THE USE OF BIOLOGICAL INDICATORS IN ASSESSING POLLUTION STATUS IN SELECTED RIVERS IN LIMPOPO PROVINCE, SOUTH AFRICA

MASTER OF SCIENCE (AQUACULTURE)

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THE USE OF BIOLOGICAL INDICATORS IN ASSESSING POLLUTION STATUS IN SELECTED RIVERS IN LIMPOPO PROVINCE, SOUTH AFRICA

by

LIVHUWANI EVA NEPHALE

DISSERTATION

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SUPERVISOR: Prof. N.A.G. Moyo
CO-SUPERVISOR: Dr. M.M. Rapatsa

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DECLARATION

I declare that **THE USE OF BIOLOGICAL INDICATORS IN ASSESSING POLLUTION STATUS IN SELECTED RIVERS IN LIMPOPO PROVINCE, SOUTH AFRICA** dissertation hereby submitted to the University of Limpopo, for the degree of **MASTER OF SCIENCE IN AQUACULTURE** has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

_____ _____

Nephale, L.E (Ms.) Date

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DEDICATION

In memory of my dear son Israel

ABSTRACT

The aim of the study was to assess the extent to which biological indicators and biomarkers can be used to monitor the pollution status of the Sand and Blood Rivers. Physico-chemical parameters were assessed as a primary approach in assessing pollution status of the Sand and Blood Rivers. Temperature, pH, dissolved oxygen (DO), biological oxygen demand (BOD), turbidity, total dissolved solids (TDS), total nitrogen, phosphorus and ammonia were assessed during the rainy and dry seasons. Physico-chemical parameters showed spatial and temporal variation. The hierarchical average linkage cluster analysis grouped the reference sites (S1 and B1) into one group and sites after points of discharge (B2, S2, S3, S4 and S5) into another group. This grouping was due to the pollution status of each site, with the reference sites less polluted than the sites downstream of the wastewater treatment plant effluent points of discharge. The canadian council of ministers of the environment water quality index (CCME WQI) categorized reference sites as good, whilst sites after points of discharge were categorized as poor. This shows that the Sand and Blood Rivers are degrading.

Heavy metal contamination in water, sediment and grass from the Sand and Blood Rivers was evaluated during the rainy season and dry season. All assessed heavy metals (Cadmium, Chromium, Copper, Iron, Lead, Manganese, Nickel, and Zinc) were below the detection limit in water, with an exception for Iron and Manganese. All metals assessed were also below the probable effect levels (PEL) according to the CCME. Geo-accumulation Index showed that the Sand and Blood Rivers were not contaminated with heavy metals. The enrichment factor (EF) further showed that only site B2 was enriched with manganese from anthropogenic activities. Heavy metal assessment in *Cyperus exaltatus* showed that this grass is a poor candidate for phytoremediation.

Macroinvertebrates were used as biological indicators to assess pollution status of the Sand and Blood Rivers. Macroinvertebrates were sampled according to South African scoring system (SASS) and identified using aquatic invertebrates of South African rivers field guide. The SASS and average score per taxon (ASPT) categorized all sampling sites as critically modified. The family-level biotic index (FBI) also showed that the Sand and

Blood Rivers are enriched with organic pollution. The reference sites recorded high macroinvertebrates diversity, compared to the sites after points of discharge. All indices used showed spatial and temporal variation in the water quality of the Sand and Blood Rivers. The relationship between macroinvertebrates and physico-chemical parameters were explored with the use of canonical correspondence analysis (CCA). The CCA triplots showed that the reference sites were associated with pollution sensitive taxa, whilst pollution tolerant taxa were associated with sites after points of discharge. Moreover, sites after points of discharge strongly correlated with phosphorus, nitrogen, ammonia, BOD and TDS.

Clarias gariepinus gills and liver histology were used as biomarkers in assessing pollution status in the Sand and Blood Rivers. Gills and liver samples were qualitatively and semi-quantitatively assessed. Fish from the reference sites had less gill lesions than fish from sites downstream. Alterations such as hyperplasia of interlamellar, fusion of secondary lamellae, epithelial lifting and hyperplasia of secondary lamellae were observed in fish collected from downstream. Fish from downstream also showed more liver alterations than fish from the reference sites. Alterations such as melano-macrophage centers (MMC), macrovesicular steatosis, sinusoid congested with kupffer cells, nuclei pleomorphism and vacuolation were identified in liver of fish from sites S4 and S5. This was further confirmed by the high gill and liver indices of fish from downstream.

Acetylcholinesterase enzyme (AChE) enzyme in brain and lactate dehydrogenase (LDH) in liver of *Clarias gariepinus* were used as biomarkers in assessing pollution status of the Sand and Blood Rivers. Both AChE and LDH enzymes were lower on fish from downstream compared to fish from the reference sites. This shows that AChE and LDH enzymes on fish from downstream were inhibited. This was attributed to the use of pesticides on farms surrounding the Sand and Blood Rivers and also the discharge of poorly treated sewage effluent from the Polokwane and Seshego wastewater treatment plants (WWTP's). The Sand and Blood Rivers are surrounded by farms that utilize pesticides in their practice. This study showed that biological indicators and biomarkers can be used to assess pollution status of the Sand and Blood Rivers. However, biological

indicators and biomarkers should be used in concurrence with physico-chemical parameters.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 GENERAL INTRODUCTION

Biomonitoring is any activity in which inferences on the status and quality of the environment are drawn from structural or functional attributes of individuals or communities (Hart, 1994). Biomonitoring in aquatic ecosystems uses organisms as biological indicators and provides information on the possible effects of water pollution due to anthropogenic activities (Oertel and Salanki, 2003). The use of biological indicators is fast, cost effective and provides integrated pollution status of an aquatic ecosystem (Mangadze *et al*., 2019). Biological indicators are able to reflect the extent of damage on the health of an organism and can serve as an early warning of reversible pollution induced stress (Depledge and Fossi, 1994). They further reflect the cumulative effects of factors affecting ecosystems over time (Dallas and Day, 1993). Some of the most commonly used biological indicators are macroinvertebrates and fish species.

Macroinvertebrates have been widely used in assessing pollution status of rivers and streams (Dallas and Mosepele, 2020; Izegaegbe *et al*., 2020; Mereta *et al*., 2019; Odountan *et al*., 2019). They are preferred biological indicators due to their sedentary behavior, ubiquity and relatively long lifespan (Abel, 2014). They are visible to the naked eye, easy to identify and have rapid life cycles (Dickens and Graham, 2002). Furthermore, they are sensitive to altered flow rates, water quality deterioration and habitat alteration (Uys, 1996). In South Africa, the most used tool in biomonitoring of aquatic ecosystems using macroinvertebrates is the SASS which was developed by Chutter (1998). The South African Scoring System is intended to be a rapid and inexpensive tool in assessing the degradation of an aquatic ecosystem (Chutter, 1998). It is based on three principal indices (SASS score, number of taxa and ASPT), which reflect changes in macroinvertebrate community structure (Vos *et al*., 2002). The SASS score and ASPT are used in SASS interpretation and analysis. However, the ASPT values have been reported to show more reliable results than the SASS score values (Ollis *et al*., 2006). Since SASS was developed, it has also been used in other Southern African countries including Zimbabwe (Bere and Nyamupingidza, 2014; Mudjazhezha and Ngoshi, 2014)

and Swaziland (Mthimkhulu *et al*., 2005; Dlamini *et al*., 2010). This has made macroinvertebrates the most useful community in biomonitoring studies. Some of the limitations of SASS is that it does not consider macroinvertebrates relative abundance and only reflects organic pollution (Bere and Nyamupingidza, 2014). It was therefore deemed prudent in this study to compare the use of macroinvertebrates to fish histology and fish enzyme activity.

Fish species have also been widely used in biomonitoring studies, due to their special biological characters such as large body size, long life cycle and they are also easy to identify (Zhou *et al*., 2008). Moreover, fish species have a high public profile and are thus preferred organisms as biological indicators (Heath and Claassen, 1999). The use of fish as biological indicators in biomonitoring studies was initiated by Karr (1981), by developing the Index of Biotic Integrity (IBI). However, the IBI was unsuccessful when employed in Southern Africa (Hocutt *et al*., 1994). This prompted the development of Fish Assemblage Integrity Index (FAII) for biomonitoring in Southern African rivers (Kleynhans, 1999). The FAII was successful when applied in Southern Africa, Nyagui River in Zimbabwe (Kadye, 2008). However, the use of FAII in biomonitoring studies has been largely abandoned due to a lot of assumptions involved. The effect of anthropogenic activities on fish community is always preceded by changes at the lower level of biological organization (Figure 1.1) (van der Oost *et al*., 2003). Thus, lower biological organizations can be useful early warning signs in assessing pollution status of an aquatic ecosystem. Therefore, it is important to assess pollution status using different biological organizations.

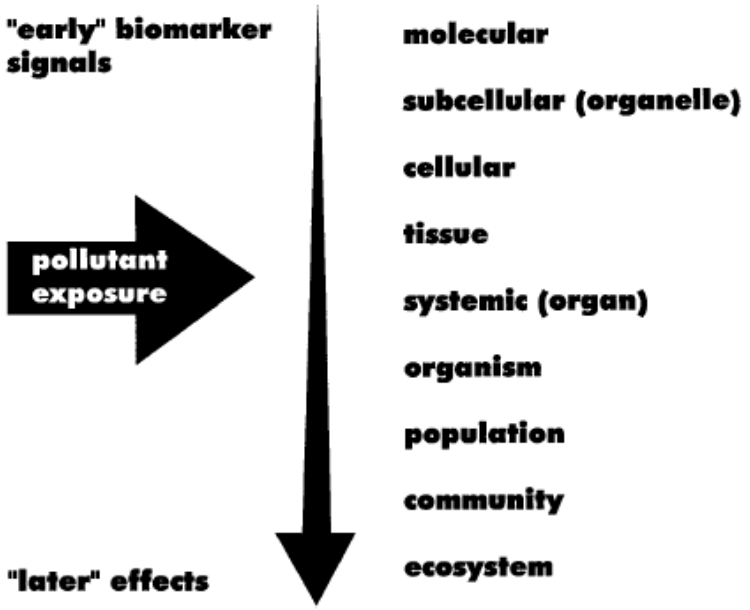

Figure 1.1: Schematic representation of the sequential order of responses to pollution in a biological system (van der Oost *et al*., 2003).

Fish histology has recently gained increasing attention in assessing pollution status of aquatic ecosystems (Yancheva *et al*., 2016; van Dyk *et al*., 2012; Wepener *et al*., 2011). This is because fish histology is a rapid, cost-effective method that can detect adverse acute and chronic effects of pollution at different tissues and fish organs (Hinton and Lauren, 1990). Moreover, histopathological changes in fish tissues and organs can show that the organism has been or is being exposed to pollution. One of the benefits of using fish histology in assessment of aquatic ecosystem pollution is that, specific organs that play a vital role in the health of fish such as the liver, gills and kidney can be examined (Bernet *et al*., 2004; Gernhofer *et al*., 2001). Fish gills are used to assess pollution in biomonitoring studies. They are multifunctional and are involved in ion transport, gas exchange, acid-base regulation and waste excretion (Genten *et al*., 2009; Dang *et al*., 2001). Gills are the main route of toxicants entry point into fish species and are in direct contact with environmental pollutants. Hence, they are useful in identifying the effects of aquatic pollution. Moreover, because of their large surface area, which is in contact with the external environment, the gills are sensitive to minor changes in the physical and chemical parameters of an aquatic ecosystem (Strzyzewska *et al*., 2016; Cerqueira and

Fernandes, 2002;). Fish liver has also been widely used in histopathological studies. It is a major organ responsible for accumulation, biotransformation and excretion of contaminants in fish species (Authman *et al*., 2013). Fish liver is preferred as a biomarker because it is regarded as a detoxification organ, thus makes it a target organ for various xenobiotic substances (Hinton and Lauren, 1990). Its microscopic structure is an integrator of biochemical functions which may produce biomarkers of prior exposure to toxicants when altered (Hinton and Lauren, 1990). The use of fish histology to assess the pollution status of aquatic ecosystems that receives poorly treated sewage effluent may be a useful approach.

Biomonitoring of aquatic ecosystems at subcellular level is a useful biomarker of pollution status (Wepener *et al*., 2011). Fish enzymes such as AChE, LDH, Pyruvate kinase (PK) and Glucose-6-phosphate dehydrogenase (G6PDH) have been reported to be inhibited by pollutants, such as pesticides and herbicides (Pundir *et al*., 2019; Mahboob *et al*., 2014). AChE enzyme activity in fish species is specifically used as a biomarker of exposure to organophosphates and carbamates pesticides due to its sensitivity to these compounds (Wepener *et al*., 2011; de la Torre *et al*., 2000). Although the use of certain pesticides and herbicides have been banned in a number of developed countries, they are still intensively used in agriculture in developing countries (Bouwman, 2004). Pesticides and herbicides have the potential to pollute aquatic ecosystems if not properly managed. These compounds produce many physiological and biochemical changes in freshwater organisms by inhibiting specific fish enzymes (Sancho *et al*., 1998). LDH enzyme activity is also useful in biomonitoring studies (Osman *et al*., 2010). Wu and Lam (1997) stated that lactate dehydrogenase can be used as a biomarker in fish which are exposed to low oxygen concentrations. Lactate dehydrogenase enzyme activity may also be used as a biomarker in muscle tissue damage of fish, especially those exposed to aquatic ecosystems that receives sewage effluent (Das *et al*., 2004a).

The discharge of poorly treated sewage effluent is associated with high levels of nutrients, organic and inorganic pollution (Moyo and Mtetwa, 2002). Nutrients that are of major concern in aquatic ecosystems are nitrogen and phosphorus. Although they are the essential elements in aquatic ecosystems, when one or both of these nutrients are in

excess, they fuel the overgrowth of algae which leads to eutrophication. Eutrophication has been regarded as one of the most serious threat facing freshwater ecosystems globally (Fetahi, 2019; Wanjohi *et al*., 2019; de Villiers and Thiart, 2007). Eutrophication may occur naturally, but it is accelerated by anthropogenic activities such as sewage effluent and agricultural run-off. Inorganic pollution is also associated with effluent from sewage, industrial and surface run-off and plays a role in the degradation of aquatic ecosystems (Camargo and Alonso, 2006). The principal inorganic pollutants in aquatic ecosystems are metals. Heavy metals are present in the environment in trace amounts. However, when these metals exceed the normal concentration, they may have detrimental long-term effects on water quality and the aquatic flora and fauna (Jackson *et al*., 2009). Hence, there is a need to assess the pollution status of threatened aquatic ecosystems. Aquatic pollution has been widely assessed by analyzing physico-chemical parameters (Roux *et al*., 1993). The use of physico-chemical parameters in assessing pollution status is an accurate method. However, this approach can be costly, time consuming and only shows conditions at the time of sampling (Holt and Miller, 2011). Moreover, this approach does not show the effect of pollution on the biota in that particular ecosystem. This has resulted in the adoption of the biomonitoring method.

The degradation of aquatic ecosystems due to anthropogenic activities is a major concern worldwide. One of the major causes of degradation of aquatic ecosystems is urbanization. Urbanization is one of the major challenges faced by developing countries. Many cities in South Africa, including the Polokwane City are experiencing rapid urbanization. The City of Polokwane is the capital city and economic hub of the Limpopo Province. It has a population exceeding 700 000 (StatsSA, 2016). Moreover, the population has a growth rate of 2.8% since 2007 (StatsSA, 2016). The Seshego Township, which is situated on the northwest side of the City of Polokwane is also experiencing rapid urbanization. The township lies 10 km from the Polokwane City. The rapid urbanization in this township may be due to its proximity to the city. According to the Limpopo census, in 2011 the Seshego population was 83 863. Urbanization is associated with high population growth and an increase in anthropogenic activities, leading to a production of a large quantity of waste.

This usually results in wastewater treatment plants (WWTP) in urban areas receiving influents exceeding their designed capacity.

1.2 PROBLEM STATEMENT

The Polokwane WWTP and Seshego WWTP discharge sewage effluent into the Sand River and Blood River, respectively. The Sand River is an urban stream which passes through the City of Polokwane and the Blood River flows from the western side towards the northern side of the Seshego Township. The Blood River further joins the Sand River from the left, north of Polokwane City. The Polokwane WWTP was commissioned in 1978, with a treatment capacity of 23 ML/day and was later rerated to 28 ML/day in 2005 (DWA, 2014). However, the Polokwane WWTP was reported to be operating at 34 ML/day in 2016 (City of Polokwane, 2019). Thus, the Polokwane City is producing domestic and industrial waste beyond the Polokwane WWTP designed capacity. The Polokwane WWTP cannot accommodate the envisaged waste emanating from the city's rapid urbanization and expansion. The Seshego WWTP designed capacity is 7 ML/day and has been reported to be exceeding its designed capacity by 0.8% (DWA, 2009). Many WWTP's in developing countries are poorly managed, overloaded and facing difficulties in handling large volumes of waste due to rapid urbanization (Mangadze *et al*., 2019). The discharge of sewage effluent into aquatic ecosystems is also common in developing countries, especially in urban waterbodies (Moyo and Rapatsa, 2016; Mthembu *et al*., 2012; Moyo and Phiri, 2002; Morrison *et al*., 2001). This has resulted in the degradation of these aquatic ecosystems.

1.3 RESEARCH JUSTIFICATION

Assessment of aquatic ecosystems has been primarily based on physico-chemical analysis. The Polokwane Municipality irregularly monitors the Sand River water quality using physico-chemical parameters. However, physico-chemical parameters are costly and only show the water quality condition at the time of sampling. In developing countries such as South Africa, the assessment of aquatic ecosystems may be limited due to monitory challenges. Thus, it is important to apply different biological indicators and

biomarkers at different biological organization to assess the pollution status of the Sand and Blood Rivers.

1.4 MAIN OBJECTIVE / AIM

The main objective of this study was to assess the pollution status of the Sand and Blood Rivers using physico-chemical parameters, biological indicators and biomarkers at different biological organizations.

1.5 SPECIFIC OBJECTIVES

I. To determine spatial and temporal variation of selected physico-chemical parameters in the Sand and Blood Rivers.
II. To determine spatial and temporal variation of selected heavy metals in water, sediment and grass in the Sand and Blood Rivers.
III. To determine spatial and temporal variation of macroinvertebrates abundance and macroinvertebrates diversity in Sand and Blood Rivers.
IV. To determine the relationship between water quality parameters and macroinvertebrates assemblage in Sand and Blood Rivers.
V. To determine spatial variation of histopathological alterations in gills and liver of *Clarias gariepinus* from the Sand and Blood Rivers.
VI. To determine spatial variation of acetylcholinesterase (AChE) enzyme activity in brain tissue of *Clarias gariepinus* and lactate dehydrogenase (LDH) enzyme activity in liver tissue of *Clarias gariepinus* from the Sand and Blood Rivers.

1.6 DISSERTATION LAYOUT

The use of biological indicators and biomarkers in assessing pollution status in Sand and Blood Rivers was assessed. The dissertation has been divided into eight chapters.

Chapter 1

The chapter introduced the problem and also highlighted the aim of the study. Specific objectives and the research hypothesis have also been outlined.

Chapter 2

The literature on the use of physico-chemical parameters in assessing pollution status of aquatic ecosystems was reviewed in this chapter. Literature on using macroinvertebrates, fish histology and fish enzyme in assessing pollution status was also reviewed.

Chapter 3

Physico-chemical parameters were used to determine the pollution status of the Sand and Blood Rivers. The water quality of the Sand and Blood Rivers were summarized using a water quality index. Spatial and temporal variation in physico-chemical parameters were also assessed.

Chapter 4

Heavy metal contamination in water, sediment and grass in Sand and Blood Rivers was assessed during the rainy and dry season across sampling sites

Chapter 5

This chapter assessed the use of macroinvertebrates as biological indicators in the Sand and Blood Rivers. The chapter further applied different indices to determine the spatial and temporal variation of macroinvertebrates in these rivers.

Chapter 6

Using fish histology as a biomarker in assessing pollution status of the Sand and Blood Rivers was evaluated.

Chapter 7

The chapter evaluated the use of fish enzymes as biomarkers in assessing pollution status of the Sand and Blood Rivers

Chapter 8

The potential of using biological indicators and biomarkers was highlighted in this chapter. Recommendations were also suggested and the conclusion of the study was highlighted.

CHAPTER 2: LITERATURE REVIEW

2.1 LITERATURE REVIEW

2.1.1 The use of physico-chemical parameters in assessing pollution status.

Physico-chemical parameters are widely used as a basic approach to assess pollution status in aquatic ecosystems (Hawkes, 1998; Roux *et al*., 1993). A number of studies have been conducted in South Africa to assess the water quality of aquatic ecosystems using physico-chemical parameters (e.g. Chetty and Pillay, 2019; Jordaan and Bezuidenhout, 2016; Chigor *et al*., 2013; Dabrowski and Klerk, 2013 Osode and Okoh, 2009). In South Africa, studies that assess the health of aquatic ecosystems using physico-chemical parameters largely rely on the South African water quality guidelines. These guidelines cater for domestic; recreational and industrial water use; agricultural water use for irrigation; agricultural water use for livestock watering; agricultural water use for aquaculture and guidelines for aquatic ecosystems (DWAF, 1996a). The South African water quality guidelines were developed by the Department of Water Affairs and Forestry, which is currently the Department of Water and Sanitation (DWS). Water quality guidelines serves as the primary source of information for determining the water quality requirements of different water uses and also protect and maintain the health of aquatic ecosystems (DWAF, 1996a).

Aquatic ecosystems have been reported as the most endangered ecosystems in the world, due to anthropogenic activities (Vörösmarty *et al*., 2010; Dudgeon *et al*., 2006; Sala *et al*., 2000). The water quality in aquatic ecosystems may be affected by both natural and anthropogenic activities such as geological structure, rainfall, urbanization, agriculture and industrialization (Chigor *et al*., 2012). These activities have both gradual and catastrophic effects on aquatic ecosystems that supports aquatic life (Scheffer *et al*., 2001). However, in developing countries such as South Africa, aquatic ecosystem water quality is mainly affected by anthropogenic activities such as sewage effluent, agriculture, industrial and mining effluents (Mangadze *et al*., 2019; Dalu *et al*., 2017; Munyika *et al*., 2014). In South Africa, most WWTP's have been reported to be mismanaged, ineffective and operating beyond their designed capacity (Mangadze *et al*., 2019; Mwedzi *et al*.,

2016; Odjadjare and Okoh, 2010). This has resulted in the discharge of raw or semi-treated sewage effluent into nearby waterbodies which alters the water quality of aquatic ecosystems. Assessment of physico-chemical parameters is important for protection of both aquatic environment and the public health (Chigor *et al*., 2013; Okoh *et al*., 2007). Moreover, according to the National Water Act, 1998 (Act No. 36 of 1998), national water resources ought to be protected, conserved, managed and controlled in a sustainable and equitable manner to benefit all persons (DWAF, 1999).

Chigor *et al*., (2013) conducted a study where physico-chemical parameters of the Buffalo River in the Eastern Cape Province, South Africa, was evaluated. Physico-chemical parameters such as temperature, pH, turbidity, electrical conductivity, total dissolved solids and salinity were assessed over 12 months. The Buffalo River has been reported to be affected by a partially treated and untreated sewage effluent. From the six sampling sites selected along the Buffalo River, the site (Eluxolzweni) after the WWTP showed a significantly decrease in dissolved oxygen levels, which was attributed to high organic load. All assessed physico-chemical parameters showed spatial variation across all sites. The study also assessed and observed temporal variation, where seasons were categorized as summer, spring, autumn and winter. A similar study was conducted in Johannesburg, South Africa, were physico-chemical parameters of two main tributaries within the Klein Jukskei catchment were assessed (van der Hoven *et al*., 2017). These tributaries were reported to be impacted by land uses such as informal settlement, urban residential area and an industrial area. Physico-chemical parameters such as temperature, pH, dissolved oxygen, electrical conductivity, turbidity and total dissolved solids were assessed in winter and spring. The land use that contributed most to poor water quality was informal settlement due to lack of proper sanitation facilities. Selected sampling sites showed spatial variation and temporal variation was also observed between winter and spring. However, none of these studies (Chigor *et al*., 2013; van der Hoven *et al*., 2017) assessed temporal variation in terms of rainy season and dry season. Rainfall plays an important role in the concentration of pollutants in aquatic ecosystems, due to dilution factor.

Physico-chemical parameters were also used to assess the pollution status of the Mohokare River in Lesotho (Chatanga *et al*., 2019). The Mohokare River, which is the

major tributary of the Senqu-Orange River has been reported to be impacted by pollution from the Maseru City, which is the most industrialized city in Lesotho. Physico-chemical parameters such as nitrate, phosphate, total suspended solids, total dissolved solids, electrical conductivity, turbidity, pH, temperature, dissolved oxygen and biochemical oxygen demand were assessed. All assessed parameters fluctuated along the river, with an exception of pH. Significant changes in parameters downstream shows that the Mohokare River water quality is impacted by anthropogenic activities such as industries, agriculture and settlement of the Maseru City. The study further stated that the ecosystem integrity of the Mohokare River should be protected by strengthening implementation of environmental policies. However, the study ignored temporal variation assessment and the study was only conducted for a period of three months. Furthermore, most studies that assess aquatic pollution using physico-chemical parameters ignore the use of water quality indices (Chatanga *et al*., 2019; Gondwe and Masamba, 2016; Bagalwa, 2006).

Water quality Indices are beneficial in summarizing and disseminating information to different stakeholders responsible for mitigation of aquatic pollution (Rangeti *et al*., 2015). They further show the degree to which natural water quality has been affected by anthropogenic activities (Wanda *et al*., 2012). The current study will make use of physico-chemical parameters and different biological indicators to assess the pollution status of Sand River and Blood River, which receives poorly treated sewage effluent from the Polokwane WWTP and Seshego WWTP. Although a number of studies are able to show the degree of pollution on aquatic ecosystems using physico-chemical parameters, this approach only reflects momentary water quality conditions, without indicating the effect of pollution on aquatic flora and fauna. Moreover, water bodies that receives sewage effluent are also associated with elevated levels of heavy metals.

2.1.2 Heavy metal contamination in aquatic ecosystems.

Heavy metals are one of the major sources of aquatic pollution and have been widely assessed in South African waterbodies (Moyo and Rapatsa, 2019, Chetty and Pillay, 2019; Edokpayi *et al*., 2017; Lebepe *et al*., 2016; Edokpayi *et al*., 2016; Jackson *et al*., 2009; Jackson *et al*., 2007). Heavy metals are the major example of inorganic pollution in aquatic ecosystems and their main source is mining, industries, agricultural activities,

sewage effluents and surface run-off (Perumalsamy and Arumugam, 2013). These land use activities have the potential to introduce metals into aquatic ecosystems, especially urban waterbodies.

Heavy metal contamination of an urban river (Sand River) in Limpopo Province, South Africa has been assessed (Moyo and Rapatsa, 2019). The Sand River is an urban river that receives poorly treated sewage effluent from the Polokwane WWTP. Metals assessed included iron, manganese, lead, copper, zinc and cadmium. The study assessed contamination of these metals in surface water, sediment, grass and fish from the Sand River. According to the Geo-accumulation Index, the study showed that the Sand River is not contaminated with trace metals. The health risk assessment Index showed that consuming fish from this river is risky, due to high levels of lead bioaccumulated in fish from the assessed river. However, the study did not further assess the source of metals, which can be achieved by determining the Enrichment Factor (Buat-Menard and Chesselet, 1979). It is important to identify the source of metal pollution, before abatement measures are put in place.

Nhiwatiwa *et al*., (2011) also assessed heavy metal concentration in water, sediment and *Clarias gariepinus* from three peri-urban rivers in Zimbabwe. Zinc, cadmium, chromium, nickel, lead, copper and iron were measured in surface water, sediment and fish tissues from Manyame, Mukuvisi and Gwebi Rivers. The study found levels of zinc, iron, copper, nickel and lead from fish tissues relatively higher than usual in all three rivers. These findings positively correlated with metal concentration observed in water and sediments. The study further concluded that metal pollution is still a major challenge in the three assessed rivers. Furthermore, fish from these rivers also recorded high levels of metals bioaccumulated in different organs. However, the study did not further assess the human health risk assessment of fish from the three rivers, although thousands of families consume fish from these rivers. The study also did not determine the degree of metal contamination in water, sediment and fish and also the source of assessed metals.

The metal contamination of the Berg River in the Western Cape Province, South Africa has been investigated (Jackson *et al*., 2007). The investigation was prompted by the decline in the water quality of this river. The study determined the concentrations of

aluminium (Al), zinc (Zn), copper (Cu), iron (Fe), lead (Pb), nickel (Ni) and manganese (Mn) in surface water, sediments and biofilm suspensions. The study reported that aluminium and iron concentrations were consistently higher than all the other metals assessed in water, sediment and biofilm samples. Aluminium and iron were significantly higher than the recommended guidelines stipulated by DWAF (1996a). The source of these elevated metal concentrations was attributed to pesticides used in farms along the Berg River. However, these studies (Moyo and Rapatsa, 2019; Nhiwatiwa *et al*., 2011; Jackson *et al*., 2007) ignored assessing temporal variation of heavy metal contamination. Heavy metals state of occurrence and concentration is influenced by factors such as pH, redox potential and bioturbation (Bryan and Langston, 1992). All these factors are highly dependent on season (rainy or dry). It is thus important to assess heavy metal temporal variation, with special focus on rainy and dry period.

2.1.3 The use of aquatic macroinvertebrates as biological indicators in assessing pollution status of an aquatic ecosystem.

Aquatic macroinvertebrates have been widely used as biological indicators in assessing pollution status of aquatic ecosystems (Dallas and Mosepele, 2020; Ochieng *et al*., 2019; Rasoloariniaina, 2017; Abong'o *et al*, 2015; Dickens and Graham, 2002; Chutter, 1994; Dallas and Day, 1993; Hilsenhoff, 1988; Lenat, 1988). Macroinvertebrates species differ in sensitivity to pollution and they are quick to react to pollutants. Their sedentary and ubiquitous behavior makes them the preferred biological indicators in changes in environmental conditions (Bere *et al*., 2016; Akindele and Olutona, 2015; Odume *et al*., 2012). Aquatic macroinvertebrates are quick and easy to sample and also require inexpensive equipment. Therefore, they are ideal for monitoring pollution status in aquatic ecosystems in developing countries, where funds availability is often a challenge (Arimoro *et al*., 2015).

The benefits of using macroinvertebrates in assessing pollution status has resulted in development of a number of biotic indices. Biotic indices are tools used in assessing environmental changes, based on different response of organisms (Borisko *et al*., 2007). Some of the widely known and used biotic indices include Trent Biotic Index (Woodiwis,

1964), Biological Monitoring Working Party Score System (BMWP) (ISO-BMWP, 1980; Armitage *et al*., 1983), Family-level biotic Index (Hilsenhoff, 1988), Chutter's Biotic Index (CBI) (Chutter, 1972), Ephemeroptera, Plecoptera and Trichoptera (EPT) Index (Lenat, 1988), Belgian Biotic Index (BBI) (De Pauw and Vanhooren, 1983), Okavango Assessment System (OKAS) (Dallas, 2009) and the South African Scoring System (SASS) (Dickens and Graham, 2002; Chutter, 1994). In Africa, different countries developed their own biotic indices which were adopted from the SASS. These include Namibian Scoring System (NASS) (Palmer and Taylor, 2004; Taylor, 1999), the Zambian Invertebrate Scoring System (ZISS) (Dallas, 2018) and Tanzania River Scoring System (TARISS) (Kaaya *et al*., 2015).

In South Africa, the South African Scoring System is currently on version 5 and has been developed by modifying and adapting the Biological Monitoring Working Party (BMWP) score system (Dickens and Graham, 2002; Chutter 1998; Chutter, 1994). It has been reported to be a rapid, easy and inexpensive tool in assessing aquatic water quality changes, especially due to organic pollution (Dallas, 2002; Dickens and Graham, 2002; Chutter 1998). The SASS has a pre-defined list of taxa that have been allocated sensitivity scores based on their sensitivity to pollution and disturbances (Dickens and Graham, 2002). Taxa scores range from 1 to 15, with 1 representing an extremely pollution-tolerant taxon and 15 an extremely pollution-sensitive taxon. The South African Scoring System forms an integral component of River Ecostatus Monitoring Programme (REMP), which is formerly known as River Health Programme (RHP). Since SASS was developed, it has been extensively used in bioassessment studies in South Africa and also outside South Africa (Odume, 2019; Niba and Sakwe, 2018; Tate and Husted, 2016; Mwedzi *et al*., 2016; Bere *et al*., 2016; Fourie *et al*., 2015; Munyika *et al*., 2014; Wolmarans *et al*., 2014; Odume and Muller, 2011).

Macroinvertebrates were used to assess the health of an urban river in the Eastern Cape, South Africa (Odume and Mgaba, 2016). The Bloukrans River has been reported to be impacted by the discharge of wastewater, informal settlement run-off and agricultural activities. The study used macroinvertebrates diversity, abundance, composition and richness to evaluate the health of the Bloukrans River. The SASS score and ASPT value

were also used. The SASS score and ASPT value also were able to differentiate the control and impaired sites. The study further assessed the relationship between physico-chemical parameters and macroinvertebrates assemblage, with the use of Canonical Correspondence Analysis (CCA). The CCA showed that dissolved oxygen, electrical conductivity, nutrients and turbidity strongly influenced the assemblage of macroinvertebrates. The study concluded that macroinvertebrates were sensitive to deteriorating water quality of the Bloukrans River. However, the study did not assess the sensitivity of macroinvertebrates based on seasons. Dallas (2007) reported that season plays a major role in macroinvertebrates assemblage and should thus be incorporated when using macroinvertebrates to assess pollution status of an aquatic ecosystem.

The water quality of the Swartkops River in Eastern Cape, South Africa was assessed with the use of diversity and structure of Chironomidae (Odume and Muller, 2011). The Swartkops River is impacted by anthropogenic activities such as industrial and domestic effluent discharges, deforestation and agricultural activities. Chironomidae larvae community structure was used to evaluate the water quality status of the Swartkops River, due to their ecological range and environmental sensitivity. The reference site in the study showed high diversity and richness as compared to impaired sites downstream. Moreover, the reference site was characterized by Orthocladiinae, Tanypodinae and Tanytarsini subfamilies, whilst sites downstream were characterized by Orthocladiinae and Chironomini subfamilies. The study further showed that different Chironomidae species differ in their sensitivity to pollution. The species identified were able to differentiate the pollution status between the reference site and sites impacted by anthropogenic activities. Chironomid community structure was influenced by physico-chemical parameters such as biological oxygen demand, dissolved oxygen, electrical conductivity, orthophosphate-phosphorus and total nitrogen. The study concluded that the family Chironomidae, when identified to species or genus levels, can be used to assess water quality of South African freshwater ecosystems (Odume and Muller, 2011). However, the study only focused on Chironomid taxa in assessing the water quality of the Swartkops River. Moreover, identifying chironomids to genus and species level is time-consuming and requires an expertise in taxonomic classification. Thus, using Chironomid species in assessing pollution status of aquatic ecosystems may not be sustainable.

Several studies in South Africa have employed SASS and ASPT values in biomonitoring studies (Dalu and Chauke, 2020; Odume and Mgaba, 2016; Watson and Dallas, 2013). Like any other rapid bioassessment tool, SASS has its own limitations. It does not consider macroinvertebrates abundance, which can also reflect the water quality of an aquatic ecosystem. It is based on macroinvertebrates sensitivity score to pollution. However, when macroinvertebrates are exposed to pollutants for a long time, they start adapting to pollutants in aquatic ecosystems. Macroinvertebrates adaptation to pollutants may results in misleading SASS score and ASPT. Gratwicke (1998) stated that seasonality of South African rivers flow has led to macroinvertebrates adaptations. Thus, it is important to apply different aquatic organisms and biological indicators and biomarkers at different biological organization in assessing pollution status of aquatic ecosystems.

2.1.4 The use of fish histology as biomarkers in assessing pollution status.

The use of fish histology in different fish tissues has been widely employed in biomonitoring studies to monitor acute and chronic changes and also to supplement physico-chemical parameter analyses (Yancheva *et al*., 2016; van Dyk *et al*., 2009a). Histological changes in fish species are more susceptible and offer early warning signs of physiological disturbances, due to toxicants in aquatic ecosystem (Jabeen *et al*., 2018). Histology is also useful in assessing both long and short term effects of toxicants at a cellular level (Meyers and Hendricks, 1985; van Dyk *et al*., 2009a; Reddy and Rawat, 2013).

In Southern Africa, *Claris gariepinus* is one of the species that has been widely used as a sentinel species in histopathological studies (van Dyk *et al*., 2009a; Marchand *et al*., 2008; Marchand *et al*., 2012; Mabika and Barson, 2013; Utete *et al*., 2019). *Clarias gariepinus* is endemic to South Africa and it inhabits most freshwater systems (Skelton, 2001). Its wide distribution and tolerance to harsh conditions makes it a suitable sentinel species in biomonitoring studies. Moreover, *Clarias gariepinus* is an important angling species. The benefits of assessing pollution status using fish histology is that, different organs such as liver, kidney, gills, skin, testis, and gonads can be used.

However, the liver and gills have been reported to be the most widely used organs in fish histology (Jabeen *et al*., 2018; van Dyk *et al*., 2007).

The liver is a target organ for toxicants since it plays a major role in biochemical transformations of pollutants in detoxification process (Reddy and Rewat, 2013). Alterations in liver structure may be useful markers to indicate exposure to environmental stressors (Hinton and Lauren, 1990). van Dyk *et al*., (2009b) categorized histological alterations as circulatory disturbances, regressive changes, progressive changes, inflammatory response and neoplasms. Histological alterations that are commonly found in the liver exposed to toxicants includes necrosis, vacuolar degeneration, congestion, hypertrophy and hyperplasia (Hinton and Lauren, 1990; Takashima and Hibiya, 1995).

Fish gills have also been widely used in histopathological studies due to their large surface area and their role in gaseous exchange and osmoregulation (Strzyzewska *et al*., 2016). They are preferred biological indicators since they are always in contact with the external environment (Roberts, 2001). Hinton *et al*., (1992) stated that they are sensitive indicators to environmental stress and exposure to harmful toxicants present in the aquatic ecosystem. Common histological alterations that can be observed in gills of fish exposed to pollutants includes telangiectasia, lamellar fusion, epithelial lifting, club-shaped lamellar and branching of the secondary lamella (Roberts, 2001; Takashima and Hibiya, 1995).

Histopathological alterations in the liver of *Clarias gariepinus* was used to assess the pollution status of two aquatic ecosystems in South Africa (Marchand *et al*., 2008). The two dams are known to be impacted from sewage treatment plants, industries and informal settlements on their upstream (Barnhoorn *et al*., 2004). A quantitative and qualitative histology based assessment was used to determine the effects of pollutants in fish health. Liver histological alterations such as hepatic cord disarray, plasma alterations, fatty degeneration of hepatocytes, an increase in melanomacrophage centers, and necrosis of liver tissue were identified in *Clarias gariepinus* from the two dams. The study further showed that fish liver from Dam 1 were more affected than fish liver from Dam 2.

Clarias gariepinus liver histopathology was also used to assess the pollution status in 13 impoundments, 12 located in the north regions of South Africa and one in the Okavango

River in northern Botswana (van Dyk *et al*., 2012b). The study showed a higher prevalence of toxicopathic non-neoplastic and pre-neoplastic alterations in the liver of *Clarias gariepinus* from the polluted impoundments. Furthermore, fish collected from the polluted impoundments showed more macroscopic liver abnormalities. The study concluded that the liver histopathology of *Clarias gariepinus* could be a useful biological indicator in assessing pollution in freshwater ecosystems. However, each of these studies used only one organ (liver) as a biomarker. Different organs respond differently to toxicants exposure. Thus, it is important to use more than one organ to affirm the effects of pollutants in fish histology. According to van Dyk *et al*., (2012b), liver histological alterations are not toxicant specific. Thus, it is difficult to identify the source of pollution responsible for the formation of a specific alteration.

Histopathological changes of *Clarias gariepinus* and *Oreochromis mossambicus* from Roodeplaat Dam were assessed on gills, liver, ovaries, testes, kidney and heart (Marchand *et al*., 2012). With the use of semi quantitative and qualitative histopathological assessment protocol, alterations were identified in selected organs, with the highest number of alterations identified in the liver. The study also showed that histopathological alterations were highest in *C. gariepinus* than in *O. mossambicus*. Thus, it is important to use more than one fish species and also different organs in assessing pollution status in aquatic ecosystems. Although the study used different organs and different fish species, there is no biomarker that is totally reliable in assessing pollution status of aquatic ecosystems. Thus, it is important to employ different biomarkers that can support or affirm the observations of other biological indicators.

Histological studies in South Africa has focused much on lentic ecosystems, while lotic ecosystems have been largely ignored. Although histological assessment offers early warning signs of exposure to toxicants (Bernet *et al*., 1999), interpretation may be misleading since fish species are mobile. Moreover, histological alterations may not only be due to anthropogenic activities. van Dyk *et al*., (2012b) also stated that histological alterations are not toxic specific.

2.1.5 Fish enzymes as biomarker in assessing pollution status of aquatic ecosystems.

Biomarkers are found at different levels of biological organization. It has been reported that early effects of pollution usually occur at the lower levels of biological organization before they can be identified at higher levels of biological organization (Bae *et al*., 2020; Jiri *et al*., 2018; Dalzochio *et al*., 2016; Hamza-Chaffai, 2014; Venter *et al*., 2004). This promoted the use of biomarkers at lower biological levels such as fish enzyme activity in biomonitoring studies (Elarabany and Bahnasawy, 2019; Fu *et al*., 2018; Vaseem and Banerjee, 2016; Barbieri and Ferreira, 2011; Monteiro *et al*., 2005). Enzyme activities are easily affected when the organism is outside its optimal conditions. In aquatic ecosystems, pollutants are the major factor that affect the activity of enzymes. Enzymes may be deactivated by the pollutant binding to the active site of an enzyme, pollutant competing with the cofactor for the active site and the presence of a toxic metabolite may inhibit the enzyme activity. Some of the commonly used fish enzymes in biomonitoring studies are AChE, LDH, G6PDH, PK and Glutathione-S-transferases (GST) (Wepener *et al*., 2011; Winzer *et al*., 2002).

AChE and LDH have become the most preferred enzymes in assessing pollution status of aquatic ecosystems (Stoyanova *et al*., 2020; Beltran and Pocsidio, 2010). Acetylcholinesterase belong to the group of serine esterases and its major role is the hydrolysis of acetylcholine in cholinergic synapses of the nervous system (Hook *et al*., 2014; Assis *et al*., 2012; Nunes, 2011; Kozlovskaya *et al*., 1993). Acetylcholinesterase enzyme in fish species is primarily known as a biomarker for organophosphates and carbamates (Bream *et al*., 2017; Hook *et al*., 2014; Barbieri and Ferreira, 2011; Monteiro *et al*., 2005). When fish are exposed to organophosphates and carbamates pesticides, toxins binds to the active site of the enzyme, phosphorylating the enzyme. Thus, inhibiting the binding of the substrate (Pundir *et al*., 2019; Assis *et al*., 2012) (Figure 2.1). Inhibition results in accumulation of acetylcholine in synapses with disruption of the nerve function, which may lead to death of fish species (Pohanka, 2009).

Pesticides have been widely used in agricultural practices (Ansara-Ross, 2012). South Africa is one of the counties that utilize high percentage of pesticides (Naidoo and

Buckley, 2003). Pesticides play a major role in food production. However, their use also contributes to environmental pollution. In South Africa, Dallas and Day, 2004 and Kwok *et al*., (2007) are some of the authors who have assessed the effect of pesticides contamination in aquatic ecosystems. Pesticides residues enter the aquatic ecosystem thorough surface run-off. Thus, affecting aquatic organisms. Acetylcholinesterase enzyme activity may be assessed from different fish organs such as liver, gills, brain, muscle and kidney (Zinkl *et al*., 1991). However, AChE activity is known to be dominant in fish brains (Kozlovskaya *et al*., 1993; Sturm *et al*., 1999).

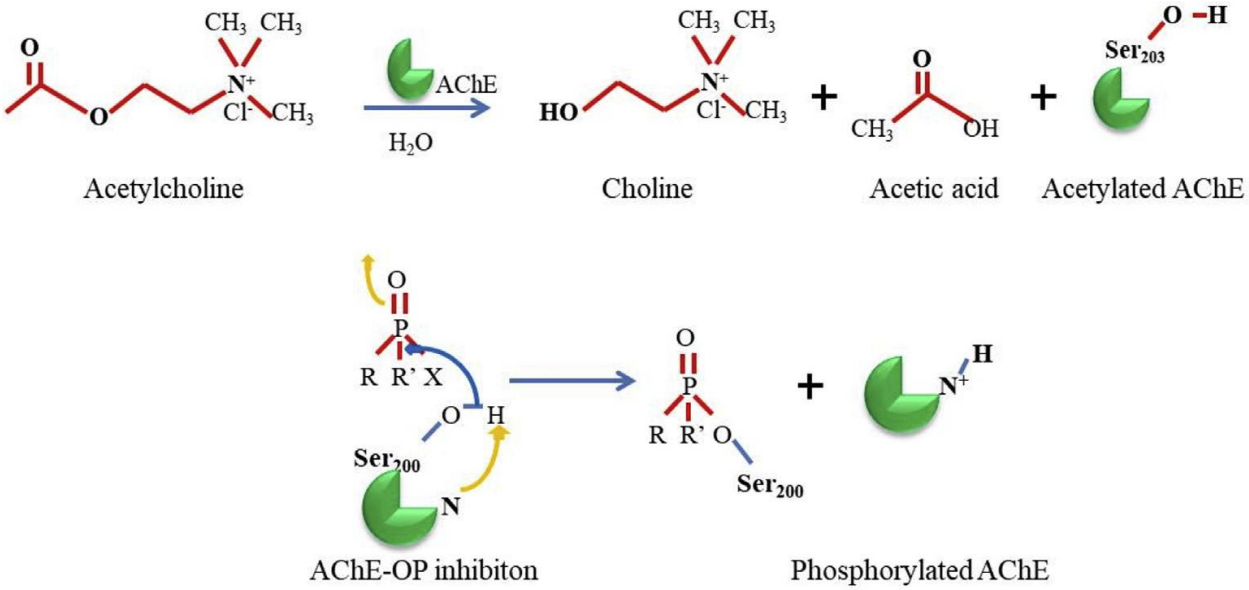

Figure 2.1: Mode of action of organophosphate pesticide on the activity of acetylcholinesterase enzyme (Pundir *et al*., 2019).

In a study conducted by Moyo and Rapatsa (2016), AChE was used as a biomarker of pollution in two rivers (Mukuvisi and Gwebi) in Zimbabwe. It was stated that the Mukuvisi River, which passes through an urban area is more polluted than the Gwebi River which passes along a non-urbanized area. *Clarias gariepinus* collected from the Mukuvisi River had much lower AChE enzyme activity. This was attributed to the metals and herbicides pollutants in the Mukuvisi River interfering with the AChE enzyme activity. The study reported that fish collected from the Gwebi River had significantly higher AChE enzyme

activity mean (58 nmol min^{-1} mg $protein^{-1}$) than those collected from Mukuvisi River (22.54 nmol min^{-1} mg $protein^{-1}$). The study further reported that AChE activity can not only indicate exposure to pesticides and insecticides, but it may also be inhibited by metals and herbicides. However, the study only used one biomarker from the lower biological organisation (AChE), which may show biased results.

In South Africa, Acetylcholinesterase enzyme activity was used as one of the biomarker in assessing the influence of multiple stressors on the Vaal River (Wepener *et al*., 2011). The Vaal River receives treated wastewater from the largest metropolitan area in South Africa and is situated in the mining and industrial heartland of South Africa. The aim of the study was to determine the risk that fish are exposed to in selected regions of the Vaal Barrage using a suite of biomarkers. Four regions (A-D) were selected in the Vaal River, based on the location of barriers and anthropogenic activities taking place around the region. The acetylcholinesterase enzyme activities were significantly lower in risk regions A and B. Moreover, acetylcholinesterase enzyme activity increased from risk region A, B, C to risk region D. However, the study showed that the suite of biomarkers used in the study were not able to identify the specific cause of mass fish mortalities that occur periodically at the Vaal River. Although the study used different biomarkers at different biological organization, the study did not have a reference site to compare with the impacted sites. This has been a major challenge in studies that use organisms in the wild, especially fish since they are mobile. One of the major land use taking place along the Sand and Blood Rivers is agricultural practice. This activity is the major source of pesticides contamination in aquatic waterbodies. The effect of pesticides on aquatic organisms of the Sand and Blood Rivers is unknown.

LDH is one of the enzymes used as a biomarker in biomonitoring studies (Diaz-Sosa *et al*., 2020; Parveen *et al*., 2017; Das *et al*., 2004; Winzer *et al*., 2012). It is a metabolic key factor and a terminal enzyme of anaerobic glycolysis in the cellular cytoplasm (Cohen *et al*., 2005). It catalyzes the conversion of pyruvate to lactate, along with NADH to NAD^+ under anaerobic conditions (Chayen *et al*., 1969) (Figure 2.2). LDH activity can reflect stress, tissue damage and exposure to different toxicants on aquatic organisms (Wu and Lam, 1997). LDH has also been reported to reflect hypoxic conditions in an organism

(Das *et al*., 2004). The effect of heavy metals on the LDH activity has been widely assessed (Barnhoorn and van Vuren, 2004; Teodorescu *et al*., 2012).

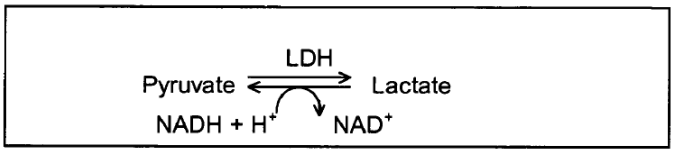

Figure 2.2: Conversion of pyruvate to lactate.

Elarabany and Bahnasawy (2019) conducted a study where *Clarias gariepinus* was exposed to sub-lethal concentrations of cadmium (Cd) and lead (Pb) to determine changes in activity of different enzymes. One of the selected enzymes was LDH, which was assessed on fish gills, liver and kidney. Fish samples that were exposed to Cd and Pb concentrations showed a significantly decrease in LDH activity as compared to the control group. The study stated that inhibition of LDH in fish exposed to heavy metals was due to stress and also possible tissue damage caused by heavy metals toxicity (Long *et al*., 2003). Almeida *et al*., (2002) also recorded a decrease in LDH activity of fish exposed to Cd and Pb concentrations.

Teodorescu et al., (2012) also conducted a study where changes in LDH activity in *Carassius auratus gibelio* was determined in kidney, gills and intestine after exposure to copper. *Carassius auratus gibelio* was exposed to two copper concentrations (100 µg/l and 250 µg/l) and was evaluated at different time intervals (24, 48 and 72 hours). The study noted a decrease in LDH activity in gills at both copper concentrations and all time intervals. However, LDH activity in the fish intestines increased at 100 µg/l and decreased at 250 µg/l concentration. The LDH activity on fish kidney only started decreasing after 72 hours of exposure. The study stated that the inhibition of LDH activity was due to stress, while stimulation might have been due to an increase in dependence on anaerobic metabolism. However, these studies (Elarabany and Bahnasawy, 2019; Teodorescu *et a*l., 2012) were conducted *in vitro*, and does not fully represent the actual conditions that take place in aquatic ecosystems. Although LDH activity has been reported to be highly concentrated in the liver, Teodorescu *et al*., (2012) ignored analysis of LDH in fish liver.

Wepener *et al*., (2005) conducted a study in the Rietvlei Wetland system, Gauteng, South Africa. The system has been reported to receive effluent from a wastewater treatment plant and an industrial complex. Active biomonitoring exposure was conducted and freshwater mollusk and *Oreochromis mossambicus* where exposed to the river for 28 days. Different cellular biomarkers, including lactate dehydrogenase enzyme were used to determine the effects of field exposure. Three sampling sites where selected, where site 1 was polluted with organic pollution, site 2 and site 3 were polluted with both organic and metallic pollution. The activity of LDH in *O. mossambicus* was reported to be significantly lower at Site 1 compared to sites 2 and 3. The LDH activity was significantly reduced at site 1 when compared to site 2 and site 3. The study reported that site 1 had high concentrations of pollutants that inhibited LDH activity in *O. mossambicus*. The study further speculated that LDH enzyme in fish is more inhibited by organic than metallic pollution. Assessing pollution status using LDH on fish from the aquatic ecosystem has been largely ignored. Organisms living in that particular waterbody should be used since they reflect previous and current exposure to different pollutants. Due to land use activities taking place along the Sand and Blood Rivers, LDH enzyme activity on fish from these rivers may be a useful biomarker in assessing pollution status at a cellular biological organization.

The current study will use *Clarias gariepinus* (Burchell, 1822) from the Sand and Blood Rivers to assess histological and enzymatic changes. This species is commonly known as the African Sharptooth catfish from the Clariidae family. It is scaleless, bony elongated body with long dorsal and anal fins and has multi-branched accessory air-breathing organ (Figure 2.3) (Skelton, 2001). *Clarias gariepinus* is widely distributed in Africa, and occurs mostly in any habitat but prefers floodplains, large sluggish rivers, lakes and dams. Due to its ability to withstand high turbidity and low dissolved oxygen levels, it is often the last or only fish species found in remnant pools of drying rivers (Skelton, 2001). The Sand and Blood Rivers have many isolated pools. Hence, this makes it the preferred candidate to use in rivers that receives poorly treated sewage effluent. Moreover, Skelton (2001) stated that *Clarias gariepinus* is probably the most widely distributed fish in South Africa.

Figure 2.3: *Clarias gariepinus* (Skelton, 2001).

2.1.6 Literature summary.

Physico-chemical parameters are the primary approach in assessing pollution status of aquatic ecosystems worldwide. Although this method is accurate, it is costly and only reflect momentary conditions. This prompted the use of biological indicators and biomarkers in assessing pollution status of aquatic ecosystem. Macroinvertebrates have also been widely used as biological indicators in biomonitoring studies. However, most studies ignored assessing pollution status with respect to rainy and dry season. Fish histology and enzyme activity have gained an increasing attention as biomarkers in assessing pollution status of aquatic ecosystems and also serve as early warning signs of aquatic degradation. However, fish histology and fish activity have limitations. Thus, it is important to assess pollution status using biological indicators and biomarkers at different biological organizations.

CHAPTER 3: AN ASSESSMENT OF SELECTED PHYSICAL AND CHEMICAL STATUS OF SAND RIVER AND BLOOD RIVER

3.1 INTRODUCTION

Physico-chemical parameters have been the primary approach in assessing pollution status in aquatic ecosystems. Changes in physico-chemical parameters in aquatic ecosystems are mainly influenced by anthropogenic activities such as agricultural, industrial and sewage effluent (Meyer *et al*., 2005; Akpor and Muchie, 2011). WWTP's in most Southern African countries are overloaded due to rapid urbanization (Mangadze *et al*., 2019; Mwedzi *et al*., 2016; Beyene *et al*., 2009). This has resulted in the discharge of substandard sewage effluent into aquatic ecosystems, especially streams in urban areas. Physico-chemical parameters that are associated with the discharge of sewage effluent into aquatic ecosystems includes low levels of dissolved oxygen and elevated levels of phosphorus, nitrogen, ammonia, biological oxygen demand, total dissolved solids, chemical oxygen demand, turbidity and electrical conductivity (Odjadjare and Okoh, 2010; Igbinosa and Okoh, 2009; Rono, 2017). The current study will assess spatial and temporal variation in the levels of total nitrogen, phosphorus, ammonia, dissolved oxygen, biological oxygen demand, turbidity, total dissolved solids, pH and water temperature across sampling sites in the Sand and Blood Rivers.

Sewage effluent is discharged in the Sand and Blood Rivers from the Polokwane WWTP and Seshego WWTP, respectively. Polokwane WWTP receives sewage influent from the suburbs in the Polokwane City, Central Business District (CBD) and industries around the city. On the other hand, Seshego WWTP receives influent from the township. In instances such as these, it is important to evaluate the spatial variation of pollutants at different sites, as this will give an indication of the self-purification capacity of these rivers. Self-purification capacity is influenced by factors such as river velocity, the load of nutrients entering the aquatic ecosystem, dissolved oxygen, temperature, turbulence and retention time of water in the catchment (Ifabiyi, 2008; Wilk *et al*., 2018). Kowalkowski (2009) reported that pollutants may reach a certain level where it may be impossible for an aquatic ecosystem to recover back to its original water quality condition.

Although spatial variation in the Sand River has been assessed by previous studies, temporal variation was ignored with respect to rainy and dry seasons (Seanego and Moyo, 2013; Moyo and Rapatsa, 2019). It is important to assess the effect of rainfall in lotic ecosystems because rainfall often results in changes in flow regime of lotic ecosystems. River velocity, flow volume and water temperature have been reported to be some of the primary factors that affect the distribution and diversity of freshwater flora and fauna (Knouft and Ficklin, 2017). The flow volume may further cause a dilution factor in aquatic ecosystems, which may influence the concentrations of total nitrogen, phosphorus and other pollutants. Whilst there is limited data on pollution status of the Sand River, the Blood River lacks such data. The objective of this chapter was to determine spatial and temporal variation of selected physico-chemical parameters in the Sand and Blood Rivers.

3.2 MATERIALS AND METHODS

3.2.1 Study area

The Sand River is a right-hand tributary of the Limpopo River in South Africa. The source of this river is in Mokopane, which lies 45 km from the City of Polokwane. The Sand River is regarded an urban river since it flows on the western edge of the Polokwane City, which is the capital city of the Limpopo Province. The Blood River, which is also known as the Mulaudzi River, flows from the western side of the Seshego Township and further joins the Sand River from the left, north of the Polokwane City (Figure 3.1). The Polokwane WWTP discharges sewage effluent into the Sand River and the Seshego WWTP discharges into the Blood River. In the Sand River, the reference site was selected before the Polokwane WWTP and was designated Site S1 (Figure 3.1). This site was selected based on its location, which is outside the CBD and not close to anthropogenic activities. The second site was selected just after the Polokwane WWTP effluent point of discharge and was designated Site S2 (Figure 3.1). The third site in Sand River was selected at the point of confluence, where the Blood River joins the Sand River and was designated Site S3 (Figure 3.1). The fourth site was selected further downstream in the Sand River and was designated Site S4 (Figure 3.1). The last site was selected downstream of S4 and was designated Site S5 (Figure 3.1). In the Blood River, the reference site was selected before the Seshego WWTP effluent point of discharge and was designated Site B1 (Figure 3.1). This site was also selected based on its location, which is outside the Seshego residential area with no known point source pollution. The second site was selected after the Seshego WWTP effluent point of discharge, just before it joins the Sand River and was designated site B2 (Figure 3.1). The Blood River is very short, thus only two sites were chosen.

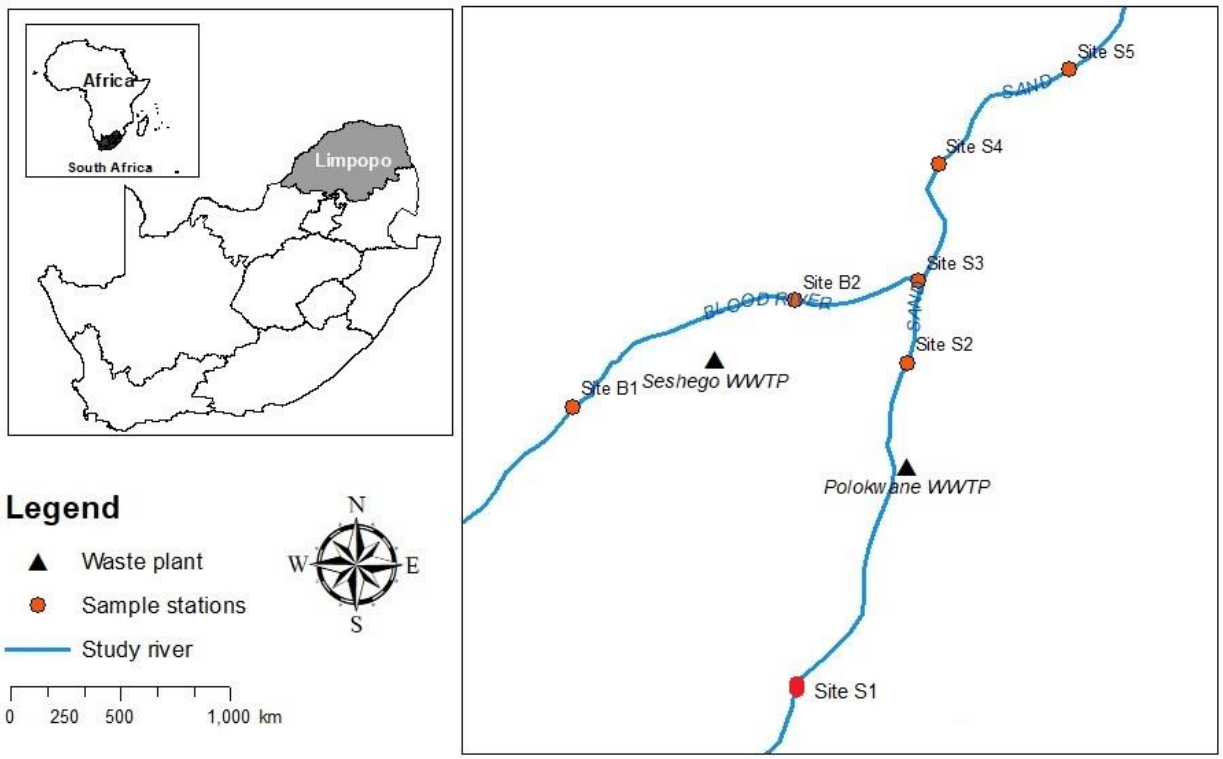

Figure 3.1: Map of the Sand River and Blood River showing sampling sites, Polokwane WWTP and Seshego WWTP. Sites B1 and B2 are in the Blood River and sites S1, S2, S3, S4 and S5 are in the Sand River.

3.2.2 Physico-chemical parameters

Water sampling was conducted once a month in triplicates during the rainy season (January to April, 2018) and dry season (May to August, 2018). Water temperature, dissolved oxygen (DO) and pH were measured *in situ* using a handheld multiparameter meter (Professional plus YSI 605000). Turbidity was measured using a WTW turbidity meter (Turb 430 IR). Polyethylene sampling bottles (1L) washed with de-stilled water were used to collect water samples at each site. The water samples were collected just below the surface at a depth of 10 cm and stored in ice during transportation from the sampling sites to the Aquaculture Research Unit (ARU) Laboratory. These water samples were used to analyze for nitrogen, phosphorous and ammonia according to APHA (2005).

For Biological Oxygen Demand (BOD), polyethylene sampling bottles were used to collect water samples 10 cm below the surface at different sites. Samples were collected in triplicates. The bottles were then wrapped in black tape immediately after sampling and were kept in the dark from sampling sites to the ARU Laboratory. On arrival, bottles were incubated for five days at room temperature (20°C). After five days, dissolved oxygen was recorded for each sample. The BOD was then calculated using the equation below:

BOD^{20}_{5}=D1-D2 (Viessman and Hammer, 1993), where D1 is the dissolved oxygen (mg/l) on the first day, D2: dissolved oxygen (mg/l) after day five. Twenty (20) is the incubation temperature (°C) and 5 is the number of incubation days.

Total dissolved solids (TDS) were determined according to Eaton *et al*., (1995). Water samples were collected in triplicates at each site using 250 ml polyethylene sampling bottles and transported to the ARU Laboratory. In the laboratory, a sample of 100 ml was filtered through a filtration apparatus where a glass fibre filter (Whatman, 1.6 µm) was used. After draining the water sample, complete drainage was accomplished by filtering 10 ml of distilled water for three minutes. The filter paper was then placed in a clean glass petri-dish and dried at 85°C. Total dissolved solids were then calculated using the equation below:

$$\text{TDS (mg/l)} = \frac{(\text{A}-\text{B})\times 1000}{\text{Sample volume(ml)}}$$

Where A is the weight of dried filter paper + dish (mg) and B is the weight of the dish (mg)

The Canadian Council of Ministers of the Environment Water Quality Index (CCME WQI) was used to summarize physico-chemical parameters in the Sand and Blood Rivers at different sites. Physico-chemical parameters that were considered included water temperature, pH, dissolved oxygen, biological oxygen demand, turbidity, total dissolved solids, phosphorus, total nitrogen and ammonia.

The CCME WQI was calculated using the formula:

$$CCME\ WQI = 100 - \left(\frac{\sqrt{{F_1}^2 + {F_2}^2 + {F_3}^2}}{1.732} \right)$$

Where F_1 (scope) represent the percentage of variables that do not meet their objectives at least once during the time period under consideration, relative to the total number of variables measured. F_2 (frequency) represents the percentage of individual tests that do not meet objectives. F_3 (amplitude) represents the amount by which failed test values do not meet their objectives (CCME, 2001a). Once the CCME WQI values were determined, water quality was ranked using categories in Table 3.1.

Table 3.1: CCME WQI categories (CCME, 2001a).

CCME WQI	Condition	Description
95-100	Excellent	Water quality is protected with a virtual absence of threat or impairment; conditions very close to natural or pristine levels
80-94	Good	Water quality is protected with only a minor degree of threat or impairment; conditions rarely depart from natural or desirable levels
65-79	Fair	Water quality is usually protected but occasionally threatened or impaired: conditions sometimes depart from natural levels
45-64	Marginal	Water quality is frequently threatened or impaired; conditions often depart from natural levels
0-44	Poor	Water quality is almost always threatened; conditions usually depart from natural levels

3.3 DATA ANALYSIS

Normality and homogeneity of variance for water quality parameters were tested with the use of Shapiro-Wilk and Levene's test, respectively. One-way analysis of variance (ANOVA) was used to test any significant differences in water quality parameters across sites in the Sand and Blood Rivers.

A hierarchical method, average linkage cluster analysis was used to cluster sites on the Sand and Blood Rivers in relation to physico-chemical parameters (IBM SPSS version 25)

3.4 RESULTS

3.4.1 Physico-chemical parameters used to assess the pollution status of the Sand and Blood Rivers.

During the rainy season, all assessed physico-chemical parameters showed spatial variation (ANOVA, $P<0.05$), with an exception of pH (Table 3.2a). Site S1, which is the reference site in Sand River recorded the lowest phosphorus levels (0.30 mg/l), which was not significantly different (ANOVA, $P>0.05$) to 0.50 mg//l recorded at the reference site in Blood River (B1). Site S1 also recorded the lowest nitrogen level (0.85mg/l), followed by site B1 (1.20 mg/l) (Table 3.2a). Site S1 also recorded the lowest levels of ammonia (0.30 mg/l), followed by site B1 (0.96 mg/l). Turbidity, BOD and TDS levels followed a similar trend (Table 3.2a). Site S3, which is the point of confluence recorded the highest levels of phosphorus (4.77mg/l), which was significantly different (ANOVA, $P<0.05$) to all sampling sites. Site S3 also recorded the highest levels of nitrogen (7.57 mg/l).

The highest dissolved oxygen levels were recorded at site S1 (4.15 mg/l), which was not significantly different (ANOVA, $P>0.05$) to site B1 (3.45 mg/l). Sites after points of discharge (S2, B2, S3 and S4) recorded a significant (ANOVA, $P<0.05$) decrease in dissolved oxygen levels, whilst the last site downstream (S5) recorded an increase in dissolved oxygen levels (2.25 mg/l). The lowest dissolved oxygen levels were recorded at site B2 (0.64 mg/l), which is the site immediately after the Seshego WWTP point of discharge (Table 3.2a).

During the dry season, all physico-chemical parameters also showed spatial variation (ANOVA, $P<0.05$), with an exception of pH and water temperature (Table 3.2b). Site S1 recorded the lowest levels of phosphorus (0.52 mg/l), nitrogen (0.87 mg/l), ammonia (0.44 mg/l), followed by site B1. The site at the point of confluence (S3) recorded the highest levels of phosphorus (11.65 mg/l) and nitrogen (36.6 mg/l), whilst the highest levels of ammonia were recorded at site B2 (12.50 mg/l).

All assessed physico-chemical parameters showed a temporal variation between the rainy season and dry season (Figure 3.2). Phosphorus, nitrogen and ammonia levels were higher during the dry season than rainy season at all sampling sites (Figure 3.2).

Dissolved oxygen levels decreased during the dry season at all sampling sites, with an exception of S1 (Figure 3.2).

During the rainy season and dry season, water temperature, pH and TDS levels were within the targeted water quality range (TWQR) for aquatic ecosystems at all sampling sites (Table 3.2a and Table 3.2b). Nitrogen, phosphorus and ammonia levels were above the TWQR for aquatic ecosystems at all sampling sites and dissolved oxygen levels were below the TWQR at all sampling sites with an exception of site S1 during the rainy season.

Table 3.2a: Physico-chemical parameters measured at seven sampling sites in the Sand and Blood Rivers during the rainy season expressed as mean ± SD. Different superscripts in a row indicate statistically significant difference (P<0.05, ANOVA). *n*= 84

Sites	**S1**	**S2**	**S3**	**S4**	**S5**	**B1**	**B2**	**TWQR***
Parameters								
Temp (°C)	21.35±0.92^{a}	22.42±0.90^{a}	24.00±0.36^{b}	24.42±0.91^{b}	23.95±0.59^{b}	21.42±0.94^{a}	23.35±0.34^{b}	5-30
pH	7.32±0.31^{a}	7.76±0.22^{a}	7.76±0.13^{a}	7.66±0.32^{a}	7.87±0.10^{a}	7.80±0.18^{a}	7.54±0.40^{a}	6-8
DO (mg/l)	4.15±0.04^{a}	1.81±0.11^{b}	1.33±0.05^{b}	1.44±0.06^{b}	2.25±0.26^{c}	3.45±0.12^{a}	0.64±0.03^{d}	4-5
BOD (mg/l)	0.18±0.01^{a}	0.37±0.03^{b}	0.83±0.05^{c}	0.31±0.02^{b}	0.25±0.02^{d}	0.23±0.02^{d}	0.91±0.05^{e}	-
Turbidity (NTU)	4.19±0.85^{a}	15.65±1.04^{b}	22.45±2.14^{c}	21.15±0.59^{c}	15.57±0.99^{b}	11.37±1.52^{b}	26.0±0.81^{c}	<100
TDS (mg/l)	353.75±19.53^{a}	626.00±10.03^{b}	660.75±9.63^{c}	609.50±2.88^{d}	556.50±6.55^{e}	403.00±2.58^{f}	624.50±3.1^{b}	200-1100
Phosphorus (mg/l)	0.30±0.08^{a}	3.52±0.17^{b}	4.77±0.35^{c}	2.87±0.38^{d}	2.44±0.13^{e}	0.50±0.08^{a}	4.17±0.17^{g}	<0.005
Nitrogen (mg/l)	0.85±0.07^{a}	5.85±0.91^{b}	7.57±0.55^{b}	6.40±0.43^{b}	5.62±0.18^{b}	1.20±0.08^{c}	6.45±0.31^{b}	<0.5
Ammonia (mg/l)	0.30±0.06^{a}	5.05±0.96^{b}	5.30±0.35^{b}	4.45±0.34^{b}	3.35±0.12^{b}	0.96±0.03^{c}	5.95±0.12^{b}	0.007

*****Targeted Water Quality Range (TWQR) for aquatic ecosystems (DWAF, 1996)

Table 3.2b: Physico-chemical parameters measured at seven sampling sites in the Sand and Blood Rivers during the dry season expressed as mean ± SD. Different superscripts in a row indicate statistically significance difference (P<0.05, ANOVA). *n*=84

Sites	**S1**	**S2**	**S3**	**S4**	**S5**	**B1**	**B2**	**TWQR***
Parameters								
Temp (°C)	16.57±0.45^{a}	16.25±0.28^{a}	16.72±0.33^{a}	16.42±0.71^{a}	16.17±0.86^{a}	16.32±0.53^{a}	16.52±0.35^{a}	5-30
pH	7.18±0.18^{a}	7.24±0.01^{a}	7.77±0.15^{a}	7.37±0.43^{a}	7.57±0.17^{a}	7.75±0.12^{a}	7.70±0.18^{a}	6-8
DO (mg/l)	3.94±0.54^{a}	0.52±0.10^{b}	0.48±0.09^{b}	0.65±0.06^{c}	1.02±0.02^{d}	2.62±0.33^{a}	0.41±0.01^{e}	4-5
BOD (mg/l)	0.28±0.02^{a}	1.22±0.05^{b}	1.35±0.03^{b}	1.28±0.05^{b}	1.17±0.02^{b}	0.40±0.07^{c}	1.83±0.08^{d}	-
Turbidity (NTU)	4.54±0.33^{a}	27.02±2.52^{b}	32.87±2.09^{b}	27.80±0.75^{b}	19.75±1.25^{c}	16.75±1.50^{c}	32.00±2.44^{b}	<100
TDS (mg/l)	417.75±13.72^{a}	640.25±15.5^{b}	670.75±10.68^{c}	651.75±5.73^{d}	636.50±5.06^{b}	451.00±6.63^{e}	674.75±3.59^{c}	200-1100
Phosphorus (mg/l)	0.52±0.09^{a}	7.07±0.20^{b}	11.65±0.70^{b}	8.97±1.02^{b}	6.25±0.35^{b}	0.77±0.09^{a}	8.55±0.50^{b}	<0.005
Nitrogen (mg/l)	0.87±0.06^{a}	21.01±1.23^{b}	36.6±3.02^{c}	26.65±1.06^{b}	18.0±0.81^{d}	1.60±0.14^{a}	26.1±1.13^{b}	<0.5
Ammonia (mg/l)	0.44±0.07^{a}	9.25±1.07^{b}	9.87±1.11^{b}	9.05±0.93^{b}	7.42±0.51^{b}	1.27±0.17^{a}	12.50±1.91^{d}	0.007

* Targeted Water Quality Range (TWQR) for aquatic ecosystem (DWAF, 1996)

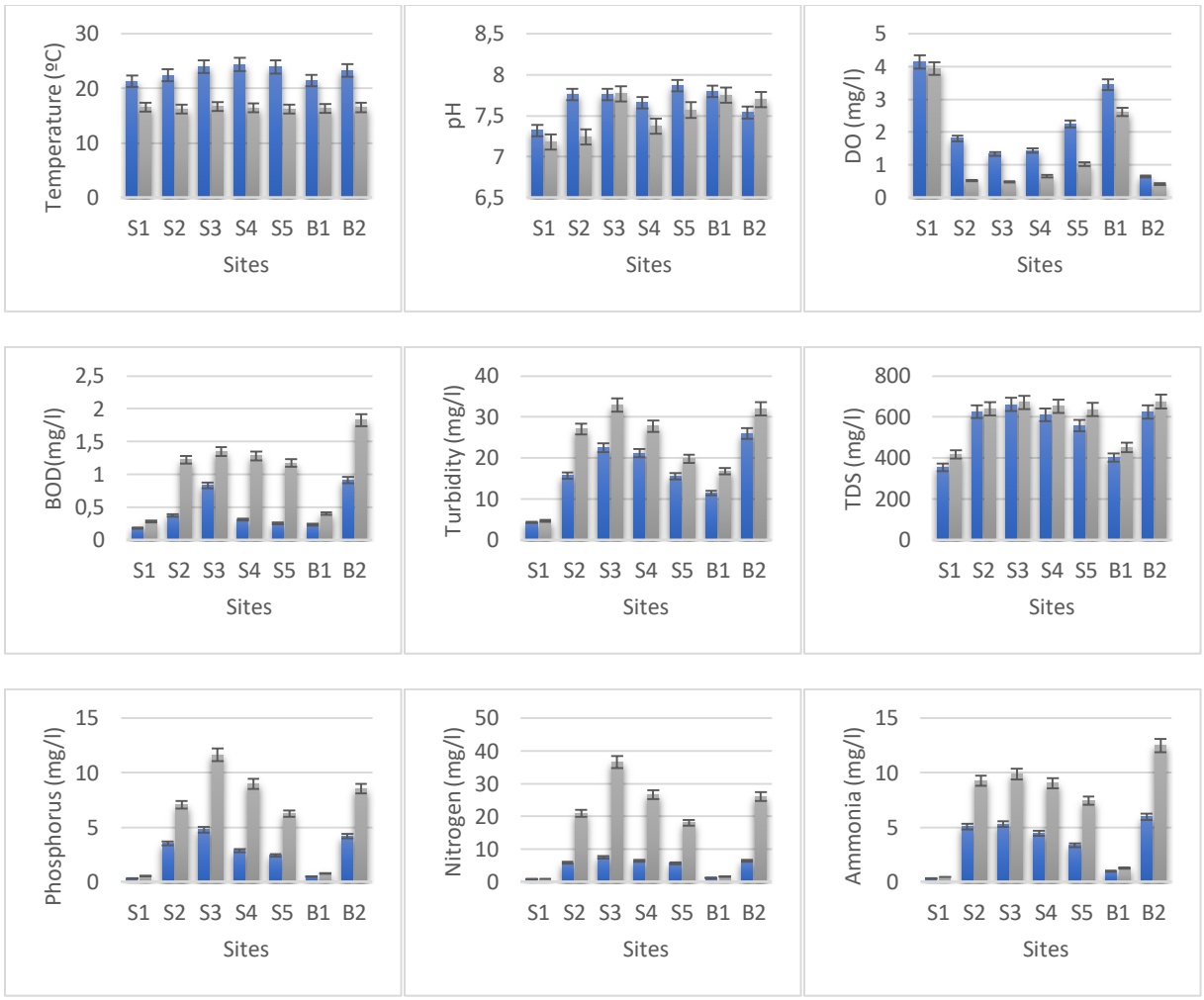

Figure 3.2: Physico-chemical parameters showing temporal variation between the rainy season (blue bars) and dry season (grey bars) at different sampling sites in the Sand and Blood Rivers.

Hierarchical average linkage cluster analysis on the assessed physico-chemical parameters produced two major clusters during both the rainy (Figure 3.3) and dry (Figure 3.4) seasons. Cluster one consisted of the reference site in the Sand River and the reference site in the Blood River (S1 & B1), while the second cluster consisted of all the sites after points of discharge in the Sand and Blood Rivers (S2, B2, S3, S4 & S5).

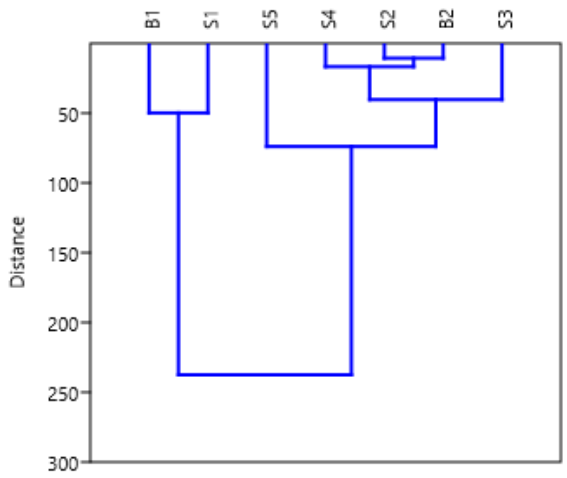

Figure 3.3: Dendrogram showing sampling sites clusters during rainy season in the Sand and Blood Rivers

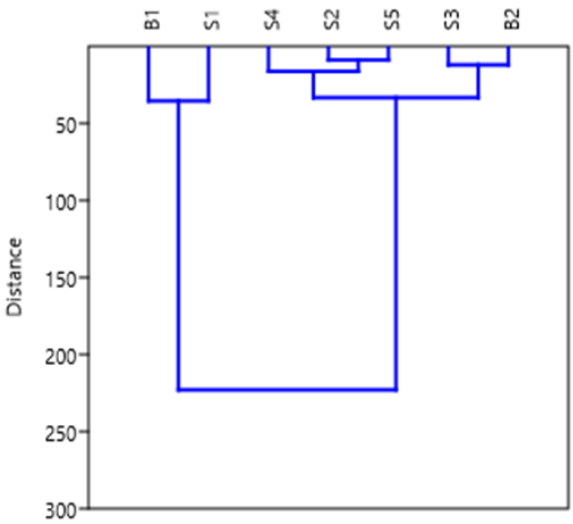

Figure 3.4: Dendrogram showing sampling sites clusters during dry season in the Sand and Blood Rivers.

The CCME WQI showed that the reference site in Sand River (S1) and Blood River (B1) had good water quality during the rainy and dry seasons (Table 3.3a and Table 3.3b). All sites after points of discharge had poor water quality, with as exception of site S5, which had fair water quality during the rainy season and dry season (Table 3.3a and Table 3.3b). The poorest water quality was recorded at S3 during the rainy season and dry season (Table 3.3a and Table 3.3b). The CCME WQI score was higher during the rainy season than dry season at all sampling sites (Table 3.3a and Table 3.3b).

Table 3.3a: CCME Water Quality Index score and conditions for sites along the Sand River and Blood River during rainy season.

Sites	**S1**	**S2**	**S3**	**S4**	**S5**	**B1**	**B2**
CCME WQI Score	86	36	27	41	69	83	29
Condition	Good	Poor	Poor	Poor	Fair	Good	Poor

Table 3.3b: CCME Water Quality Index score and conditions for sites along the Sand River and Blood River during dry season.

Sites	**S1**	**S2**	**S3**	**S4**	**S5**	**B1**	**B2**
CCME WQI Score	82	28	23	36	65	80	25
Condition	Good	Poor	Poor	Poor	Fair	Good	Poor

3.5 DISCUSSION

Total nitrogen, phosphorus, ammonia, turbidity, TDS, dissolved oxygen and BOD showed spatial variation between sampling sites in the Sand and Blood Rivers during the rainy and dry seasons. In both seasons, the reference site in the Sand River (S1) and the reference site in the Blood River (B1) recorded the lowest levels of phosphorus, nitrogen, ammonia, BOD, TDS and turbidity, compared to sites after points of discharge. The low levels of pollutants recorded at site S1 shows that the site is less impacted by anthropogenic activities. Site S1 is located just outside the Polokwane CBD. It is also secluded with dense riparian and aquatic vegetation. Moreover, aquatic vegetation at this site may have also played a role in low levels of phosphorus and nitrogen by absorbing these nutrients. The low levels of pollutants at site B1 may also be due to its location and lack of known point source pollution. Although S1 and B1 had low levels of nitrogen, phosphorus, ammonia and BOD, concentrations at site B1 were slightly higher than those recorded at site S1. This may be due to the informal settlement, which is not connected to a sewer system, illegal dumping take place near site B1. These activities may affect the water quality at site B1. The significant low levels of pollutants at the sites before WWTP's have been reported elsewhere (Akpen *et al*., 2016; González *et al*., 2014; Bere, 2007).

The sites immediately after points of discharge (S2 and B2) recorded elevated levels of phosphorus, nitrogen, ammonia, TDS and BOD during the rainy season and dry season. The elevated levels of pollutants at these sites may be due to the discharge of poorly treated sewage effluent from the Polokwane and Seshego WWTP's. These wastewater treatment plants have both been reported to be poorly managed, operating beyond their designed capacity (Seanego and Moyo, 2013; DWA, 2009). The Seshego WWTP designed capacity is 7 ML/day and receives influent from the Seshego Township, which is more populated than the Polokwane City. Although the Polokwane WWTP designed capacity is 28 ML/day, it receives influent from several suburbs, CBD and major industries such as SAB Miller, Coca-Cola Fortune, Granor Passi and two smelters around the city. This has resulted in the discharge of substandard sewage effluent into the Sand and

Blood Rivers, which is associated with an increase in nitrogen, phosphorus and ammonia levels.

The point of confluence (S3) recorded the highest levels of phosphorous, nitrogen and TDS. Ammonia, turbidity and BOD were also elevated at site S3 during the rainy and dry seasons. An increase in these parameters may be due to the combination of effluents from the Seshego and Polokwane WWTP's. Effluent discharge from WWTP's has been described as the major source of increased nutrients levels in lotic ecosystems in South Africa (Odume *et al*., 2016; Okoh *et al*., 2012; Oberholster *et al*., 2008; Jordaan and Bezuidenhout, 2016). Moreover, site S3 is located less than a kilometer from sites S2 and B2, which is not an ample distance for self-purification. Lack of aquatic vegetation at S3 may have limited the uptake of nutrients from the water column. However, an improvement in water quality was observed at sites S4 and S5, respectively. A decrease in pollutants concentration at sites downstream has been observed in other studies (González *et al*., 2014). This observation is associated with self-purification capacity, which is influenced by factors such as topography, sedimentation, vegetation, rivers hydraulic characteristics and the retention time of water in the catchment (Wilk *et al*., 2018; González *et al*., 2014). Seanego and Moyo (2013) reported that the Sand River still has the ability to maintain self-purification capacity, even though the water quality is degraded.

A temporal variation in physico-chemical parameters was observed in the current study. At all sampling sites, nitrogen, phosphorous, ammonia, TDS and BOD were higher during the dry season than rainy season. Temporal variation in physico-chemical parameters was also observed in other water bodies (Chetty and Pillay, 2019; Pullanikkatil *et al*., 2015; González *et al*., 2014; Chigor *et al*., 2013). Elevated levels of pollutants during the dry season may be due to dilution factor. In Limpopo Province, the dry season is associated with low or lack of rainfall. This often results in concentrated levels of pollutants in aquatic ecosystems during the dry season. Irrespective of season, sites after points of discharge were more polluted than sites before points of discharge. This was further confirmed by the hierarchical average linkage cluster analysis that grouped sites before points of discharge as one cluster and sites after points of discharge as another cluster in both the rainy and dry seasons.

Phosphorus, nitrogen, ammonia and dissolved oxygen did not comply with the TWQR for aquatic ecosystems in South Africa during the rainy and dry seasons (DWAF, 1996a). In South Africa, most studies conducted in streams that are affected by land use activities have reported physico-chemical parameters that do not comply with South African water quality guidelines (van der Hoven *et al*., 2018; Namugize *et al*., 2018; Singh and Lin, 2015; Olaniran *et al*., 2014). However, dissolved oxygen levels complied only at site S1 during the rainy season. Anthropogenic activities such as rapid urbanization, agriculture, industrial and sewage effluent have been reported to play a major role in affecting the nutrient load of aquatic ecosystems (Dalu *et al*., 2019; Odjadjare and Okoh, 2010).

The spatial and temporal variation of physico-chemical parameters in the Sand and Blood Rivers was further assessed using CCME WQI. The reference sites were categorized as good, whilst sites after points of discharge were categorized as poor during both seasons. Site S5 showed fair water quality. This shows that the CCME WQI was able to differentiate the reference sites from sites which are impaired by land use activities.

Physico-chemical parameters were able to show the current pollution status of the Sand and Blood Rivers. This was affirmed by the discrimination of the reference sites from the sites downstream of the sewage effluent discharge points. Furthermore, physico-chemical parameters showed that the Sand and Blood Rivers water quality do not comply with the South African targeted water quality guideline for aquatic ecosystem.

CHAPTER 4: EVALUATION OF HEAVY METAL CONTAMINATION IN WATER, SEDIMENT AND GRASS IN SAND AND BLOOD RIVERS

4.1 INTRODUCTION

Heavy metal contamination is a major concern in aquatic ecosystems (Madu *et al*., 2017; Jooste *et al*., 2014; Mohammed *et al*., 2011; Zhou *et al*., 2008). Heavy metals are present in trace amounts in aquatic ecosystems, originating from rocks and soil weathering (Reza and Singh, 2010). The level of these metals is often elevated due to anthropogenic activities (Teta *et al*., 2017; Martin *et al*., 2015). Anthropogenic activities that play a major role in metal contamination in aquatic ecosystems includes mining effluent, industrial effluent, agricultural run-off and the discharge of sewage effluent into aquatic waterbodies, especially those in urban areas (Gao *et al*., 2009; Agbozu *et al*., 2007). The discharge of sewage effluent into aquatic ecosystems in urban areas is very prominent in developing countries (Agbozu *et al*., 2007). Sewage effluent also contain trace amounts of heavy metals, which may contaminate aquatic ecosystems.

In aquatic ecosystems, sediment act as a sink and a repository for heavy metals (Javed, 2005). Heavy metals in sediment may be resuspended into water column through chemical, physical and biological processes (Cloete *et al*., 2017). The resuspended heavy metals in the water may be toxic to aquatic organisms such as fish, macroinvertebrates and plants (Siwela *et al*., 2010; Chatterjee *et al*., 2009). Heavy metal concentration in sediment has been reported to be higher than in the water column (Nyamangara *et al*., 2008). Thus, heavy metal contamination is better monitored in sediment than surface water (Qiao *et al*., 2013; Iwegbu *et al*., 2007). This has led to formulation of several sediment quality guidelines in different countries. In Canada, the Canadian council of ministers of the environment developed sediment quality guidelines for the protection of aquatic life (CCME, 2001b). Due to lack of sediment guidelines in South Africa, studies that assess heavy metal contamination adopt the CCME sediment guidelines.

Heavy metal contamination in aquatic ecosystems can also be assessed using contamination factor, pollution load index, Geo-accumulation Index and Enrichment Factor. Heavy metal contamination has been evaluated in the Sand River previously (Moyo and Rapatsa, 2019). However, the study did not establish the source of heavy metal contamination. It is important to identify the source of heavy metal contamination before any abatement measures are put in place. Furthermore, heavy metal contamination status of the Blood River is unknown.

Heavy metal contamination in aquatic ecosystems also influence bioaccumulation in aquatic flora and fauna. This has led to the adoption of phytoremediation. Phytoremediation is a cost-effective, environmental-friendly process, whereby plants with the ability to uptake heavy metals are used to control or abstract heavy metals from sediment. Phytoremediation includes a range of plant-based remediation techniques such as phytoextraction, phytostabilization, phytoimmobilization, rhizofiltration, and phytovolatilization, which focus on reduction of the aquatic pollution level. In the Sand River, Moyo and Rapatsa (2019) used *Ischaemum fasciculatum* grass as a phytoremediation grass. However, the study showed that *Ischaemum fasciculatum* grass is not an effective phytoremediation grass due to its low transfer factor value. It is thus paramount to assess other plants in the Sand and Blood Rivers to test their potential in phytoremediation. The current study will use *Cyperus exaltatus* as a potential candidate for phytoremediation in the Sand and Blood Rivers. The objective of this chapter was to determine spatial and temporal variation of selected heavy metals in water, sediment and grass in the Sand and Blood Rivers.

4.2 MATERIALS AND METHODS

4.2.1 Heavy metal assessment in water, sediment and grass

Water, sediment and grass samples for heavy metal analysis were collected once a month in triplicates from the Sand and Blood Rivers sampling sites (S1, S2, S3, S4, S5, B1 and B2) during the rainy season (January to April, 2018) and dry season (May to August, 2018). Heavy metals analyzed in these samples included: cadmium (Cd), copper (Cu), iron (Fe), manganese (Mn), lead (Pb), zinc (Zn), chromium (Cr) and nickel (Ni). Inductively Coupled Plasma-optical Emission Spectrometry (ICP-OES, Perkin Elmer: Optima 2100 DV) was used to analyze heavy metals in water, sediment and grass.

Heavy metal analysis in water

Pre-cleaned (1L) polyethylene sampling bottles were used to collect water 10 cm below the surface. Sampling bottles were stored on ice during transportation from the sites to the ARU Laboratory. Samples were filtered through 0.45 µm pore size Whatman filters before analysis. Water samples were then preserved by adding 10 ml of 65% nitric acid to keep heavy metals in solution and then stored at 4°C until analysis. Heavy metals in surface water were analyzed according to ISO 11885 (2007). During analysis, 100 ml of each sample was used, 10 ml of aqua regia HNO_3: HCL in the ratio of 3:1 was added in a culture test tube. The solution was then incubated at 80°C for 5 minutes. After incubation, the solution was allowed to cool and diluted to 50 ml with deionized water. The solution was then analyzed for selected heavy metals using an ICP-OES (Ogoyi *et al*., 2011). Analysis was done in triplicates and one blank containing 10 ml of 65% nitric acid was included. Quality assurance and quality control procedures were conducted using Ultraspec multi-element aqueous certified reference material from De Bruyn spectroscopic solutions. Recoveries ranged from 92.7 % to 98.4% (Table 4.1). Detection limit for cadmium was 0.003 mg/l, for chromium, iron, manganese, nickel and zinc was 0.025 mg/l and for copper and lead detection limit was 0.010 mg/l. All chemicals used were analytical reagent grade.

Table 4.1: Heavy metal concentration in Ultraspec multi-element aqueous certified reference material, water samples and percentage recoveries.

Metals	Certified values (mg/l)	Obtained values (mg/l)	Recovery (%)
Cd	0.001±0.00	Bdl	-
Cr	0.001±0.00	Bdl	-
Cu	0.001±0.00	Bdl	-
Fe	0.333±1.25	0.309±1.22	92.7
Pb	0.001±0.00	Bdl	-
Mn	0.130±1.08	0.128±1.04	98.4
Ni	0.001±0.00	Bdl	-
Zn	0.001±0.00	Bdl	-

Bdl: below detection limit; -: not available

Heavy metal analysis in sediment

Sediment samples were collected from each site at 20 cm below the water surface using a hand trowel and placed in 250 ml polyethylene sampling bottles. The samples were kept in ice during transportation to the ARU Laboratory. On arrival, samples were immediately transferred to a freezer (-20°C) until analysis. Heavy metals in sediment were determined according to ISO 11466 (1995) method. During analysis, each sample was freeze-dried, finely crushed and homogenized using pestle and mortar. About 0.5 g of the homogenized sample was digested in 10 ml of aqua regia (1:3 HNO_3:$HClO_4$) in a culture test tube. The samples were incubated in a water bath at 80°C until dry. After digestion, the solution was allowed to cool and diluted to 50 ml with deionized water. The solution was then used to determine the concentrations of selected metals using an inductively coupled plasma-optical emission spectrometry (ICP-OES) (Ogoyi *et al*., 2011). Analysis was done in triplicates and one blank containing 10 ml of 65% nitric acid was included. Quality control and quality assurance procedures were conducted by using ultraspec multi-element aqueous certified reference material from De Bruyn spectroscopic solutions. Recoveries ranged from 72.0% to 99.7% (Table 4.2). Detection limit for cadmium, chromium, copper, nickel, lead and zinc was 0.010 mg/kg and for iron and manganese, detection limit was 0.025 mg/kg. All chemicals used were analytical reagent grade.

Sediment particle size was determined by wet sieving technique as described by Switzer and Pile (2014). Sieves with different mesh sizes were stacked in decreasing order from top to bottom (2 mm, 0.5 mm, 0.25 mm, 0.125 mm and 0.062 mm). A weighed sediment sample (100 g) per site was placed on the top sieve stack and water was run through the stacks for 15-20 minutes. Weights of fractions from each sieve were recorded and the fraction with the highest weight was recorded as the dominant sediment particle size. Sediment particle size was determined in triplicates.

Table 4.2: Heavy metal concentration in Ultraspec multi-element aqueous certified reference material, sediment samples and their recoveries.

Metals	Certified values (mg/kg)	Obtained values (mg/kg)	Recovery (%)
Cd	0.25±0.02	0.18±0.01	72.0
Cr	30.15±2.63	28.90±1.99	95.8
Cu	15.22±0.45	14.60±1.28	95.9
Fe	3150.33±5.88	3141.99±4.23	99.7
Pb	5.46±1.20	5.33±1.06	97.6
Mn	200.08±2.60	195.00±1.35	97.4
Ni	9.18±1.28	9.05±1.34	98.5
Zn	23.51±1.00	21.51±1.08	91.4

Geo-accumulation Index

Heavy metal contamination was assessed with the use of the Geo-accumulation Index (*Igeo)*. This index has been widely applied in assessment of soil and sediment contamination. Geo-accumulation Index (Muller, 1969) is calculated using the following equation:

$$Igeo=\log_2\frac{Cn}{1.5\ \mathrm{Bn}} \qquad \text{(Muller, 1969)}$$

Where Cn is the measured concentration of metal in the sediment, Bn is the geochemical background value of the element, and the 1.5 is the factor used to minimize the possible variations in the background values which may be due to lithogenic effects. Geochemical background values used were Fe: 50 000 mg/kg, Mn: 950 mg/kg, Zn: 70 mg/kg, Ni: 75 mg/kg, Cr: 100 mg/kg, Pb: 13 mg/kg, Cu: 55 mg/kg and Cd: 0.2 mg/kg (Krauskopf and Bird, 1995). The Geo-accumulation Index has its specific grades (Table 4.3)

Table 4.3: The Geo-accumulation Index grades (Muller, 1969).

Grades	Description
Igeo≤0	Uncontaminated
0≤*Igeo*≤1	Uncontaminated to moderately contaminated
1≤*Igeo*≤2	Moderately contaminated
2≤*Igeo*≤3	Moderately to heavily contaminated
3≤*Igeo*≤4	Heavily contaminated
4≤*Igeo*≤5	Heavily to extremely contaminated
5<*Igeo*	Extremely contaminated

Enrichment factor (EF)

The extent of heavy metal contamination compared to the background area was assessed using the Enrichment Factor (Buat-Menard and Chesselet, 1979). In this study, iron was used as a reference metal for normalizing metal concentrations. The EF was calculated using the formula below:

$$EF = \frac{X/Fe(Sediment)}{X/Fe(Reference)}$$ (Buat-Menard and Chesselet, 1979)

Where X is the metal assessed and X/Fe is the ratio of the concentration of the assessed metal to iron. The enrichment factor can be categorized into seven classes (Table 4.4). The heavy metal concentrations of sediment at site S1 were used as background reference when calculating EF of sites S2, S3, S4 and S5. Site B1 sediment concentrations were used as background reference when calculating EF of site B2.

Table 4.4: The degree of metal pollution based on seven enrichment factor classes (Taylor, 1964).

EF Value	Designation of sediment quality
50	Extremely severe enrichment
25-<50	Very severe enrichment
10-<25	Severe enrichment
5-<10	Moderately severe enrichment
3-<5	Moderately enrichment
1-<3	Minor enrichment
<1	No enrichment

Heavy metal analysis in grass

Cyperus exaltatus grass was sampled at each site along both the Sand and Blood Rivers. The grass was placed in clean brown paper bags and transported to the ARU Laboratory. Heavy metal analysis in grass was determined according to ISO 11466 (1995) method. Grass samples were dried at 80°C for 48h and ground into a powder. About 0.5 g of the grass sample was placed in a beaker with 20 ml reverse aqua regia (3:1 HNO_3: HCL) and digested on a hot plate for 3 hours at 125°C. The grass samples were then reconstituted with de-stilled water after digestion and the heavy metal concentrations were analyzed using ICP-OES (Bourioug *et al*., 2015). Analysis was done in triplicates and one blank containing 10 ml of 65% nitric acid was included. For quality assurance and quality control, rye grass from Sigma-Aldrich was used as a certified reference material. Recoveries ranged from 70.0% to 95.0% (Table 4.5). Detection limit for cadmium, chromium, copper, nickel, lead and zinc was 0.010 mg/kg and for iron and manganese, detection limit was 0.025 mg/kg. All chemicals used were analytical reagent grade.

Table 4.5: Heavy metal concentration in rye grass certified reference material, grass samples and their recoveries.

Metals	Certified values (mg/kg)	Obtained values (mg/kg)	Recovery (%)
Cd	0.10±0.01	0.07±0.08	70.0
Cr	2.63±0.15	2.20±0.02	83.65
Cu	2.99±0.87	2.48±0.03	82.94
Fe	266.08±1.36	252.85±1.25	95.0
Pb	0.29±0.02	0.22±0.04	75.8
Mn	128.25±2.11	119.52±2.57	93.1
Ni	1.45±0.07	1.33±0.06	91.72
Zn	8.44±1.09	7.41±1.07	87.7

Plant transfer factor (PTF)

The Plant Transfer Factor (PTF) of *Cyperus exaltatus* was calculated using the transfer factor formula below:

$$\mathrm{TF} = \frac{Cplant}{Csoil}$$ (Sheppard and Evenden, 1990)

Where TF is transfer factor, Cplant is the metal concentration in plant (mg/kg) and Csoil is the metal concentration in sediment/soil (mg/kg).

4.3 DATA ANALYSIS

Normality and homogeneity of variance for heavy metal concentration in water, sediment and grass was tested with the use of Shapiro-Wilk and Levene's test, respectively. One-way analysis of variance (ANOVA) was used to test any significant differences in heavy metal concentration in water, sediment and grass across sites in the Sand and Blood Rivers.

4.4 RESULTS

4.4.1 Heavy metal concentration in water, sediment and grass

Heavy metal concentration in water

Cadmium, chromium, copper, lead, nickel and zinc concentrations in water were below detection limit during the rainy season and dry season (Table 4.6a and Table 4.6b).

Iron and manganese concentrations showed spatial variation during the rainy season and dry season (ANOVA, $P<0.05$). During the rainy season, the highest iron concentration was recorded at site S5 (0.477 mg/l), which is the last site downstream. The lowest iron concentration was recorded at the reference site (S1) in Sand River (0.206 mg/l), which was not significantly (ANOVA, $P>0.05$) different to concentration recorded at reference site (B1) in Blood River (0.211 mg/l). A similar trend was observed during the dry season (Table 4.6b). Both iron and manganese concentration increased at sites after points of discharge (Table 4.6a and Table 4.6b).

Iron and manganese concentrations were higher during the rainy season than dry season at all sampling sites (Table 4.6a and Table 4.6b). Manganese concentrations were below the TWQR for aquatic ecosystem during the rainy season and dry season (Table 4.6a and Table 4.6b).

Table 4.6a: Heavy metal concentrations in mg/l from surface water in the Sand and Blood Rivers during rainy season, expressed as means ± SD. Different superscripts in a row shows statistically significant difference (P<0.05, ANOVA). *n*= 84

Sites	S1	S2	S3	S4	S5	B1	B2	TWQR
Heavy metals								
Cadmium	<0.003*	<0.003*	<0.003*	<0.003*	<0.003*	<0.003*	<0.003*	0.00015
Chromium	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	0.012
Copper	<0.010*	<0.010*	<0.010*	<0.010*	<0.010*	<0.010*	<0.010*	0.0003
Iron	0.206±0.10^{a}	0.314±0.11^{b}	0.330±0.06^{c}	0.471±0.07^{d}	0.477±0.17^{d}	0.211±0.11^{a}	0.444±0.13^{e}	-
Lead	<0.010*	<0.010*	<0.010*	<0.010*	<0.010*	<0.010*	<0.010*	0.0002
Manganese	0.146±0.09^{a}	0.154±0.11^{b}	0.155±0.12^{b}	0.166±0.05^{c}	0.168±0.08^{c}	0.153±0.14^{b}	0.162±0.09^{c}	0.180
Nickel	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	-
Zinc	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	0.002

- : Not available, * : no SD because values were below the detection limit in all triplicates

Table 4.6b: Heavy metal concentrations in mg/l from surface water in the Sand and Blood Rivers during dry season, expressed as means ± SD. Different superscripts in a row shows statistically significant difference (P<0.05, ANOVA). *n*= 84

Sites	S1	S2	S3	S4	S5	B1	B2	TWQR
Heavy metals								
Cadmium	<0.003*	<0.003*	<0.003*	<0.003*	<0.003*	<0.003*	<0.003*	0.00015
Chromium	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	0.012
Copper	<0.010*	<0.010*	<0.010*	<0.010*	<0.010*	<0.010*	<0.010*	0.0003
Iron	0.198±0.09^{a}	0.275±0.12^{b}	0.266±0.13^{c}	0.338±0.10^{d}	0.404±0.20^{e}	0.194±0.17^{a}	0.263±0.08^{c}	-
Lead	<0.010*	<0.010*	<0.010*	<0.010*	<0.010*	<0.010*	<0.010*	0.0002
Manganese	0.138±0.04^{a}	0.147±0.05^{b}	0.146±0.07^{b}	0.156±0.06^{c}	0.147±0.07^{b}	0.141±0.00^{a}	0.152±0.04^{c}	0.180
Nickel	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	-
Zinc	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	<0.025*	0.002

- : Not available, * : no SD because values were below the detection limit in all triplicates

Heavy metals in sediment

Sediment fraction

Sampling sites in the Sand River (S1, S2, S3, S4 and S5) were dominated by gravel sediment fraction (>2 mm) and sites in the Blood River (B1 and B2) were dominated by fine sand sediment fraction (>0.125 mm).

Heavy metal concentration in sediment

There was a significant difference ($P<0.05$, ANOVA) in chromium, iron and manganese concentrations in sediment during the rainy season between some sites (Table 4.7a). Cadmium, copper, lead, nickel and zinc showed no significant difference ($P>0.05$, ANOVA) during the rainy season across sites (Table 4.7a).

During the dry season, chromium, copper, iron and manganese were significantly different ($P<0.05$, ANOVA) amongst some sites (Table 4.7b). Cadmium, lead, nickel and zinc were not significantly different ($P>0.05$, ANOVA) across sites (Table 4.7b).

All assessed metals in sediment did not show temporal variation between the rainy season and dry season (Table 4.7a and Table 4.7b).

Cadmium, chromium, copper, lead and zinc concentrations were below the Probable Effect Level (PEL) during the rainy season and dry season (Table 4.7a and Table 4.7b)

Table 4.7a: Heavy metal concentrations (mean ± SD) in mg/kg from sediment at different sampling sites in the Sand and Blood Rivers during the rainy season. Different superscripts in a row shows statistically significant difference (P<0.05, ANOVA). *n*=84

Metals	S1	S2	S3	S4	S5	B1	B2	CCME (PEL)
Cadmium	0.25±0.12^{a}	0.22±0.18^{a}	0.24±0.12^{a}	0.25±0.15^{a}	0.24±0.11^{a}	0.30±0.18^{a}	0.27±0.22^{a}	3.5
Chromium	50.71±7.43^{a}	85.71±6.55^{b}	63.42±4.46^{c}	60.42±8.61^{c}	49.28±7.01^{a}	33.14±9.92^{d}	20.71±6.72^{e}	90
Copper	16.85±4.94^{a}	17.84±4.72^{a}	20.6±1.63^{a}	16.25±7.86^{a}	15.70±2.56^{a}	15.24±3.62^{a}	17.28±5.64^{a}	197
Iron	2568±229.11^{a}	4364.57±198.26^{b}	4902.85±150.67^{c}	3637.71±127.54^{d}	3162.57±129.28^{e}	1743±130.80^{f}	2621.71±116.46^{g}	-
Lead	12.00±3.55^{a}	6.54±2.73^{a}	7.29±3.71^{a}	7.56±2.80^{a}	7.49±2.51^{a}	6.23±2.54^{a}	6.95±4.14^{a}	91.3
Manganese	203.28±0.80^{a}	224.57±2.37^{b}	313.85±10.73^{c}	166.14±9.00^{d}	231.42±9.53^{e}	138.28±11.84^{f}	252.14±8.74^{g}	-
Nickel	10.09±3.33^{a}	9.57±1.26^{a}	10.71±1.49^{a}	10.26±1.68^{a}	10.56±0.78^{a}	12.42±1.61^{a}	11.85±1.06^{a}	-
Zinc	20.00±1.73^{a}	23.14±2.79^{a}	22.42±2.69^{a}	20.00±2.16^{a}	20.42±3.15^{a}	21.42±2.14^{a}	20.57±2.99^{a}	315

PEL: Probable Effect Level; - : not available

Table 4.7b: Heavy metal concentrations (mean ± SD) in mg/kg from sediment at different sampling sites in the Sand and Blood Rivers during the dry season. Different superscripts in a row shows statistically significant difference (P<0.05, ANOVA). *n*=84

Metals	S1	S2	S3	S4	S5	B1	B2	CCME (PEL)
Cadmium	0.21±0.10^{a}	0.19±0.14^{a}	0.26±0.09^{a}	0.24±0.10^{a}	0.22±0.13^{a}	0.25±0.08^{a}	0.22±0.14^{a}	3.5
Chromium	55.63±5.50^{a}	71.71±8.41^{b}	58.21±6.11^{a}	62.12±11.52^{a}	36.60±7.01^{c}	42.11±11.16^{c}	21.61±5.41^{d}	90
Copper	15.12±3.20^{a}	20.61±7.05^{b}	26.30±6.71^{b}	22.25±6.99^{b}	16.58±7.14^{a}	21.33±6.70^{b}	20.11±5.55^{b}	197
Iron	2443±100.12^{a}	3956.55±155.20^{b}	4563.74±122.65^{c}	4005.36±166.41^{d}	3325.99±111.65^{e}	3958±121.63^{b}	4541.28±141.28^{f}	-
Lead	09.24±2.00^{a}	8.13±1.05^{a}	9.21±3.21^{a}	10.36±3.45^{a}	8.33±2.91^{a}	5.02±1.22^{a}	5.21±3.25^{a}	91.3
Manganese	200.63±0.98^{a}	198.21±6.55^{a}	232.71±12.11^{b}	213.01±5.63^{c}	222.23±6.98^{d}	145.09±09.85^{e}	211.65±14.66^{c}	-
Nickel	07.00±4.13^{a}	10.66±3.98^{a}	12.23±2.21^{a}	08.11±0.77^{a}	08.55±0.86^{a}	10.77±3.00^{a}	12.16±2.05^{a}	-
Zinc	21.44±2.25^{a}	20.12±3.88^{a}	22.61±3.25^{a}	21.01±3.41^{a}	20.78±2.14^{a}	20.00±2.69^{a}	22.54±3.28^{a}	315

PEL : Probable Effect Level; - :not available

Geo-accumulation Index

Cadmium showed the highest Geo-accumulation Index values, whilst nickel had the lowest Geo-accumulation values during the rainy season and dry season across sites (Figure 4.1 and Figure 4.2).

The Geo-accumulation Index values of cadmium, copper, lead, manganese, nickel and zinc showed no spatial variation across sites during the rainy season and dry season (Kruskal-Wallis, $P>0.05$). The Geo-accumulation Index for chromium and showed spatial variation amongst sites (Kruskal-Wallis, $P<0.05$) during both the rainy and dry seasons (Figure 4.1 and Figure 4.2).There was no temporal variation in Geo-accumulation index across all sampling sites (Figure 4.1 and Figure 4.2).

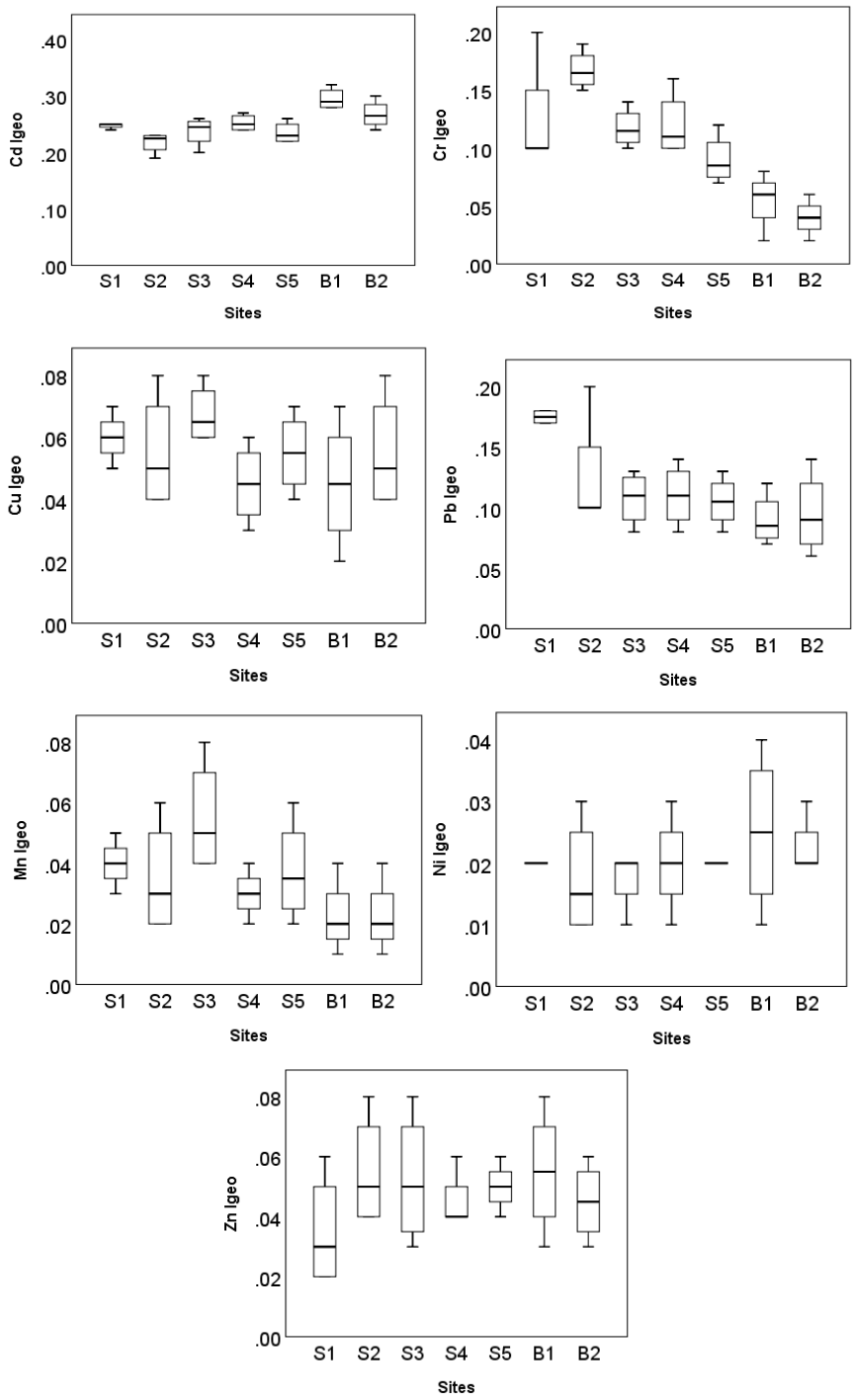

Figure 4.1: Geo-accumulation Index of assessed metals in sediments from the Sand and Blood Rivers during the rainy season.

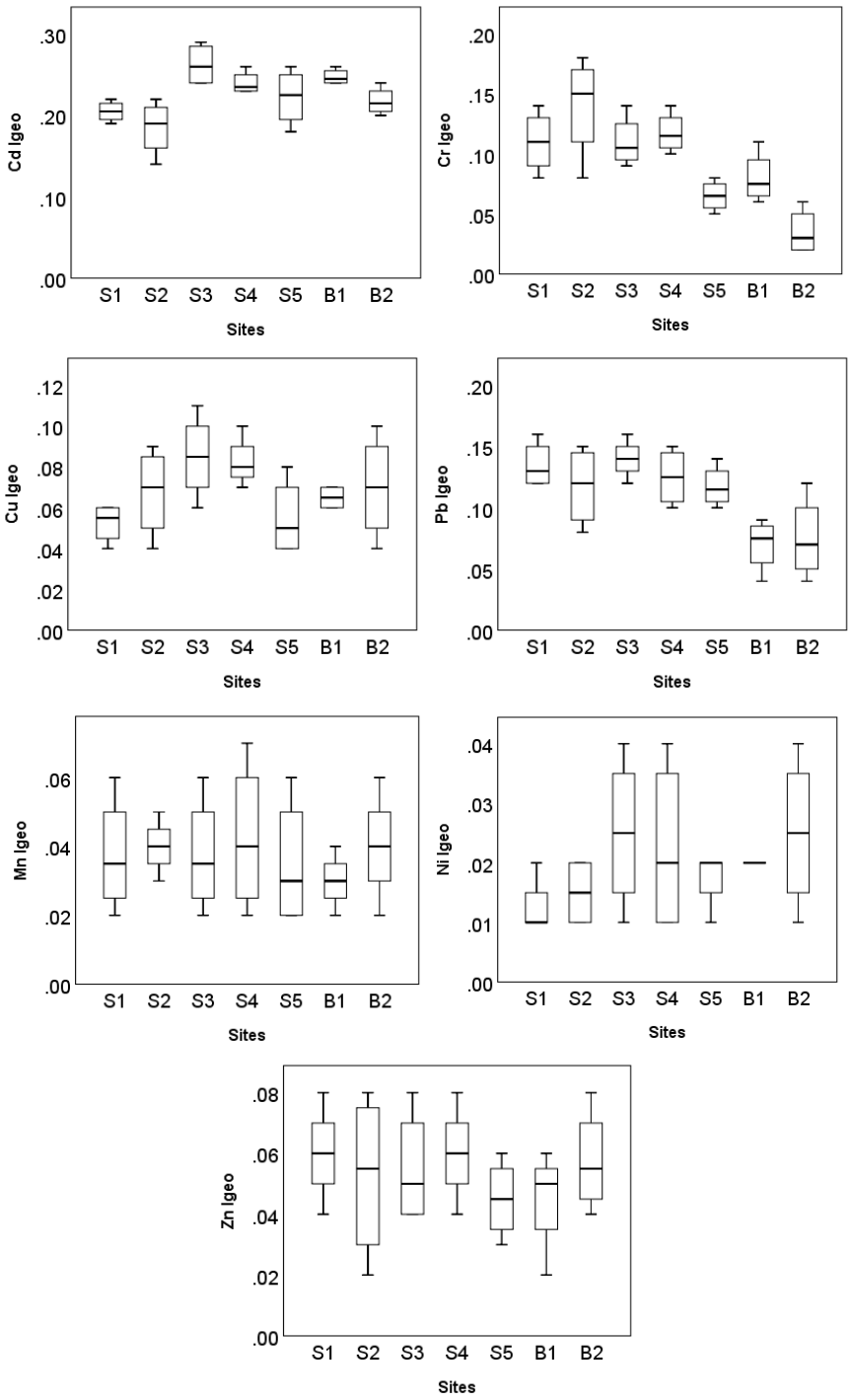

Figure 4.2: Geo-accumulation Index of assessed metals in sediments from the Sand and Blood Rivers during the dry season.

Enrichment factor

Cadmium, chromium, copper, lead, nickel and zinc showed Enrichment Factor below 1 at all sampling sites during the rainy season and dry season (Figure 4.3 and Figure 4.4). Manganese showed Enrichment Factor above 1 only at site B2 during rainy and dry season.

All assessed heavy metals showed spatial variation (Kruskal-Wallis, $P<0.05$) during the rainy season and dry season (Figure 4.3 and Figure 4.4). There was no temporal variation in Enrichment Factor of assessed metals (Figure 4.3 and Figure 4.4).

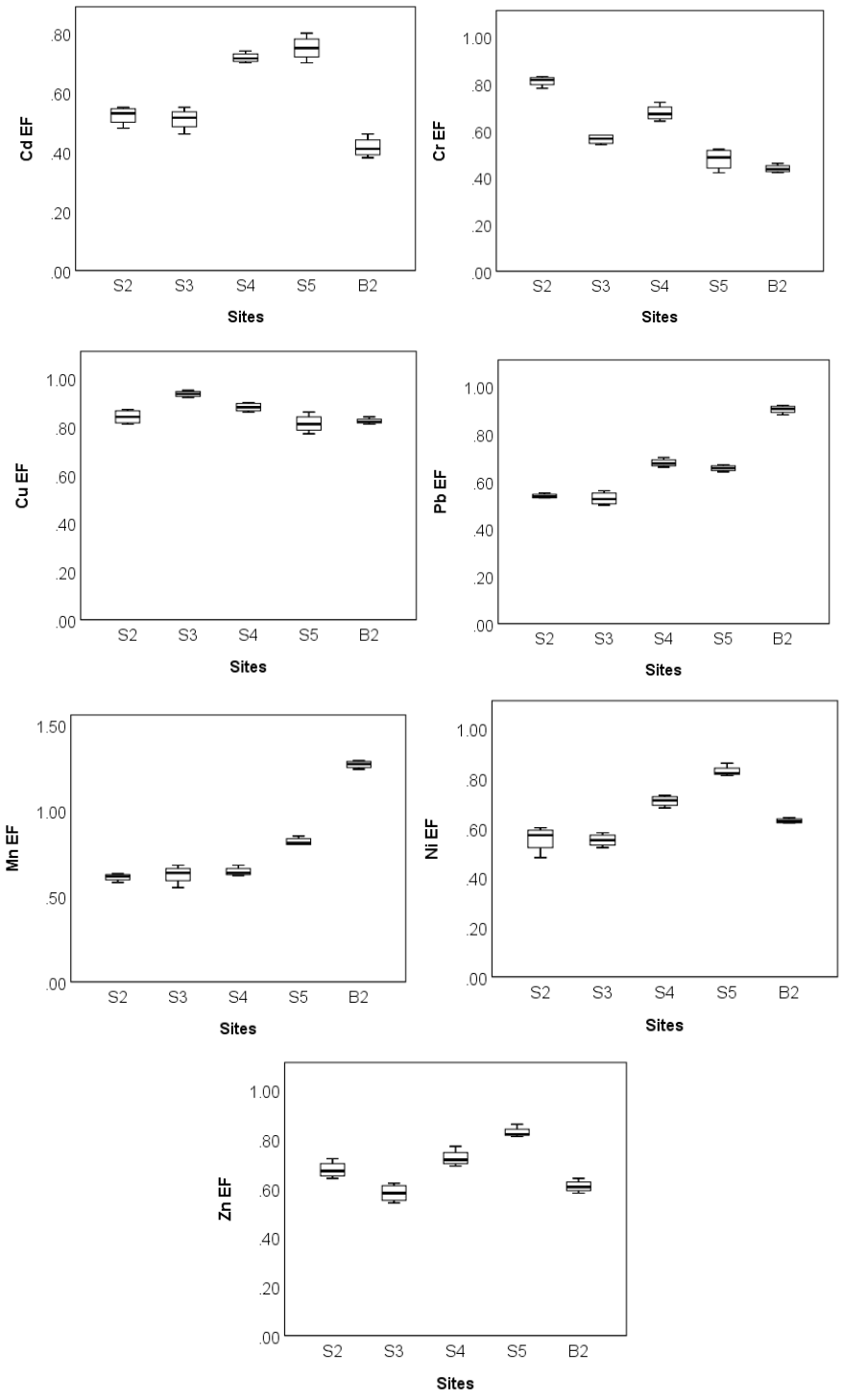

Figure 4.3: Enrichment Factor of assessed metals in sediments from the Sand and Blood Rivers during the rainy season.

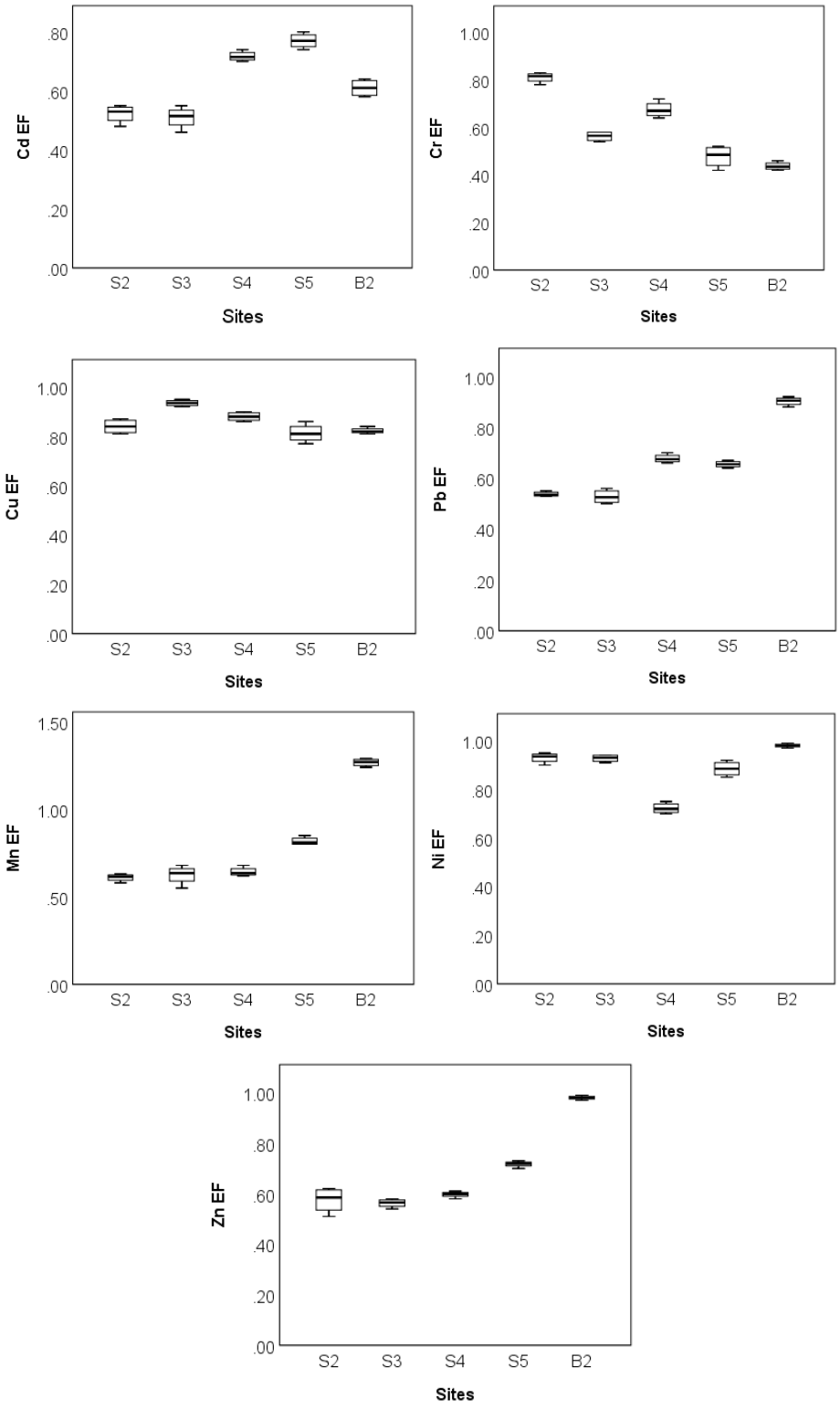

Figure 4.4: Enrichment Factor of assessed metals in sediments from the Sand and Blood Rivers during the dry season.

Heavy metal concentrations in grass

There was a significant difference ($P<0.05$, ANOVA) in iron and manganese concentration during the rainy season and dry season between sites. Cadmium, chromium, copper, lead, nickel and zinc did not differ significantly ($P>0.05$, ANOVA) across sites (Table 4.8a and Table 4.8b).

All assessed heavy metals did not show temporal variation between the rainy season and dry season (Table 4.8a and Table 4.8b).

Table 4.8a: Heavy metal concentrations (mean ± SD) in mg/kg from grass at different sampling sites. Different superscripts in a row shows statistically significant difference during the rainy season (P<0.05, ANOVA). *n*= 84

Heavy metals	S1	S2	S3	S4	S5	B1	B2
Cadmium	0.07±0.03^{a}	0.08±0.02^{a}	0.06±0.03^{a}	0.07±0.065^{a}	0.07±0.01^{a}	0.08±0.01^{a}	0.07±0.02^{a}
Chromium	1.36±0.67^{a}	1.45±0.84^{a}	1.20±0.13^{a}	1.45±0.89^{a}	1.33±1.00^{a}	1.30±0.43^{a}	1.39±0.67^{a}
Copper	2.57±1.14^{a}	2.66±0.82^{a}	2.67±1.13^{a}	2.58±1.33^{a}	2.28±1.17^{a}	2.27±0.60^{a}	2.48±1.27^{a}
Iron	243.14±25.58^{a}	371.14±14.98^{b}	165.71±9.89^{c}	155.85±24.92^{d}	252.14±31.96^{e}	356.85±11.45^{f}	357.00±26.96^{f}
Lead	0.27±0.08^{a}	0.19±0.11^{a}	0.28±0.12^{a}	0.22±0.07^{a}	0.25±0.17^{a}	0.21±0.18^{a}	0.23±010^{a}
Manganese	150.71±23.92^{a}	141.00±26.37^{b}	130.71±18.53^{c}	211.85±2.67^{d}	201.00±2.88^{e}	119.42±39.66^{f}	114.71±20.54^{f}
Nickel	1.51±0.33^{a}	1.45±0.46^{a}	1.59±0.62^{a}	1.44±0.92^{a}	1.42±1.11^{a}	1.46±0.77^{a}	1.57±0.77^{a}
Zinc	5.14±1.01^{a}	5.71±2.21^{a}	6.85±3.13^{a}	7.00±1.91^{a}	6.85±5.33^{a}	7.42±2.69^{a}	5.71±2.62^{a}

Table 4.8b: Heavy metal concentrations (mean ± SD) in mg/kg from grass at different sampling sites. Different superscripts in a row shows statistically significant difference during the dry season (P<0.05, ANOVA). *n*= 84

Heavy metals	S1	S2	S3	S4	S5	B1	B2
Cadmium	0.06±0.01^{a}	0.10±0.02^{a}	0.08±0.03^{a}	0.07±0.02^{a}	0.06±0.01^{a}	0.09±0.03^{a}	0.06±0.02^{a}
Chromium	1.28±0.21^{a}	1.36±0.19^{a}	1.19±0.11^{a}	1.36±0.15^{a}	1.44±1.22^{a}	1.36±0.17^{a}	1.29±0.30^{a}
Copper	3.01±0.06^{a}	2.79±0.11^{a}	2.61±1.08^{a}	2.65±0.10^{a}	2.87±1.14^{a}	2.52±0.14^{a}	2.49±1.20^{a}
Iron	255.22±9.25^{a}	360.10±9.11^{b}	164.45±6.77^{c}	159.88±11.52^{c}	244.13±14.14^{d}	346.23±19.28^{e}	357.36±17.08^{b}
Lead	0.20±0.04^{a}	0.22±0.13^{a}	0.21±0.11^{a}	0.23±0.09^{a}	0.27±0.12^{a}	0.21±0.17^{a}	0.21±08^{a}
Manganese	144.52±15.96^{a}	151.88±14.09^{b}	122.46±14.11^{c}	200.13±10.00^{d}	209.18±16.52^{e}	125.15±19.00^{c}	117.22±18.79^{f}
Nickel	1.36±0.14^{a}	1.47±0.16^{a}	1.60±0.19^{a}	1.49±0.22^{a}	1.45±0.08^{a}	1.61±0.12^{a}	1.55±0.23^{a}
Zinc	7.22±1.17^{a}	6.48±1.88^{a}	5.74±1.61^{a}	6.10±1.00^{a}	5.88±3.19^{a}	7.96±1.02^{a}	5.00±2.58^{a}

Plant transfer factor (PTF)

The PTF values for cadmium, iron, manganese and zinc showed spatial variation (Kruskal-Wallis, $P<0.05$) amongst some of the sampling sites in Sand and Blood Rivers during the rainy season (Figure 4.5), whilst the PTF values for chromium, copper, lead and nickel showed no spatial variation (Kruskal-Wallis, $P>0.05$) (Figure 4.5).

During the dry season, the PTF values of cadmium, manganese and zinc showed spatial variation (Figure 4.6).

The PTF values for all metals assessed showed no temporal variation between the rainy season and dry season (Figure 4.5 and Figure 4.6)

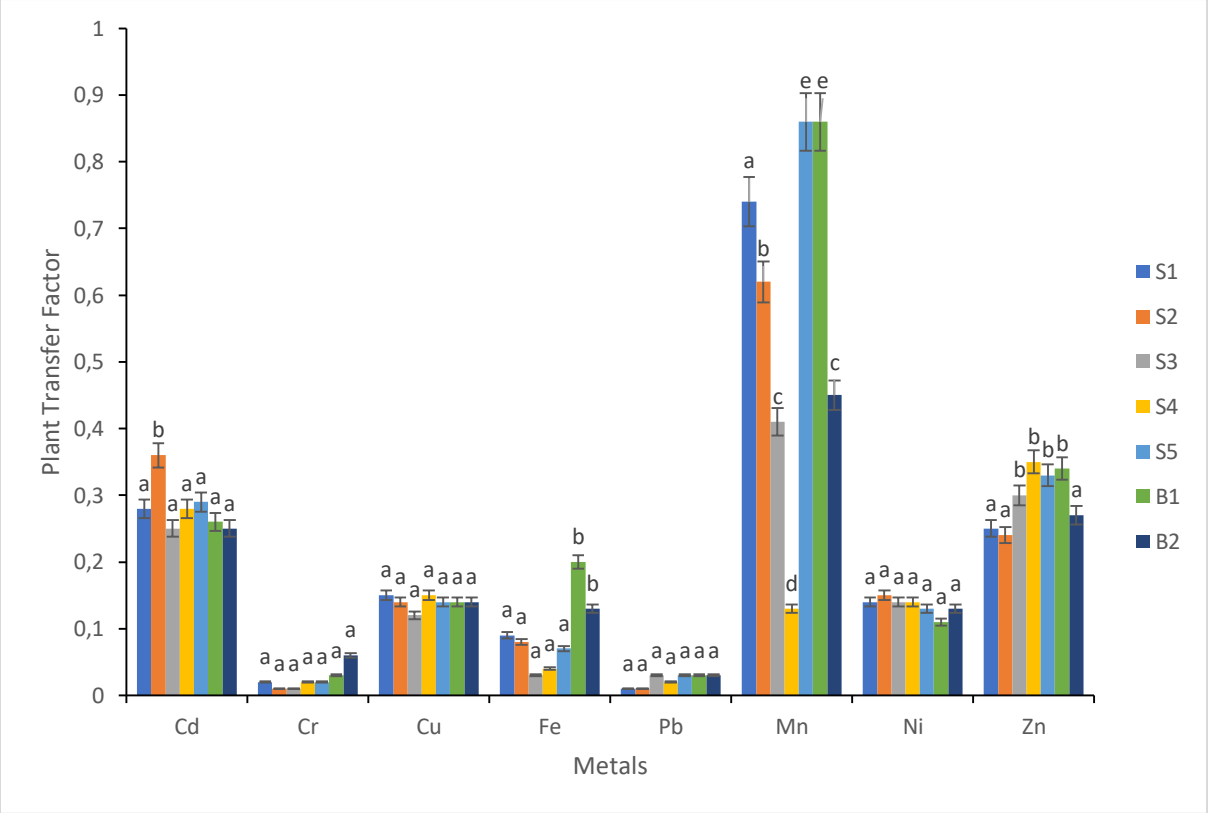

Figure 4.5: The plant transfer factor values for all assessed metals at sampling sites in the Sand and Blood Rivers during the rainy season. Error bars denotes standard error (*n*=84).

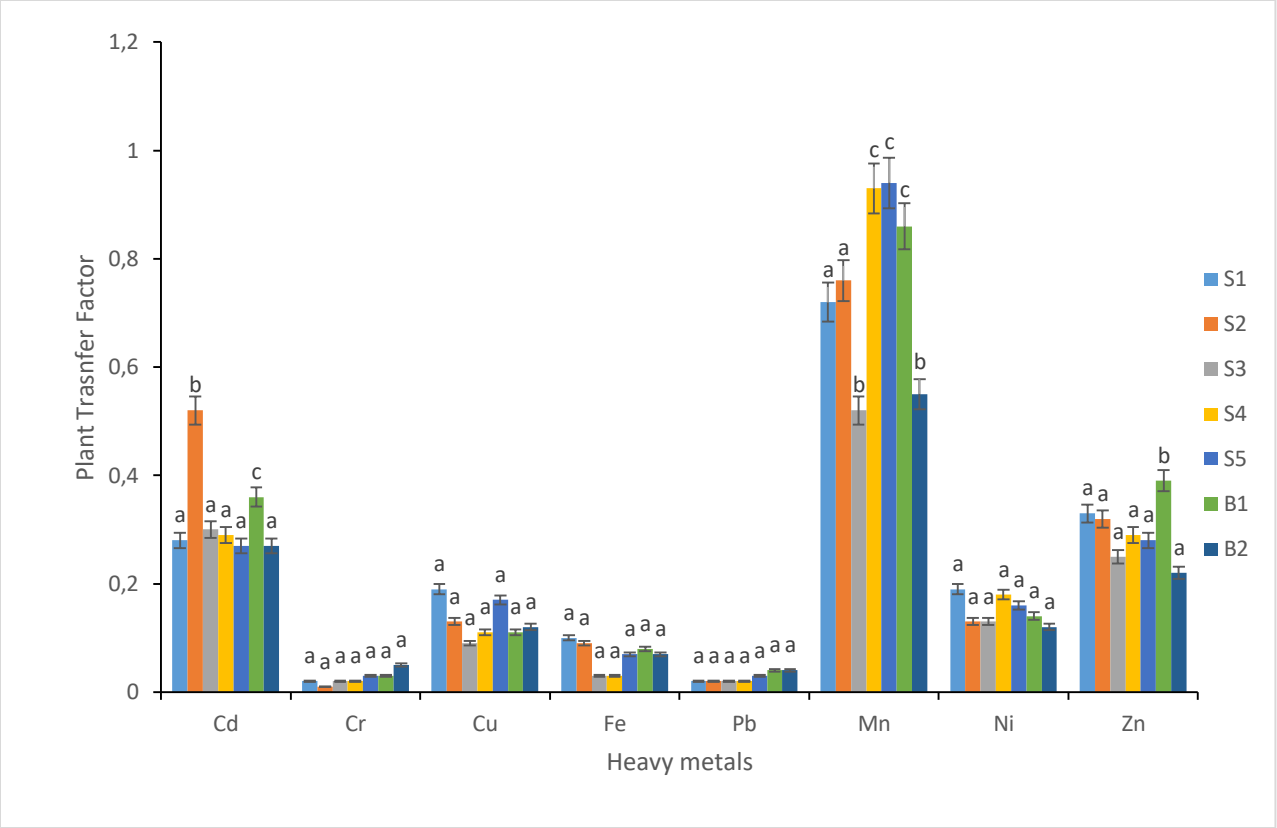

Figure 4.6: The plant transfer factor values for all assessed metals at sampling sites in the Sand and Blood Rivers during the dry season. Error bars denotes standard error (*n*=84).

4.5 DISCUSSION

Heavy metal concentrations in water were assayed across all sampling sites in the Sand and Blood Rivers. All metals assayed in water were below the detection limit, with an exception of iron and manganese during both the rainy and dry seasons. The Polokwane City and Seshego Township have no major industries that has the potential to discharge traces of heavy metals into the Sand and Blood Rivers. This may be the reason most metals assessed were below the detection limit in water. The spatial variation in iron and manganese may be due to their dominance in the composition of heavy metals assessed. The dominance of iron and manganese may also be due to local lithology (Fosso-Kankeu *et al*., 2017). Moreover, the chemistry of iron is closely related with that of manganese (DWAF, 1996a). The dominance of iron and manganese was also observed by Moyo and Rapatsa (2019) in the Sand River. Although manganese concentration dominated the metal composition in these rivers, its concentration was below the TWQR for aquatic ecosystem during both seasons. Iron and manganese concentration were higher during the rainy season than dry season. This is because during the rainy season, metals are readily available in water due to bioturbation. Moreover, high water flow during the rainy season may release iron and manganese traces that has precipitated out during low flow periods (Greenfield *et al*., 2012).

Since heavy metal contamination is better monitored in sediment than water (Nyamangara *et al*., 2008), sediments from the Sand and Blood Rivers were also assessed across all sampling sites. A spatial variation was observed in iron, chromium and manganese between sites during the rainy season and dry season. Polokwane City has a number of stormwater channels that discharge into the Sand River through the Sterkloop Spruit and open stormwater channels. Stormwater may contain traces of metals from tar roads, fuel garages and surface run-off from the Silicon and Platinum Smelters in Polokwane. However, there was no notable difference in metal concentration of sediment from the reference sites and from sites after discharge. This shows that WWTP's are not the major source of heavy metal concentration recorded in sediments. It is then speculated that the source of metals in sediments is the chemical geological

weathering. Iron and manganese continued to dominate the metal composition in sediments as observed in water.

Heavy metal concentration recorded in sediments was higher than those recorded in surface water. This trend is very common in heavy metal studies (Nhiwatiwa *et al*., 2011; Sultan and Shazili, 2009). Heavy metal concentration often occurs in greater quantity in sediments than in water (Dalu *et al*., 2017; Akele *et al*., 2016). This is because metals tend to amass in bottom sediment (Bhuyan *et al*., 2017; Nobi *et al*., 2010). Moreover, sediments act as a sink of heavy metals and can influence the concentration of metals in both water and biota (Milenkovic *et al*., 2005).

Although sediment has been reported to indicate heavy metal pollution better than water, South Africa has no guideline for heavy metals in sediments. Hence, the Canadian sediment quality guidelines for the protection of aquatic life were used to test the compliance of heavy metal concentrations in sediments from the Sand and Blood Rivers (CCME, 2001b). All metals across sampling sites, with an exception of iron, manganese and nickel were below the (PEL during the rainy season and dry season. The PEL is the lower limit of the range of metal concentrations that is usually associated with adverse biological effects. The PEL of iron, manganese and nickel are not available in the Canadian sediment guidelines for the protection of aquatic life (CCME, 2001b). The compliance of heavy metal concentration in sediments confirms that the Sand and Blood Rivers are not contaminated with heavy metals. Moreover, sampling sites on the Sand and Blood Rivers were dominated by gravel and sand sediment particle sizes which have low surface area compared to silt/clay fraction. Gravel and sand fractions are poor in adsorbing metals.

Contamination of heavy metals in the Sand and Blood Rivers was further assessed by Geo-accumulation Index. All assessed heavy metals had Geo-accumulation Index below 1 at all sampling sites. According to Geo-accumulation Index grades, values between 0 and 1 implies that there is no metal contamination in that particular ecosystem (Muller, 1969). These findings are similar to those observed in a previous study conducted in Sand River (Moyo and Rapatsa, 2019). However, the study did not further assess the source of heavy metal concentration recorded in sediments. In the current

study, this was achieved by assessing the Enrichment Factor. All assessed heavy metal concentration in sediment had Enrichment Factor values below 1 at all sites, with an exception of manganese at site B2 during the rainy season and dry season. The EF values below 1 implies that heavy metal concentration recorded in sediments from the Sand and Blood Rivers were naturally enriched from geological weathering. Enrichment of heavy metals from local background is very common in aquatic ecosystems (Binning and Baird, 2001; Singh *et al*., 2017; Davies *et al*., 1991). This observation was the same during both seasons. The EF values below 1 are in conformity with the Geo-accumulation Index findings. However, the EF further showed that manganese had minor enrichment, which means the recorded manganese concentration at site B2 was enriched from anthropogenic activities. The South African water quality guidelines for aquatic ecosystems stated that, elevated levels of manganese in aquatic ecosystem may be due to steel, fertilizer and chemical industries discharge (DWAF, 1996a). It is important to note that Site B2 has informal settlement, commercial farms and a number of illegal panel beating garages on its upstream. It is thus speculated that these activities may be the source of elevated levels of manganese at this site. Geo-accumulation and EF values of all metals were not influenced by season.

Heavy metal contamination may be abated by using aquatic plants in a process called phytoremediation (Ebenebe *et al*., 2017; Matache *et al*., 2013). *Cyperus exaltatus* was assessed for metal concentration and spatial variation was observed. However, the plant showed PTF values below 1 for all heavy metals assessed across sites during rainy season and dry season. A PTF below 1 suggests that a plant is not a good candidate for phytoremediation. Of all metals assessed, manganese, zinc and cadmium were easily bioaccumulated by *Cyperus exaltatus*. Different plant species bioaccumulate metals at different concentration. Plants such as *Typha capensis* and *Cyperus articulates* have been reported to have PTF values above 13 (Mganga *et al*., 2011). A good candidate plant for phytoremediation is a plant that has high growth rate, widely branched shoot, high bioaccumulation and translocation capacity, easy to cultivate and harvest (Usman *et al*., 2018). It is thus suggested that other plant species be assessed for phytoremediation.

Evaluation of heavy metal contamination in Sand and Blood Rivers showed that these two rivers are not contaminated with heavy metals. However, the current study did not

assess the bioaccumulation of heavy metals in fish species and also the human health risk. People from surrounding areas fish from the Sand and Blood River for family consumption. However, the human health risk of consuming fish, especially *Clarias gariepinus* from the Sand and Blood River is unknown. It is thus recommended that such studies be conducted in these rivers.

CHAPTER 5: THE USE OF MACROINVERTEBRATES IN ASSESSING POLLUTION STATUS OF THE SAND AND BLOOD RIVERS IN LIMPOPO PROVINCE, SOUTH AFRICA.

5.1 INTRODUCTION

Aquatic pollution has been primarily monitored by analyzing physico-chemical parameters (Davies and Day, 1998; Roux *et al*., 1993). This approach was used to assess the pollution status of the Sand River and Blood River in the current study (Chapter 3), and it showed that these rivers are degraded, due to anthropogenic activities. However, this approach is costly and only reflects momentary conditions of aquatic ecosystems without showing the effect of pollution on the biota (Rosenberg and Resh, 1993). In developing countries such as South Africa, aquatic monitoring may be limited due to financial constraints. This prompted the use of alternative methods such as biological indicators. Biological indicators are cost effective and reflect the ecological integrity by integrating the effects of different pollutants over time (Gyedu-Ababio and van Wyk, 2004).

One of the most used aquatic biota in assessing the health status of aquatic ecosystems is macroinvertebrates (Hawkes, 1998; Chutter, 1995; Metcalfe-Smith, 1994; Dallas and Day, 1993; Hilsenhoff, 1988; Washington, 1984). Macroinvertebrates have been primarily used as biological indicators of organic pollution (Dickens and Graham, 2002; Hilsenhoff, 1988; Davis, 1995; Washington, 1984; Fenoglio *et al*., 2002). Thus, macroinvertebrates may be useful biological indicators in the Sand and Blood Rivers, which are severely polluted by organic pollution. Benthic macroinvertebrate communities are easily affected by alterations in water quality. They are ubiquitous, sedentary in nature and different taxa respond differently to a variety of pollutants (Odume and Muller, 2011; Bonada *et al*., 2006; Rosenberg and Resh, 1993; Dallas, 1995).

Different biotic indices such as the SASS, ASPT, Shannon Weiner diversity Index (H') and the FBI have been used in biomonitoring studies using macroinvertebrates (Dickens and Graham, 2002; Chutter, 1998; Hilsenhoff 1988). The SASS, which is the commonly used biotic index in biomonitoring studies in Southern Africa, was developed to assess

organic pollution, which makes it a suitable tool to use in the Sand and Blood Rivers. Each taxon in SASS has been allocated a sensitivity score, based on their sensitivity or tolerance to pollution (Dickens and Graham, 2002). Taxa such as Chironomidae, Oligochaeta and Culicidae have been allocated low sensitivity score due to their tolerance to a wide range of pollutants (Dickens and Grahams, 2002). The Family-level Biotic Index (FBI), which is extensively used in North America, was also developed to evaluate organic and nutrient pollution in freshwater streams (Hilsenhoff, 1988; Reynoldson & Metcalfe-Smith, 1992). The Sand and Blood Rivers receives substandard sewage effluent from the Polokwane and Seshego WWTP's. Thus, the FBI may also be a useful biotic index in assessing pollution status in Sand and Blood Rivers. The FBI estimates the total tolerance of the community in a sampled site, weighted by the relative abundance of each taxonomic group.

Although macroinvertebrates have been successfully used as biological indicators, most studies have focused on spatial variation, while temporal variation has been largely ignored (Cox *et al*., 2019; Mereta *et al*., 2019; Muli, 2005; Niba and Mafereke, 2015; Dlamini *et al*., 2010; Munyika *et al*., 2014). Dallas (2007) stated that season plays a major role in macroinvertebrates assemblage. It is important to also determine the temporal variation of benthic macroinvertebrates in aquatic ecosystems.

The objective of this study was to determine spatial and temporal variation of macroinvertebrates abundance and diversity in Sand and Blood Rivers and also to determine the relationship between water quality parameters and macroinvertebrates assemblage in Sand and Blood Rivers.

5.2 MATERIALS AND METHODS

5.2.1 Macroinvertebrates sampling

Macroinvertebrates were collected at different sites as depicted in (Figure 3.1: Chapter 3) in Sand and Blood Rivers once a month during rainy season (January to April, 2018) and dry season (May to August, 2018). Sampling was conducted according to the South African scoring system, Version 5 (SASS5) sampling protocol (Dickens and Graham, 2002). A hand net with a mesh size of 2 mm, secured to a 30 cm square frame was used to collect macroinvertebrates.

Samples were collected from the three pre-defined biotope groups (i.e. stones in-current; marginal and aquatic vegetation and sand in current) and analyzed separately. As per protocol, stones-in-current (SIC) were sampled for two minutes, gravel, sand and mud (GSM) for a total of one minute, while marginal and aquatic vegetation were sampled for two minutes. Macroinvertebrates were collected at 1 square meter at each site. Samples collected at each site from each of the biotopes were placed in separate trays for sorting and identification. Trays of approximately 30 × 45 cm in size with a depth of 10 cm, were used. Each biotope was assessed for 15 minutes. Macroinvertebrates were identified and scored on the SASS5 score sheet. After recording the samples on the SASS5 score sheet, all macroinvertebrates were counted, and the total number of macroinvertebrates collected at each site was recorded. Unidentified samples were preserved in 10% formalin for identification in the laboratory. Uncertain organisms were confirmed using optical microscope. Identification was undertaken to the family level, using a photographically illustrated identification guide and aquatic invertebrates of South African rivers field guide (Gerber and Gabriel, 2002).

5.2.2 The South African Scoring System and Average Score per Taxon determination

The South African Scoring System (SASS) and Average Score per Taxon (ASPT) were calculated according to Dickens and Graham (2002). The SASS score was calculated by summing the sensitivity scores of all taxa collected at each site. The ASPT was calculated by dividing the SASS5 score with the number of taxa collected at each site. The SASS

scores and ASPT were interpreted with the use of biological bands classification derived from the Limpopo plain ecoregion as depicted by Dallas (2007) (Figure 5.1; Table 5.1).

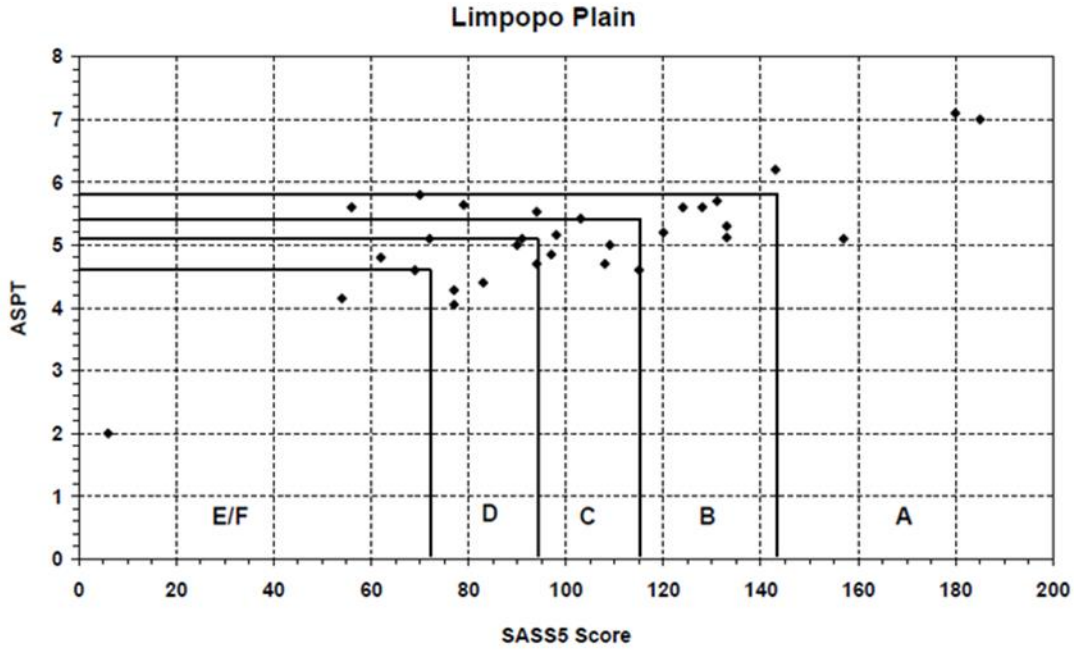

Figure 5.1: Biological bands for interpreting SASS5 and ASPT in the Limpopo Plain aquatic ecoregion (Dallas, 2007).

Table 5.1: Biological Bands (A-F), SASS5 score and ASPT range used to categorize the water quality condition in Sand and Blood Rivers.

Biological Band	Ecological condition description	SASS5 Score range	ASPT values range
A	Unmodified/ Natural	>144	>5.8
B	Largely natural with few modifications	115-143	5.4-5.7
C	Moderately modified	94-114	5.1-5.3
D	Largely modified	72-93	4.6-5.0
E/F	Seriously/ critically modified	<71	<4.6

5.2.3 Macroinvertebrates abundance

Macroinvertebrates abundance was calculated by summing the total number of all individuals collected per square meter at each site.

Macroinvertebrates abundance was further used to assess the water quality condition in Sand and Blood Rivers sampling sites using the Family-level Biotic Index (FBI) (Table 5.2). The FBI was calculated using the equation below:

$$FBI = \sum \frac{xi.ti}{n}$$

Where *xi* is the number of individuals in the taxon, *ti* is the tolerance score for taxon and *n* the total number of individuals in a sample (Hilsenhoff, 1988).

Table 5.2: Water quality classification system based on the Family-level Biotic Index values (Hilsenhoff, 1988).

Family Biotic Index	Water quality	Degree of organic pollution
0.00-3.75	Excellent	Organic pollution unlikely
3.76-4.25	Very good	Possible slight organic pollution
4.26-5.00	Good	Some organic pollution probable
5.01-5.75	Fair	Fairly substantial pollution likely
5.76-6.50	Fairly poor	Substantial pollution likely
6.51-7.25	Poor	Very substantial pollution likely
7.26-10.00	Very poor	Severe organic pollution likely

5.2.4 Macroinvertebrates diversity and evenness.

The diversity of macroinvertebrates at each site in both rivers was determined using the Shannon Wiener diversity index (Shannon and Wiener, 1949). The index was calculated using the following equation:

$$H' = \frac{n \log n - \sum(fi \log fi)}{\mathrm{n}}$$

Where H' is the Shannon diversity Index, n is the total number of frequencies and fi is the frequency of occurrence.

5.3 STATISTICAL ANALYSIS

Data was tested for normality and homogeneity of variance using Shapiro-Wilk test and Levene's test, respectively. Kruskal-Wallis (non-parametric test) was used to test the significant difference ($P<0.05$) in SASS scores, ASPT and Shannon Weiner Diversity Index across sampling sites in Sand and Blood Rivers using SPSS version 25.

Mann-Whitney test was used to test the significant difference ($P<0.05$) in SASS scores, ASPT and Shannon Weiner Diversity between the rainy season and dry season across sites.

The canonical correspondence analysis (CCA) was used to determine the relationship between the selected water quality parameters and macroinvertebrates assemblage. The relationship was statistically analyzed using CANOCO 4.5 package (ter Braak and Šmilauer, 2002). Physico-chemical parameters used were water temperature, pH, dissolved oxygen (DO), biological oxygen demand (BOD), turbidity, total dissolved solids (TDS), phosphorus (P), total nitrogen (N) and ammonia (NH_3) (Table 3.2a and Table 3.2b). Macroinvertebrates taxa with less than 10 individuals were excluded from the analysis.

5.4 RESULTS

5.4.1 The South African Scoring System and Average Score Per Taxon.

The SASS score and ASPT showed spatial variation during the rainy season and dry season (Kruskal-Wallis, $P<0.05$) (Table 5.3). The reference site in the Blood River (B1) and the reference site in the Sand River (S1) recorded the highest SASS score during the rainy season (Table 5.3), whilst the highest ASPT was recorded at sites B1 and S4, respectively (Kruskal-Wallis, Table 5.3). The lowest SASS score was recorded at S3, which was not significantly different ($P>0.05$) to sites immediately after points of discharge (S2 and B2). Moreover, the lowest ASPT was also recorded at S3 during the rainy season (Table 5.3). During dry season, the reference sites also recorded the highest SASS score and ASPT (Table 5.3). The site at the point of confluence also recorded the lowest SASS score, which was not significantly different (Kruskal-Wallis, $P>0.05$) to sites S2, B2 and S4.

The SASS and ASPT scores where higher during the rainy season than dry season at all sampling sites in the Sand and Blood Rivers (Table 5.3).

According to the biological bands (Table 5.3), all sampling sites in the Sand and Blood Rivers were categorized as E/F, which shows a critically modified ecological condition (Table 5.3).

Table 5.3: The SASS and ASPT scores (mean ± SD) and biological band recorded in Sand and Blood Rivers during rainy and dry seasons. Different superscripts in a column shows statistically significant difference (P<0.05, Kruskal-Wallis).

	Rainy season			Dry season		
Sites	**SASS**	**ASPT**	**Biological band**	**SASS**	**ASPT**	**Biological band**
S1	26.75±7.27[a]	3.93±0.51[a]	E/F	22.50±2.64[a]	3.48±0.58[a]	E/F
S2	9.75±6.02[b]	3.58±1.62[a]	E/F	2.25±0.50[b]	1.50±1.00[b]	E/F
S3	6.00±4.76[b]	2.18±0.85[b]	E/F	1.25±0.50[b]	1.00±0.00[b]	E/F
S4	17.25±7.13[a]	4.40±0.49[a]	E/F	5.00±1.82[b]	2.33±1.08[a]	E/F
S5	20.00±5.59[a]	3.81±0.14[a]	E/F	11.25±3.30[a]	2.98±0.51[a]	E/F
B1	27.50±5.68[a]	4.61±0.32[a]	E/F	24.50±10.50[a]	4.57±0.86[a]	E/F
B2	9.50±4.04[b]	2.45±0.86[b]	E/F	1.50±0.57[b]	1.00±00[b]	E/F

5.4.2 Macroinvertebrates abundance in Sand River and Blood River.

A total number of 27 taxa from 10 orders were recorded during the rainy season in Sand River and Blood River sampling sites (Table 5.4). During the rainy season, macroinvertebrates abundance followed the order B1>S5>S1>S4>S2>B2>S3 (Table 5.4). Site S1 was dominated by oligochaeta and Chironomidae taxa, whilst site B1 was dominated by Atyidae and Coenagrionidae taxa (Table 5.4). The sites after points of discharge (S2 and B2) where both dominated by Chironomidae and Dytiscidae taxa. The site at the point of confluence was dominated by Chironomidae and Oligochaeta taxa. Site S4 was dominated by Chironomidae and Dytiscidae taxa, whilst the last site downstream was dominated by Chironomidae and Culicidae taxa (Table 5.4).

During the dry season, 26 taxa from 10 orders were recorded (Table 5.5). Macroinvertebrates abundance followed the order B1>S1>S5>B2>S4>S2>S3 (Table 5.5). The reference site in the Sand River (S1) was dominated by Coenagrionidae and Pleidae taxa, whilst the reference site in the Blood River was dominated by Atyidae and Oligochaeta (Table 5.5). Sites after points of discharge (S2 and S3) and a site after point

of confluence where dominated by Culicidae and Syrphidae taxa. Sites S4 and S5 where both dominated by Culicidae and Chironomidae taxa (Figure 5.5).

The total number of macroinvertebrates collected was higher (1673/m^2) during the rainy season than during the dry season (988/m^2).

Table 5.4: Macroinvertebrates abundance (no/ m^2) along Sand and Blood Rivers sampling sites during the rainy season.

Order	Taxa	S1	S2	S3	S4	S5	B1	B2
ANNELIDA	Oligochaeta	71	34	33	25	45	63	
CRUSTACEA	Potamonautidae	17					13	
	Atyidae						149	
PLECOPTERA	Perlidae						26	
EPHEMEROPTERA	*Baetidae* sp.	10			1			
ODONATA	Calopterygidae	3					31	
	Coenagrionidae	33			16	9	73	
	Platycnemidae	21				2		
	Aeshnidae	4			2			
	Gomphidae	14						
	Libellulidae						7	
HEMIPTERA	Belostomatidae				25	10	4	
	Gerridae	12						
	Naucoridae					2		
	Pleidae	8						
TRICHOPTERA	*Hydropsychidae* sp.		5					
COLEOPTERA	Dytiscidae	7	43		30	32		45
	Elmidae		2		6	2		
	Gyrinidae					5		
	Haliplidae							7
DIPTERA	Ceratopogonidae		22			4		
	Chironomidae	63	64	46	86	145	30	43
	Culicidae	21	20		25	70	16	23
	Empididae	2						
	Syrphidae		1	2				23
GASTROPODA	Lymnaeidae						4	
	Physidae	9				1	6	
SASS		26.75	9.75	6.00	17.25	20.00	27.50	9.50
ASPT		3.93	3.58	2.18	4.40	3.81	4.61	2.45
Total abundance		295	191	81	216	327	422	141

Table 5.5: Macroinvertebrates abundance (no/ m^2) along Sand and Blood Rivers sampling sites during the dry season.

Group	Taxa	S1	S2	S3	S4	S5	B1	B2
ANNELIDA	Oligochaeta	19					35	
CRUSTACEA	Potamonautidae	11					4	
	Atyidae						189	
PLECOPTERA	Perlidae							
EPHEMEROPTERA	*Baetidae* sp.	12					28	
ODONATA	Calopterygidae						28	
	Coenagrionidae	80						
	Platycnemidae	10					4	
	Aeshnidae							
	Gomphidae							
	Libellulidae	1					24	
HEMIPTERA	Belostomatidae						8	
	Corixidae						4	
	Gerridae	24					1	
	Nepidae							
	Notonectidae	6				3		
	Pleidae	44				1	4	
	Veliidae	3						
TRICHOPTERA	*Hydropsychidae* sp.							
COLEOPTERA	Dytiscidae	16	1			26		
DIPTERA	Chironomidae	33			13	61	5	
	Culicidae	18	7	7	28	79		62
	Simuliidae				5	6		
	Syrphidae		22	6	8	8		25
GASTROPODA	Physidae						3	
	Thiaridae						6	
SASS		22.50	2.25	1.25	5.00	11.25	24.50	1.50
ASPT		3.48	1.50	1.00	2.33	2.98	4.57	1.00
Total abundance		277	30	13	54	184	343	87

The Family-level biotic Index (FBI) showed that the water quality during the rainy season was fairly poor at all sampling sites, with an exception of sites S3 and B2 (Table 5.6). Site S3 had a fair water quality whilst site B2 had very poor water quality. The FBI further showed that the water quality condition during the dry season was poor at the reference site in Sand River and fair at reference site in Blood River (Table 5.6). All sites after points of discharge were categorized as very poor during the dry season.

Table 5.6: The water quality condition in the Sand and Blood Rivers based on the Family-level Biotic Index.

	Rainy season		**Dry season**	
Sites	**Water quality Condition**	**FBI values**	**Water quality Condition**	**FBI values**
S1	Fairly poor	5.88	Poor	7.13
S2	Fairly poor	5.97	Very poor	9.51
S3	Fair	5.69	Very poor	8.92
S4	Fairly poor	6.32	Very poor	7.79
S5	Fairly poor	6.40	Very poor	7.28
B1	Fairly poor	6.23	Fair	5.66
B2	Very poor	7.51	Very poor	8.57

5.4.3 Macroinvertebrates diversity in Sand and Blood Rivers.

During the rainy season, the reference sites (S1 and B1) recorded the highest macroinvertebrates diversity (Figure 5.2). Sites immediately after points of discharge (S2 and B2) showed a significant (Kruskal-Wallis, $P<0.05$) decrease in macroinvertebrates diversity, whilst the lowest diversity was recorded at the point of confluence (S3), which was significantly different to all sampling sites (Figure 5.2). Sites further downstream (S4 and S5) showed an increase in macroinvertebrates diversity and they did not differ significantly (Kruskal-Wallis, $P>0.05$) with other sites (Figure 5.2).

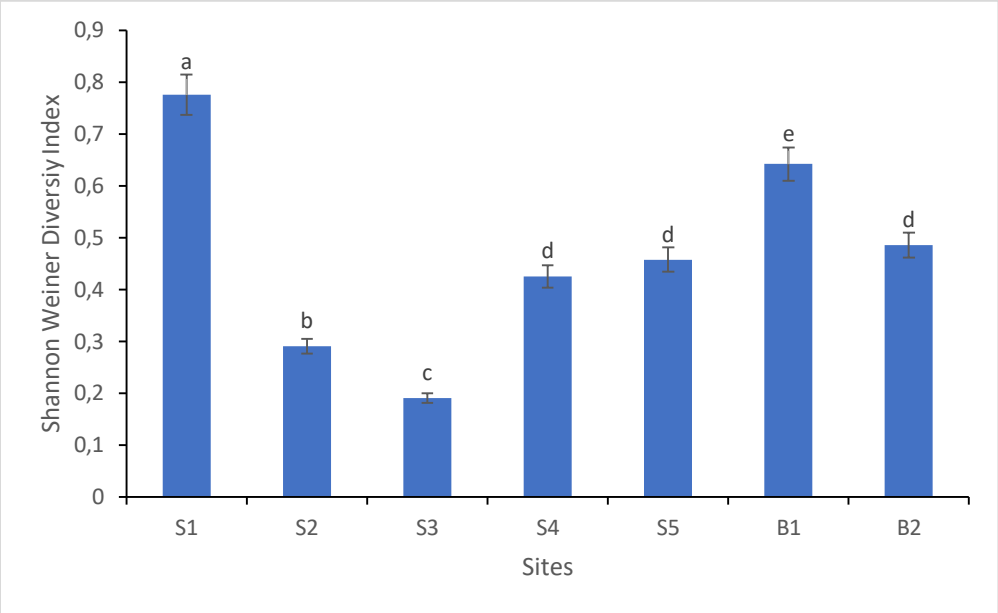

Figure 5.2: Shannon Weiner Diversity Index (H') during rainy season in Sand River and Blood River sampling sites. Bars with different letters are significantly different (Kruskal-Wallis, $P<0.05$). Error bars denote standard error.

During the dry season, the reference sites (S1 and B1) also recorded the highest macroinvertebrates diversity (Figure 5.3). Sites after points of discharge (S2, B2, S3) showed a significant decrease in macroinvertebrates diversity ($P<0.05$), with the lowest diversity recorded at site S2 (Figure 5.3). Sites S4 and S5 showed an increase in macroinvertebrates diversity (Figure 5.3).

Macroinvertebrates diversity was higher during the rainy season than dry season (Figure 5.2 and Figure 5.3)

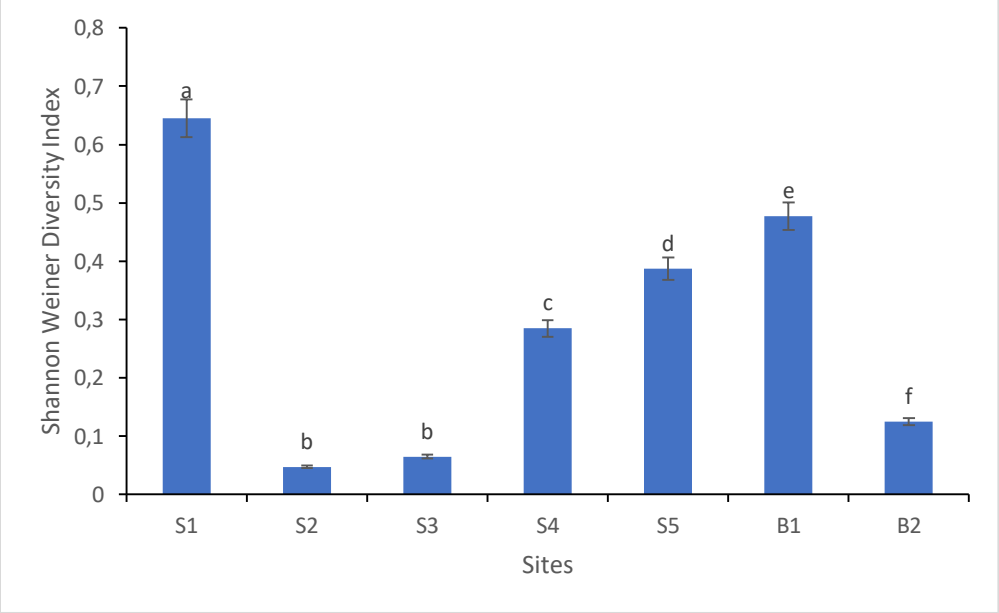

Figure 5.3: Shannon Weiner Diversity Index (H') during dry season in Sand River and Blood River sampling sites. Bars with different letters are significantly different ($P<0.05$, Kruskal-Wallis). Error bars denote standard error.

5.4.4 The Canonical Correspondence Analysis (CCA).

During the rainy season, CCA showed that Axis 1 and Axis 2 accounted for 65.5 % of the variation in macroinvertebrates assemblage (Table 5.7). The correlation matrix showed a positive loading for dissolved oxygen and the rest of water quality parameters showed a negative loading in both Axis 1 and Axis 2 (Table 5.8). The reference site in the Sand River (S1) was associated with pollution sensitive taxa (Gerridae and Gomphidae) and the reference site in the Blood River (B1) was also associated with pollution sensitive taxa (Perlidae and Atyidae) (Figure 5.4). All sites after points of discharge (S2, B2, S3, S4 and S5) were associated with pollution tolerant macroinvertebrates such as Culicidae, Chironomidae, Syrphidae, Elmidae and Ceratopogonidae (Figure 5.4). The assemblage of these macroinvertebrates was associated with total nitrogen, phosphorus, ammonia, water temperature and TDS (Figure 5.4). Oligochaeta taxa was located at the center of ordination. Potamonautidae and Physidae taxa were lying on the dissolved oxygen gradient and Belostomatidae on the pH gradient (Figure 5.4).

Table 5.7: Eigenvalues of the correlation matrix of water quality and macroinvertebrates recorded during the rainy season in the Sand and Blood Rivers.

	Axis 1	**Axis 2**	**Axis 3**	**Axis 4**
Eigenvalues	0.435	0.280	0.181	0.098
Cumulative variance percentage	39.9	65.5	82.1	91.1
Total variation				1.091

Table 5.8: The correlation Matrix of water quality parameters and macroinvertebrates during rainy season in Sand River and Blood River.

Parameters	Axis 1	Axis 2	Axis 3	Axis 4
Water temperature (Temp)	-0.7313	-0.2123	-0.3266	-0.4264
pH	-0.1473	-0.8405	-0.3539	0.1768
Dissolved oxygen (DO)	0.8619	0.4306	-0.0361	0.2249
BOD	-0.6451	-0.2554	0.6787	-0.1081
Turbidity	-0.7336	-0.5522	0.1031	-0.3368
TDS	-0.8941	-0.3811	-0.1157	-0.0430
Phosphorous (P)	-0.9229	-0.2919	0.0901	0.0079
Total nitrogen (N)	-0.9073	-0.2862	-0.1480	-0.1233
Ammonia (NH_3)	-0.9207	-0.3522	0.0252	-0.0615

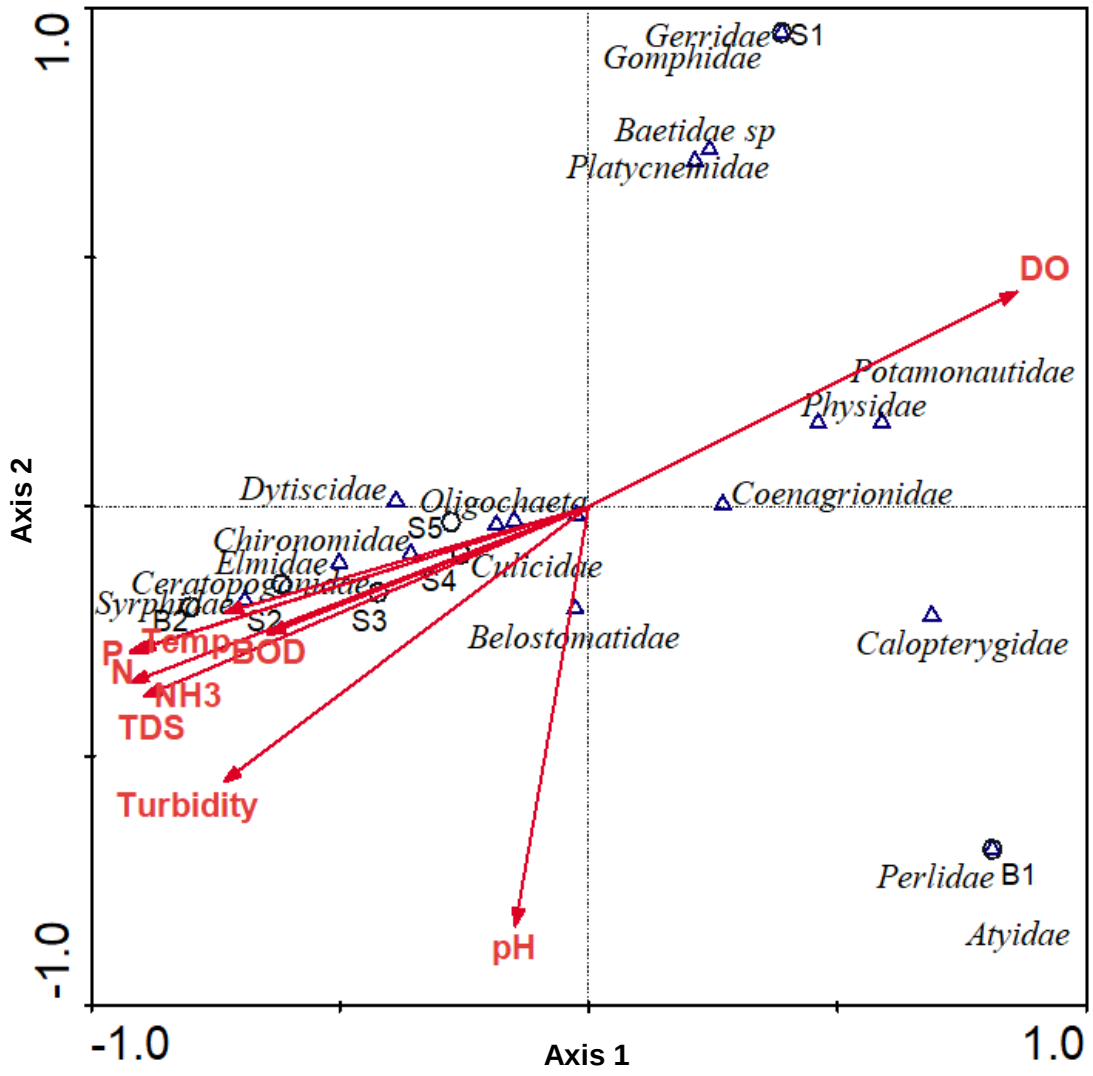

Figure 5.4: The CCA triplot of the relationship between water quality parameters and macroinvertebrates in the Sand and Blood Rivers during the rainy season.

During the dry season, CCA showed that Axis 1 and Axis 2 accounted for 80.9 % of the variation in macroinvertebrates assemblage (Table 5.9). The correlation matrix showed negative loadings for water temperature, pH and dissolved oxygen, whilst the rest of the assessed physico-chemical parameters showed a positive loading in Axis 1(Table 5.10). Axis 2 showed negative loadings of all assessed parameters, with an exception of dissolved oxygen and water temperature which showed a positive loading (Table 5.10). The reference site in the Sand River (S1) was still associated with pollution sensitive macroinvertebrates (Pleidae and Coenagrionidae) and the reference site in the Blood River was associated with Atyidae and Calopterygidae (Figure 5.5). Both the reference sites were not defined by any physico-chemical parameter. Sites after points of discharge (S2, B2, S3 and S4) were associated with pollution tolerant macroinvertebrates (Culicidae and Syrphidae). These macroinvertebrates were associated with total nitrogen, phosphorous, ammonia, TDS and BOD (Figure 5.5).

Table 5.9: Eigenvalues of the correlation matrix of water quality and macroinvertebrates recorded during the dry season in the Sand and Blood Rivers.

	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.730	0.360	0.194	0.052
Cumulative variance percentage	54.2	80.9	95.3	99.2
Total variation				1.347

Table 5.10: The correlation Matrix of water quality parameters and macroinvertebrates during dry season on the Sand and Blood Rivers.

Parameters	Axis 1	Axis 2	Axis 3	Axis 4
Water temperature (Temp)	-0.0455	0.3925	0.6650	0.5706
pH	-0.0847	-0.8433	-0.2004	-0.1590
Dissolved oxygen (DO)	-0.7825	0.5576	0.2614	0.0870
BOD	0.9010	-0.3538	-0.1111	-0.0155
Turbidity	0.6795	-0.7212	-0.1052	0.0625
TDS	0.9006	-0.3401	-0.2481	-0.0730
Phosphorus (P)	0.9250	-0.3020	-0.1428	0.0927
Total nitrogen (N)	0.9231	-0.3083	-0.1159	0.1008
Ammonia (NH_3)	0.9202	-0.3547	-0.0906	-0.0034

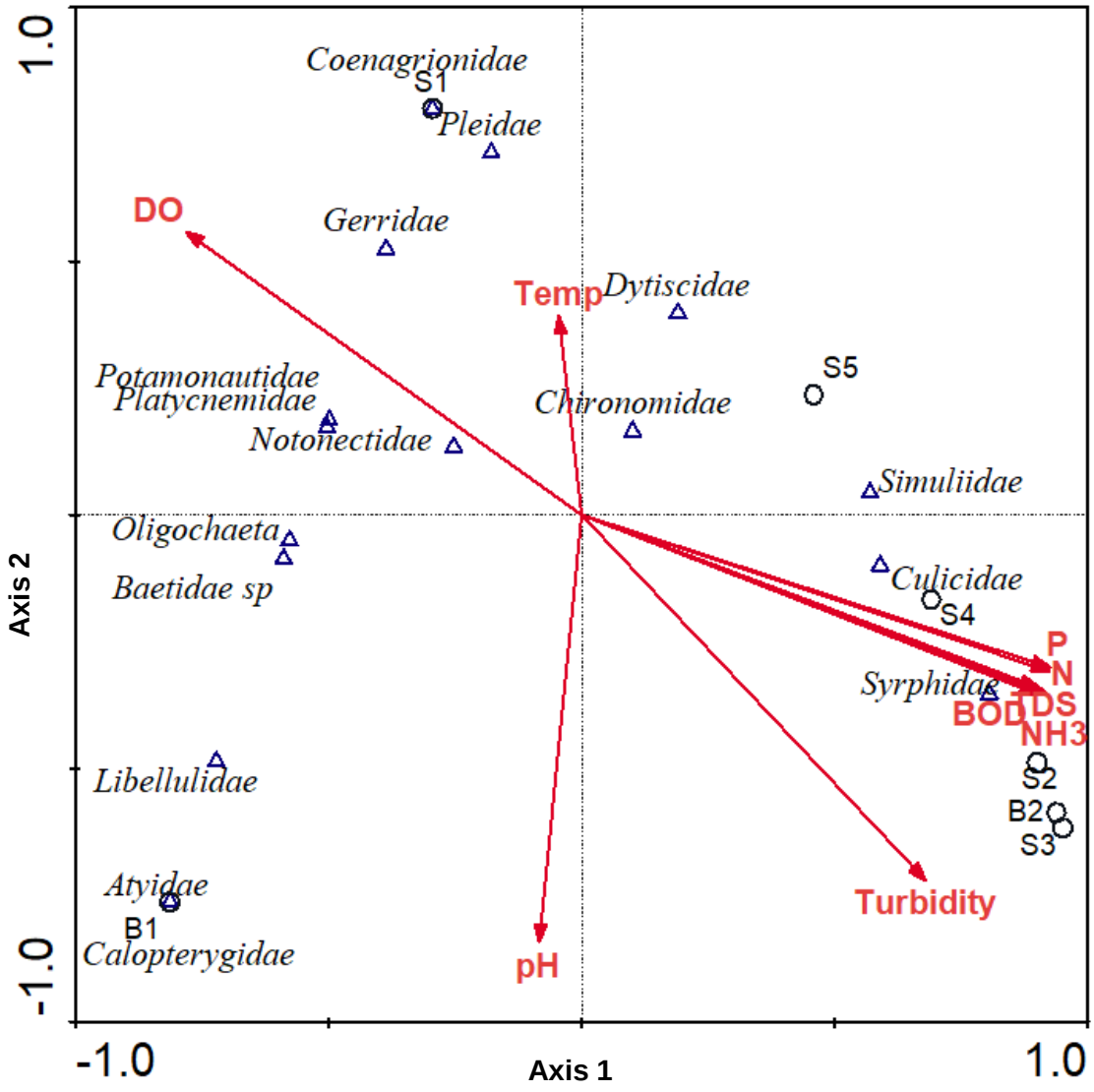

Figure 5.5: The CCA triplot of the relationship between water quality parameters and macroinvertebrates in the Sand and Blood Rivers during the dry season.

5.5 DISCUSSION

The South African Scoring System (SASS5) and Average Score Per Taxon (ASPT) showed spatial variation during the rainy season and dry season. During both seasons, the reference sites (S1 and B1) recorded the highest SASS score and ASPT. This may be attributed to high levels of dissolved oxygen observed at these sites, which favored the presence of different taxa. Water quality with a high level of dissolved oxygen create an optimum environment for a variety of taxa. Moreover, these sites have no know point source of pollution. Thus, the water quality has high levels of dissolved oxygen. Reference sites recorded dissolved oxygen levels between 2.62 mg/l to 4.15 mg/l throughout the study period. The highest SASS score and ASPT at the reference sites may also be due to biotope availability. Watson and Dallas (2013) stated that the biotope with the highest SASS score is stones. The reference sites were largely composed of vegetation and stones biotopes. The SASS score and ASPT decreased at sites after points of discharge during the rainy and dry seasons. This may be attributed to the different habitat type and trophic status that macroinvertebrates occupy (Beneberu *et al*., 2014). Sites after points of discharge were largely composed of vegetation and sand biotope. According to Dallas (2002), sand biotope is not a suitable habitat to support diverse macroinvertebrates. Organic pollution may also result in loss of macroinvertebrates, resulting in low SASS and ASPT. A decrease in SASS and ASPT in rivers that receives sewage effluent is a common phenomenon in developing countries (Odume and Mgaba, 2016; Odume *et al*., 2012; Mudyazhezha and Ngoshi, 2014). The SASS and ASPT scores categorized all the sampling sites as critically modified. A critically modified ecological condition shows that the water quality is poor. One of the major challenge in SASS is that it does not consider macroinvertebrates abundance. Thus, sites that have good water quality may be wrongly categorized.

The SASS and ASPT scores where higher during the rainy season than dry season. The major influencing factor in temporal variation at sampling sites may be the dilution factor. Physico-chemical parameters recorded in chapter 3 show that during the dry season, water quality was poorer compared to rainy season. This was further confirmed by the

CCME WQI scores, which were higher during the rainy season than dry season at all sampling sites.

Macroinvertebrates abundance in Sand and Blood Rivers reference sites were higher than at sites after points of discharge during dry season. The most dominating taxa recorded from the reference sites were Atyidae, Coenagrionidae, Oligochaeta and Chironomidae during the rainy and dry seasons. Atyidae and Coenagrionidae are pollution sensitive taxa (Dickens and Graham, 2002; Gerber and Gabriel, 2002). These taxa are sensitive to low dissolved oxygen and high levels of nitrogen and phosphorus. The reference sites had dissolved oxygen levels ranging from 2.62 mg/l to 4.15 mg/l. Thus, dissolved oxygen range at these sites was ideal for survival of Atyidae and Coenagrionidae taxa. High macroinvertebrates diversity is associated with good water quality. High macroinvertebrates abundance at the reference sites has been recorded elsewhere by Odume *et al*., (2016) and Tate and Husted (2016). Pollution tolerant taxa such as Oligochaeta and Chironomidae were also found in abundance at the reference sites. Oligochaeta and Chironomidae are some of the most dominating taxa in aquatic ecosystems and are found in both impacted and non-impacted sites (Emere and Narisu, 2007; Tyokumbur *et al*., 2002).

Macroinvertebrates abundance at sites after points of discharge decreased drastically, with the lowest abundance recorded at site S3 during the rainy and dry seasons. The decrease in macroinvertebrates abundance at sites after points of discharge are corresponding with the significant decrease in SASS and ASPT scores recorded at sites after points of discharge. The most dominating taxa recorded at sites after points of discharge during the rainy and dry seasons were Chironomidae and Culicidae. These macroinvertebrates taxa are regarded as pollution tolerant (Dickens and Graham, 2002). Chironomidae and Culicidae belong to the Order Diptera, which is often the most common occurring order in aquatic ecosystems (Shimba and Jonah, 2016). Chironomidae is also known to occur in greater abundance even in low dissolved oxygen and nutrient enriched environments (Johnson *et al*., 1993; Quinn *et al*., 1997). Sites after points of discharge recorded dissolved oxygen levels as low as 0.41 mg/l (Chapter 3, Table 3.2a and Table 3.2b). Chironomidae taxon possess hemoglobin, which allows them to respire effectively

in low oxygen conditions. The dominance of Chironomidae and Culicidae taxa at sites after points of discharge may also be due to rich organic matter. Chironomidae and Culicidae taxa feed on organic matter. This explains their dominance at sites after points of discharge. Moreover, water quality parameters showed that sites after points of discharge had poor water quality and where nutrient enriched.

Macroinvertebrates abundance showed temporal variation between the rainy season and dry season. The rainy season recorded higher abundance than the dry season at all sites. Moreover, the total number of macroinvertebrates was 1673/m^2 during the rainy season and 988/m^2 during the dry season. During the dry season, physico-chemical parameters such as ammonia were twice as high compared to levels recorded during the rainy season (Table 3.7a). Thus, ammonia levels recorded during the dry season may have influenced the low number of macroinvertebrates. Similar findings have been reported at different aquatic environments in biomonitoring studies (Shimba and Jonah, 2016; Masese *et al*., 2019). Macroinvertebrates are influenced by prevailing physico-chemical parameters in a waterbody (Chiu *et al*., 2013; Leunda *et al*., 2009). Dallas (2004) stated that seasonal changes in water flow, dissolved oxygen and water temperature may have an effect on the distribution and abundance of macroinvertebrates. The rainy season and dry season had different dissolved oxygen levels and water temperature (Chapter 3). The dry season showed more degraded water quality than the rainy season. Thus, the rainy season was able to support more macroinvertebrates than the dry season.

Macroinvertebrates abundance was used to determine the water quality of Sand and Blood Rivers with the use of Family-level Biotic Index (FBI). The FBI showed that all sampling sites (reference and after discharge) had fairly poor water quality during the rainy season, with an exception of site S3, which was fair and site S4 which was categorized as very poor. A fairly poor water quality condition shows that the site has substantial likelihood of organic pollution (Hilsenhoff, 1988). These water quality conditions correspond to those observed using SASS and ASPT biological bands. The FBI is an index originally developed for rivers in North America (Hilsenhoff, 1998; Reynoldson and Metcalfe-Smith, 1992). Thus, some taxa found in South African aquatic systems are not allocated the tolerance value used in calculating the FBI. This may result

in inaccurate description of the water quality condition at samplings sites. Furthermore, the FBI requires 100 or more macroinvertebrates for identification and determination of an index value for a site (Hilsenhoff, 1998). The FBI further showed a very poor water quality condition at all sites after points of discharge during the dry season. The reference sites showed poor (S1) and fair (B1) water quality conditions during the dry season. These findings confirm the temporal variation observed in physico-chemical parameters, SASS and ASPT and macroinvertebrates abundance observed during the current study. This further showed that the dry season had poor water quality than the rainy season. Both SASS and FBI shows that the Sand and Blood Rivers are enriched with organic pollution.

Macroinvertebrates diversity was assessed with the use of Shannon Weiner diversity Index, and spatial variation was observed during the rainy season and dry season. The reference sites (S1 and B1) showed high diversity index during the rainy season and dry season. Sites with high diversity are usually associated with high dissolved oxygen levels (Arimoro and Ikomi, 2008; Mazgebu *et al*., 2019). The high levels of dissolved oxygen at the reference sites resulted in an elevated macroinvertebrates diversity. High diversity at reference sites was also influenced by biotope availability. Similar findings have been reported by other authors (Dlamini *et al*., 2010; Beyene *et al*., 2009). The high diversity at the reference sites correlated with high macroinvertebrates abundance recorded during both the rainy and dry seasons.

A decrease in diversity was recorded at sites after points of discharge (S2, B2 and S3), Sites further downstream (S4 and S5) showed an improvement in diversity during the rainy season and dry season. Loss of macroinvertebrates at sites after points of discharge may be due to organic enrichment, which has been driven by the discharge of substandard sewage effluent. The nitrogen and phosphorus levels recorded at sites after points of discharge where above TWQR. The use of fertilizers and pesticides in farms downstream of Sand and Blood Rivers may have resulted in increased amount of nutrients entering the Sand and Blood Rivers. This may have further contributed to a significant decrease in dissolved oxygen levels downstream. Macroinvertebrates diversity at all sampling sites were below 1. According to Mason (2002) and Wilhm and Doris (1968), macroinvertebrates diversity value less than 1 reflect a stressed community and

also organic pollution. This further confirms the water quality condition reported from SASS biological bands and the FBI.

Shannon Weiner diversity Index further showed a temporal variation between the rainy season and dry season. This may be attributed to the dilution factor that takes place during the rainy season. Physico-chemical parameters such as lower nitrogen, phosphorus and ammonia levels recorded during the rainy season may have resulted in the presence of diverse taxa.

The association between macroinvertebrates assemblage and physico-chemical parameters was evaluated with the use of CCA. The first and second axes of CCA explained 65.5 % of cumulative variance during the rainy season. This shows the presence of a relationship between macroinvertebrates and physico-chemical parameters. The CCA triplot showed that the reference site in the Sand River was associated with pollution sensitive macroinvertebrates (Gerridae and Gomphidae), whilst the reference site in the Blood River was associated with Atyidae and Perlidae taxa. This confirms the abundance of pollution sensitive taxa at these sites. The presence of Gerridae, Gomphidae, Atyidae and Perlidae taxa was influenced by dissolved oxygen levels recorded at these sites. The CCA further confirmed the association of pollution tolerant taxa with sites after points of discharge. Taxa such as Culicidae, Chironomidae, Syrphidae, Ceratopogonidae and Elmidae were dominating sites after points of discharge (S2, B2 S3, S3 and S5). Taxon such as Chironomidae are capable of surviving in polluted environmental conditions. Moreover, these taxa were associated with total nitrogen, phosphorus, ammonia, BOD, TDS and water temperature. This is because these taxa can tolerate elevated levels of nitrogen, phosphorus and ammonia. The physico-chemical parameters showed that sites after points of discharge also had low dissolved oxygen levels and were nutrient enriched. Furthermore, most of the taxa found at sites after points of discharge can breathe atmospheric oxygen (Lillie *et al*., 2003). Oligochaeta was located at the center of the ordination. Thus, it was ubiquitous, and its assemblage was not defined by any of the assessed physico-chemical parameters (Nicacio and Juen, 2015; Rosenberg and Resh, 2008). Seanego and Moyo (2013) observed similar findings in the Sand River.

During the dry season, axis 1 and 2 accounted for 80.9 % of the variation in macroinvertebrates assemblage. This shows that the association between macroinvertebrates assemblage and physico-chemical parameters was strong during the dry season as compared to the rainy season. Physico-chemical parameters, biological bands and Family-level Biotic Index showed that the dry season had poor water quality as compared to the rainy season. This further reflect that the high concentration of nutrients during the dry season highly influenced macroinvertebrates assemblage. Furthermore, the reference sites were still associated with pollution sensitive macroinvertebrates whereas sites S2, B2, S3 and S4 were lying on the total nitrogen, phosphorus, ammonia and BOD gradients. The taxa Culicidae and Syrphidae were found at sites after points of discharge. This shows that Syrphidae and Culicidae can withstand highly nutrient enriched environments. Macroinvertebrates taxa such as Potamonautidae, Platycnemidae, Notonectidae and Physidae were influenced by dissolved oxygen levels. This is because these taxa naturally inhabits ecosystems with saturated or high levels of dissolved oxygen and these sites are often less polluted.

Macroinvertebrates recorded in the current study were dominated by taxa from the Order Diptera during the rainy season and dry season. The abundance of macroinvertebrates from this order is because most of them possess a mechanism for breathing atmospheric oxygen and are therefore not affected by organic pollution (Odume *et al*., 2016). The CCA showed that recorded taxa from this order are strongly correlated with total nitrogen, phosphorus, ammonia, BOD and TDS. Thus, the order Diptera is a poor biological indicator since it is ubiquitous and cannot discriminate between the reference sites and impacted sites. Macroinvertebrates such as Atyidae and *Baetidae sp*. may be potential biological indicators in impacted rivers such as the Sand and Blood Rivers. These macroinvertebrates were only found at less impacted sampling sites and their absence may signal alteration in water quality of an aquatic ecosystem. It is thus suggested that Atyidae and Baetidae may be potential biological indicator macroinvertebrates in biomonitoring studies.

Macroinvertebrates indices used were able to discriminate the reference sites from the polluted sites. The SASS and FBI both confirmed the organic pollution in Sand and Blood

Rivers. However, each biological index has its own limitations. Thus, multiple biological indicators from different biological organization must be employed in assessing pollution status of an aquatic ecosystem (Wepener *et al*., 2011).

CHAPTER 6: FISH HISTOLOGY AS A BIOMARKER IN ASSESSING POLLUTION STATUS OF THE SAND AND BLOOD RIVERS, LIMPOPO PROVINCE, SOUTH AFRICA

6.1 INTRODUCTION

The use of biological indicators and biomarkers in assessing pollution status of aquatic ecosystem has gained increasing attention in South Africa (Dickens and Graham, 2002; Richardson *et al*., 2010; Marchand *et al*., 2012; Wepener *et al*., 2011). This approach has been widely adopted, since it integrates assessment of pollution status of an aquatic environment (Taylor *et al*., 2009). Some of the most used biological indicators are macroinvertebrates and fish species (Dallas and Day, 1993; Roux *et al*., 1993). The use of macroinvertebrates in assessing pollution status was applied in Chapter 5.

Fish species have been used as biological indicators due to their sensitivity to different pollutants (Karr, 1981; Zhou *et al*., 2008; Zimmerli *et al*., 2007). Fish occupy a wide variety of habitats and trophic positions and can thus provide an indication of pollution at different levels of trophic organization (Whitfield and Elliot, 2002). However, aquatic biota may only reflect changes in community assemblage. Wepener *et al*., (2011) stated that aquatic health is better monitored at different levels of biological organizations, ranging from subcellular to ecosystem. The current chapter will employ fish histology as biomarker of aquatic pollution.

Histopathology is widely used in assessing pollution status in aquatic environments (Wepener *et al*., 2011; McHugh *et al*., 2011; Barnhoorn *et al*., 2004; Wagenaar *et al*., 2012). This approach shows histological alterations in tissues and organs of fish exposed to xenobiotics, which usually occur before macroscopic changes and changes in community assemblage can be identified (van Dyk *et al*., 2009b; van Dyk *et al*., 2012). Moreover, histopathological changes also reflect changes in physiological and or biochemical function (Hinton *et al*., 1992). Thus, they reflect early warning signs of exposure to pollutants (Hinton *et al*., 1992; Gerber *et al*., 2017; Marchand *et al*., 2008).

One of the most used fish species in histopathology studies is *Clarias gariepinus* (van Dyk *et al*., 2007; Marchand *et al*., 2009; van Dyk *et al*., 2009a; Barnhoorn *et al*., 2004).

This is due to its wide distribution across South Africa and its abundance in aquatic ecosystems (Skelton, 2001). *Clarias gariepinus* can tolerate very low dissolved oxygen levels and can endure harsh aquatic conditions (Skelton, 2001). Previous chapters showed that the Sand and Blood Rivers are severely degraded. However, *Clarias gariepinus* is one of the species that can tolerate the poor water quality conditions in these aquatic ecosystems. One of the advantages of using fish histology as a biomarker is that different organs such as gills and liver can be used (Mumford *et al*., 2007; van Dyk *et al*., 2009a; Marchand *et al*., 2008; van Dyk *et al*., 2012). These are the most preferred organs due to the vital roles they play in the health of a fish. Different alterations may be observed on these organs due to exposure to different pollutants. Pollutants such as heavy metals, organochlorine pesticides, high levels of nutrients, sewage effluent, industrial effluent and agricultural runoff may cause alterations on the gills and liver of fish species.

The use of fish histology in assessing pollution status will be employed for the first time in Sand River and Blood River, which receive substandard sewage effluent. The use of biomarkers at different biological organizations has been reported to reflect early warning signs and also invaluable information to use in assessing the degradation of aquatic ecosystems (Wepener *et al*., 2011). This chapter's main objective was to determine spatial variation of histopathological alterations in gills and liver of *Clarias gariepinus* from the Sand and Blood Rivers.

6.2 MATERIALS AND METHODS

6.2.1 Fish collection

Fish sampling was undertaken randomly from January to August, 2018 in Sand and Blood Rivers. Seine nets with different mesh sizes (30 mm, 50 mm), rod and line and electrofisher (LR-24, Smith Root Company, USA) were used to collect fish samples at all sampling sites in Sand and Blood Rivers (Figure 3.1). After collection, fish were placed in 30L tanks containing water from that particular site, aerated by diffusing oxygen with air stones and then transported to the ARU Laboratory. Fish samples were then placed in ice and sacrificed by cutting the spinal cord. Fish liver and gills were excised and separately preserved in sampling bottles containing 10% neutral buffered formalin for 24 hours. The fish were mostly caught in isolated pools at sampling sites. A total number of 20 *Clarias gariepinus* were caught upstream of the WWTP's and another 20 fish were caught at sites/pools downstream of the WWTP's.

6.2.2 Histological analysis

Liver samples where cut into approximately $1cm^3$ and washed in running tap water for 2 hours to remove fixing agent. The samples were dehydrated in a series of increasing concentration of ethanol (70%, 80%, 96% and 100%) for 1 hour in each concentration. Xylene was used to clear samples until transparent, followed by infiltration through increasing concentration of Tissue-Tek® III wax in 60°C oven. Samples were then embedded in Tissue-Tek® III wax blocks and left to cool.

Each block was sectioned at 4-5 µm thickness using a wax microtome (Leica: RM2125 RTS). The sections were placed on microscope slides and stretched using albumin solution (Humason, 1979). The slides were then dried on a hot plate and kept in the oven overnight (30°C). Dried slides were stained with haematoxylin and eosin (H&E) according to van Dyk and Pieterse (2008). Stained slides were mounted with cover slips using Entellan® (Merck).

6.2.3 Histological assessment

A qualitative histological assessment was carried out on gills and liver slides. The slides were examined and photographed under light microscope (Leica Microsystems model DM750, Leica, Bannockburn, IL, USA) at different magnification to identify any histological alterations present.

If any alterations were identified, they were semi-quantitatively assessed following van Dyk *et al*., (2009a) protocol which was adapted from Bernet *et al*., (1999). For each alteration, an Important Factor from 1 to 3 representing the potential of the alteration to affect the health of the fish was allocated as follows:

1: Alteration is reversible

2: Alteration is reversible if the stressor is neutralized

3: Alteration is irreversible

A Score Value ranging between 0 and 6 representing the severity of occurrence of the alteration was allocated as follows:

Score 0: Absent

Score 2: Mild

Score 4: Moderate

Score 6: Severe

The Important Factor was then multiplied by the Score Value to determine the Organ Index (I_{org}). The Organ Index (gills and liver) represented the histological alteration in each organ.

The mean organ Indices values were classified according to a scoring scheme by (van Dyk *et al*., 2009a) which was adapted from Zimmerli *et al*., (2007). The classes were:

Class 1: (Index <10) Normal tissue structure with slight histological alterations

Class 2: (Index 10-25) Normal tissue structure with moderate histological alterations

Class 3: (Index 26-35) Pronounced alterations of organ tissue

Class 4: (Index >35) Severe alterations of organ tissue

Furthermore, an overall Fish Index (I_{fish}) was calculated by adding the sum of the Liver Index and Gill Index, which represent the histological response of each fish individually (van Dyk *et al*., 2009a).

6.3 DATA ANALYSIS

Normality and homogeneity of variance was tested using Shapiro Wilk test and Levene's test, respectively. Kruskal-Wallis was used to test the significant difference in organ indices (Gill and liver) at sampling sites. Kruskal-Wallis test was also used to test the significant difference in Fish Index across sampling sites in the Sand and Blood Rivers.

6.4 RESULTS

5.5.1 Histology assessment

Qualitative histological assessment of *Clarias gariepinus* gills

Fish samples were only found in pools at sites S1, B1, S4 and S5 throughout the study period. At the reference sites (S1 and B1), 70% of fish samples had normal gill structure (Figure 6.1 A and B). Hyperplasia of the secondary lamellae was also observed in fish from the reference sites (Figure 6.1 C and D).

Fish samples from sites downstream (S4 and S5) showed more alterations than fish from the reference sites. Identified alterations included: fusion of the secondary lamellae (A), hyperplasia of the interlamellar cells (B), epithelial lifting of secondary lamellae (C) and hyperplasia of the secondary lamellae (D) (Figure 6.2).

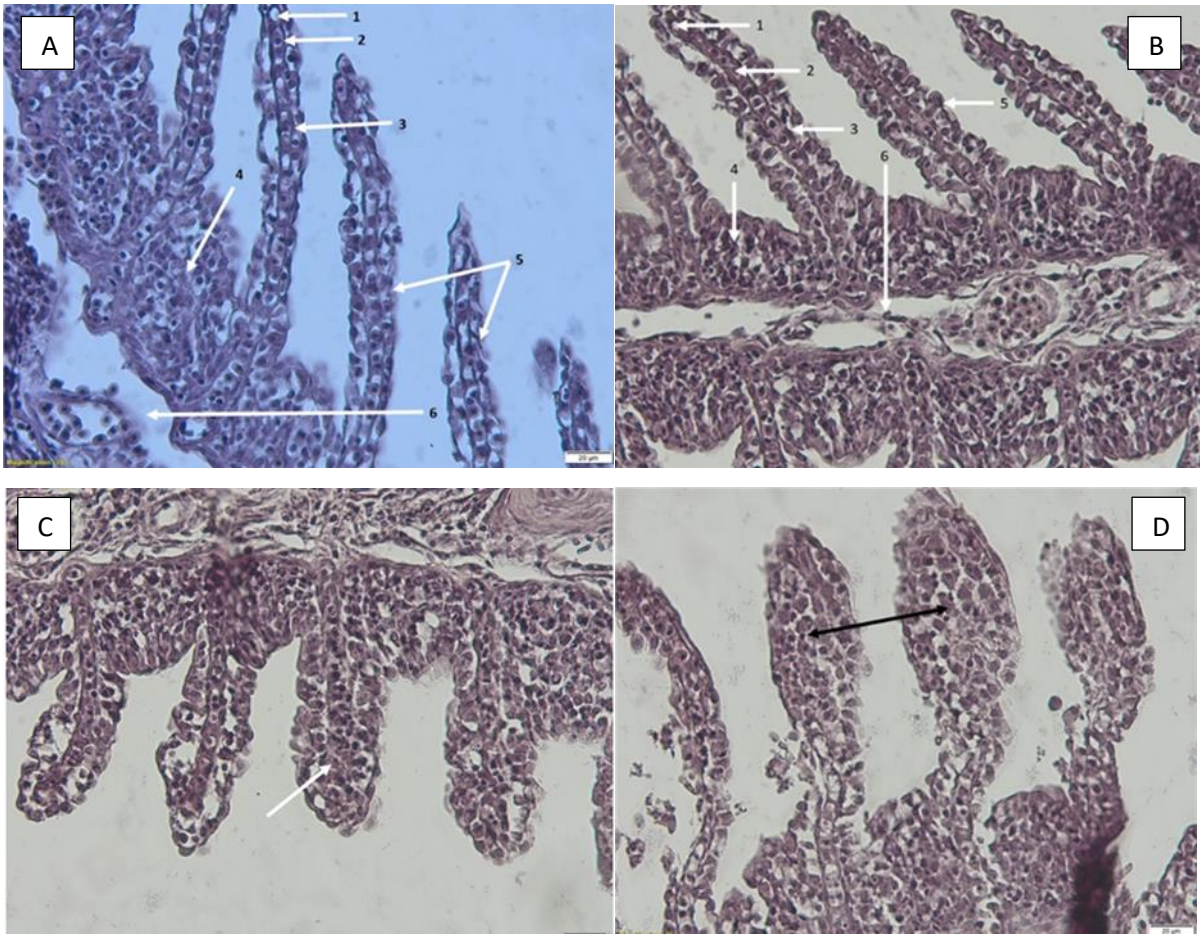

Figure 6.1: Micrograph of normal gill on *Clarias gariepinus* from the reference site in the Sand River (A) and reference site in the Blood River (B) stained with H&E. 1-Capillary lumen, 2-Pillar cell, 3-Epithelium, 4- Interlamellar cells, 5-Secondary lamella, 6-Primary lamella; and alterations observed in fish from the Sand River (C) and Blood River (D) reference sites showing hyperplasia of secondary lamella (arrows) (40X).

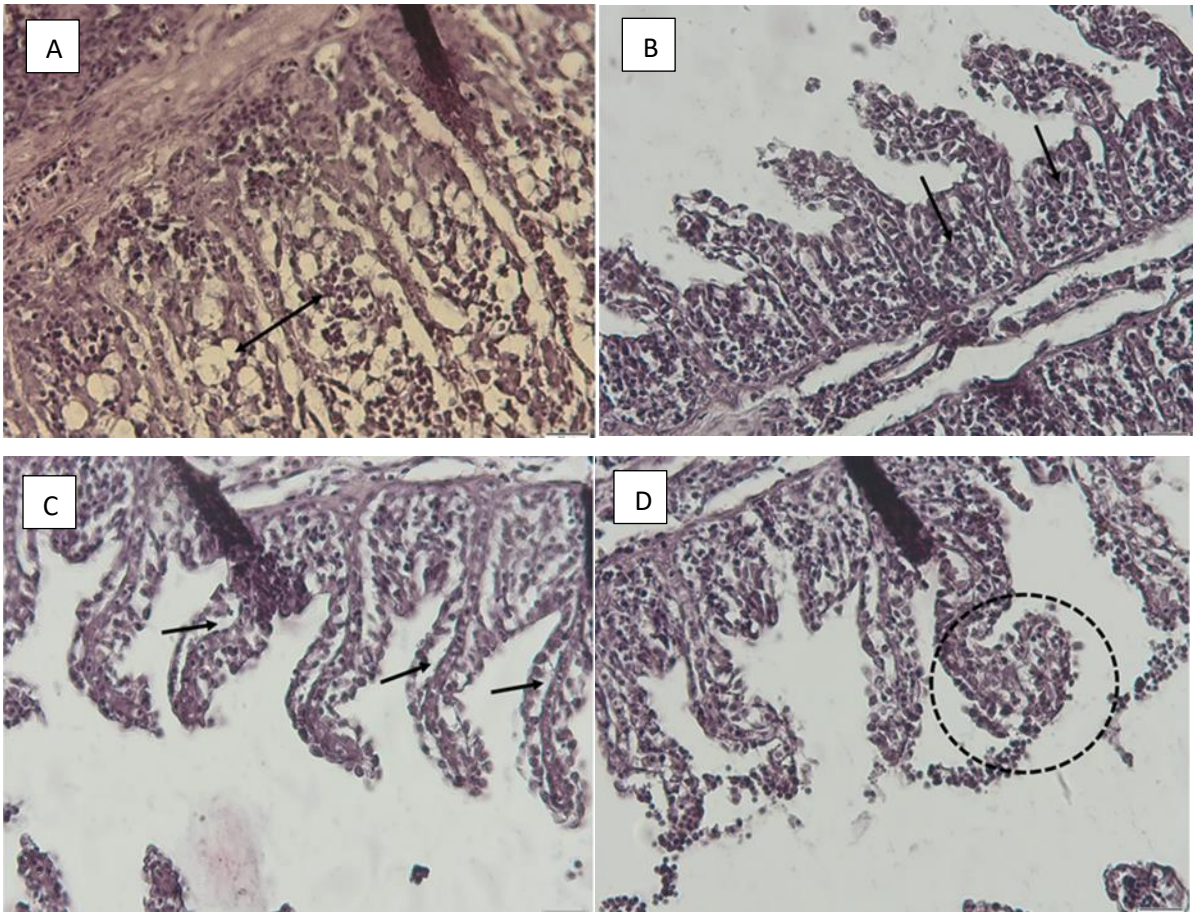

Figure 6.2: Micrographs of *Clarias gariepinus* gills from sites downstream (S4 and S5) in Sand River showing some of the observed alterations stained with H&E. A-Fusion of the secondary lamellae (arrows), B-Hyperplasia of the Interlamellar cells (arrows), C-Epithelial lifting of secondary lamellae (arrows), D-Hyperplasia of secondary lamellae (encircled) (20X)

Qualitative histological assessment of *Clarias gariepinus* liver

Normal liver structures were observed in fish from the reference sites (Figure 6.3 A and B). Nuclei pleomorphism (C), vacuolation (C) and central vein congested with red blood cells (D) were also observed on fish from the reference sites (Figure 6.3 C and D).

Fish from sites downstream (S4 and S5) showed more histological alterations as compared to those from the reference sites. Alterations identified included: Melano-macrophage centers (MMC's) (A), Macrovesicular steatosis (A), sinusoid congested with Kupffer cells (B), nuclei pleomorphism (C) and vacuolation (D) (Figure 6.4 A-D).

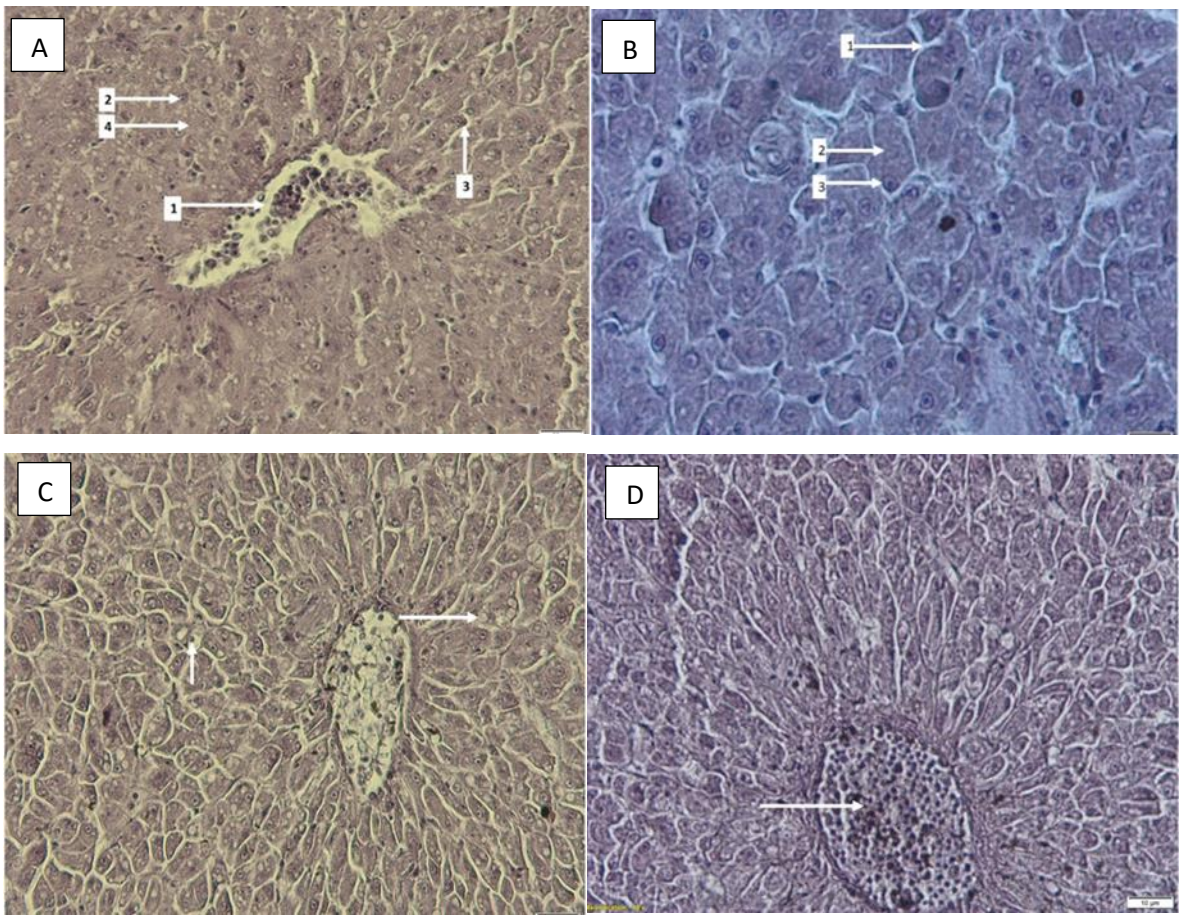

Figure 6.3: Micrographs of normal liver of *Clarias gariepinus* from the reference site in the Sand River (A) and reference site in the Blood River (B) and alterations observed in fish from the Sand River (C) and Blood River (D) reference sites. A: 1: Central vein with red blood cells, 2: Nucleus, 3: Sinusoid, 4: Hepatic plate. B: 1: Sinusoid, 2: Hepatic plate, 3: Nucleus. C: Nuclei pleomorphism (short arrow), Vacuolation (long arrow). D: Central vein congested with red blood cells. (H&E, 40X).

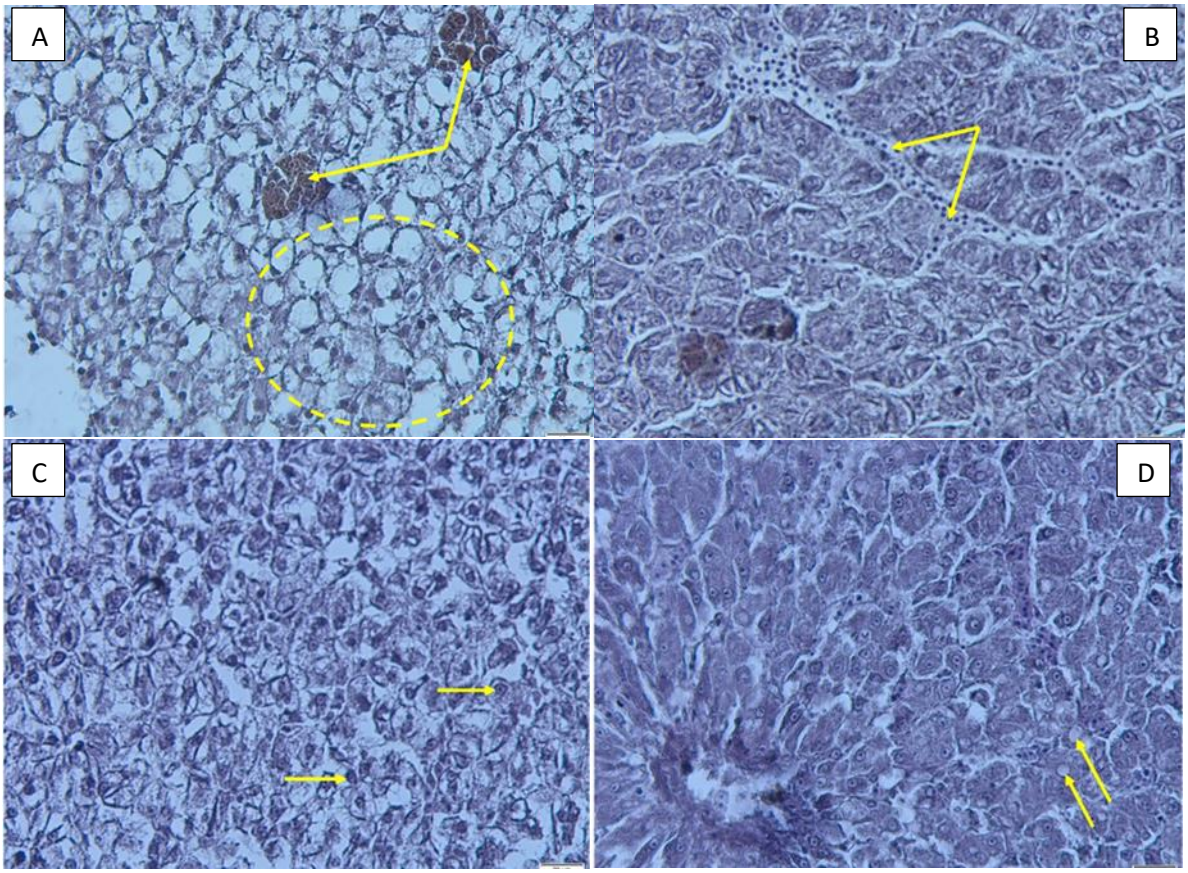

Figure 6.4: Micrographs of *Clarias gariepinus* liver from sites downstream in the Sand River showing observed alterations stained with H&E. A: Melano-macrophage centres (MMCs) (arrows), Macrovesicular steatosis (encircled), B: Sinusoid congested with kupffer cells (arrow), C: Nuclei pleomorphism, D: Vacuolation (arrows) (20X)

Semi-quantitative histological assessment of gills and liver

The gill and liver indices showed spatial variation (P<0.05, Kruskal-Wallis) (Figure 6.5). Gill and Liver Indices were significantly lower at the reference site in the Sand River (S1) and highest at site S4 downstream (Figure 6.5).

The gill index was classified as class 1 at site S1 and class 2 at sites B1, S4 and S5 (Table 6.1). The liver index was classified as class 2 at site S4, and Class 1 at sites B1, S4 and S5.

The fish index showed significant difference across sites (P<0.05, Kruskal-Wallis) (Figure 6.6). The lowest fish index was recorded at site S1, whilst the highest was recorded at site S4 (Figure 6.6).

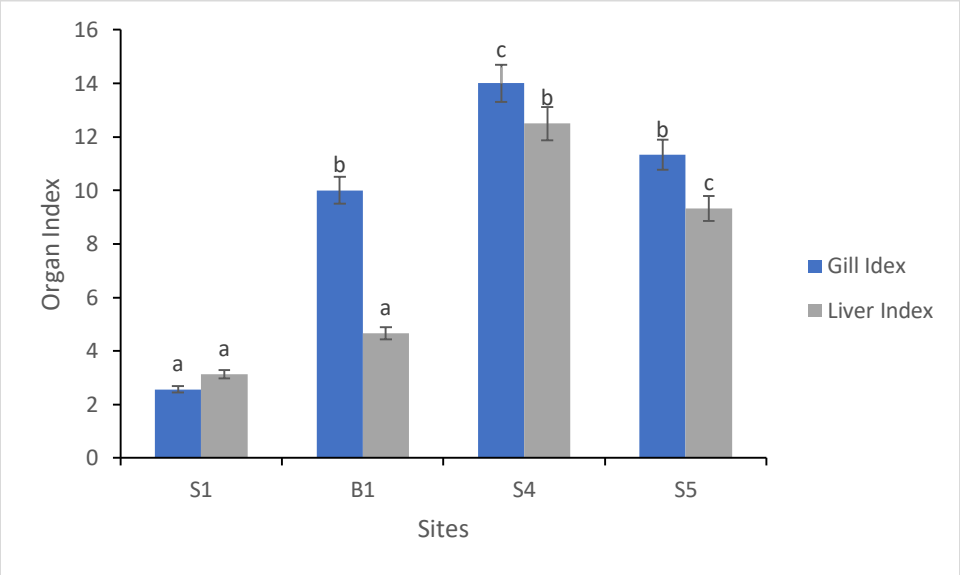

Figure 6.5: Gill and liver Indices of *Clarias gariepinus* from the reference sites (S1 and B1) and sites downstream (S4 and S5) in Sand and Blood Rivers. Gill index bars with different letters are significantly different (P<0.05, Kruskal-Wallis test). Liver Index bars with different letters are significantly different. Error bars denote standard error (SE)

Table 6.1: The mean organ indices values classified according to a scoring scheme (van Dyk *et al*., 2009; Zimmerli *et al*., 2007).

	S1	B1	S4	S5
Gill Index	2.57	10	14	11.33
Class	Class 1	Class 2	Class 2	Class 2
Liver Index	3.14	4.66	12.5	9.33
Class	Class 1	Class 1	Class 2	Class 1

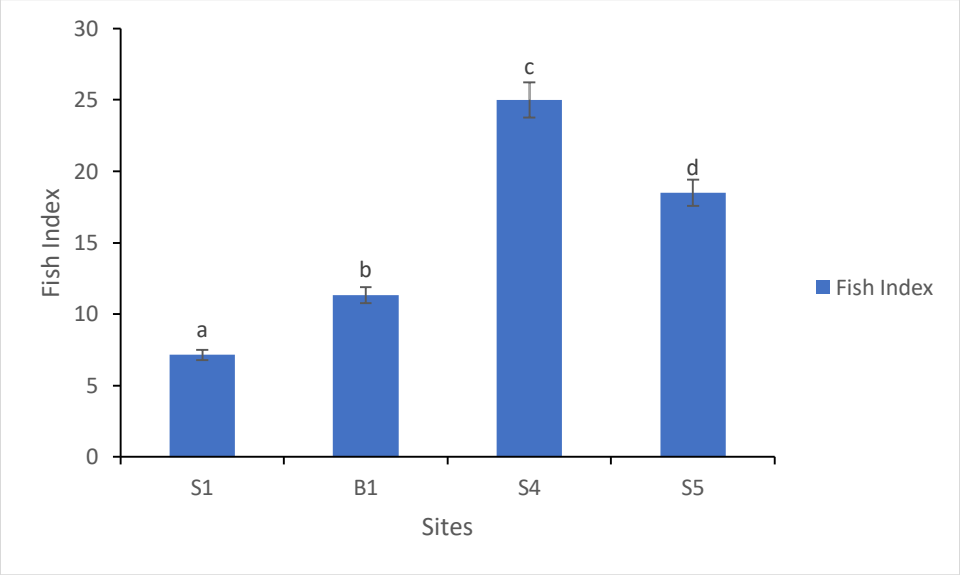

Figure 6.6: The Index of *Clarias gariepinus* from the reference sites (S1 and B1) and sites downstream (S4 and S5) in Sand and Blood Rivers. Different letters on bars shows significant difference ($P<0.05$, Kruskal-Wallis test). Error bars denote standard error (SE).

6.5 DISCUSSION

Most fish caught from the reference sites showed normal gill structure. The normal gill histology of fish from these sites may be due to the low ammonia levels recorded at sites S1 and B1. Site S1 recorded the lowest level of ammonia (0.30 mg/l), followed by site B1 (0.96 mg/l) during the rainy season. The dissolved oxygen levels recorded at these sites were also favorable to *Clarias gariepinus*. Thus, most fish from these sites had normal gill structure. Although the reference sites had favorable water quality for *Clarias gariepinus*, alterations such as hyperplasia of the secondary lamellae were observed in some of the fish. Hyperplasia is due to a substantial increase in mucous, epithelial and chloride cells in secondary lamellae, which is associated with irritants in the environment (Stryzewska *et al*., 2016; Jabeen and Chaundry, 2013). The sites upstream of the WWTP's in the Sand and Blood Rivers receives storm water and surface run-off from the Polokwane CBD, residential area and the informal settlement in Seshego Township. The run-off water has the potential to cause irritations on fish gills in Sand and Blood Rivers. Moreover, water from the storms and surface run-off may also contain pathogens and parasites, which may affect the health status of *Clarias gariepinus*. Furthermore, gills are always in contact with the external environment and are thus sensitive to even slight changes in water quality (Genten *et al*., 2009).

Sites further downstream (S4 and S5) recorded more histological alterations than those from the reference sites. Histological alterations such as hyperplasia of interlamellar, fusion of secondary lamellae, epithelial lifting and hyperplasia of the secondary lamellae were identified on gills of *Clarias gariepinus* from downstream. Hyperplasia of the secondary lamellae is categorized as a progressive alteration (Bernet *et al*., 1999). It occurs when there is an increased proliferation of cell population, which is usually triggered by an irritant (Meyers and Hendricks, 1985). This often results in an enlargement of the target organ. Hyperplasia of the secondary lamellae causes reduction in oxygen diffusion across gill epithelium, leading to hypoxia in cases where fish species has no capacity to survive with low oxygen uptake (Heath, 1987). However, *Clarias gariepinus* has a suprabranchial organ that allows it to breathe atmospheric oxygen (Skelton, 2001). Thus, *Clarias gariepinus* was able to survive even though the secondary lamellae was

altered. Hyperplasia of the secondary lamellae often leads to fusion of the secondary lamellar. Although the fusion occurs as a solid fusion of many lamellae capillaries, it can also be stimulated by pollutants such as parasitic and bacterial infections and also chemical toxicants (Roberts, 2001). The current study did not focus on parasite infections. However, there was no parasite infection observed in the gills of all fish assessed. Mabika and Barson (2013) reported that fusion of the secondary lamellar could cause a decrease in gas exchange, thus affecting the health of the fish.

Epithelial lifting is a regressive gill alteration that increase the distance between water and blood (Roberts, 2001). It is characterized by the displacement of the lining epithelium of secondary lamellae and may results in reduction of gills surface area. Thus, impairing oxygen uptake (Fernades and Mazon, 2003). The identified alterations were also observed in water bodies impacted by sewage effluent, industrial, agricultural and mining activities in South Africa (van Dyk *et al*., 2009b; Marchand *et al*., 2012; Bernet *et al*., 2004). All the observed gill alterations were not severe and were mostly associated with respiratory impairment. It is important to note that sites downstream of Sand and Blood Rivers are impacted by the discharge of substandard sewage effluent from Polokwane and Seshego WWTP's. Moreover, sites downstream recorded ammonia levels above the TWQR for aquaculture in South Africa, which is between 0.0 to 0.3 mg/l for warm water species (DWAF, 1996b). Furthermore, the low dissolved oxygen levels recorded at sites S4 (0.65 mg/l) and S5 (1.02 mg/l) increased the toxicity of ammonia, which acts as an irritant on fish gills, resulting in histological alterations. Irritants not only affect the histology of gills in fish species. Vital organs such as a liver are also altered.

Histological alterations were also assessed in the liver of fish from the reference sites and sites downstream. The liver was selected as a biomarker due to its detoxification, metabolism and excretion role in fish species. Liver of fish collected at the reference sites were mainly normal, with few showing alterations. Normal liver histology was characterized by distinguishable central vein, nucleus, sinusoid and normal hepatic plate. Central veins of some of the fish liver were congested with red blood cells. Congestion of red blood cells in central vein is categorized as a circulatory disturbance, which is associated with blood flow in tissues (Bernet *et al*., 1999). The congestion of red blood

cells in central vein is due to arterial or venous processes. This shows that the water quality condition at the reference sites was not pristine. This was confirmed by the CCME WQI, which showed that sites S1 and B1 had good water quality condition. According to the CCME WQI, good water quality means that the water has only minor degree of impairment (CCME, 2001a; Chapter 3, Table 3.1).

Fish liver from sites downstream showed more pronounced alterations compared to those collected from the reference sites. Alterations identified included: melano-macrophage centres (MMC), macrovesicular steatosis, sinusoid congested with red blood cells, nuclei pleomorphism and vacuolation. Melano-macrophage centres are distinctive groupings of pigment-containing cells found in different organs in fish (Agius and Roberts, 2003). They usually contain a variety of pigments including melanin, lipofuscin, hemosiderin and ceroid (Wolkea, 1992). Melano-macrophage centres are often associated with normal histology in fish liver (Bruslé and González, 1996; van Dyk *et al*., 2011). However, van Dyk *et al*., (2011) reported that the increase in MMC's number and size could be due to exposure to toxicants. Increase in number of MMC's was more prevalent in fish from sites downstream. The presence of MMC's in liver of fish inhabiting sewage polluted systems was also observed by Marchand *et al*., (2009).

Macrovesicular steatosis was also identified in some of the fish assayed and is a form of vacuolation that occupy the hepatocytes, which often results in pushing the nuclei to the periphery of the cell .This may result in nuclei pleomorphism, which is associated with detoxification and metabolic processes of toxicants in the liver (Begum *et al*., 2009). Vacuolations were also observed in the current study. Vacuoles contain a diluted proteinous colloid (Takashima and Hibiya, 1995). They are associated with the inhibition of protein synthesis, dissagregation in microtubule or shift in substrate utilization (Hinton and Lauren, 1990). Sinusoids congested with kupffer cells were also identified in liver of some of the fish from downstream. Severe congestion may lead to atrophy of hepatic cells in the adjacent area. Fish liver alterations are mainly due to what the fish feeds on. *C. gariepinus* occupies different trophic levels within the aquatic ecosystem. It has been reported to feed on aquatic insects, fish, plant debris and terrestrial insects. Due to its

wide diet composition, it is likely to be exposed to wide range of contaminants through biomagnification.

The semi-qualitative assessment of histology alterations shows that the liver and gill Indices of fish showed spatial variation. Site S1 recorded the lowest gill index value, followed by site B1. This may be due to the low total dissolved solids recorded at these sites. Total dissolved solids have the potential to cause irritants to gills in fish species. Moreover, site S1 was classified as class 1. According to van Dyk *et al*., (2019) and Zimmerli *et al*., (2007), class 1 means that the gill structure of fish from this site was normal with slight histological alterations. Sites B1 and S4 showed an increase in gill index, respectively. The gill index dropped at site S5. However, all these sites (B1, S4 and S5) were classified as Class 2, which means that these fish had normal gill structure with moderate histological alterations.

Fish index was lowest at site S1, followed by B1. Thus, fish from the reference sites had less histological alterations than those collected downstream (S4 and S5). Histological alterations have been observed in fish exposed to different toxicants such as heavy metals, pesticides, sewage effluent and parasites (Marchand *et al*., 2008; Wepener *et al*., 2005; van Dyk *et al*., 2007; Barnhoorn *et al*., 2004). The Sand and Blood Rivers are severely polluted with sewage effluent discharged into these rivers from the Polokwane and Seshego WWTP's. This explains histological alterations observed in fish from these rivers. Histology is a biomarker that reflect early warning signs of aquatic degradation. The major challenge with histopathology as a biomarker is that alterations lack specificity to pollutants. Thus, it is difficult to pinpoint the exact pollutants responsible for the formation of a specific histological alteration (van Dyk *et al*., 2012).

CHAPTER 7: ACETLYCHOLINESTERASE AND LACTATE DEHYDROGENASE ENZYME ACTIVITY AS BIOMARKERS IN ASSESSING POLLUTION STATUS OF SAND AND BLOOD RIVERS, LIMPOPO PROVINCE, SOUTH AFRICA

7.1 INTRODUCTION

Biological indicators and biomarkers have been reported to integrate ecological conditions over time and have become the preferred method in assessing pollution status of aquatic ecosystems (Dallas and Day, 1993; Barbour *et al*., 1999). Each biological indicator or biomarker has its own limitations. It is thus important to employ different biological indicators and biomarkers at different biological organization in assessing pollution status of aquatic ecosystems (Wepener *et al*., 2011; Adams, 2001; van der Oost *et al*., 2003). Fish enzymes have recently gained attention as biomarkers in assessing pollution status of waterbodies (Barnhoorn and van Vuren, 2004: Adedeji *et al*., 2012). Fish enzymes are at a lower level of biological organization and can reflect early warning signs of toxicants effects before they can be identified on higher biological organization (van der Oost *et al*., 2003; Lionetto *et al*., 2019; Hook *et al*., 2014). Some of the widely used fish enzymes in biomonitoring studies are acetylcholinesterase (AChE) and lactate dehydrogenase (LDH) (Al-Ghais, 2013; Frasco *et al*., 2005).

Acetylcholinesterase is known as a biomarker for organophosphate and carbamate pesticides (Richardson *et al*., 2010; Lau *et al*., 2004; Golombieski *et al*., 2009). These are the group of pesticides that are more biodegradable and less persistent than organochlorines. However, they are more toxic to non-target organisms such as aquatic flora and fauna (Arufe *et al*., 2007). Although AChE enzyme can be assessed in liver, gills, muscle, kidney and brain tissues, it is more concentrated in brain tissue (Zinkl *et al*., 1987). Thus, organophosphates and carbamates primarily inhibit the activity of AChE in brain tissue. The Sand and Blood Rivers are surrounded by a number of commercial farms, such as ZZ2 downstream. The use of pesticides in agricultural activities is very common in South Africa (Ansara-Ross *et al*., 2012). Hart and Pimentel (2002) estimated that, only 0.1% of the applied pesticides reaches the target, while the rest spreads across the environment. During surface-runoff, residues of these pesticides are washed off into

the nearby draining systems. However, the effect of these pesticides on the health of fish species in Sand and Blood Rivers is unknown.

Lactate dehydrogenase enzyme is also gaining momentum as a biomarker of aquatic ecosystems (Osman *et al*., 2007; Das *et al*., 2004). Lactate dehydrogenase can be inhibited by pollutants such as heavy metals and pesticides. However, LDH is known as a non-specific indicator of pollution (Barnhoorn and van Vuren, 2004). The inhibition of LDH can be assessed in all tissues. However, LDH is concentrated in the liver, skeletal muscle and heart of organisms (Sinha and Ray, 2018). The current study will assess LDH in liver of *Clarias gariepinus*. The use of biomarkers at a lower biological organization (cellular) in assessing pollution status in Sand and Blood Rivers has not been evaluated. Therefore, the objective of this chapter was to determine spatial variation of AChE enzyme activity in brain tissue of *Clarias gariepinus* and spatial variation of LDH enzyme activity in liver tissue of *Clarias gariepinus* from the Sand and Blood Rivers.

7.2 MATERIALS AND METHODS

7.2.1 Fish collection

Fish were collected as described in 6.3.1 (Chapter 6). Brain and liver samples were excised and immediately frozen in liquid nitrogen. Brain and liver samples were then transferred to a biofreezer (-80°C) at the Biotechnology Unit Laboratory, University of Limpopo until analysis. A total number of 10 *Clarias gariepinus* where collected from each reference site (S1 and B1) and 20 *Clarias gariepinus* downstream of the WWTP's.

7.2.2 Enzyme assay

Acetylcholinesterase (AChE)

Acetylcholinesterase activity was assayed according to Ellman *et al*., (1961). Brain samples were ground in liquid nitrogen using pestle and mortar. Samples were weighed and homogenized in 0.1M phosphate buffer, pH 8.0 (20 mg tissue per 1 ml buffer) and centrifuged at 12 000 rpm for 4 minutes. The supernatant was decanted and used for acetylcholinesterase analysis. The assay was performed in triplicate where: 25 µl of enzyme extracts, 280 µl of 0.1M phosphate buffer (pH 8.0) and 10 µl of 0.01M 5.5'-di-thiobis-2-nitrobenzoic acid (DTNB) were added into a microtiter plate wells. Blanks were similarly prepared, without enzyme extracts. The reaction was started by adding 2 µl of 0.075M acetylcholine iodine. Change in absorbance was read at 412 nm for 4 minutes at 25°C using a microplate reader (Beckman Coulter DTX 880 Multimode). The protein concentration was determined according to Lowry *et al*., (1951) using bovine serum albumin as a standard. Enzyme activity was expressed as nmol/min/mg protein.

Lactate dehydrogenase assay (LDH)

Lactate dehydrogenase was assayed according to Vassault (1983). Liver samples were ground in liquid nitrogen using pestle and mortar. Liver samples were weighed and homogenized in 50 mM Tris-HCL buffer, pH 7.4 (20 mg sample per 1 ml buffer). Samples were centrifuged at 10 000 rpm for 5 minutes at 4°C. Supernatants were decanted and used for enzyme activity assay. For enzyme assay, 75 µl of nicotinamide adenine dinucleotide (NADH) 300 mM, 25 µl pyruvate (4.5 mM) and 15µl of enzyme extracts (1/40

dilution) were added into a microtiter plate wells. Blanks were similarly prepared, without enzyme extracts. The assay was done in triplicates. The absorbance was read at 340 nm for 3 minutes at 37°C using a micro plate reader (Beckman Coulter DTX 880 Multimode Detect). The protein concentration was determined according to Lowry *et al*., (1951) using bovine serum albumin as a standard. Lactate dehydrogenase activity was expressed as unit per mg protein (U/mg protein). One unit of enzyme activity is the amount required to transform 1µmol of substrate to product per minute.

7.3 DATA AND STATISTICAL ANALYSIS

Normality and homogeneity of variance were tested with the use of Shapiro-Wilk and Levene's test, respectively. Kruskal-Wallis test was used to test significant difference in AChE enzyme activity and LDH enzyme activity of fish from the Sand and Blood Rivers sampling sites.

7.4 RESULTS

Acetylcholinesterase enzyme activity showed spatial variation across sites in the Sand and Blood Rivers (Kruskal-Wallis, P<0.05) (Figure 7.1). The highest enzyme activity was recorded at site S1 and B1, respectively. Whilst the lowest enzyme activity was recorded at site S4.

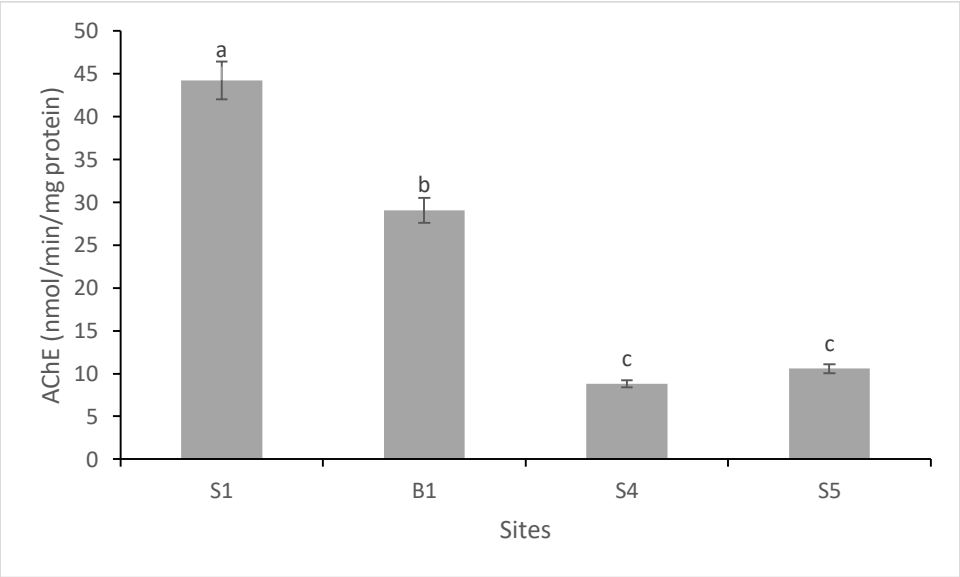

Figure 7.1 Acetylcholinesterase enzyme activity in brain tissue of *Clarias gariepinus* collected from the Sand and Blood Rivers sampling sites. Different letters on bars shows significant difference (Kruskal-Wallis, P<0.05). Error bars denotes standard error.

Lactate dehydrogenase enzyme activity (Figure 7.2) followed a trend similar to Acetylcholinesterase enzyme activity.

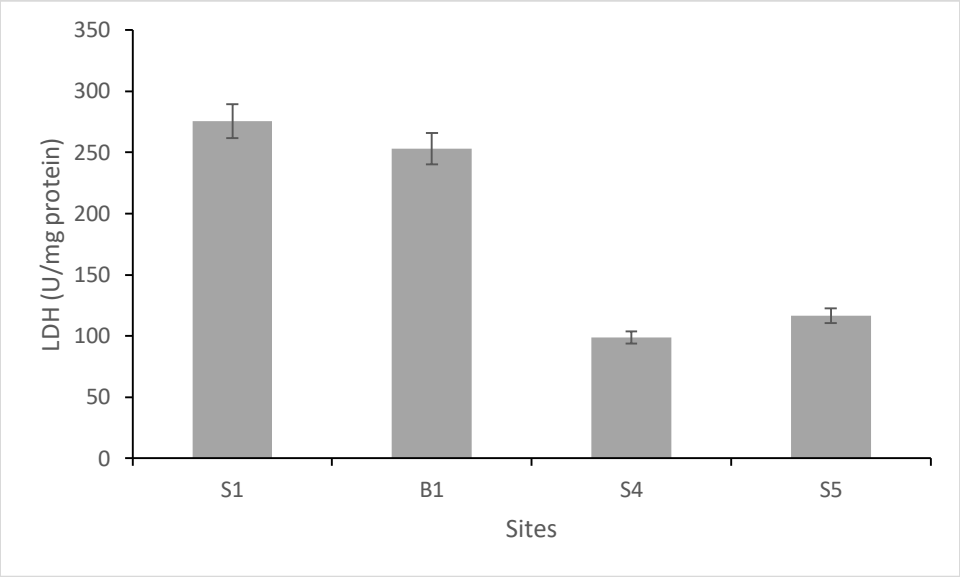

Figure 7.2: Lactate dehydrogenase enzyme activity on brain tissue of *Clarias gariepinus* collected from the Sand and Blood Rivers sampling sites. Different letters on bars shows significant difference (Kruskal-Wallis, P<0.05). Error bars denotes standard error.

7.5 DISCUSSION

The activity of Acetylcholinesterase (AChE) enzyme was higher in fish from site S1, followed by B1. This shows that fish from the reference sites were less stressed. Thus, the AChE activity was less inhibited. Acetylcholinesterase has been reported as a biomarker specifically for organophosphates and carbamates pesticides (Assis *et al*., 2012). Site S1 is located outside the Polokwane CBD and there are no agricultural activities or potential sources of organophosphates and carbamates pesticides in its vicinity. Site B1 is located on the upstream of the Blood River. Although there are no intense agricultural activities taking place on its surrounding, there are few subsistence farms in its vicinity. This explains a lower AChE activity compared to Site S1.

There was a decrease in AChE activity of fish collected from site S4. Moreover, this site recorded the lowest AChE activity. This reflects that the activity of AChE in fish from this site was highly inhibited by organophosphates and carbamates pesticides. Sites downstream of the Sand and Blood Rivers are surrounded by farms which utilize organophosphates and carbamates pesticides. These pesticides are among the most widely used, due to their ability in combating pests (Beltran and Pocsidio, 2010). Organophosphates and carbamates pesticides traces are often drained into the nearby aquatic ecosystem during surface run-off. Thus, they act as inhibitors of AChE enzyme in fish organs, which is responsible for the rapid hydrolysis of the neurotransmitter acetylcholine (ACh) (Fu *et al*., 2018). Organophosphates and carbamates pesticides binds to the active site of the enzyme through phosphorylation or carbamylated, causing inhibition (Assis *et al*., 2012). The inhibition of AChE enzyme activity of fish from polluted streams or rivers that receives substandard sewage effluent has been reported in Zimbabwe and South Africa (Moyo and Rapatsa, 2016; Wepener *et al*., 2011). Although AChE enzyme is widely known as a biomarker for organophosphates and carbamates pesticides, studies have also shown that this enzyme can also be inhibited by pollutants such as heavy metals and other emerging pollutants (de la Torre *et al*., 2002; Zinkl *et al*., 1991; Fu *et al*., 2018). Evaluation of heavy metal contamination showed that the Sand and Blood Rivers are not contaminated with heavy metals (Chapter 4).However, these rivers receive sewage effluent from the Polokwane and Seshego WWTP's.

Lactate dehydrogenase enzyme activity was also used as a biomarker in assessing pollution status of Sand and Blood Rivers. LDH activity followed a similar trend with AChE activity. The highest activity was also recorded at site S1, followed by B1. Lactate dehydrogenase is known as a biomarker of variety of toxicants in aquatic ecosystems (Almeida *et al*., 2002). The high LDH activity on fish from the reference sites strongly correlate with the water quality condition observed from physico-chemical analysis of the current (see Chapter 3) and previous studies (Seanego and Moyo, 2013). Physico-chemical parameters showed that these sites are less impacted than sites downstream. Sites S1 and B1 recorded the lowest levels of ammonia, total nitrogen and phosphorus throughout the sampling period. Thus, LDH enzyme activity in fish liver from sites upstream was less inhibited. Similar findings were observed by Osman *et al*., (2012).

The Inhibition of LDH on liver of fish from site S4 may be attributed to different anthropogenic activities taking place along the river. Some of these activities includes agriculture, informal settlements, illegal dumping sites and sand mining. It has been reported that land use activities are the major source of pollution in aquatic ecosystems (Mangadze *et al*., 2019). Sand River and Blood River are no exception. Lactate dehydrogenase enzyme is also used as a biomarker for tissue damage (Diamantino *et al*., 2001). Under stressful conditions, glycolysis rate increases (Abhijith *et al*., 2016). Pollutants tends to inhibit the aerobic and anaerobic metabolism of fish, resulting in decreased LDH activity (Tripathi and Shasmal, 2011). This further explains the high gill and liver indices and high fish index on fish from sites downstream as observed in semi-quantitative histological analysis in the current study (Chapter 6). The spatial variation observed in enzyme activities is in conformity with findings from previous chapters. Thus, AChE and LDH enzyme activities were successfully employed as biomarkers in assessing pollution status of the Sand and Blood Rivers.

CHAPTER 8: GENERAL DISCUSSION, RECOMMENDATIONS AND CONCLUSION

The aim of this study was to assess the extent to which biological indicators and biomarkers can be used to ascertain pollution levels in the Sand and Blood Rivers. Before using biological indicators and biomarkers, it was deemed important to ascertain the water quality of the Sand and Blood Rivers using physico-chemical parameters in Chapter 3. Hierarchical average linkage cluster analysis isolated the reference sites from the sites downstream of the WWTP during the dry and rainy seasons. The CCME WQI designated the water quality at the reference sites as good and sites downstream where poor, except for the last site across seasons. The poor water quality at the sites after discharge of sewage effluent require that pollution abatement measures be put on place to reduce the pollution load. This is particularly important since the water is used for irrigation. It is recommended that Sodium Adsorption Ratio (SAR) and Sodium Soluble Percentage (SSP) be calculated on a monthly basis to determine the suitability of this water for irrigation.

In Chapter 4, the status of heavy metal pollution in the Sand and Blood Rivers was determined. Inorganic pollution is emerging as one of the major problems associated with effluent from urban areas (Madu *et al*., 2017). Heavy metal analysis in the Sand and Blood Rivers was carried out in the water, sediment and grass. Heavy metal concentration in surface water was below the detection limit in all assessed metals with an exception of iron and manganese. In sediments, all assessed metals were below the PEL. Geo-accumulation and EF further confirmed that the Sand and Blood Rivers where not contaminated with heavy metals. Although the Sand and Blood Rivers are not contaminated with heavy metals, it is recommended that heavy metal speciation be assessed. The toxicity of metals depends on the form of occurrence of the individual species. Assessment of intermetallic relations is also a significant aspect. *Cyperus exaltatus* grass showed PTF values below 1 in all metals assessed. This implies that this grass is not a good candidate to use in phytoremediation. It is thus recommended that other plants along the Sand and Blood Rivers be assessed for their potential in phytoremediation.

Globally, the use of biological indicators and biomarkers is gaining reaction (Dalzochio *et al*., 2016), particularly in developed countries. Biological indicators and biomarkers have been reported to be rapid, reliable and cost-effective methods in assessing pollution status of aquatic ecosystems (Bere *et al*., 2014; Taylor *et al*., 2007). Macroinvertebrates are one of the widely used biological indicators in assessing pollution status in aquatic ecosystems. The SASS and ASPT were used and showed that the Sand and Blood Rivers water condition is critically modified. The FBI was also used to assess the pollution status of the Sand and Blood Rivers and it showed that the Sand and Blood Rivers are enriched with organic pollution. The SASS and ASPT did not discriminate the reference sites and the sites that receives poorly treated sewage effluent. This shows that SASS cannot be solely reliable in assessing pollution status. It is recommended that SASS be reviewed and also put macroinvertebrates abundance into consideration.

Fish histology and fish enzymes has also gained increasing attention as biomarkers in assessing pollution status of aquatic ecosystems (Wepener *et al*., 2011). Fish liver and gills of *Clarias gariepinus* were assessed and most alterations were observed in fish from sites downstream. The gills alterations observed where mostly associated with respiratory impairment. The alterations observed in the liver included vacuolation, sinusoids congested with kupffer cells and central vein congested with red blood cell. Gill and liver indices of fish from the reference sites was lower, whilst the gill and liver indices of fish from downstream were higher. This further classified gills and liver of fish from the reference sites as normal tissue structure with slight moderations, according to Zimmerli *et al*., (2007). Gills and liver of fish from the sites downstream were classified as normal tissue with moderate histological alterations. This shows that gills and liver of *Clarias gariepinus* from the Sand and Blood Rivers were not severely impaired. One of the limitations in using fish histology as a biomarker is that alterations are not toxic specific and fish can recover from gill or liver damage. It is recommended that fish histology be used as biomarker of pollution assessment in concurrence with other biological indicators.

The last chapter assessed AChE in brain and LDH in liver of *Clarias gariepinus*. Acetylcholinesterase enzyme is known as a biomarker for organophosphate and carbamate pesticides and LDH is a non-specific biomarker. Assessment of AChE in brain

and LDH in liver was due to the high concentrations of these enzymes in these tissues. Fish from downstream showed inhibited AChE enzyme activity. The sites downstream are prone to receiving agricultural run-off from the farms surrounding the Sand and Blood Rivers. LDH enzyme activity was also inhibited at sites downstream. AChE and LDH are generally respond to stress. When fish is caught on the hook or on gill net, these enzymes may be inhibited. This shows that the inhibition of these enzymes cannot be solely attributed to pesticides and the discharge of sewage effluent into the Sand and Blood Rivers. It is recommended that different biomarkers from different biological organization be employed in assessing pollution status in aquatic ecosystems.

This study showed that biological indicators and biomarkers should be used in concurrence with the physico-chemical parameters. Although the use of biological indicators and biomarkers is rapid, reliable and cost-effective, this biomonitoring method should not replace the use of physico-chemical parameters. The Polokwane Municipality should introduce biomonitoring methods in assessing pollution status of the Sand and Blood Rivers.

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