Water scorpions)	-	-	-	-	-	-	A	-	-	-	-	-
	Notonectidae (Backswimmers)	-	-	-	A	-	-	1	B	-	-	-	-
	Pleidae (Pygmy backswimmers)	-	-	-	-	-	-	-	-	-	A	-	-
	Veliidae (Ripple bugs)	-	-	-	-	-	-	A	-	-	-	-	A
	Hydropsychidae 1 sp	-	-	B	-	-	-	A	-	-	A	-	-

TRICHOPTERA (Caddisflies)	Cased caddis:	-	-	-	-	-	-	-	-	-	-	-	-
	Glossosomatidae	-	-	-	-	-	-	1	-	-	-	-	-
	Lepidostomatidae	-	-	-	A	-	-	-	-	-	-	-	-
	Leptoceridae	-	-	-	A	-	-	A	-	-	-	-	-
	Sericostomatidae	-	-	-	-	-	-	A	-	-	-	-	-
COLEOPTERA (Beetles)	Dytiscidae/ Noteridae (Diving beetles)	B	-	-	-	-	-	B	B	1	-	-	-
	Elmidae/Dryopidae (Riffle bettles)	-	-	-	-	-	-	A	-	-	-	-	-
	Gyrinidae (Whirligig beetles)	-	-	-	B	-	-	C	-	-	-	-	B
	Helodidae (Marsh beetles)	-	-	-	A	-	-	-	-	-	-	-	-
	Hydrophilidae (Water scavenger beetles)	-	-	-	-	-	-	-	1	A	-	-	-
DIPTERA (Flies)	Athericidae	A	-	-	A	-	-	1	-	-	C	-	-
	Blephariceridae (Mountain midges)	-	-	-	A	-	-	-	-	-	-	-	-
	Ceratopogonidae (Biting midges)	A	-	-	B	-	-	A	A	1	-	-	A
	Chironomidae (Midges)	A	D	A	C	B	B	C	C	C	B	A	C
	Culicidae (Mosquitoes)	A	A	-	-	-	-	-	-	-	-	-	A
	Dixidae (Dixid midge)	-	-	-	A	-	-	1	-	-	-	-	-
	Ephydridae (Shore flies)	-	-	-	-	-	-	-	-	-	-	-	A
	Muscidae (House flies, Stable flies)	-	-	-	-	-	-	-	-	-	-	-	-
	Psychodidae (Moth flies)	-	-	-	-	A	A	-	-	-	-	-	A
	Simuliidae (Blackflies)	-	-	C	B	-	-	C	C	1	C	-	B
	Syrphidae (Rat tailed maggots)	-	-	-	-	A	C	-	-	-	-	C	-
	Tabanidae (Horse flies)	1	-	-	A	-	-	B	-	-	-	-	-
	Tipulidae (Crane flies)	A	-	-	A	A	-	-	-	1	-	-	A
GASTROPODA (Snails)	Ancylidae (Limpets)	-	-	-	-	-	-	-	-	-	A	-	A
	Lymnaeidae (Pond snails)	-	-	-	-	-	-	A	-	-	-	-	-
	Physidae (Pouch snail)	-	1	-	-	-	-	-	1	-	-	-	-
PELECYPODA (Bivalves)	Corbiculidae	-	-	A	-	-	-	-	-	-	-	-	-
	Sphaeriidae (Pills clams)	-	-	-	-	-	-	-	1	-	-	-	-

5.5.1. Seasonal variation in the SASS score value, number of taxa and ASPT value

The SASS scores, number of taxa and ASPT values obtained during the spring season in Bloukrans River and Swartkops River were higher than the ones obtained in winter season, except for the ASPT value of Bl4, SASS score and ASPT value of S2 which were slightly lower during spring season (table: 5.5 and 5.6). The similar pattern of results was reported in Boesmanspruit River and Witrandspruit River samples during winter and spring season (Tate and Husted, 2015) and in a study which was conducted by Odume et al. (2012) at Swartkops River and Odume and Mgaba (2016) at Bloukrans River. The increase in the SASS score, number of taxa and ASPT value observed in Bloukrans River

and Swartkops River during spring season may be due to increase in water levels and improvement in the quality of water due to dilution by the rain. Changes in the climate during spring season may have an effect on macroinvertebrates biodiversity and abundance. The IHAS value was higher in spring as compared to winter season at Bloukrans River and Swartkops River except for S2 and S4 during the spring season. High IHAS value also indicates that the sampling habitat and stream conditions have improved during the spring season and was able to sustain the biodiversity community. The average for SASS score, total number of taxa and ASPT value of Bloukrans River and Buffalo River are presented in figure 5.4 and 5.5.

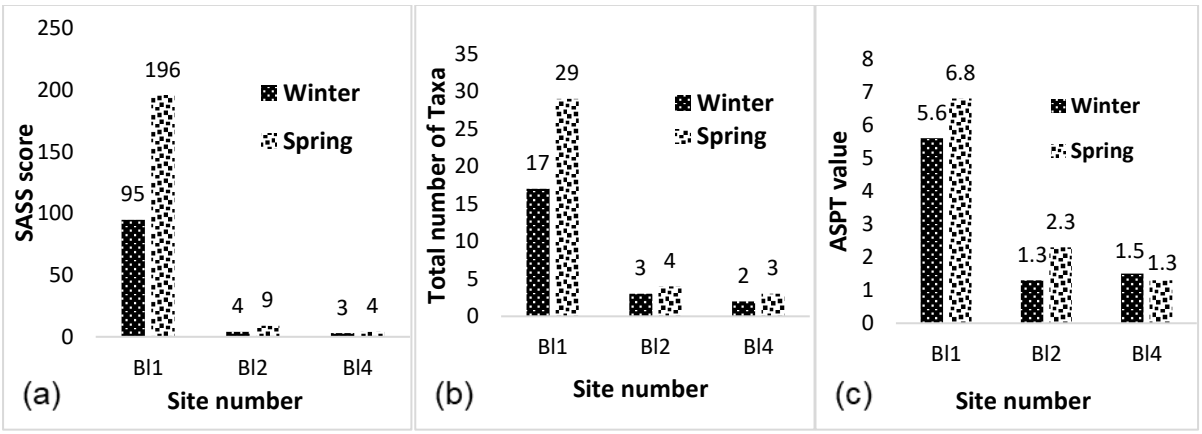

Figure 5. 4: The average SASS scores, number of taxa and ASPT value obtained during winter and spring season at Bloukrans River in the upper-stream site (Bl1), middle-stream site (Bl2) and lower-stream site (Bl4).

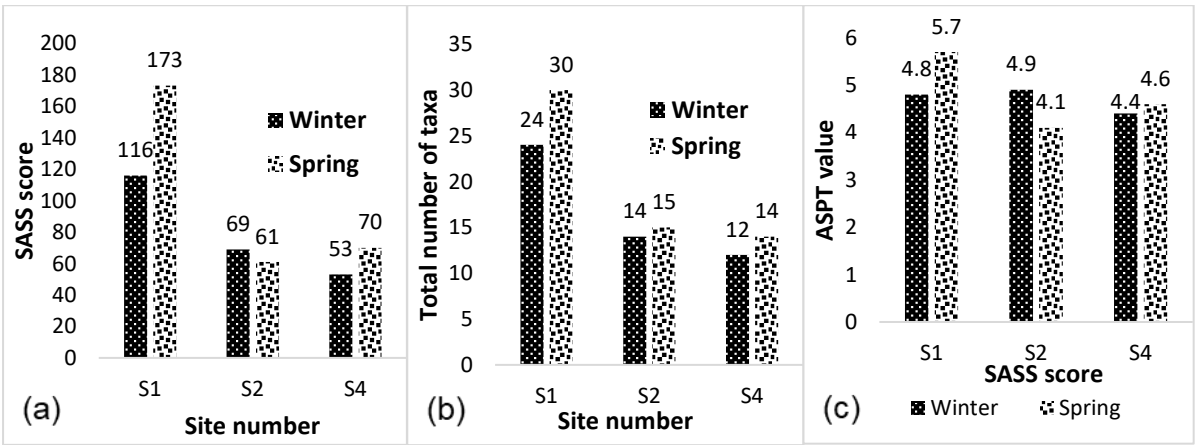

Figure 5. 5: The average SASS scores, number of taxa and ASPT value obtained during winter and spring season at Swartkops River in the upper-stream site (S1), middle-stream site (S2) and lower-stream site (S4).

The SASS scores, number of taxa and ASPT values were high during the winter season at Tyhume River as compared to spring season (Figure 5. 7: a, b and c). However, at Buffalo River, the SASS

scores were high in winter season while the number of taxa had no much variation during both seasons with lower ASPT values at site B2 and B4 during the spring season (Figure 5. 6: a, b and c). The IHAS value was higher in spring both at Tyhume River and Buffalo River. The higher IHAS value observed in both the rivers indicated that the sampling habitat and stream condition improved during the spring season.

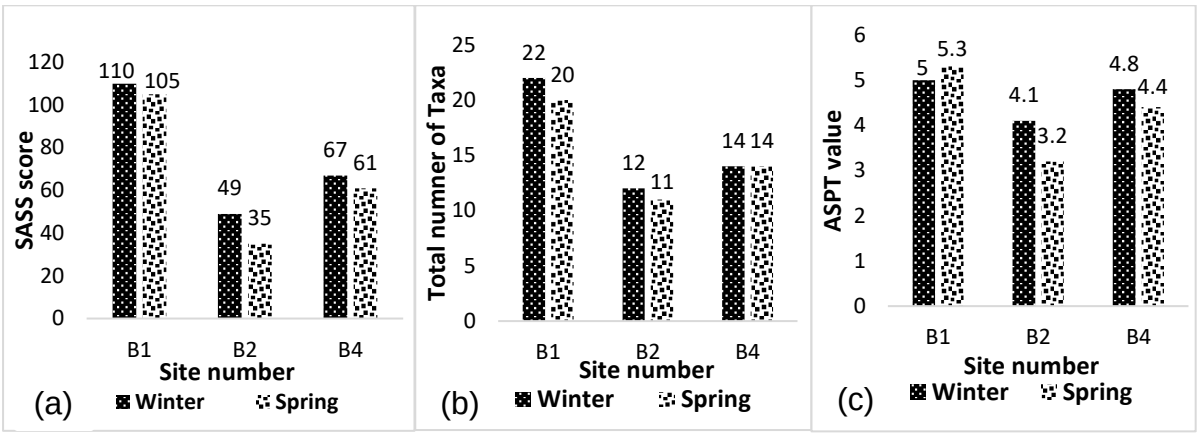

Figure 5. 6: The average SASS scores, number of taxa and ASPT value obtained during winter and spring season at Buffalo River in the upper-stream site (B1), middle-stream site (B2) and lower-stream site (B4).

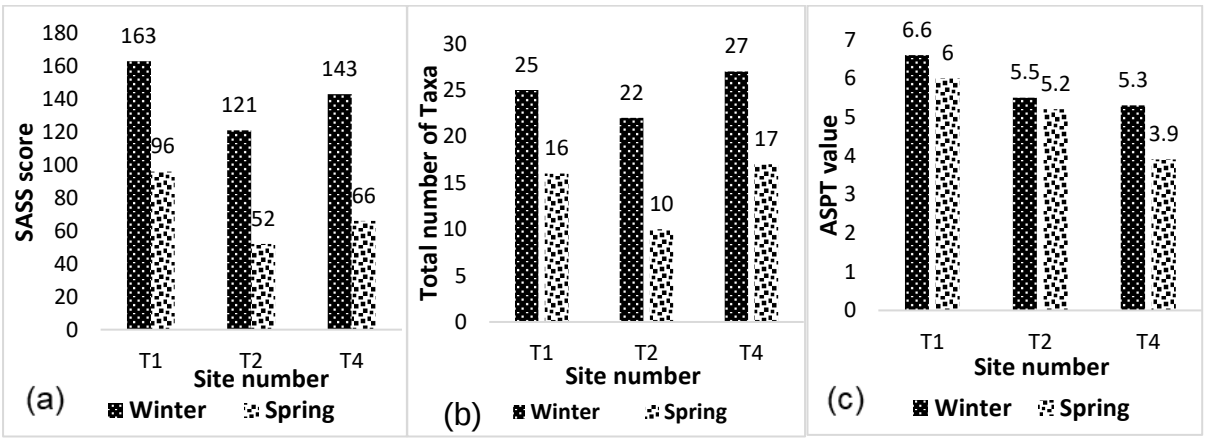

Figure 5. 7: The average SASS scores, number of taxa and ASPT value obtained during winter and spring season at Tyhume River in the upper-stream site (T1), middle-stream site (T2) and lower-stream site (T4).

5.5.2. Abundance macroinvertebrate families

The families which were in high abundance in this study are the tolerant families and survive well in water of poor quality caused by high amounts of dissolved organic substances (Thirion, 2006; Wolmarans *et al*. 2014). Those families include Oligochaeta which was found in all the sites during the winter season and 75% of the sites during the spring season, Chironomidae which was found in all

the sites during winter and spring season. Baetidae was found in 75% of the sites during winter and spring season. Potamounautidae was found in 67% of the sites during the spring season. The Coenagrionidae was found in 83% of the sites during the winter season and 58% of the sites during the spring season. Hirudinea was found in 58% of the sites during the spring season. Belostomatidae was found in 50% of the sites during spring. Ceratopogonidae was found in 58% of the sites during the winter season and 50% of the sites during the spring season. Simulidae was found in 83% of the sites during the winter season and 58% of the sites during the spring season. Caenidae was found in 67% of the sites during the winter season and 58% of the sites during the spring season. According to the results of the previous studies at Swartkops River and Bloukrans River and the results obtained in this study, the ecological state of these rivers has not improved for the past few years. The Bloukrans River is deteriorating due to constant pollutions by rounding location, Grahamstown WWTP effluent discharge and surrounding farms. This was an indication that pollution control needs to be applied at these rivers so that the ecological state of these rivers can improve.

5.6. Conclusion

In all the studied rivers the upper-stream sites were in the good/natural state except for the Buffalo River upper-stream site which was in a poor state in both winter and spring season. These observations were further confirmed by the presence of highly sensitive and moderate sensitive aquatic macroinvertebrates families belonging to the order of Plecoptera, Ephemeroptera, Trichoptera, Coleoptera, and Odonata. Thus, indicating that the river water was suitable for domestic use as the water quality was in a good state. The outcomes of biomonitoring of the ecological state of the river are a further confirmation of the results for physicochemical parameters which were reported in chapter 2. Nonetheless, the detection of the faecal coliforms and potential pathogens identified from these sites (discussed in chapter 3) indicated that the water would require pre-treatment before use such as boiling or use of bleach or even other water treatment techniques that remove or kills the potential pathogens. In addition, the presence of carbamazepine and sulfamethoxazole in these sites could be a threat since their effects is not yet known although they are present in small concentrations. The middle-stream sites and lower-sites of all the studied rivers were in a poor/seriously modified state indicating that the water was not suitable for domestic use. These observations indicate that the anthropogenic activities, WWTPs, and agricultural runoff are the main cause of pollution in these sites. Therefore, the department of water affairs and forestry in conjunction with the department of water and sanitation needs to find a way of solving this issue. Since rivers serve as a source of drinking water for both humans and animals. Resolving the issue of contamination in aquatic environments could also ease the issue of scarce drinking water. Pharmaceuticals will be included when I have the results.

5.7. References

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Chapter 6

General conclusions

Several studies (Chigor *et al*. 2013; Sibanda *et al*. 2013, Odume, 2011; Odume and Mgaba, 2016) reporting contamination of water in those four selected rivers have been documented. A study that was conducted by Chigor et al. 2013 and Sibanda et al. 2013 have also indicated that the health of people living near those rivers may be affected since river water is sometimes used as source of water for domestic purposes. This indicates that contamination of river water is a serious environmental issue that needs to be addressed for the protection of public health. This study aimed to monitor the quality of river water in Buffalo River, Tyhume River, Bloukrans River and Swartkops River located in the Eastern Cape Province. The study was motivated by the scarcity of water in South Africa and that rivers in rural areas are sometimes used as a source of water due to limited reliable and accessible clean and safe drinking water. The assumption that middle stream sites and lower stream sites are impacted when compared to the upper-stream sites was used to assess the possible health effects that might arise by using inadequately treated river water. This hypothesis was proven right when the biomonitoring results indicated that the upper-stream sites of the rivers was in good or natural state with exception of Buffalo River. The seasonal variations in the quality of river water was also investigated as sampling was conducted in autumn, winter and spring season. The physicochemical property testing results indicated that the river water of the upper-stream sites was suitable to be used as source of domestic water. Since most of assessed water parameters complied with South African Water Quality guidelines for domestic water use with exception of chloride and turbidity which were more than the target range, 0 -100 mg/L and 0 NTU respectively. In addition, the results for pharmaceutical screening indicated that the reference sites/ upper-stream sites were not critically polluted by pharmaceutical residues as carbamazepine, sulfamethoxazole and clarithromycin were detected in small concentrations (not detected to 40.9 ng/0.5L) . This indicates that pharmaceutical pollution is minimal in these sites therefore chances that these pharmaceuticals would have impact in human and animal health would be minimal. However, the continuous presence of antibiotics in the environment even at small concentrations may disturb microbial diversity and lead to antibiotic resistance. Thus, causing changes in the aquatic environment and development of pathogenic strains. The results were further confirmed by biomonitoring results, which indicated that the ecological status of the upper-stream sites of the rivers was in good or natural state during winter and spring season with exception of the upper-stream site of Buffalo River. The presence of highly sensitive and moderate sensitive macroinvertebrates families belonging to the order Plecoptera, Ephemeroptera, Trichoptera, Coleoptera, and Odonata was a confirmation that the quality of river water was good, and no recent

disturbance have occurred in the river. While the absence of highly sensitive macroinvertebrates was an indication of poor water quality in upper stream site of Buffalo River. Although physicochemical and biomonitoring results were indicating that river water was in good quality, the presence of pathogenic microorganisms such as *Klebsiella oxytoca*, *Vibrio alginolyticus*, *Providencia stuartii*, *Ewingella Americana, Providencia alcalifaciens/rustigianii*, *Enterobacter cloacae*, *Leclercia adecarboxylata, Proteus penneri*, *Aeromonas salmonicida ssp salmonicida* and *Escherichia coli* on upper stream sites of the rivers was an indication that the river water at this site was not suitable for domestic use as exposure could lead to sicknesses and deaths.

According to the results obtained in the middle stream sites, WWTPs and lower stream sites, the river water was not suitable as sources of drinking water as most of the water parameters did not comply with the South African Water Quality guidelines for domestic water use described in chapter 2. Contamination of river water was further confirmed by high turbidity, COD and EC levels indicating high concentrations of inorganic contaminants. Pollution has negative effect on aquatic macroinvertebrates as high COD levels causes low dissolves oxygen concentration in water. Only iron and sulphate complied with the South African Water Quality guidelines in all seasons, while phosphate, nitrates, nitrite, turbidity, temperature, pH and electric conductivity were above the acceptable target in some of the sites during the sampling seasons. High turbidity levels are usually associated with microbial contamination and water that are turbid may not be safe for human consumption. The presence of pathogens such as *Aeromonas spp. Kluyvera spp. Klebsiella pneumoniae ssp pneumoniae, Citrobacter freundii, Klebsiella oxytoca, Raoultella ornithinolytica, Escherichia coli, Shigella spp., Vibrio cholerae* and *Salmonella spp*. in river water was a confirmation that the river water was faecal polluted and that could result in illnesses and deaths in exposed individuals. Pharmaceutical pollution was also observed as carbamazepine, sulfamethoxazole, clarithromycin and erythromycin residues were detected in high concentrations at these sites. Higher concentrations of pharmaceutical residues detected in WWTP effluents was an indication that they are the main source of pharmaceuticals in surface waters. High concentration of carbamazepine and erythromycin in river samples was an indication that these two pharmaceuticals are one of the commonly used pharmaceuticals. Contamination of these sites was further confirmed by the absence of highly sensitive and moderate sensitive macroinvertebrates. Although the results indicated that river water at this site was not suitable to be used for domestic purposes, this river water was still suitable for agricultural purposes as it was nutrient rich and would probably stimulate plant growth. However, pre-treatment may be necessary as the water contains faecal pathogens. There were no seasonal variations observed on the physicochemical parameters of water and microbial quality of water.

Although no significant seasonal variation on the physicochemical properties of river water was not observed in this study, constant monitoring of surface waters is important to determine the quality of water for the protection of public health since the downstream sites are used for irrigation and domestic purposes. The findings of this study clearly indicated that rivers are in constant stress due to variety of pollutants and that the quality of water is gradually declining each year. It is clear that WWTPs are one of the main sources of pollutants in aquatic environments since WWTPs are incapable of removing contaminants such as pathogens, organic and inorganic contaminants in wastewater. This therefore serves an indication that the Department of Water and Sanitation need to find strategies to improve the WWTP processes and the South African Department of Water Affairs need to ensure that guideline for standard water are meet. In addition, the pollution point source needs to be controlled for ecological status of the rivers to improve.

In this study, the quality of water and biological status of the Eastern Cape rivers was well studied as this study combined the physicochemical parameters of river water with the microbial quality of river water, pharmaceuticals screening and biomonitoring using aquatic macroinvertebrates. Although some studies in those rivers have been done before, this is the first study that reports on the presence of pharmaceutical contaminants in river water and highlights the concentrations of those pharmaceuticals in river water. The obtained results cause environmental concern on the pathway and toxicology of those pharmaceuticals in aquatic systems.

Limitation and recommendations

Further investigations need to be done to determine the actual sources of pollution in those rivers. Monitoring physicochemical parameters of water in all the seasons may be advantageous in determination of the seasonal effects on the quality of water. The iron content remained undetected in all the samples and therefore atomic absorbance spectrum should have been used to validate the results. The concentration of biological oxygen demand and dissolved oxygen was not measured in this study. These parameters should also be measured when testing water quality so that full insight of the state of the river water can be determined as these parameters has great influence on the aquatic organisms. Detection of faecal coliforms in most of the samples was an indication that the river water was faecal polluted. Pathogenic organisms were identified by the use of analytical profile index 20E kit up to family level. Although analytical profile index 20E is cost effective and easy to use for screening of broad range bacteria, there are chances of miss identification since most bacteria shares the same biological properties. The use of polymerase chain reaction may be beneficial for identifying bacteria since it can identify up to species level and limit error (miss identification). Further studies need to be

conducted on toxicology, antibiotic resistance and risk assessment evaluation which involves stake holders and communities.

Appendix 1

Standard curves and spectrum for target pharmaceuticals

1. Standard curves for testing water chemical parameters

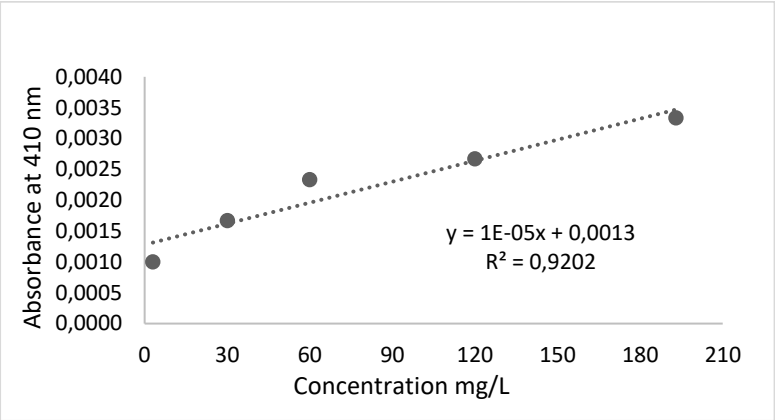


Figure 1: Ammonium standard curve.

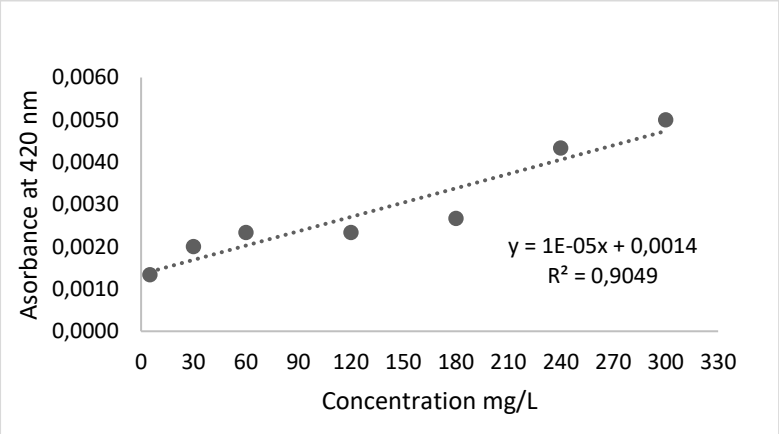


Figure 2: Sulphate standard curve.

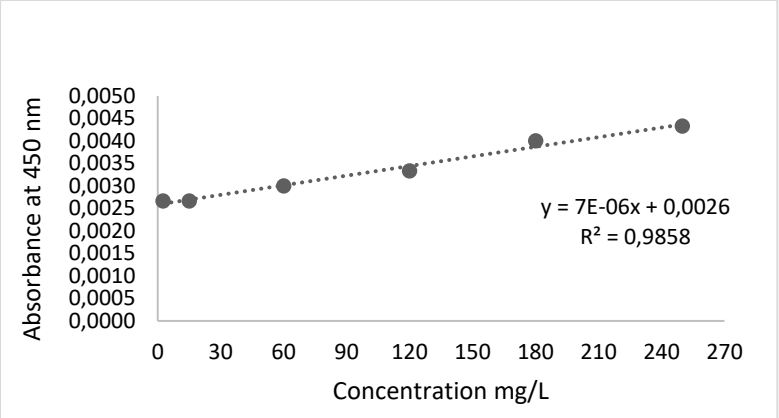


Figure 3: Chlorine standard curve.

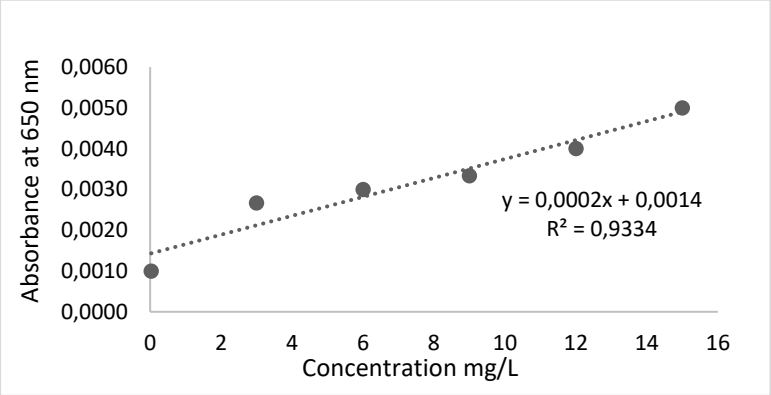


Figure 4: Phosphate standard curve.

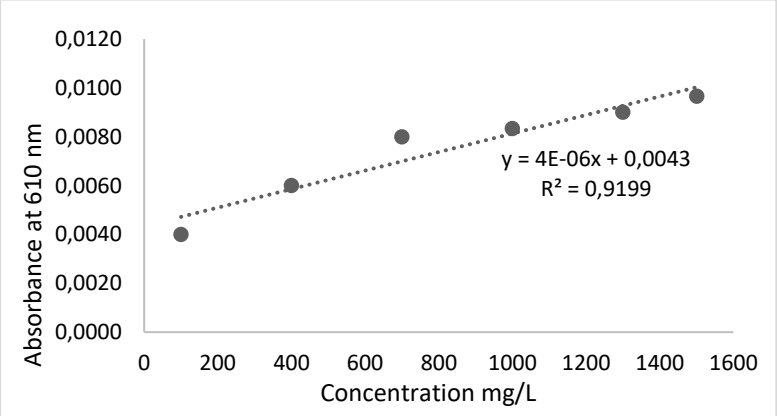


Figure 6: Chemical oxygen demand standard curve.

2. ELISA screening standard curve

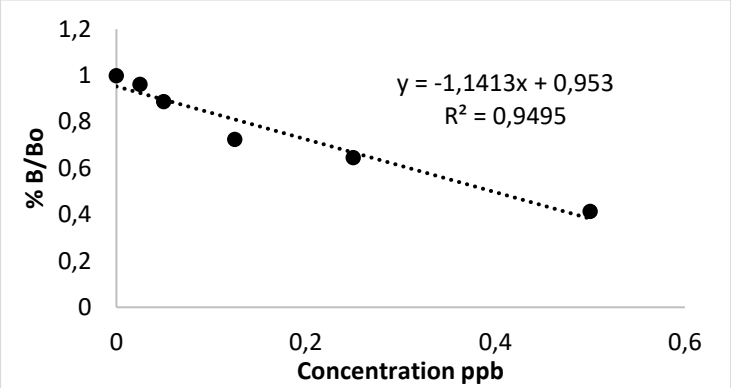


Figure 7: Fluoroquinolone standard curve for Swartkops River and Bloukrans River samples.

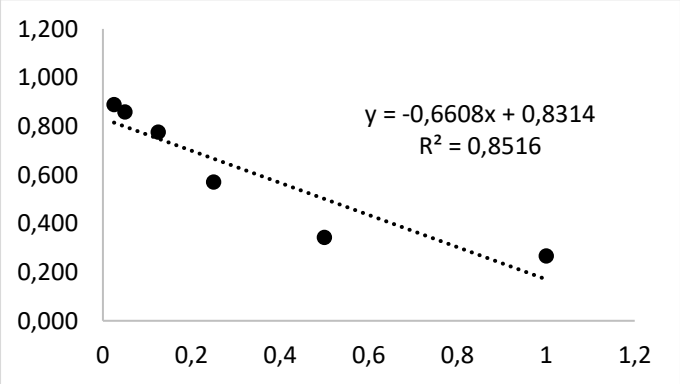


Figure 8: Fluoroquinolones standard curve for Buffalo River and Tyhume River samples

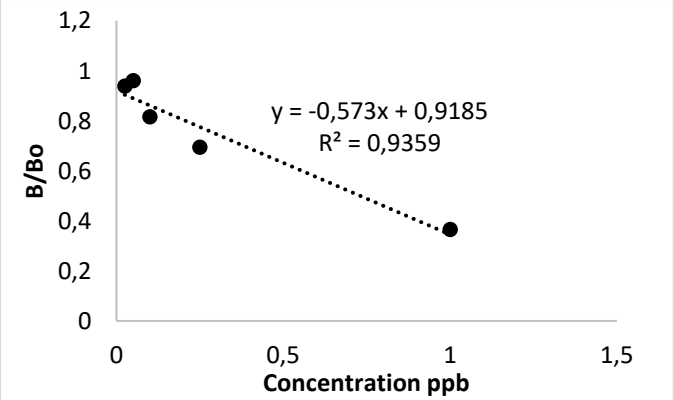


Figure 9: Sulfamethoxazole standard curve for Swartkops River and Bloukrans River samples.

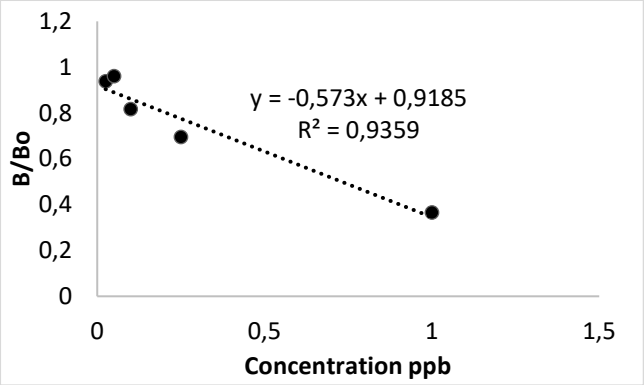


Figure 10: Sulfamethoxazole standard curve for Buffalo River and Tyhume River samples.

3. Spectrum of the target pharmaceuticals

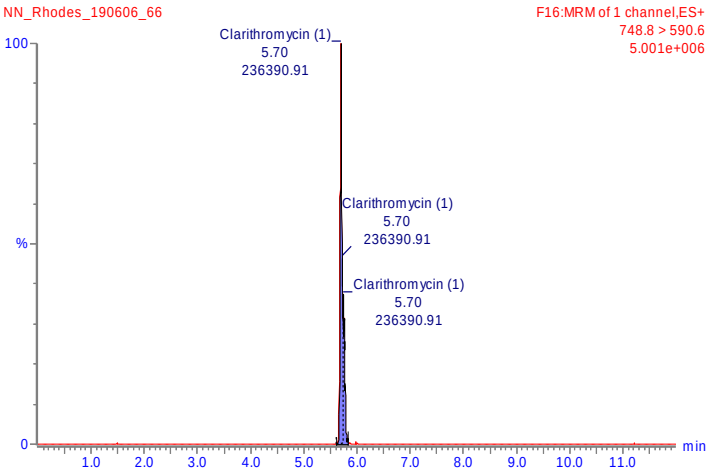


Figure 11: Spectrum for clarithromycin standard

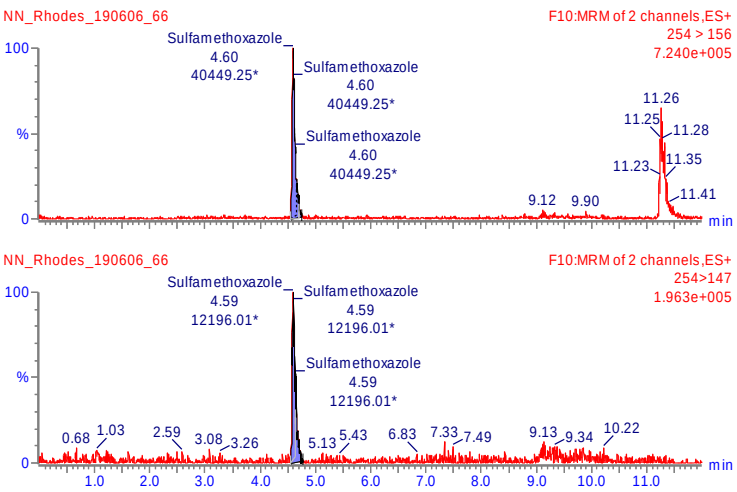


Figure 12: Spectrum for sulfamethoxazole standard.

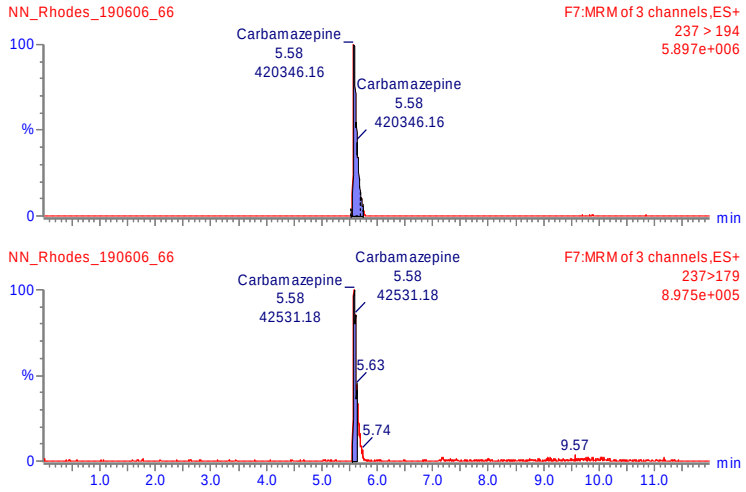


Figure 13: Spectrum for carbamazepine standard.

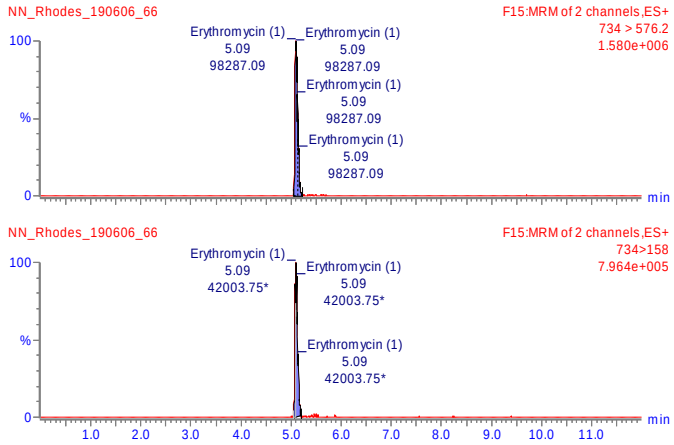


Figure 14: Spectrum for erythromycin standard.

4. Freeze drying results

Table 1: Freeze drying results of 500 ml river water samples.

River	Site number	Sample volume (mL)	Yield (g)
Tyhume	T1	500	0.1033
	T2	500	0.1441
	T3	500	0.1717
	T4	500	0.1986
Buffalo	B1	500	0.2820
	B2	500	0.2143
	B3	500	0.2725
	B4	500	0.2594
Swartkops	S1	500	0.1553
	S2	500	0.1922
	S3	500	0.7239
	S4	500	1.1441
Palmiet	P1	500	0.1501
Bloukrans	B2	500	0.4931

	B3	500	0.4159
	B4	500	0.4824

Appendix 2

Sampling permits

Province of the Eastern Cape

DEPARTMENT OF ECONOMIC DEVELOPMENT, ENVIRONMENTAL AFFAIRS AND TOURISM

Chief Directorate: Environmental Affairs Sarah Baartman Region

Collegiate Building
Cnr Athol Fugard Terrace and Castle Hill
Private Bag X500L Greenacres, Port Elizabeth 6057
South Africa

Collegiategebou
H/v Athol Fugard Terrace en Castle Hill
Privaatsak Greenacres, Port Elizabeth 6057
Suid-Afrika

Dr Helen James,
Albany Museum,
Somerset Street/
GRAHAMSTOWN,
61 39

Tel/lfoni: (041) 508 5813
Fax/lfexi: (041) 508 5850
Enquiries/lmibuzo: A. Southwood

Ref/lreferensi: CRO 38/18CR
CRO 39/18CR
Date: 27 March 201 7

Dear Helen

RE: PERMIT TO COLLECT FRESHWATER INVERTEBRATES ON PRIVATE LAND IN THE EASTERN CAPE PROVINCE: PERMIT NOS.: CRO 69/17CR AND CRO 70/17CR

YOU, Drs Alexandra Holland, Ferdinand de Moor and Lydall Pereira da Conceicoa, and Ms Ina Ferreira and Mr Musawenkosi Mlambo are hereby granted permits in terms of Section 29 of the Nature and Environmental Conservation Ordinance, 1974 (Ordinance 1 9 of 1974), Sections 24 and 25 of Environmental Conservation Decree, 1 992 (Decree No. 9 of 1 992, former Transkei) and Sections 20 and 21 of the Nature Conservation Act 1987 (Act No. 10 of 1987, former Ciskei) to collect freshwater invertebrates for research purposes as per your application.

Note the following conditions:

1.Written permission of the landowner must be obtained before entering the property to collect freshwater invertebrates. The written permission must reflect:

1.1 the full names and address of the owner of the land concerned or the person authorised to grant such permission;

1 .2 the full names and address of the person to whom permission is granted;

1 .3 the number and species, the date or dates and the name of the land in respect of which permission is granted; and 1.4is signed and dated by such owner or person authorised by him.

2. Any collecting of invertebrates must be documented, and species lists must be submitted to this Department.

3. The Department must be acknowledged in any publications that results from this project.

4. The permit is valid until 30 March 2019 and it must be returned to this office within fourteen days after the expiry date.

5. We would like to have copies of all reports and / or publications resulting from this research.

6. Issuing of further permits will depend upon the fulfilling of the above conditions. Failure to adhere to the above conditions may lead to this permit being cancelled with immediate effect.

Yours faithfully

DAYA AN GOVENDER
MANAGER: ENVIRONMENTAL AFFAIRS
SARAH BAARTMAN REGION

No. CRO 38/18CR

PROVINSIALE ADMINISTRASIE VAN DIE OOS-KAAP PROVINSIE

HOOF DIREKTORAAT OMGEWINGSAKE

PERMIT 0M WILDE DIE-RE *DEUR MIDDEL VAN VERBODE

JAGMETODE TE JAG/ ÄCI I I A N I

I A (2/ /CDQ D 'AD C \/CDCll k

Ordonnansie op Natuur- en Omgewingsbewaring, 1974 (Ordonnansie 19 van 1 974) (Arts 29 eæ33), Environmental Conservation Decree, 1992 (Decree No. 9 of 1992, former Transkei) (Sections 24 and 25) and Nature Conservation Act.
1987 (Act No. 10 of 1987, former Ciskei) (Sections 20 and 21)

PROVINCIAL ADMINISTRATION OF THE EASTERN CAPE

PROVINCE

CHIEF DIRECTORATE ENVIRONMENTAL AFFAIRS

PERMIT *TO COLLECT HUNT ANIMALS BY MEANS OF PROHIBITED HUNTING METHODÆO-H-JJ-N-T

IDD /CTAÅADCÄC IA IlA Ilk A A I C D TUC A

pos

Nature and Environmental Conservation Ordinance, 1974 (Ordinance 19 of 1974) (Section. 29 and-33) Environmental Conservation Decree, 1992 (Decree No. 9 of 1992, former
Transkei) (Sections 24 and 25) and Nature Conservation Act
1987 (Act No. 10 of 1987, former Ciskei) (Sections 20 and 21)

NIE OORDRAAGBAAR /NOT TRANSFERABLE

Drs Helen James, Alexandra Holland, Ferdinand de Moor and Lydall Pereira da Conceicoa; Ms Ina Ferreira and Mr Musawenkosi Mlambo

Alban Museum,

Somerset Street,

61 39 GRAHAMSTOWN,

word hierby kragtens Artikel *29/33 van die Ordonnansie op Natuur-en Omgewingsbewaring, 1 974 (Ordonnansie 19 van 1 974), Environmental Conservation Decree, 1992 (Decree No. 9 of 1 992, former Transkei) (Sections 24 and 25) and Nature Conservation Act 1987 (Act No. 10 of 1987, former Ciskei) (Sections 20 and 21) 'n permit uitgereik om die volgende wilde diere te *versamel/~~versteur/dryf/jaag~~

is hereby granted a permit in terms of Section *29/33 of the Nature and Environmental Conservation Ordinance (Ordinance 19 of 1974) Environmental Conservation Decree, 1 992 (Decree No. 9 of 1992, former Transkei) (Sections 24 and 25) and Nature Conservation Act. 1987 (Act No. 10 of 1987, former Ciskei) (Sections 20 and 2 1) to *collect/~~disturb/stampede~~ the following Wild animals -

Spesie	Getal	S ecie	Number

REFER TO SECTION 2.5 OF THE APPLICATION FORM

o die eiendom

on the propert

Private Land in the Eastern Ca e Province

* niddel/~~gebruik te maak~~van

b y *means/~~the use~~ Of -

Prohibited hunting method excluding fire and poison

l. Hierdie permit is geldig tot 27 March 2019.

2. Wanneer die houer van hierdie permit enige be wilde dier daarkragtens versamel moet hy, voordat hy die bogemelde eiendom verlaat, of indien hy dit nie verlaat nie, na elke dag se versameling, die besonderhede aangaande die datum, spesies en getal van elke geslag van elke spesie, of indien dit onmoontlik is om die geslagte te onderskei, die totale getal van elke spesie van sodanige wilde diere wat hy geversamel het, aanteken in die ruimte wat op die keersy verskaf word.

3. Die houer van hierdie permit moet dit binne 14 dae na die vervaldatum daarvan aan die Streeksdirekteur van Ekonomieseontwikkeling, Omgewingsake & Toerisme, Privaatsak X5001 , Greenacres, 6057t terugstuur.

4. Verkry 'n skriftelike bewys van Grond eienaar voordat enige jagtog mag plaas vind

l . This permit is valid until 27 March 2019.

2. When the holder of this permit collects wild animal in terms thereof, he shall, before leaving the abovementioned property, or if he does not leave it, after each day's collection, record in the space provided on the reverse side, the particulars regarding the date, species and number of each sex or each species, or if it is impossible to distinguish the sex, the total number of each species of such wild animals which he had collected.

3. The holder of this permit shall return it to the Regional Director of Economic Development, Environmental Affairs and Tourism, Private Bag X500L Greenacres 6057, within 14 days of the date thereof.

4. The written permission of the Land owner must be obtained before any hunting takes place.

Senior Bestuurder van Omgewingsake / Senior Manager of Environmental Affairs

27 March 2018

Datum/Date

nior Béstuurder van Omgewingsake / Senior Manager of Environmental Affairs

*Skrap wat nie van toepassing is nie

*Delete whichever is not applicable

N16

NO.: CRO 39/18CR

PROVINCIAL ADMINISTRATION OF THE EASTERN CAPE PROVINCE

CHIEF DIRECTORATE ENVIRONMENTAL AFFAIRS

PERMIT TO *IMPORT/EXPORT/TRANSPORT *WILD ANIMALS/ PROTECTED WILD ANIMALS AND TO #SELL/DONATE/BUY/RECEIVE AS A DONATION ENDANGERED/

Nature and Environmental Conservation Ordinance, 1974 (Ordinance 19 of 1974) (section 44 (1) (a) and (e)

PROVINSIALE ADMINISTRASIE VAN DIE OOS - KAAP PROVINSIE

HOOF DIREKTORAAT OMGEWINGSAKE

PERMIT OM WILDE DIERE *IN TE VOER/UIT TE VOER/TE VERVOER EN OM*BEDRIEGDE/BESKERMDE WILDE DIERE TE VERKOOP/SKENK/KOOP/AS SKENK TE ONTVANG

Ordonnansie op Natuur- en Omgewingsbewaring, 1974 (Ordonnansie 19 van 1974) (Artikel 44 (1) (a) en (e)

NOT TRANSFERABLE / NIE OORDRAAGBAAR

In terms of Section 44 (l) (a) and (e) of the Nature and Environmental Conservation Ordinance, (Ordinance 19 of 1974), a permit is hereby issued to -

'n Permit word hierby ingevolge Artikel 44 (l) (a) en 1974 (e) van die Ordonnansie op Natuur- en Omgewingsbewaring, 1974 (Ordonnansie 19 van 1 974), uitgereik aan -

Drs Helen James, Alexandra Holland, Ferdinand de Moor and Lydall Pereira da Conceicoa; Ms Ina Ferreira and Mr Musawenkosi Mlambo
Albany Museum
Somerset Street
Grahamstown, 6139

to transport in or through ondergemelde Wilde diere the Eastern Cape Province the undermentioned ia-of deur die Oos - Kaap Provinsie te vervoer animals -

S ecies / Spesie	Number / Getal	S ecies / Spesie	Number / Getal

REFER TO SECTION 2.5 OF THE APPLICATION FORM

and the lastmentioned person is hereby authorised to sell / donate/buy/receive as a donation the endangered/protected wild animals to / from him/her

en laasgenoemde persoon word hierby gemagtig om bedriedge/ beskermde wilde diere aan/van hom/haar te verkoop / skenk / koop / as geskenk te ontvang.

CONDITIONS

VOORWAARDES

This permit is valid until 27 March 2019.

Hierdie permit is geldig tot 27 March 2019.

2. The actual number of wild animals transported and the number of such animals of each species which have been unloaded alive at the destination shall be recorded on the reverse side

2. Die werkiike getal wilde diere van elke spesie wat vervoer is en die getal van sodanige wilde diere wat elke spesie wat lewend by die besteming afgelaai is moet op die keersy aangeteken word.

3. The holder of the permit shall return it to the Regional Manager of Economic Development, Environmental Affairs & Tourism, Private Bag Greenacres, 6057 within 14 days of the expiry date.

3. Die houer van hierdie permit moet dit binne 14 dae vanaf die vervaldatum aan die Streeksdirekteur van Ekonomieseontwikkeling, Omgewingsake & Toerisme , Privaatsak X5001 Greenacres 6057 terugstuur.

27 March 2018

DATE/DATUM

Delete whichever is not applicable Skrap wat nie van toepassing is nie

N18

CRO 40/17CR

PROVINCIAL ADMINISTRATION OF THE EASTERNPROVINSIALE ADMINISTRASIE VAN DIE OOS

CAPE PROVINCE KAAP PROVINSIE

CHIEF DIRECTORATE ENVIRONMENTAL AFFAIRSHOOF DIREKTORAAT OMGEWINGSAKE

PERMIT TO IMPORT THE CARCASE OF WILD PERMIT 0M DIE KARKASSE VAN DEE WILDE DIERE IN

ANIMALS INTO THE EASTERN CAPE PROVINCEDIE KAAPPROVINSIE IN TE VOER VAN 'N PLEK BUITE

FROM A PLACE OUTSIDE THE REPUBLIC DIE REPUBLIEK

Nature and Environmental Conservation Ordonnansie op Natuur en OmgewingsOrdinance, 1974 (Ordinance 19 of 1974) bewaring, 1974 (Ordonnansie 1 9 van 1974)

(Section 44(1)(b) (Artikel 44 (l)

PLEASE USE CAPITAL LETTERS / GEBRUIK ASSEBLIEF HOOFLETTER

In terms of Section 44(1)(b) of the Nature and 'n Permit word hierby ingevolge Artikel 44(1 Environmental Conservation Ordinance, 1974 van die Ordonnansie op Natuur(Ordinance 19 of 1974), a permit is hereby Omgewingsbewaring, 1974, (Ordonnansie 19 issued to — van 1974), uitgereik aan -

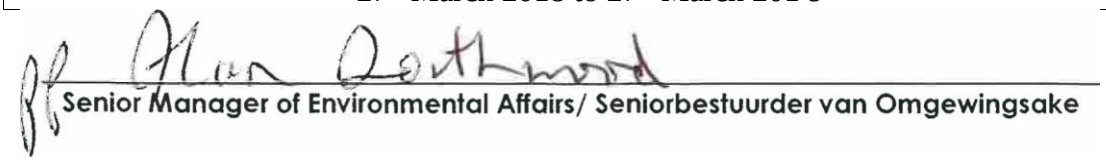
Drs Helen James, Alexandra Holland, Ferdinand de Moor and Lydall Pereira da Conceicoa; Ms Ina Ferreira and Mr Musawenkosi Mlambo
Alban Museum
Somerset Street
Grahamstown 6139

to import the carcases of wild animals as set outom die karkasse van die wilde diere soos hereunder into the Eastern Cape Province hieronder uiteengesit in die Oos — Kaap Provinsie in te voer -

Species/Spesie	Carcase or part of carcase Karkas of deel van karkas	Number/ Getal	Mass / Massa
Samples of species of vertebrates / invertebrates collected in Angola as part of the Okavango Wilderness Project	Whole Specimens	Unlimited	

during the period - gedurende die tydperk

27th March 2018 to 27th March 201 9



27th March 2018
Date/Datum

BUFFALO CITY METROPOLITAN MUNICIPALITY

MEMORANDUM

Date: **29 MAY 2018**

From: **HEAD:INFORMATION KNOWLEDGE MANAGEMENT, RESEARCH AND POLICY**

To: **S. VUMAZONKE**

Our ref:	Please ask for	Your ref:
	MR J.FINE	
	(043) 705 9742	

RE: REQUEST FOR PERMISSION TO CONDUCT RESEARCH IN BCMM: MS. SESETHU VUMAZONKE

It is hereby acknowledged that **Sesethu Vumazonke** a student at Rhodes University, completing the **Masters of Science in Pharmaceutical Chemistry,** has met the prerequisites for conducting research at Buffalo City Metropolitan Municipality (BCMM) for partial fulfillment of her degree. She has provided us with all the necessary documentation as per the BCMM Policy on External Students conducting research at the institution. With reference to the letter to the City Manager received on the 18 May 2018, permission was requested to conduct research at BCMM for her Research Report, entitled **"ENVIRONMENTAL EFFECTS OF PHARMACEUTICAL ACTIVE COMPOUNDS IN FRESHWATER"** This request was acknowledged by the Office of the City Manager, and forwarded to the Information & Knowledge Management, Research & Policy Unit for further assistance. **Ms. Vumazonke**

was asked to provide the Unit with the necessary documentation, which she subsequently did.

The relevant Officials to assist in the research were identified and duly informed about the research, and the fact that **Ms. Vumazonke** has met all the prerequisites. Their contact details have also been provided to **Ms. Vumazonke** and she was informed to contact them directly for assistance.

We wish you good luck in your studies.

pp

DR T F NORUSHE

HEAD: INFORMATION, KNOWLEDGE MANAGEMENT, RESEARCH AND POLICY

RHODES UNIVERSITY
FUCULTY OF PHARMACY
PO BOX 94
GRAHAMSTOWN
6139

Date: 23 April 2018

THE CITY MANAGER
BUFFALO CITY METROPOLITAN MUNICIPALITY
PO BOX 134
EAST LONDON
5200

Dear Sir,

RE: REQUEST FOR PERMISSION BY STUDENTS TO CONDUCT RESEARCH STUDY AT BCMM

I am a student at Rhodes University, completing Masters of Science in Pharmaceutical Chemistry. I am sure you are aware that any post graduate study involves completion of a Treatise or Dissertation or Thesis. It is for this reason that I request your personal and professional permission to partake in my research in directorates and departments within BCMM.

The title of my research Treatise or Dissertation or Thesis, (Water is essential to life in both humans and animals. Thus it is important that humans and animals have access to clean and safe water. For the past few years water supply in South Africa has been a major challenge, and this results in people who are living in rural areas specifically in the Eastern Cape Province to be heavily affected as they rely on river water as primary source of water. Mornitoring microbial quality and physicochemical properties of river water is essential to ensure the water quality fall within acceptable standards that will not lead to health complications) the title of the resrach is ENVIRONMENTAL EFFECTS OF PHARMACEUTICAL ACTIVE COMPOUNDS IN FRESHWATER, and is being undertaken under the Supervision of Dr. Nosiphiwe Ngqwala and co-supervision of Dr. Sandile Khamanga

The main objectives and aim of this study are:

- To detect the presence and concentrations of pharmaceutical active compounds (PhACs) from the samples obtained at Kowie River, Buffalo River, Tyhume River and Swartkops River using ELISA kits and Liquid chromatography Mass spectrometry (LC-MS).
- To evaluate microbial quality of river water using faecal indicators such as *E. coli* and identify possible pathogens using API 20E kit.
- To assess the health and quality of river water using benthic macroinvertebrates such as South African Scoring System version 5 and measure the physico-chemical parameters of water.
- To measure the removal rate of persistent PhACs by activated carbon and determine optimum conditions using software (Artificial neural network).

The research study will not make use of interviews/completion of questionnaries with key selected potential participants or respondents, chosen through/according to (The data needed for the study will be obtained by collecting water samples from the rivers and the effluent samples of the wastewater treatment plants). The potential participants or respondents would thus include (insert title and/or functions of chosen participants respondents).The study will be beneficial to BCMM as it will provide an insight on the pharmaceutical, physicochemical and microbial state of Buffalo river water and it will also highlight possible sources of contamination. The BCMM will also be aware if there is a need to develop strategy that will assist in management and protection of the Buffalo River. Also the results of the study will help in prevention of waterborn diseases and improve the health of both humans and animals.

The ethical research principles will be strictly adhered to thoughout the research process so as to maintain a high standard of work and a high quality of the research study. The infomation obtained will be used only for purposes of this study, and will ensure anonymity and confidentiality of potential research participants or respondents **A copy of the full research report, once approved by the University will be handed to IKM, Research & Policy Unit, BCMM.**

I thus request granting of permission to collect the necessary data/information from relevant officials (and Councillors) at BCMM for the purposes of completion of my Research Treatise or Dissertation or Thesis.

Your kind assistance in granting me permission will be highy appreciated and thank you for taking the time in allowing your staff to be part of this research study as I am sure it will not only be of benefit to me but to them as well.

Yours faithfully,

Sesethu Vumazonke

E-mail address: vumazonkesesethu@gmail.com

Cellphone: 073 7668 688

CITY MANAGER: _____ **Date:** 10/05/18

Approved	**~~Not Approved~~**