A COMPARATIVE ECOTOXICOLOGICAL STUDY OF TWO MAJOR SOUTH AFRICAN RIVER SYSTEMS: A CATCHMENT SCALE APPROACH FOR IMPROVED RISK ASSESSMENT AND ENVIRONMENTAL MANAGEMENT THROUGH THE INTEGRATION OF ABIOTIC, BIOTIC, BIOCHEMICAL AND MOLECULAR ENDPOINTS

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DECLARATION 1

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes two original papers published in peer-reviewed journals and three unpublished publications. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation, indicating the nature and extent of the contributions of the co-authors.

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ABSTRACT

Along with the prosperity of humankind, the inevitable increase in developments to support this often results in water resources becoming repositories for the waste generated by such progress. To ensure sustainable development, anthropogenic impacts need to be properly assessed and anticipated in order to respond accordingly to mitigate such events. With the Mokolo River being at the center of plans to exploit the largest remaining coalfield in South Africa, this study was initiated to improve our understanding of the range of expected water resource impacts that may occur, by taking lessons learned from the upper Olifants River system, which has been severely impacted by coal mining for more than a century.

The aim of this study was to conduct a comparative ecotoxicological study at a catchment scale on both the Mokolo River and upper Olifants River systems. Study sites in the upper Olifants and Mokolo rivers, their major tributaries, as well as major impoundments were selected and assessed seasonally for a number of years. Water and sediment samples were analyzed for various constituents, whilst biological samples (invertebrates and fish) were also sampled to gain perspectives on the impact of pollution at molecular, cellular and community levels. Lastly, a case study to examine the potential use of rehabilitation measures on ecological infrastructure to mitigate the potential impact of acid mine drainage (associated with coal mining activities) was conducted.

Important abiotic ecosystem drivers were identified, whilst the influence of the tributaries and impoundments on the main stream rivers were determined. The major exposure routes of bioaccumulation were found to be through the dietary uptake of contaminated sediment and algae. The use of the fresh water crab, *Potamonautes warreni*, provided an excellent model organism to study the fate, transport and impact of pollution and also to monitor various degrees of molecular and cellular impacts. In contrast to the general use of fish as bioindicators, the use of aquatic invertebrate community structures and freshwater crabs proved more useful to characterize the ecosystem integrity of these systems, detect turnover and determine the links with possible contaminants. From the various biochemical and molecular markers used, several endpoints proved useful in monitoring chronic impacts on biota, which may be used as early warning indicators, whilst others are linked to acute anthropogenic impacts.

A significant improvement in water quality occurred after the wetland rehabilitation case study, including increased productivity, reduced toxicity (embryotoxicity and teratogenicity) and changes in the biotic community structures. Therefore, the benefit of the rehabilitation of ecological infrastructure to mitigate the impact of coal mining associated pollution was

proven. Overall, the use of a comparative catchment approach, employing both an impacted and less impacted river system, proved extremely useful and valuable to support the future management of the Mokolo River in the face of ever increasing land use activities. This catchment scale comparative study is a rare and unique opportunity which few studies are able to utilize to assess the likely impacts of future land use change.

OPSOMMING

Tesame met die welvaart van die mensdom, veroorsaak die onvermydelike styging in ontwikkelings om dit te ondersteun dikwels dat waterbronne bewaarplekke word vir afval wat gegenereer word tydens die vordering. Ten einde volhoubare ontwikkeling te verseker, is dit nodig om antropogeniese invloede behoorlik te evalueer en te verwag om sodoende dienooreenkomstig te reageer op sulke gebeurtenisse, sodat die impak verminder kan word. Met die Mokolorivier wat te midde is van planne om die grootste oorblywende steenkoolveld in Suid-Afrika te ontgin, is hierdie studie geïnisieer om ons begrip van die omvang van die verwagte waterbron impakte wat mag voorkom, te verbeter deur te leer uit vorige ervarings in die boonste Olifantsrivier, wat erg geraak is deur steenkoolmynbou vir meer as 'n eeu.

Die doel van hierdie studie was om 'n vergelykende ekotoksikologiese studie te doen op 'n opvangsgebied skaal op beide die Mokolo- en boonste Olifantsriviere. Studie areas in die boonste Olifants- en Mokoloriviere, hulle hoof sytakke en damme is gekies en seisoenaal geassesseer vir 'n aantal jare. Water en sediment monsters is ontleed vir verskillende parameters, terwyl biologiese monsters (invertebrata en visse) ook versamel is om die uitwerking van besoedeling te bepaal op 'n molekulêre, sellulêre en gemeenskapsvlak. Laastens was 'n gevallestudie gedoen om die potensiële gebruik van rehabilitasie van ekologiese infrastruktuur om die impak van suur mynwater (geassosieer met steenkoolmyn aktiwiteite) te verminder, ondersoek.

Belangrike abiotiese ekosisteem drywers is geïdentifiseer, terwyl die invloed van die sytakke en damme op die hoofstroom riviere bepaal was. Die grootste blootstellingsroetes van bioakkumulering is gevind om deur die opname van gekontamineerde sediment en alge te wees. Die gebruik van die varswater krap, *Potamonautes warreni*, is 'n uitstekende modelorganisme om die lot, vervoer en impak van besoedeling te bestudeer, asook om verskillende grade van molekulêre en sellulêre impakte te monitor. In kontras met die algemene gebruik van visse as bio-indikators het die gebruik van akwatiese invertebraat gemeenskapstrukture en varswater krappe bewys dat dit meer bruikbaar is om ekosisteem integriteit te beskryf, omsette te bepaal, asook die verbintenisse met moontlike besoedeling te bepaal. Vanuit die verskillende biochemiese en molekulêre biomerkers wat gebruik is, het verskeie eindpunte bewys om nuttig te wees in die monitering van kroniese impakte op biota, wat gebruik kan word as vroeë waarskuwingsindikators, terwyl ander gekoppel is aan akute antropogeniese impakte.

Daar was 'n beduidende verbetering in die waterkwaliteit na die rehabilitasieproses van die vleiland, insluitend 'n toename in produktiwiteit, verminderde toksisiteit (embriotoksisiteit en

teratogenisiteit) en verandering in die biotiese gemeenskapstrukture. Hierdeur was die nuttigheid van die rehabilitasie van ekologiese infrastruktuur om die impak van steenkoolmyn geassosieerde besoedeling te verminder, bewys. Dus, die gebruik van 'n vergelykende opvangsgebied benadering, deur die gebruik van beide 'n besoedelde en onbesoedelde rivierstelsel, is baie nuttig om die toekomstige bestuur van die Mokolorivier te ondersteun in die aangesig van toenemende antropogeniese aktiwiteite. Hierdie vergelykende studie op opvangsgebied skaal is 'n seldsame en unieke geleentheid wat min studies in staat is om aan te wend om die waarskynlike impak van veranderings in toekomstige grondgebruik te evalueer.

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“Aan Hom wat vir ewig Koning is, die onverganklike, onsienlike, enigste God, kom toe die eer en die heerlikheid tot in alle ewigheid! Amen.”

1 Timoteus 1:17

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LIST OF SUPPLEMENTARY MATERIAL

LIST OF ABBREVIATIONS

Al	Aluminium
AMD	Acid Mine Drainage
ANOVA	Analysis of Variance
AOPP	Advanced Oxidation Protein Products
As	Arsenic
AVS	Acid Volatile Sulfide
BAF	Bioaccumulation Factor
Ca	Calcium
CAT	Catalase
Cd	Cadmium
cDNA	Complementary DNA
Cl^-	Chloride
COD	Chemical Oxygen Demand
Cu	Copper
DNA	Deoxyribonucleic Acid
DOC	Dissolved Organic Carbon
ELISA	Enzyme-linked Immunosorbent Assay
Fe	Iron
FETAX	Frog Embryo Teratogenesis Assay - *Xenopus*
FHAI	Fish Habitat Assessment Index
FSS	Fish Sensitivity Score
GSSG/GSH	Total Glutathione
H_2O_2	Hydrogen Peroxide
HAI	Habitat Assessment Index
HF	Hydrofluoric Acid
HNO_3	Nitric Acid
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
IDH-1	Isocitrate Dehydrogenase 1
IHAI	Invertebrate Habitat Assesment Index
ISS	Inverterbrate Sensitivity Score
K	Potassium
LoI	Loss of Ignition
Mg^{2+}	Magnesium
ML	Mega Litres
Mn	Manganese
MR	Mokolo River
mRNA	Messenger RNA
MXR	Multi-xenobiotic resistance
Na	Sodium
NH_4^-	Ammonia
Ni	Nickel
NO_2^-	Nitrite
NO_3^-	Nitrate
OR	Olifants River
p53	Tumor Protein p53
Pb	Lead
PCA	Principal Component Analysis
PSA	Particle Size Analysis

RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
ROS	Reactive Oxygen Species
Se	Selenium
SEM	Simultaneously Extracted Metals
SO_4^{2-}	Sulfate
SOD	Superoxide Dismutase
TBARS	Thiobarbituric Acid Reactive Substances
TDS	Total Dissolved Salts
TKN	Total Kjeldahl Nitrogen
TN	Total Nitrogen
TOC	Total Organic Carbon
TP	Total Phosphate
TSS	Total Suspended Solids
V	Vanadium
WWTW	Wastewater Treatment Work
Zn	Zinc

CHAPTER 1: INTRODUCTION

1.1. BACKGROUND

South Africa's water security is being jeopardized through the reduction in ecosystem services provided by impacted water resources (SANBI, 2014). The sustainable use of water is essential for water security in South Africa and entails water resources being able to cope with and recover from various anthropogenic impacts (Krantz, 2001).

It is evident from several international studies that the environmental impact of coal mining is significant and may persist for a long time resulting in it becoming the major source of freshwater pollution in places such as the United Kingdom (Young, 1997). For example, the Allegheny and Monongahela River catchments have been impacted by coal mining for more than 200 years, resulting in significant impacts on water quality (Williams et al., 1996; Sams and Beer, 2000). These long-term impacts of acid mine drainage (AMD) on aquatic ecosystems were also evident in Pennsylvania where more than 200 years of coal mining impacts resulted in changes in the regional carbon and sulfur budgets (Raymond and Oh, 2009). The U.S. Environmental Protection Agency (USEPA) found a similar trend when surveying streams in the Mid-Atlantic and Southeastern United States in that roughly 10% of the streams in the Northern Appalachians subregion were acidic as a result of AMD (Herlihy et al., 1990; Bernhardt et al., 2012). Through studies carried out in south east Ireland, Gray (1997) highlights that coal mining impacts, such as AMD, affect aquatic ecosystems in several direct and indirect ways. These impacts, however, are difficult to quantify and predict due to its complexity. It is therefore crucial to make use of information from long-term impacted sites to improve our understanding of these impacts so that the management of future mining areas may be improved.

At present a unique opportunity presents itself where the Mokolo River (situated in the Limpopo Province of South Africa) faces similar risks from coal mining that exert progressively more serious adverse impacts on the upper Olifants River (which has been impacted by coal mining for more than 100 years) in the Mpumalanga Province. To determine the existing levels of risks to the Mokolo River system and to ensure its sustainable use during an increase in coal mining, key lessons may be learned through a multiple-comparative approach on a catchment scale from the upper Olifants River system. These two study areas have been shown to be comparable (De Klerk et al., 2016) and thus this study makes use of an opportunity which very few studies are able to capitalize on. Not only does this study identify the current state of the Mokolo River compared to the upper Olifants River, but it also highlights the potential future impacts of increased coal mining in the area. Studies, for example Young (1997), have found that the long-term remediation of coal mining impacted sites is best achieved through passive means. Therefore, the current study further evaluates the use of wetland rehabilitation as a means of passive AMD treatment to reduce the negative impacts of coal mining on the receiving environment. The information gained from this comparative study is therefore of utmost importance, not only for water management in South Africa, but may also contribute to the improvement of international riverine management strategies.

1.2. RATIONALE

South Africa is known as a water scarce country and defined as such according to the United Nation's definition that water availability of less than 1 700 m^3/person/year constitutes water stressed. South Africa has an average rainfall of approximately 450 mm/year, which is almost half of the global average of ≈860 mm/year (CSIR, 2010). Most of South Africa has a negative water balance due to the evapotranspiration levels (1 500 mm – 3 000 mm) that exceed annual rainfall (McCarthy, 2011). South Africa's water resources, which include rivers, dams, wetlands, lakes and subsurface aquifers, along with rainfall, evaporation, abstraction and discharge, generally control the quantity of South Africa's inland waters (Cessford and Burke, 2005; DEAT, 2006). The mean annual runoff of South Africa is ≈43 500 million m^3/annum. The total available yield is ≈13 200 million m^3/annum, and for the year 2000 the water demand was ≈12 900 million m^3/annum, whilst the minimum water needed to sustain the aquatic ecosystems was ≈9 500 million m^3/annum (DWAF, 2004). Most of South Africa's water is obtained from rivers and dams, but groundwater has also been used extensively to supply water to South African citizens. Unfortunately, these resources are constrained as well, with only ≈20% of South Africa's groundwater being present in major aquifers that can be used for such purposes (Cessford and Burke, 2005). As the population in South Africa increases, the water resources are likely to be put under further pressure, as this is inextricably linked to urbanization and industrialization to meet economic growth targets (Strydom et al., 2006). This is especially important in the face of climate change.

Besides supplying water, water resources also provide a variety of services which either directly and / or indirectly benefit the various water users. As a result, these naturally functioning water resources (also referred to as ecological infrastructure) are the natural equivalent of built infrastructure (Figure 1-1) (SANBI, 2014). The ecosystem services provided by ecological infrastructure may be divided into four categories, namely supporting, regulating, provisioning and cultural services (Millenium Ecosystem Assessment, 2005). Examples of these services include:

- **Supporting services**: soil formation, photosynthesis and nutrient cycling;

- **Regulating services**: these services influence climate, floods, disease, wastes and water quality which may reduce the severity of impacts;
- **Provisioning services**: providing food, water, timber and fiber; and
- **Cultural services**: the provision of recreational, aesthetic and / or spiritual benefits to humans.

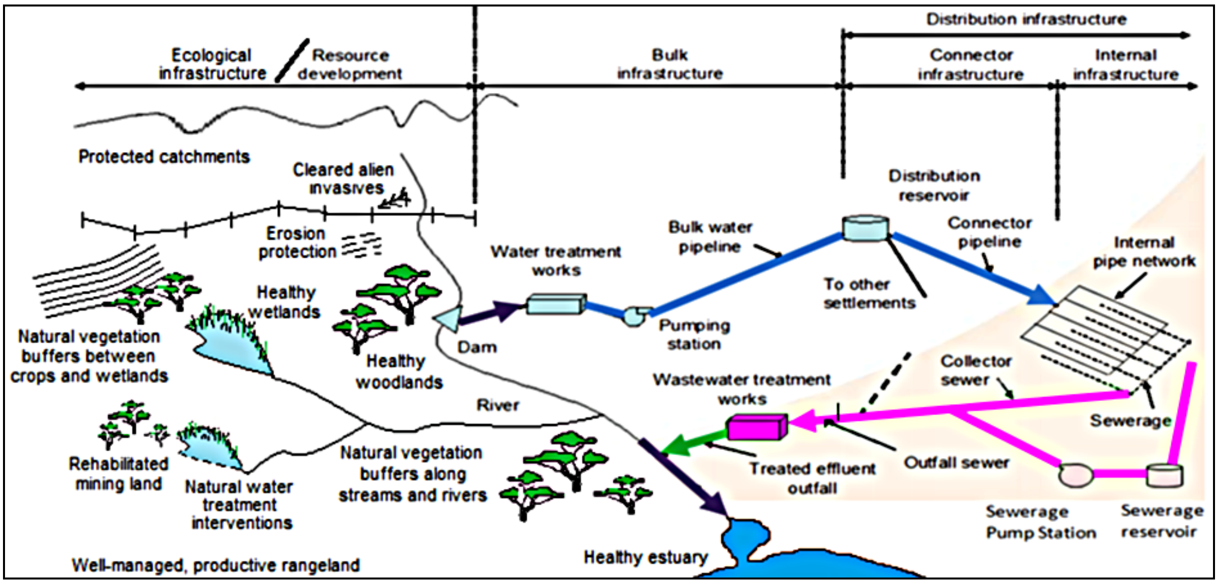

Figure 1-1: A schematic representation of the relationship between ecological infrastructure and built infrastructure (DEA, 2014).

Other direct / indirect services that may also be provided by ecological infrastructure, include sediment trapping, phosphate and nitrate removal, toxicant removal, erosion control, carbon storage, the maintenance of biodiversity, as well as education and research (Kotze et al., 2009). As a result of the significant benefits derived from water resources, they are frequently impacted upon by different activities. These resources thus need to be maintained and managed, and in some cases restored to ensure that maximum benefits are gained from them (SANBI, 2014). Arguable, the improvement of water quality is one of the most important services provided by water resources from which users benefit. Ironically, this is also the one aspect that frequently gets impacted upon by various land uses.

1.2.1. Land Use in South Africa

Changing land use is known to affect water flows, availability and quality. This can occur through increased runoff, which consequently may reduce groundwater recharge. Hydrological patterns could also be significantly transformed through affecting flow speeds and volumes (i.e., through the construction of dams or mining), whilst increased land disturbance often results in erosion. This in turn could lead to an increased introduction of sediment (and its associated pollutants) into receiving waterbodies, which will ultimately degrade the ecosystem and change the flow dynamics. Finally, disturbed unnatural land is known to affect water flows by, for example, allowing the colonization of alien vegetation which generally uses greater volumes of water compared to indigenous vegetation (Cessford and Burke, 2005).

The most significant land uses in South Africa and the impact they have on water resources that are of primary concern, are well-documented in for example DWAF (2004), DEAT (2006) and CSIR (2010). A text analysis of this information is summarized in Figure 1-2. From the text analysis results it is evident that mining operations, agricultural practices, urbanization / wastewater treatment works (WWTWs) and industrial activities are of the critical role players when it comes to land use impacts on receiving water resources. Each of these activities will be discussed in detail below.

Figure 1-2: A word cloud produced from text analyzed from various sources (DWAF, 2004; DEAT, 2006; CSIR, 2010).

Water resources may be polluted from these activities either through point-source releases or non-point / diffuse source inputs. Point source pollution occurs when certain activities (i.e., sewage works or industries) directly release wastewater into a receiving waterbody. Alternatively, non-point source pollution pollutes a water resource indirectly through various routes, for example air pollution or surface runoff (i.e., mining operations or agricultural practices).

1.2.2. Mining Operations

Globally, the mining sector is relatively small (economically speaking), but it produces a variety of different mineral commodities. Some of the biggest producers of the mining commodities worldwide include United States of America, Canada, Australia, Russia, Brazil, South Africa, China and the European Union (Azapagic, 2004). Overall, approximately 30 million people are involved in large-scale mining, whilst ≈13 million take part in small-scale mining (IIED and WBCSD, 2002). Coal also provides ≈27% of the global primary energy needs and generates about 36% of the world's electricity. As a result, coal production has significantly increased (Tiwary, 2001).

Water resources are known to be affected by mining through the use of water when processing ore, discharged mine effluent, as well as seepage from tailings dams and waste rock impoundments (McClure and Schneider, 2001). This may lead to the contamination of water resources with pollutants such as metals, sulfates, etc. (Sams and Beer, 2000; Rohasliney and Jackson, 2008). Certain types of mineral mining are also associated with AMD that leads to the long-term acidification of water resources, which negatively affects aquatic ecosystem health and causes an irreversible loss of biodiversity (CEC, 2000). Acid mine drainage is produced through the oxidation of sulfide minerals, mainly iron pyrite or iron disulfide, which occurs when these minerals are exposed to air and water, leading to acidic conditions that can lead to a significant impact on water quality and overall ecological integrity (Bigham and Nordstrom, 2000; Ravengai et al., 2007; Kidd, 2011; Truter et al., 2014). Acid mine drainage is a natural process, but anthropogenic activities such as mining enhance the rate at which it takes place, leading to significant impacts (Jennings et al., 2008). Acid mine drainage is acknowledged to be a multi-factor pollutant and when coupled with metal pollution, it can affect water chemistry and aquatic ecosystems for decades (Gray, 1997; Kambole, 2003; Lupankwa et al., 2004; Oberholster and De Klerk, 2014).

Minerals obtained through mining are important for everyday life and as such are important in the production of various products. These minerals are used, inter alia, in construction during urbanization, raw materials for the production of chemicals and fertilizers needed in agricultural practices to produce food for a growing population, as well as essential elements for other industrial activities, namely electronics, paint, plastics, etc. (CEC, 2000; Azapagic, 2004). During the exploitation of virgin coalfields it is not only the mining operations that are faced with sustainable development challenges and environmental issues, but also the ancillary activities and / or knock on effects which follow, for example urbanization, agriculture and industry. Mining and agricultural practices are also further inextricable linked through competition for available water resources as both activities usually occur in the same area (Ashton et al., 2001; Musiwa et al., 2004).

1.2.3. Agricultural Practices

As South Africa's population increases, so will the demand for food, and this requires adequate agricultural land (Verheul, 2012). Agriculture uses approximately 66% of the available water supplies in the world and as such is also one of the main contributors to degraded water qualities (Robertson, 2006). A similar trend occurs in South Africa, with approximately 50% of water used by the irrigation sector (Dennis and Nell, 2002). Approximately 18 million ha (≈ 14.75%) of South Africa's surface area has the potential to be used as cultivated land (Dennis and Nell, 2002). Aquatic impacts arising from agricultural practices may be as point source / direct impact or as non-point source / indirect which may affect the water quality of receiving waterbodies and compromise aquatic organisms' ability to reproduce, grow and survive (Stone et al., 2005; Tu, 2011).

Soil erosion and sedimentation, as a result of agriculture, is a major contributor to water quality degradation and may affect the temperature, turbidity and ultimately the chemistry of a waterbody (Ferreira, 2008). On the other hand, the hydrological properties of a catchment may also be altered by compaction that may be caused by grazing and vegetation removal which may reduce water infiltration and increase surface runoff (Strauch et al., 2009). Furthermore, the utilization of a significant amount of fertilizers may indirectly affect water resources through an increase in inorganic nitrogen and phosphorous compounds which may lead to eutrophication (Kremser and Schnug, 2002). This stimulates primary productivity and can result in algal blooms as well as an increase in toxic ammonia (Basu and Pick, 1996; Paisley et al., 2003). Eutrophication is one of the main role players in the degradation of water resources and can contribute to the loss of ecosystem services provided by these systems (CSIR, 2010). Pesticides / herbicides are also used readily by agricultural practices and may end up in receiving water bodies through surface runoff which may affect an ecosystem's structure and function (Dabrowski et al., 2002). Examples of this include imidacloprid, where sub-lethal levels have been found to affect the reproductive success of invertebrates (Alexander et al., 2007). This is one of the reasons why agricultural practices are known as one of the leading sources of chemical pollution in water resources (Tu, 2011).

The cumulative effect of agricultural practices is also important to keep in mind as individual farms may not contribute significantly to the degradation of water resources, but cumulatively, multiple farms in a catchment may result in a significant impact further downstream (Schröder et al., 2004).

1.2.4. Urbanisation and Wastewater Treatment Works

Population growth is estimated to increase significantly in the near future, with ≈60% of the population anticipated to live in urban areas by the year 2030 (Paul and Meyer, 2001). Urbanization is the process where areas such as natural lands, croplands, grasslands and pastures are transformed to urban settlements (OECD, 1998; Nuissl et al., 2009). Because of the construction of buildings, roads, etc. in urban areas, the impermeability of that area increases which affects the water cycle and ultimately impacts on the water resources in the catchment (Braud et al., 2013).

As a result of the increase in surface runoff, natural water flowpaths are transformed and transports human generated pollutants into these receiving waterbodies (Randhir, 2003; Marsalek et al., 2006; Matteo et al., 2006; Li et al., 2008). Some of the most common pollutants associated with urbanization include phosphate, ammonia, pesticides, faecal coliforms and bacteria (Bartram and Ballance, 1996). Studies have confirmed the relationship between settlement growth (which increases urban surfaces) and a noticeable decrease in quality of water of a catchment (Roberts and Prince, 2010; Carey et al., 2011). Urbanization may also contribute to a decrease in the quality of water resources through reductions in water residence times and the introduction of new contamination sources (Collin and Melloul, 2003; Wang et al., 2005; Dietz and Clausen, 2008). These effects may be ascribed to water and waste management considerations in urban developments, which impact on the hydrology (or water balance) in a catchment. These management interventions include the construction of stormwater drains, flood protection and WWTWs and its associated sewerage networks (Collin and Melloul, 2003; Ott and Uhlenbrook, 2004; Barron et al., 2013).

The construction of suitable WWTWs is of particular concern, as an inflow of people concentrated in a small built-up / urban area results in an increase in human waste that needs to be managed for health and safety reasons. In South Africa, these WWTWs are often not sufficient to deal with the volume of waste it receives, resulting in untreated or partially treated wastewater entering a watercourse (Barnes and Taylor, 2004; Snyman et al., 2006; Oberholster et al., 2013; Seanego and Moyo, 2013). This then result in dissolved oxygen depletion, as well as increase in nutrients and faecal contamination in water resources (Moyo and Mtetwa, 2002). Ultimately, these impacts have been reported to lead to, inter alia, major fish kills, cholera outbreaks and eutrophication (Momba et al., 2006; De Villiers, 2007; Wepener et al., 2011).

Worldwide, WWTWs are known to contribute to 50 – 90% of annual nutrient loads in rivers (Marti et al., 2004) which lead to diminished and modified biological diversity that supports the dominance by tolerant species (Ogbogu and Hassan, 1996; Warwick, 2001).

1.2.5. Industrial Activities

The first industrial revolution (1760's) in England, resulted in the development and rapid growth of several industries, including mining, after which a second industrial revolution (1800 – 1900's) saw industrialization spread to other parts of the world which allowed for the improved mining and refinement of coal (Ashton, 1966). The major restructuring of the business sector (1970's – 1990's) occurred due to several factors and are bringing about what is known as the third industrial revolution (Jensen, 1993).

Although industrialization is able to realize various social and economic objectives, it is also associated with climate change, biodiversity reduction, impacts on natural resources, pollution, species extinctions, etc. (Suthar et al., 2009). The problem with industrial wastewater (as with sewage wastewater as well) is that it represents a constant pollution of waterbodies and does not only occur seasonally, as is the case with surface runoff (Singh et al., 2005). Thus, industrial development may significantly affect the quality of surface water resources (Singh and Singh, 2007). This wastewater is often associated with various toxic substances that are frequently discharged into rivers where it pollutes the water resources and therefore increased industrialization is putting growing pressures on the environment (Suthar et al., 2009).

Metals are some of the most problematic pollutants in industrial wastewater that negatively impact the receiving waterbodies. The increased demand for metals for the production of goods has resulted in significant decreases in the quality of water resources (Aleem et al., 2003). Chromium is a good example of such a metal pollutant. It has various industrial uses and therefore large amounts of this pollutant are released into water resources. Chromium pollution takes place mostly due to mining, leather tanning, textile dyeing, electroplating, etc. (Tamie et al., 2005). Chromium (VI) occurs most often in water and is of significant concern due to its toxicity to human beings, animals, plants and other organisms (Wepener et al., 1992; Cieślak-Golonka, 1996). Industrial wastewater is also a major contributor to toxic organic compounds that may be found in the nearby water resources. Industries that can be associated with such pollutants include tire, dye and paper manufacturing plants (Groisman et al., 2004; Stackelberg et al., 2004). Some of these compounds have the potential to cause serious chronic effects when exposed to low levels over a long period (Rajkumar and Palanivelu, 2004).

It can thus be seen that industrial wastewater is often a blend of different substances that, on their own, can be harmful, but may also result in synergistic / antagonistic effects that are not always well understood.

1.3. PROBLEM STATEMENT

The planned increase in mining and power generating activities in the Waterberg area, Limpopo Province, may result in an increase in solid, liquid and gaseous emissions that will have a range of possible impacts on receiving waters and aquatic ecosystems. The Mokolo River is at the center of these activities and hence future coal mining impacts will need to be properly anticipated, monitored and suitable management actions set in place to ensure that impacts are kept to a minimum.

In order to achieve this, the importance of understanding the impact of coal mining, and the associated activities that increase as a result of coal mining (e.g., urbanization), is vital. Therefore, by comparatively studying the Mokolo River with the upper Olifants River, which has been impacted by coal mining since the late 1800s, provides for a unique and important opportunity. This information may provide a sound and defensible scientific basis for the assessment of likely impacts, the evaluation of the significance of these impacts and the design of remedial and preventative measures related to the Mokolo River.

Very few studies ever get the opportunity to be able to comparatively study two river systems that are impacted by coal mining on different ends of the spectrum. Most studies on coal mining only focus retrospectively, but during this study we were able to not only look retrospectively at the impacts of coal mining in the upper Olifants River, but also the potential future changes in the Mokolo River as a result of an increase in coal mining. This was achieved through not only understanding the interplay of several drivers in upper Olifants River better, but also through putting it in context with the current situation occurring within the Mokolo River. The information from this study therefore goes a long way in improving water resource management linked to coal mining and its associated activities.

1.4. AIMS AND OBJECTIVES

The aim of this study was to collect, process and analyze multiple sets of data using a comparative catchment approach from both the upper Olifants River, as well as the Mokolo River. Through this our aim was to better understand the current state of the Mokolo River, as well as to have unique insights into a impacted system such as the upper Olifants River. Thus, this information may aid in developing a baseline for the Mokolo River relative to the upper Olifants River. This may assist in predicting the potential future impacts of increased land use activities (e.g., coal mining) along with improved monitoring and management.

In order to achieve the main aim of this study, the following objectives were established:

1. Identifying key abiotic ecosystem drivers in both the upper Olifants and Mokolo rivers in support of river management;
2. Understanding the dynamics (i.e., fate, transport and impact) of metal pollution of these two river systems;
3. Examining the ecotoxicogenomic links in the upper Olifants and Mokolo rivers using *Potamonautes warreni* as model organism;
4. Investigating invertebrate and fish community structure changes in these two rivers using multiple lines of evidence; and
5. Determining the effect of wetland rehabilitation measures in response to AMD from coal mining activities as a possible tool to mitigate pollution.

1.5. RESEARCH DESIGN

The study areas selected for this study were the upper Olifants River catchment within the Mpumalanga Province of South Africa, with the main focus being the main channel of the upper Olifants River, as well as the Mokolo River catchment situated in the Waterberg Region within the Limpopo Province of South Africa (Figure 1-3). These two catchments were found to be comparable in terms of the specific underlying geology, as well as the type of land use activities occurring / anticipated to occur within the catchment (De Klerk et al., 2016). The extent and duration of these activities (especially coal mining) and their associated impacts, however, differ quite substantially.

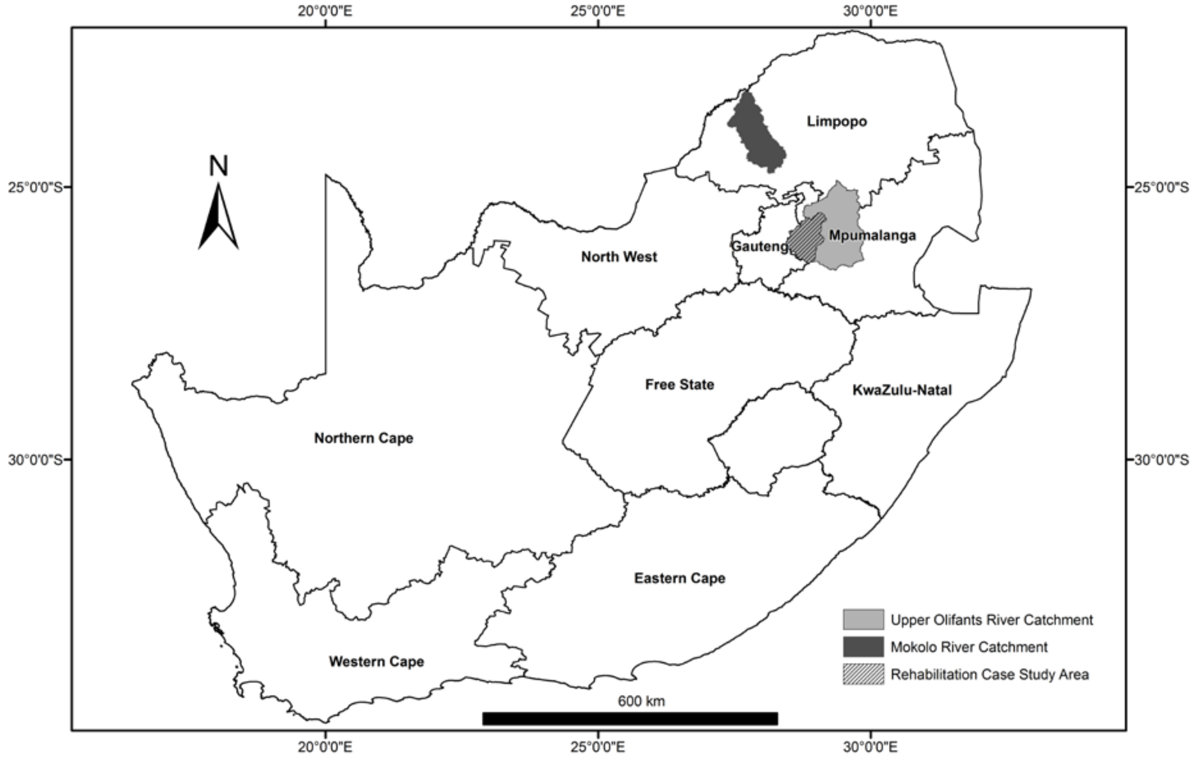

Figure 1-3: The location of the two study areas selected for this case study, namely the Mokolo River catchment and the upper Olifants River catchment, with the portion in the upper Olifants catchment where the rehabilitation case study was conducted.

This study was designed to collect data over multiple years within both the Mokolo River and the upper Olifants River catchments. Seasonality was taken into account to ensure that representative samples were obtained from various hydrological extremes. Data were obtained from within the main channel of both these rivers, but also from the main tributaries draining the catchment into each of these rivers, as well as a major impoundment located within each of the study areas. Study sites were selected to represent the different variations within each catchment (namely degree of impact, composition, etc.) so as to obtain a realistic overall representation.

For the rehabilitation experiment a study area was selected within the upper Olifants River catchment that is impacted by AMD where the case study took place. Seasonality was also taken into account with sites selected above and below the rehabilitated area, accompanied by a suitable reference site further upstream, which is surrounded mainly by natural land.

1.6. BRIEF CHAPTER OVERVIEW

This thesis has been constructed as follows:

- **Chapter 2:** A literature review of the available information relating to this study, specifically focusing on the background information of the upper Olifants and Mokolo River catchments, as well as the various endpoints used during this study.
- **Chapter 3:** A watershed approach in identifying key abiotic ecosystem drivers using data obtained from the main stream river, major tributaries, as well as the prominent impoundments in each catchment.
- **Chapter 4:** Understanding the dynamics (fate, transport and impact) of metal pollution of the two selected catchments.
- **Chapter 5:** Evaluating the ecotoxicogenomical links in the upper Olifants and Mokolo rivers, using *Potamonautes warreni* as model organism.
- **Chapter 6:** Investigating invertebrate and fish community structure changes in two rivers using multiple lines of evidence.
- **Chapter 7:** The effect of rehabilitation measures on ecological infrastructure in response to AMD from coal mining.
- **Chapter 8:** Overall conclusions from the study with potential recommendations of future work needed to improve the sustainability of South Africa's water resources.

Since this dissertation has been structured as separate articles which have either been published or submitted for publication, duplication in terms of specific types of information (e.g., background, methodologies, etc.) was unavoidable. Abiotic data from the same study sites (e.g., water quality) were integrated across Chapters 3 - 6 and therefore selectively duplicated.

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CHAPTER 2: LITERATURE REVIEW

2.1. SOUTH AFRICAN WATER RESOURCES

Globally, South Africa is generally ranked as 30th in terms of water scarcity (Figure 2-1) (Aphane and Vermeulen, 2015). Many of South Africa's water resources are shared across political boundaries which support the importance of proper water resource management and protection (Cessford and Burke, 2005). This is further emphasized due to the ongoing increase in land use activities which result in pollution. This further complicates the water scarcity problems in South Africa (CSIR, 2010). All of these issues relating to South Africa's water resources, especially in the face of climate change, are complex and need to be properly addressed to ensure sufficient water quantities of suitable qualities to meet not only human needs, but also aquatic ecosystem requirements, because the availability and quality of fresh water is crucial for sustaining life on earth, human health, as well as social and economic development (DWAF, 2004a).

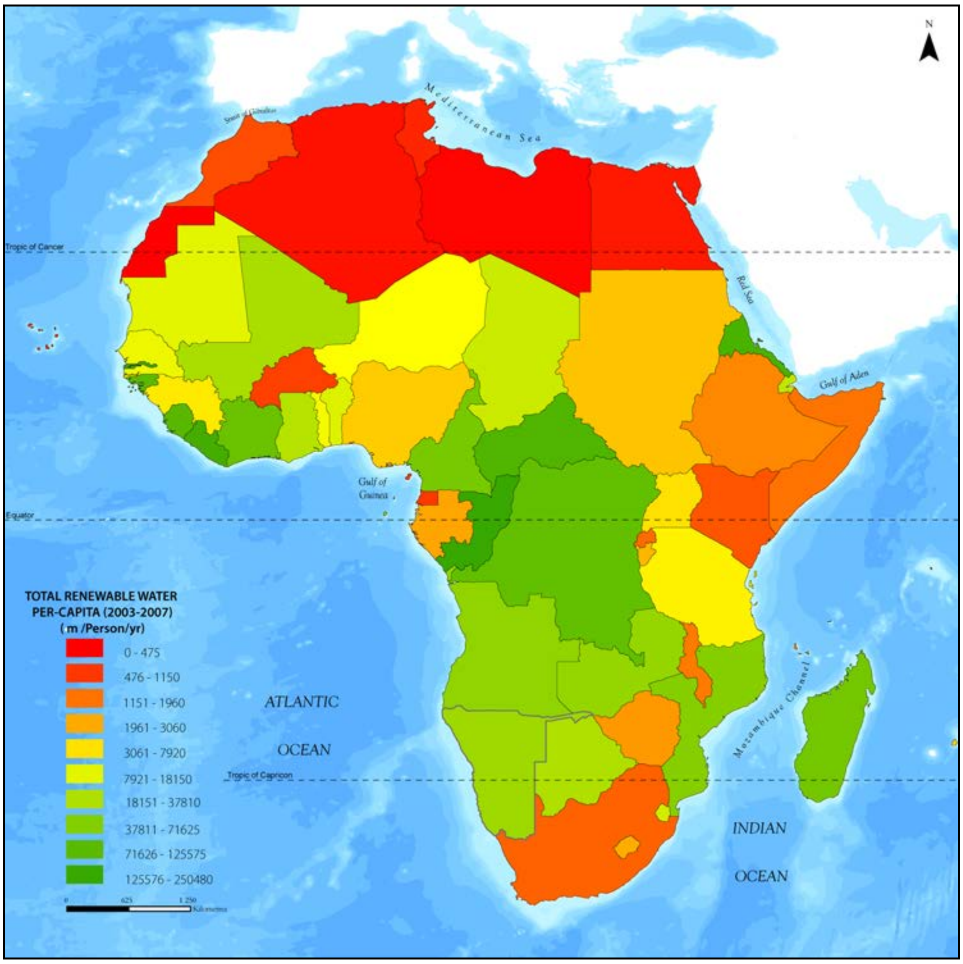

Figure 2-1: A map of Africa, indicating how water stressed South Africa is in comparison to the rest of Africa, by reflecting the amount of renewable water per capita (UNEP, 2008).

2.2. UPPER OLIFANTS RIVER CATCHMENT

2.2.1. Background

The Olifants River catchment is the largest of all the different catchments that make up the Limpopo River basin which expands over four different countries, namely Botswana, Mozambique, South Africa and Zimbabwe. The surface area of the entire Olifants River catchment is ≈85 000 km^2, with the Mozambique portion ≈11 500 km^2, whilst the South African portion is ≈73 500 km^2 (Ashton and Dabrowski, 2011). The Olifants River originates in the east of South Africa in the Mpumalanga Province and flows in a northern direction into Mozambique and finally into the Indian Ocean. It is a major tributary of the Limpopo River. Not only is the Olifants River one of South Africa's main river systems, but it is also recognized as one of the most polluted rivers in southern Africa (Grobler et al., 1994). This situation is compounded by the fact that the Olifants River was historically a strong perennial river, but has become a very weak perennial river with frequent periods of no flow (Joubert and Van Gogh, 2007). Important impoundments associated with the Olifants River include the Phalaborwa Barrage, Loskop Dam and Flag Boshielo Dam. The larger tributaries of the Olifants River include the Wilge, Elands, Steelpoort, Blyde and Ga-Selati rivers (Joubert and Van Gogh, 2007; Dabrowski et al., 2013, 2014).

Due to the differences in climate and water availability in the Olifants River catchment, this catchment has been divided into four sub-areas for more effective water management (Basson and Rossouw, 2003). These areas are as follows (Figure 2-2):

- The upper Olifants catchment, which comprises the entire upstream catchment up to Loskop Dam.
- The Middle Olifants catchment, which comprises everything downstream of Loskop Dam up to the Steelpoort River confluence.
- The Steelpoort catchment, which covers the catchment of the Steelpoort River.
- The lower Olifants catchment, which signifies everything below the Steelpoort River confluence after which the Olifants River flows through the Kruger National Park (KNP) before entering Mozambique (Mussagy, 2008).

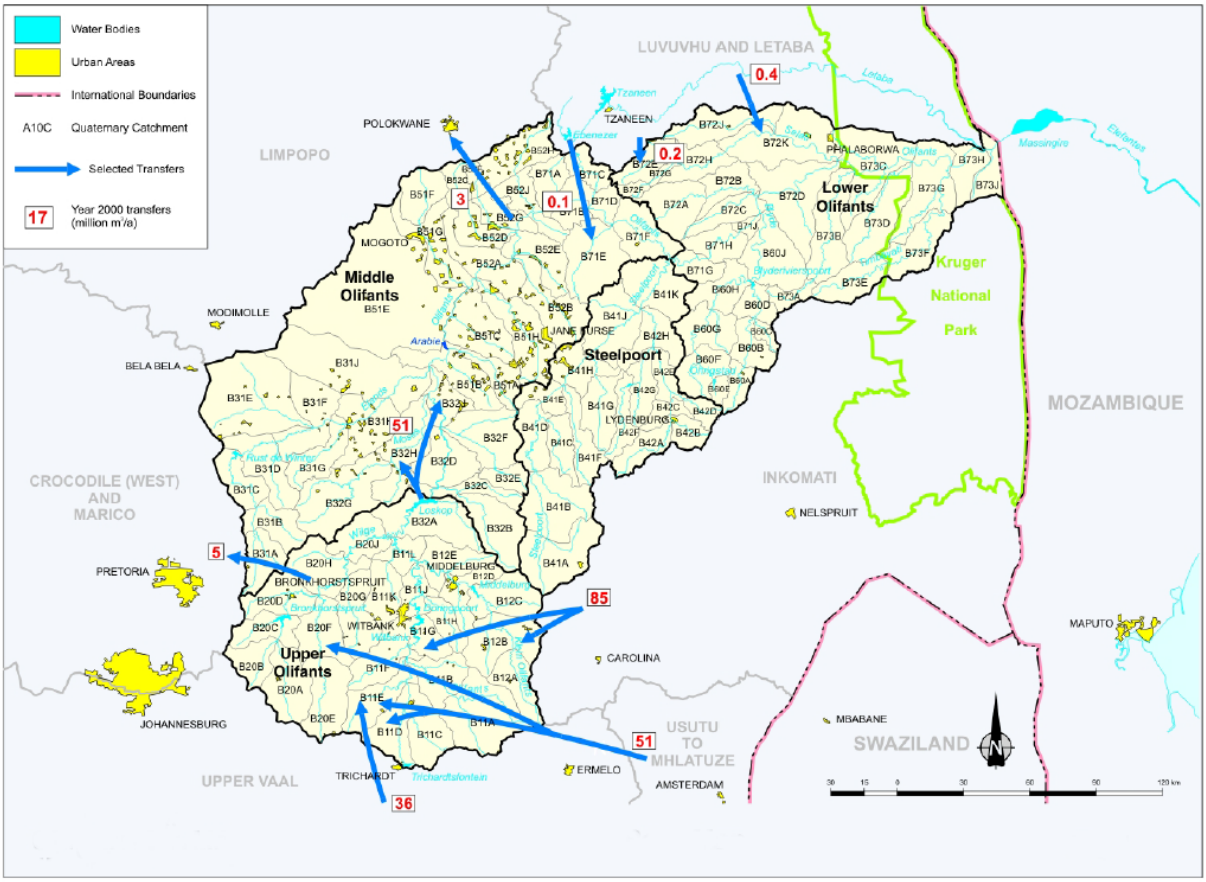

Figure 2-2: The four water management areas of the Olifants River catchment, also indicating the selected transfers taking place (Basson and Rossouw, 2003).

The upper Olifants River catchment houses ≈4 million people and has approximately 201 water storage dams with a combined capacity of ≈4 688 ML (Middleton and Bailey, 2009; Van Vuuren, 2009). Most of the pollutants originate in the upper reaches of the Olifants River which flow into Loskop Dam. The cities of Witbank and Middelburg are the main economic centers in the upper Olifants River catchment and are located in the concentrated area of mining and industrial activities (McCartney and Arranz, 2007). As a result of these intense activities, the upper Olifants River has experienced various stresses and impacts for just more than 100 years and these impacts are still occurring today. In turn, these activities generate pollutants which may affect water resources through acid mine drainage (AMD) (Bell et al., 2001; Hobbs et al., 2008), acid rain (Rodhe et al., 2002), complex industrial effluents and nutrient enrichment (De Villiers and Thiart, 2007; Oberholster et al., 2010a), partially treated or untreated sewage / wastewater, as well as runoff and habitat destruction (Seymore et al., 1994; Buermann et al., 1995; Robinson and Avenant-Oldewage, 1997). This has resulted in the river being characterized by high levels of inorganic and organic pollution, as well as significant erosion (Buermann et al., 1995; Bollmohr et al., 2008). Pollution from coal-fired power generation, chemical manufacturers, as well as chrome and steel smelters has also added to the environmental stresses in the catchment (Dabrowski et al., 2008; Dabrowski and De Klerk, 2013). Thus, it is no surprise that the Olifants River has seen major pollution driven events such as the mass mortalities of, inter alia, fish, birds and crocodiles not so long ago (2008 – 2009) (Driescher, 2007; Myburgh and Botha, 2009; Ashton, 2010; Ferreira and Pienaar, 2011).

In the upper Olifants River catchment, land use is predominated by large-scale coal mining, mineral processing, power generation, as well as agricultural activities. With the mining sector and other ancillary industries expanding in the region, urbanization and the demand for water is also rapidly increasing (Driescher, 2007; Hobbs et al., 2008). The numerous activities within the upper Olifants River catchment are extremely important to South Africa and its economy and these activities depend heavily on the benefits derived from the water resources in the catchment (Dabrowski and De Klerk, 2013). For this reason, water is augmented from elsewhere to address the shortfalls in the water supply experienced in the upper Olifants River catchment to sustain these activities (Basson et al., 1997).

The estimated total available water in the upper Olifants River catchment is ≈409 million m^3/a, whilst the requirement is ≈410 million m^3/a. Although availability and demand appear to be balanced, the water balance in the catchment is actually significantly negative. This is because water availability in the catchment is already fully allocated. The deficit is made up through transferring the exact quantities needed from other areas (≈22% of the total water available is transferred) (Basson and Rossouw, 2003). This transferred water is mainly used for power stations due to the poor water quality in the Olifants River and because power generation is the largest user of water (≈57%) in the area. Currently, transfers are occurring from the Inkomati, Usutu to Mhlatuze and upper Vaal Water Management Areas (172 million m^3/a). With the planned increase in certain sectors over the next ten years, this deficit is expected to increase, thus requiring increased water transfers, water re-use or other means of efficient water use (Basson and Rossouw, 2003).

One of the major pollution problems is the large quantities of AMD discharged into the river. This is due to the upper Olifants River catchment being extensively mined for coal (McCarthy, 2011). Mining is one of the largest contributors of point source pollution in the upper Olifants River (Nussey, 1998). In the course of extracting the coal reserves (i.e., mining) the surface area is greatly disturbed, resulting in an increased rate of AMD production (McCarthy, 2011). Large amounts of calcite or dolomite have the ability to neutralize this effect, but in the upper Olifants River catchment, this is not enough and the large amounts of AMD are released into the rivers and streams (McCarthy, 2011). Acidic mine water has the potential to increase the solubility of metals such as aluminium and results in water resources that are toxic to varying degrees. Usually, some neutralization does occur due to dilution and reactions with sediment. Even though this may aid in the removal of some pollutants, certain compounds with high solubility may remain, namely sulfate (McCarthy, 2011). This is one of the main reasons why the upper Olifants River is characterized by exceedingly high levels of metals (Nussey, 1998). These include copper and zinc (Grobler et al., 1994; Seymore et al., 1994) and this is of concern since they are classified as highly toxic (Hellawell, 1986) and with the potential to be bioaccumulated (Seymore et al., 1996). One of the main contributors to metal pollution in South Africa and especially in the upper Olifants River catchment, is mining operations (Coetzee, 1996). Metals are known to accumulate in the sediment and water column of water storage dams and contain dangerously high concentrations of trace metals and nutrients (Driescher, 2007; Oberholster et al., 2010a).

The main dams in the upper Olifants River catchment and its tributaries are Bronkhorstspruit, Witbank, Doornpoort, Middelburg and Loskop dams. Bronkhorstspruit Dam was completed in 1948 with a capacity of ≈58 million m^3, whilst Witbank Dam was competed in 1949 with a capacity of ≈104 million m^3. Two other dams, namely Doornpoort and Middelburg dams were completed somewhat later (1951 and 1966, respectively) and are somewhat smaller, with a capacity of ≈9.2 and ≈49 million m^3, respectively. The oldest (1937) and largest (≈375 million m^3) dam in the upper Olifants River catchment, and as a result the most important, is Loskop Dam, which is located at the bottom end of the upper Olifants River catchment (Basson and Rossouw, 2003).

2.2.2. Climatic and Topographical Characteristics

The upper Olifants River catchment has a mean annual precipitation of ≈680 mm, mean annual runoff of ≈10 800 million/m^3, and a mean annual evaporation of ≈1 600 mm (Figure 2-3) (Midgley et al., 1994). The prevailing wind systems are known to strongly influence the climate of the Olifants River catchment (Ashton and Dabrowski, 2011), with the primary rain-bearing winds coming from the south-east, producing the summer rainfall pattern in the catchment (Tyson, 1986). The elevated areas in the catchment receive the most rainfall and as a result the summer rainfall exceeds the summer evaporation rates, with very little rainfall received during the drier winter months (Schulze, 1997).

The topography of the Olifants River catchment is extremely varied, ranging from approximately 150 m above sea level where it joins the Limpopo River in Mozambique, to over 2 000 m in the mountainous region, marking the transverse position of the northern extension of the Drakensberg Mountains (Ashton and Dabrowski, 2011).

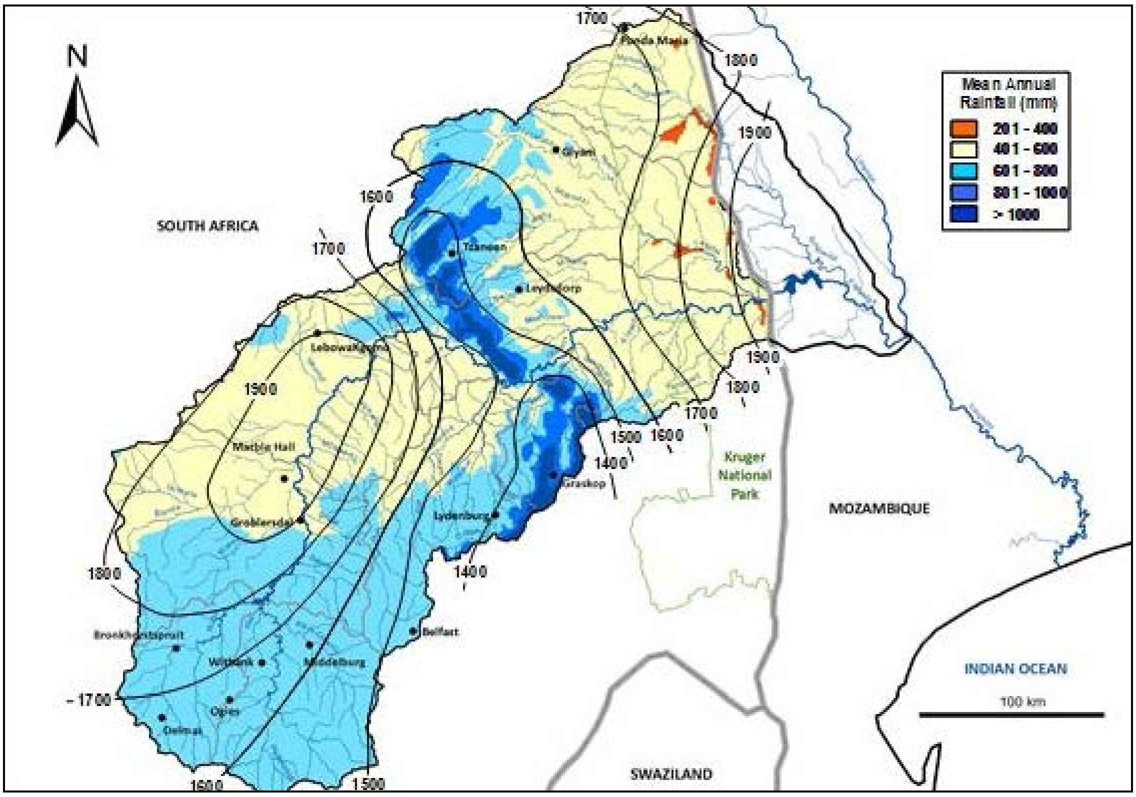

Figure 2-3: The mean annual rainfall and mean annual evaporation of the Olifants River catchment in South Africa (Ashton and Dabrowski, 2011).

2.2.3. Geological Characteristics

The entire Olifants River catchment (Figure 2-4) overlies a portion of the Kalahari Craton, which forms part of the Limpopo basin. The Karoo System rocks overlie extensive areas in the southwestern (upper) portion of the catchment that is associated with sandstones, mudstones, conglomerates and shales (Johnson et al., 2006; Ashton and Dabrowski, 2011). The coal seams in the Witbank Coalfield occur within the Vryheid Formation that forms the midpart of the Ecca Group, which, in turn, is part of the Karoo Supergroup (Bell et al., 2001). In the southern portion of the basin, the extensive, carbon-rich sedimentary rocks of the Karoo System contain enormous economic reserves of coal and as a result host intensive coal mining activities (Bullock and Bell, 1997).

At the northern margin of the coalfield, the Vryheid Formation rests on the basement rocks of the Transvaal Supergroup, the Waterberg Group and volcanic rocks associated with the Bushveld Igneous Complex. The Transvaal Super Group consists mainly of dolomite, quartzite and shale, with further intrusion by diabase dykes (Nussey, 1998), whilst the Waterberg Group consists mainly of coarse-grained, red sedimentary rocks, consisting mainly of sandstone and quartzite. Regional metamorphism has taken place through the intrusion of the Bushveld Complex into the Transvaal Supergroup (Watson, 1998).

The five recognized coal seams in the Witbank area are named consecutively from 1 to 5, with coal seam 5 being the youngest. The weathered zone ranges between 5 and 10 m and consists of weathered sandstones and shales with an abundance of ferricrete (Bell et al., 2001).

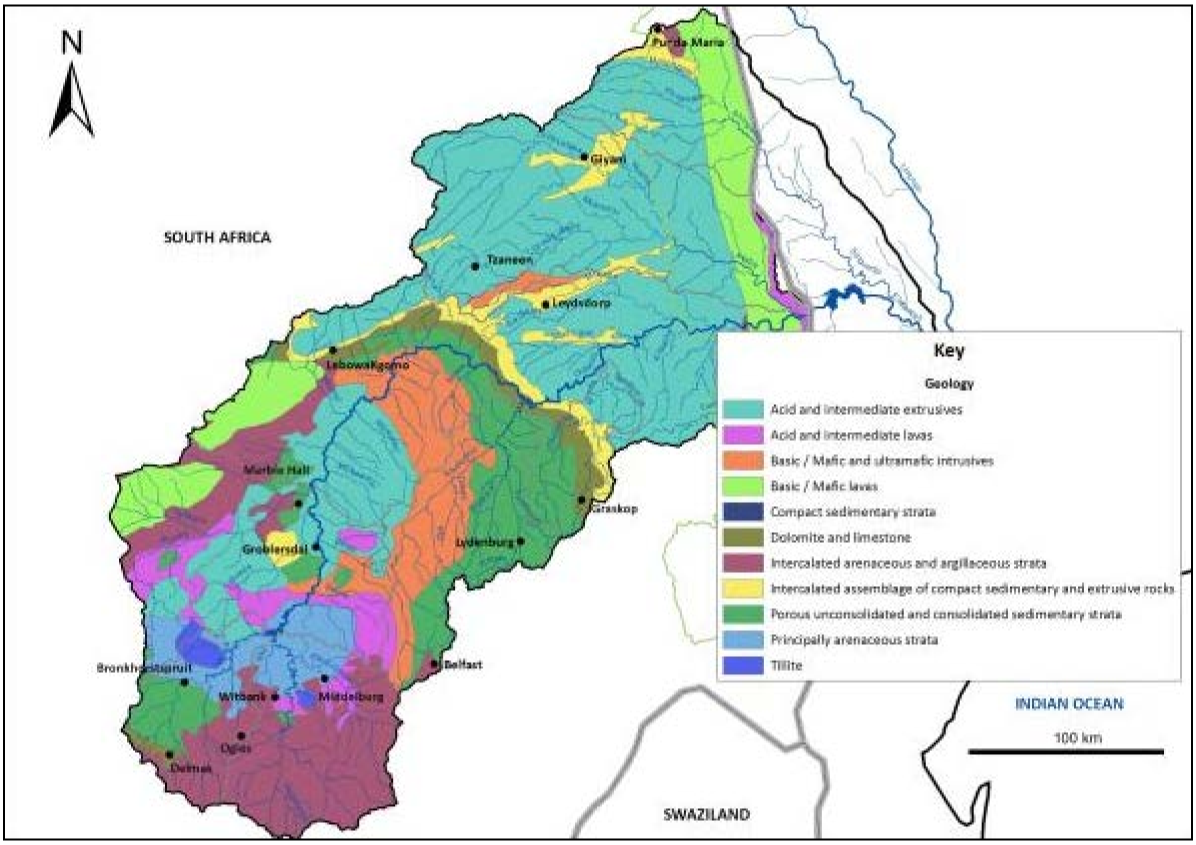

Figure 2-4: The geological variation of the Olifants River catchment in South Africa (Ashton and Dabrowski, 2011).

2.2.4. Coal Mining

Various different commodities are mined in the Olifants River catchment (Figure 2-5), with the coal mining industry being second only to the gold mining industry in South Africa (Hobbs et al., 2008). The Witbank coalfield, along with the Highveld and Ermelo coalfields, signifies the largest active coal mining area in South Africa and produces ≈48% of the country's total power generating capacity (Tshwete et al., 2006). Coal mining in Witbank and Middelburg started around 1895 to support the gold and diamond mining industries. Many of these mines are now abandoned, whilst some have caught fire and others have collapsed. But one thing that most of the mines have in common is that they are decanting AMD (Wilson and Anhaeusser, 1998; McCarthy, 2011). Numerous underground coal mines have also been flooded, resulting in large volumes of AMD decanting into the rivers and streams of the upper Olifants River catchment since the 1990s (Oberholster et al., 2010a).

Coal mining is an extremely important activity in South Africa and 81% of the coal in South Africa is produced by the Witbank-Highveld coalfield, thereby contributing significantly to the South African economy (DMR, 2009). This was evident from a study in 1997 where more than 5% of South Africa's gross domestic product (GDP) came from the Olifants River catchment. The largest contributing sectors in this area include mining (≈22%), manufacturing (≈18%), electricity generation (≈16%) and government (≈15,6%) (Basson and Rossouw, 2003). The eight largest collieries in the area produced ≈152 MT in 2005, whilst a further 21 medium-sized mines produced 67 MT and 38 small mines produced 27 MT (Prevost, 2007). This equates to a total saleable production of 246 MT in 2005, which is a significant increase from 1995 (100 MT) (Wilson and Anhaeusser, 1998). The strategic significance of these activities for South Africa can thus not be over emphasized.

Coal is extracted in different ways in the Olifants River catchment. During a study in 2005 it was found that ≈53% of the run of mine (ROM) was provided by opencast mines, whilst bord and pillar accounted for 37%, stoping accounted for 7% and 3% from longwall mining techniques (Hobbs et al., 2008). Coal mining in the area is one of the largest contributors of pollutants in the Olifants River and has a variety of ecological consequences (Coetzee, 1996; Nussey, 1998). This is of particular importance after a mine has been depleted. The reason for this is that during operations most mining companies have suitable remedial plans in place, but once a mine closes a lack of management, planning and financial provision results in various environmental impacts. A recent study estimates that coal mines in the Olifants basin have the potential to be able to produce ≈17 ML of minewater per day (Idowu et al., 2008). If not dealt with properly, this may lead to more significant impacts in future.

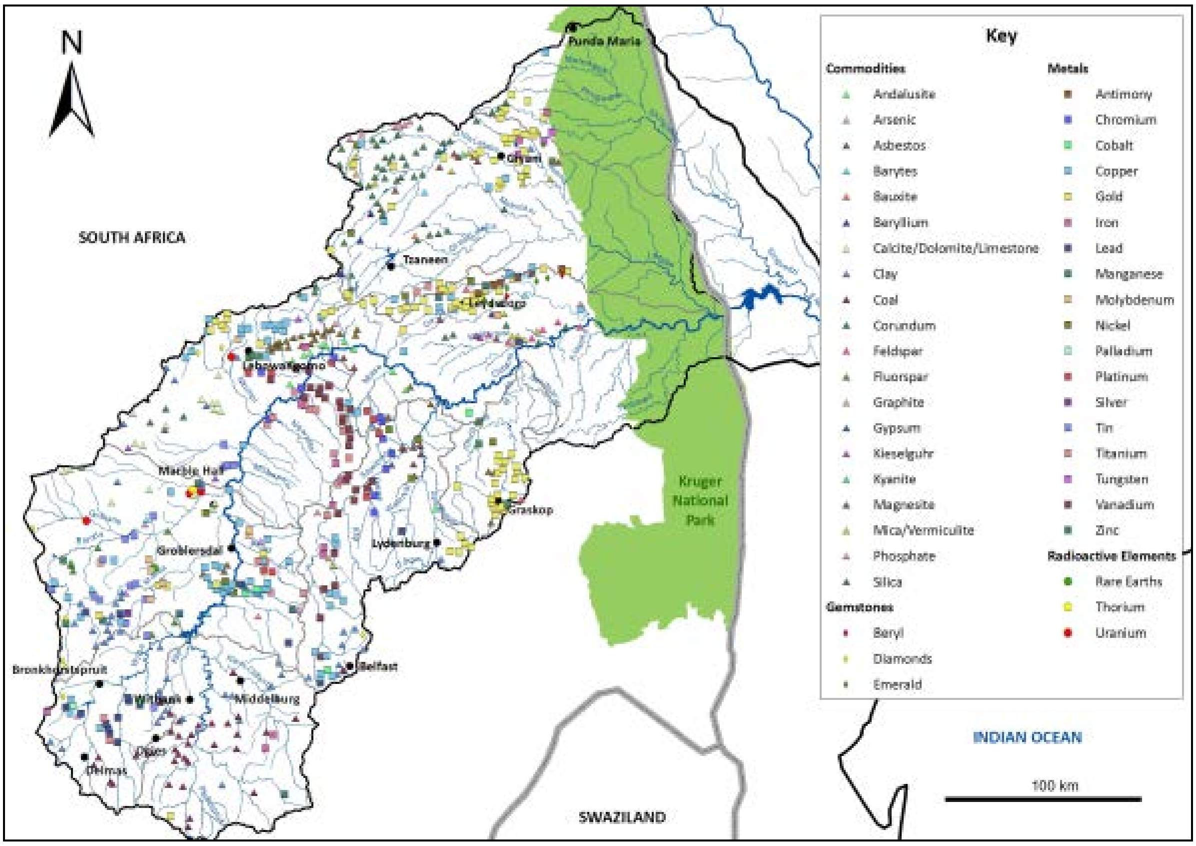

Figure 2-5: The distribution of various minerals mined in the Olifants catchment (Ashton and Dabrowski, 2011).

2.2.5. Other Land Uses

Besides coal mining, a wide variety of other developments occur in the upper Olifants River catchment (Figure 2-6). These include agricultural practices, rural and urban development, industrial activities, aforrestation and power generation (McCartney and Arranz, 2007). Agriculture is considered to be the largest user of water within the catchment and is mainly characterised by maize and potatoes (Nussey, 1998). Dry-land cultivation is also quite extensive with grains, cotton and sunflower being the predominant products produced (Nussey, 1998; Basson and Rossouw, 2003). The crops produced by the agricultural sector in the upper Olifants River catchment are sold on both the local and international market, especially the European Union (Oberholster et al., 2013). The demand for water in rural areas includes all domestic water requirements other than those for urban use. This water use may be for livestock, subsistence irrigation or personal use. On the other hand, the water demand for urban areas includes the water requirements for industrial, commercial, institutional and municipal uses. Linked to urban expansion is the development of wastewater treatment works (WWTWs) that are also prevalent in the catchment. Although WWTWs are meant to reduce the impact of human waste on receiving waterbodies, most of these are not functional in the area (Dabrowski et al., 2013; Dabrowski and De Klerk, 2013). Industrial activities such as chemical manufacturers and metal smelters occur in the catchment (Driescher, 2007), producing effluent containing a variety of potential pollutants.

Afforestation is known to affect the hydrology of a catchment by reducing runoff and increasing evapotranspiration (Basson and Rossouw, 2003). Roughly more than 17 500 ha of afforestation occurs within the upper catchment (Nussey, 1998). Due to the high prevalence of coal in the area, most of South Africa's coal-fired power generation also occurs in this area (Dabrowski et al., 2008). The large quantities of coal being burnt are known to emit large amounts of chemicals (Zunckel et al., 2000) that may enter the upper Olifants River or its tributaries (Ashton and Dabrowski, 2011).

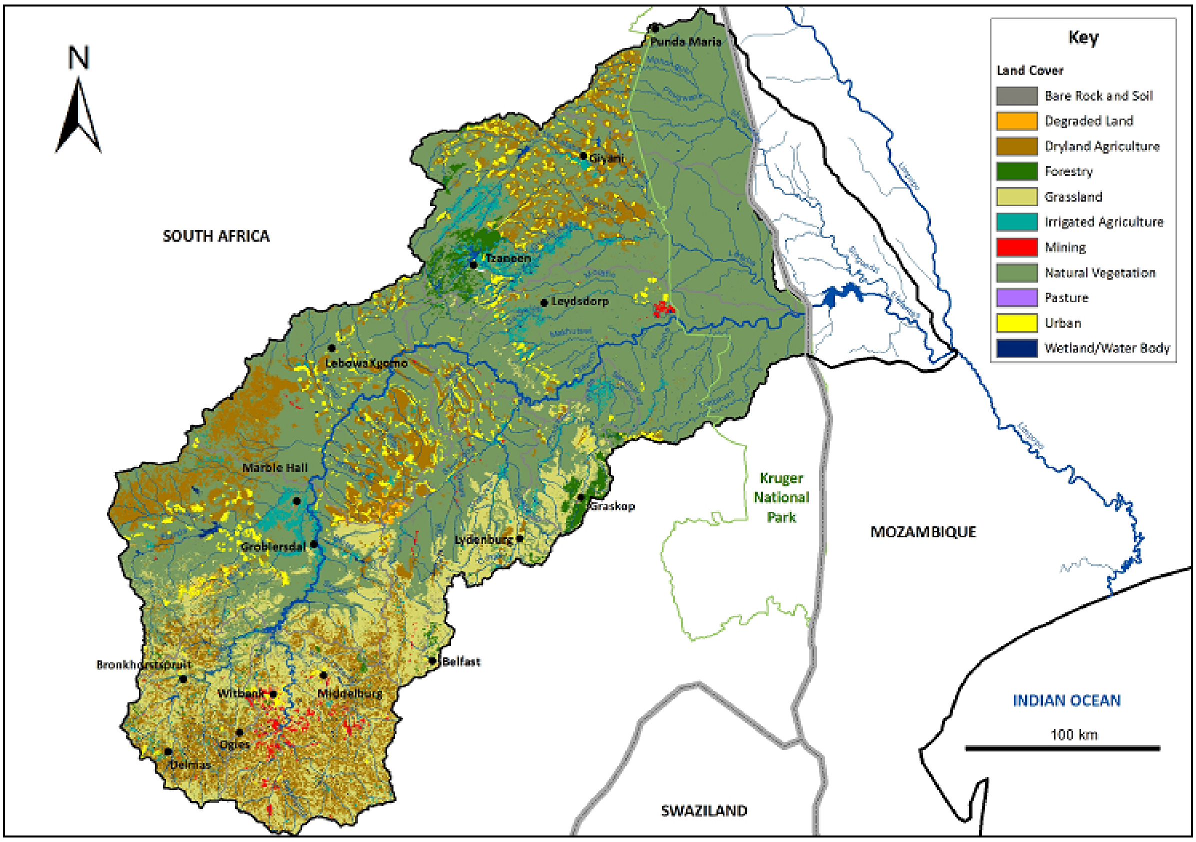

Figure 2-6: An overview of the different land uses within the Olifants River Catchment (the upper, middle and lower parts of the South African portion) (Ashton and Dabrowski, 2011).

2.2.6. Overall

Due to this region being heavily impacted by coal mining for more than 100 years, progressive urbanization, industrialization and mining along the Olifants River have placed substantial pressures on this aquatic ecosystem (Swanepoel, 1999; Steyn, 2008; Oberholster et al., 2010a). Ironically, this has provided researchers with a unique opportunity to study this river to gain insights into the long-term effects of coal mining on South Africa's water resources. This is particularly useful since numerous new coal mining leases have been granted in the upper Olifants River catchment (DME, 2004). These operations will most likely prolong and exacerbate the already deteriorated nature of the upper Olifants River through, inter alia, increased AMD. Although every effort should be made to restore the Olifants River, this does provide a significant opportunity to study the interaction of the complex blend of mining, industrial, agricultural, sewage and urban wastewater and its interaction with the water resources. If done properly, this should provide researchers with the ability to identify those limited controlling factors that regulate a multitude of variables so that management and monitoring efforts may be designed more optimally to ensure the sustainability of South Africa's water resources when threatened in this way. This is especially important since the anticipated future growth in water demand (DWAF, 2004b) will require a more efficient use of available water resources.

2.3. MOKOLO RIVER CATCHMENT

2.3.1. Background

Globally, South Africa ranks approximately ninth in terms of proven coal reserves that are expected to last for more than 200 years. This is important since 77% of South Africa's energy needs are obtained from coal-fired power stations. With the Witbank / Middelburg coalfields in decline, the Waterberg coalfield is estimated to supply coal for roughly 40% of South Africa's energy needs in the near future (Aphane and Vermeulen, 2015). The Waterberg coalfield, discovered in the 1920s, was located relatively too far from South Africa's economic hub and thus has not been mined extensively. With the energy crisis that South Africa is faced with, along with the fact that the Witbank / Middelburg coalfields are in decline, stakeholders are considering the Waterberg coalfield to secure South Africa's future energy needs for coal (CSIR, 2009).

The Waterberg forms a wide basin draining four main rivers into the Limpopo River. These rivers are the Lephalala, Mokolo, Matlabas and Mogalakwena rivers (CSIR, 2009). Currently, most of the mining related activities are occurring in the catchment of the Mokolo River, which is one of the larger tributaries (Schachtschneider and Reinecke, 2014). Most of the plans for future mining will be in this catchment, so the Mokolo River is expected to be at the center of all these planned activities and faces the greatest risks. The Mokolo River catchment is one of the most important tributaries of the Limpopo River and extends approximately ≈8 400 km^2 (Jacobsen and Kleynhans, 1993; DWA, 2012). Some of the main tributaries of the Mokolo River are the Grootspruit, Klein Sandspruit, Heuningspruit, Malmanies, Poer-se-Loop and Rietspruit.

The catchment of the Mokolo River is considered to have the most potential for further development (DWAF, 2003), due to the higher availability in surface water compared to the other three rivers (DWAF, 2009; DWA, 2012). The mean annual runoff of the Mokolo River catchment is ≈315 million m^3/a (Tshikolomo et al., 2013), whilst the mean annual runoff of the Mokolo Dam is estimated to be ≈240 million m^3/a (Midgley et al., 1994). The storage capacity of Mokolo River catchment is ≈145.9 million m^3. There is thus an available mean annual runoff in the Mokolo River catchment of ≈154 million m^3 (Tshikolomo et al., 2013).

The Mokolo River catchment is divided into six units (Figure 2-7), based on catchment layout and different ecoregions, of which the mainstem Mokolo River make up three of these units and the other three are main tributary regions (River Health Programme, 2006). The six regions in the Mokolo River catchment are:

- Upper Mokolo: This is the upper most (southern) portion of the Mokolo River which stretches from the confluence with the Klein Sand River up to approximately the confluence with the Sterkstroom.
- Middle Mokolo: This middle portion of the Mokolo River stretches from the confluence with the Sterkstroom to just before the confluence with the Tambotie River.
- Lower Mokolo: Everything below the middle Mokolo River until where the Mokolo River enters the Limpopo River is categorized as the lower Mokolo River.
- Sand tributaries: This area comprises all the tributaries that combine to form the Mokolo River near the town of Alma.
- Sterkstroom catchment: This sub-catchment entails all the streams that drain into the Sterkstroom that eventually enters the Mokolo River.
- Rietspruit: This region encompasses parts of the Sandspruit system, as well as the Rietspruit.

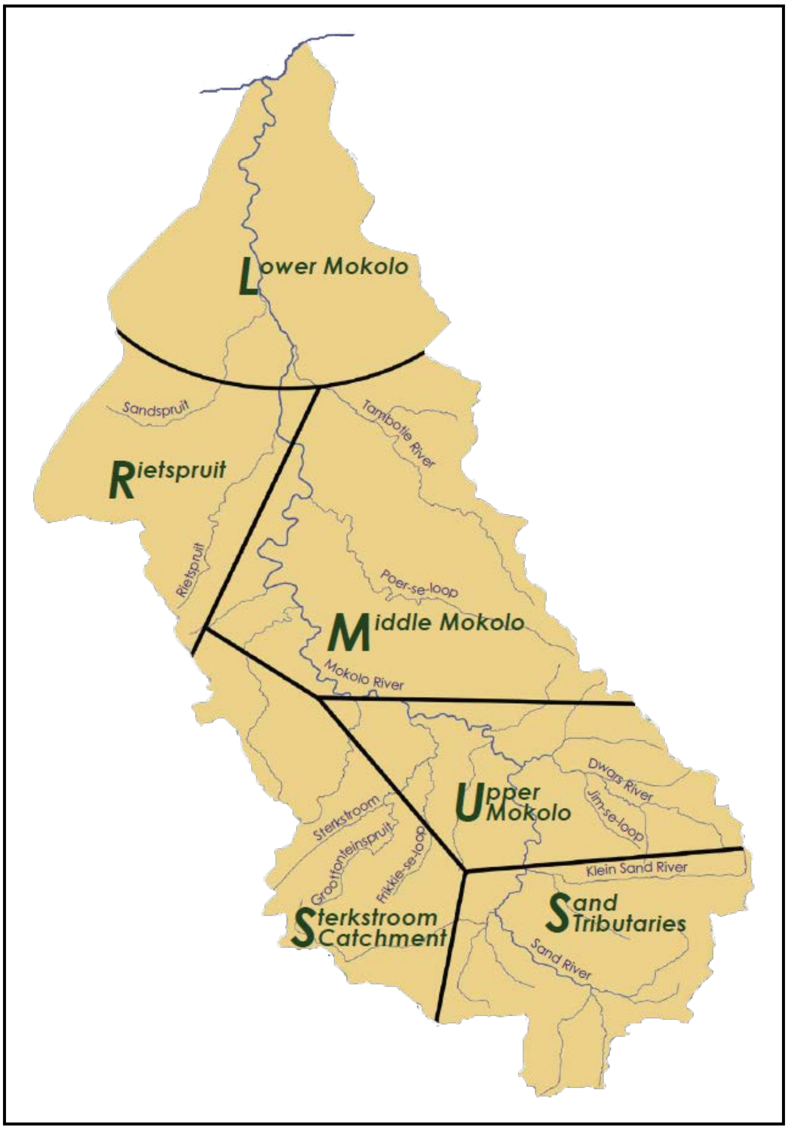

Figure 2-7: The six regions into which the Mokolo River catchment is divided (River Health Programme, 2006).

The upper Mokolo River is mostly perennial, whilst lower parts are generally semi-permanent to intermittent. With the relatively low volumes of water available in this catchment, a host of different activities needs to be supported (River Health Programme, 2006). A couple of relatively small towns are located in the Mokolo River catchment, as well as informal settlements around these towns. The towns of Lephalale, Alma and Vaalwater are the major ones in the catchment. All of these developments rely on the water in the Mokolo River supplied either from impoundments, river abstraction points or boreholes. Commercial agriculture is currently the most important economic activity along the Mokolo River, with game farming to a lesser extent (Seaman et al., 2013). As a result, the most extensive water user in the catchment is formal irrigation, whilst the power stations located in the lower catchment also uses significant amounts of water. In addition, numerous small-scale subsistence farmers also rely on the river. A variety of drought-tolerant crops are produced in this catchment (Ashton et al., 2001). Due to the limited water available in the area, many farmers have opted to convert irrigation practices in favor of cattle or game farming (DWAF, 2007).

The main industrial land use is restricted to the lower part of the catchment and is mainly centered on the Grootegeluk Coal Mine, as well as the Matimba and Medupi Power Stations, all of which are in the vicinity of the town of Lephalale. The Grootegeluk Coal Mine is the largest coal mine in South Africa (DWAF, 2008) and is currently expanding its operations. Extensive sand mining operations also occur along the lower Mokolo River that changes the flow of the river (Seaman et al., 2013; Ramoelo et al., 2015). As a result, riparian vegetation is destroyed, riverbanks are modified and increased erosion is having negative impacts on the Mokolo River (LEDET, 2012; Ramoelo et al., 2015). Matimba Power Station, the largest dry cooling power station in the world, was commissioned between 1988 and 1993. This power station is provided with coal from the Grootegeluk Mine and currently uses its total allocation of ≈7.3 million m^3/annum of water (DWAF, 2004c, 2008). In 2007, the construction of the Medupi Power Station was started by Eskom (South Africa's state-owned electricity utility) in the Lephalale region in the Waterberg (Fouilloux and Otto, 2009). The Medupi Power Station operates (although not at full capacity yet) alongside the Matimba Power Station and uses water from the Mokolo Dam for cooling (Busari, 2008). Ultimately, this may result in the Grootegeluk Mine to use most of its water allocation (≈9.9 million m^3/annum) to be able to provide sufficient coal for Medupi as well. This will undoubtedly affect the assurance of water for other sectors (e.g., agriculture) further downstream, as Grootegeluk is currently using only about half (≈4 million m^3/a) of its water allocation from the Mokolo Dam (Seaman et al., 2013).

The Mokolo Dam is the largest reservoir in the Mokolo River catchment and is situated approximately 50 km north-west from Vaalwater. The building of the Mokolo Dam was completed in 1980, mainly to supply the Matimba Power Station and to support irrigation activities downstream (DWAF, 2004c). The Mokolo Dam is approximately ≈9 km^2 large with a full storage capacity of 145 ML (Oberholster et al., 2012). After construction, the dam produced a yield of about 39 million m^3/annum (RSA, 1970), but due to the increase in agriculture upstream the yield decreased to ≈23 million m^3/annum (DWAF, 1992). Apart from the mining and power generation allocation, approximately 1 million m^3/annum is allocated for the town of Lephalale and ≈10.4 million m^3/annum for the irrigation practices downstream (DWAF, 2001). The Mokolo Dam is thus already fully allocated (DWAF, 2004c). With largely unexploited coal reserves remaining in the Waterberg, various mining operations are envisaged for the area, which could further compound the water demand situation in the catchment. Thus, the Mokolo and Crocodile (West) Water Augmentation Project is currently underway to transfer water to the Mokolo River from the Crocodile (West) River. This water will be used to address the deficit between the water demand and availability near the town of Lephalale (Van Niekerk and Du Plessis, 2013).

Currently, the Mokolo River is classified as endangered (LEDET, 2012) and it is anticipated that mine expansion in the area will not only affect the water demand in the area, but may also have a significant impact on the quality of the surrounding water resources. However, due to the low rainfall, low groundwater recharge and high evapotranspiration rates, the effect of, for example AMD, may be reduced (Bester and Vermeulen, 2010). The potential impact of AMD in the Waterberg has been studied to some degree using techniques such as acid base accounting to try and determine the acid generating potential or neutralizing potential of the geology in the area (Aphane and Vermeulen, 2015). According to Vermeulen et al. (2011), a high acid generating potential does exist. This has been supported by a study by Bester and Vermeulen (2010) that also showed that the rocks in the area are susceptible to acid generation, but also has a potential degree of neutralization. With this high potential for AMD in the Mokolo River catchment, it is important to note that no method for the proper remediation of AMD impacts has as yet been obtained (Aphane and Vermeulen, 2015). Thus, proper monitoring is needed to ensure sustainable development as mining may also impact on the water resources not only through pollution, but also through depleting the already limited amount of water available in the area (Bester and Vermeulen, 2010).

2.3.2. Climate and Topographical Characteristics

The Waterberg is a very water stressed catchment with an annual rainfall of ≈500 mm, whilst the estimated evaporation is approximately 2 000 mm per annum (Figure 2-8) (Schulze, 1997). The Mokolo River (being a tributary of the Limpopo River) and the Limpopo River (the border of South Africa) are the two important water resources in the area, as most of the other water resources only have a strong seasonal characteristic (Aphane and Vermeulen, 2015).

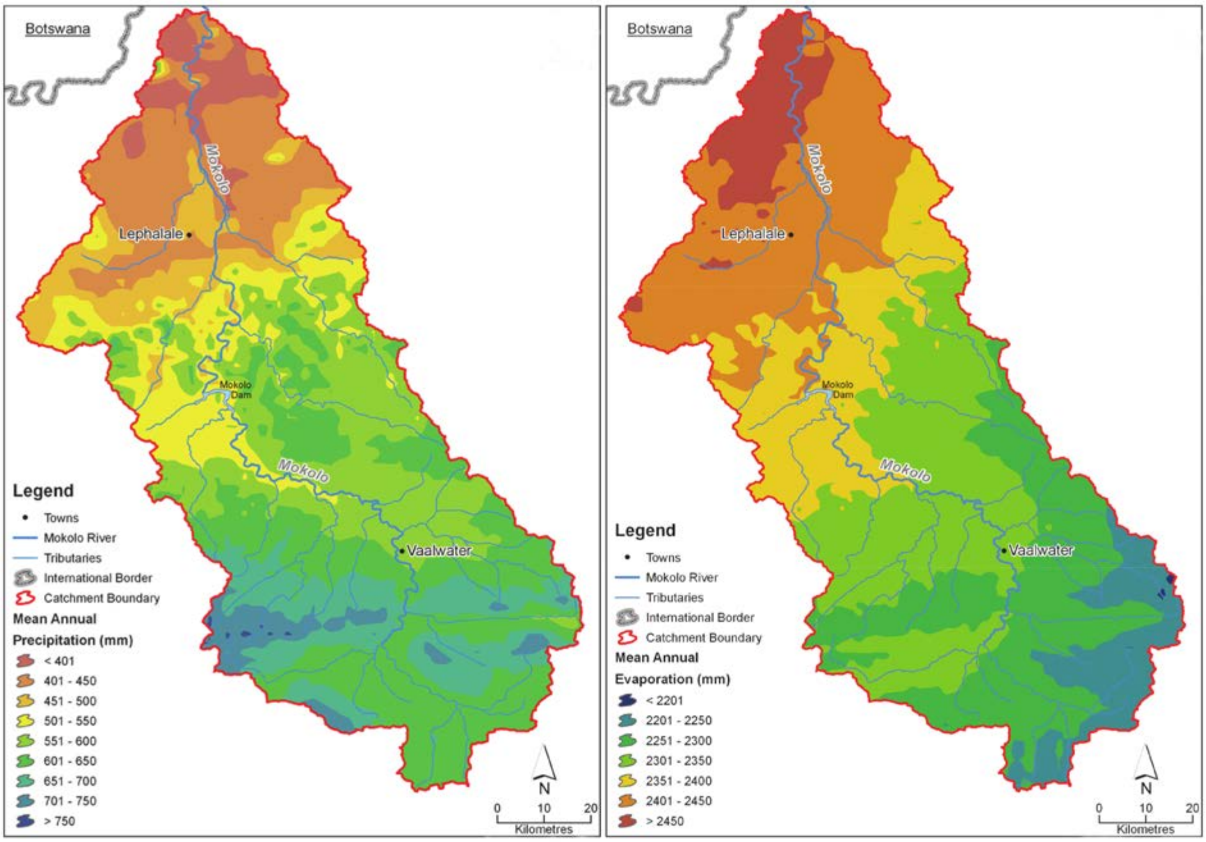

Figure 2-8: The mean annual rainfall (left) and mean annual evaporation (right) of the Mokolo River catchment (Seaman et al., 2013).

The Mokolo River originates in the Waterberg mountains and varies quite substantially in elevation and slope from south to north (Figure 2-9). The south of the area ranges between 1 500 – 1 700 m above sea level and drains into the Limpopo River at an altitude of between 800 – 1 000 m (Partridge et al., 2010), producing a relief of roughly 1 000 m. The slope in the Mokolo River catchment ranges from lowest in the northern region and the steepest in the middle and upper parts in the southern region (Seaman et al., 2013).

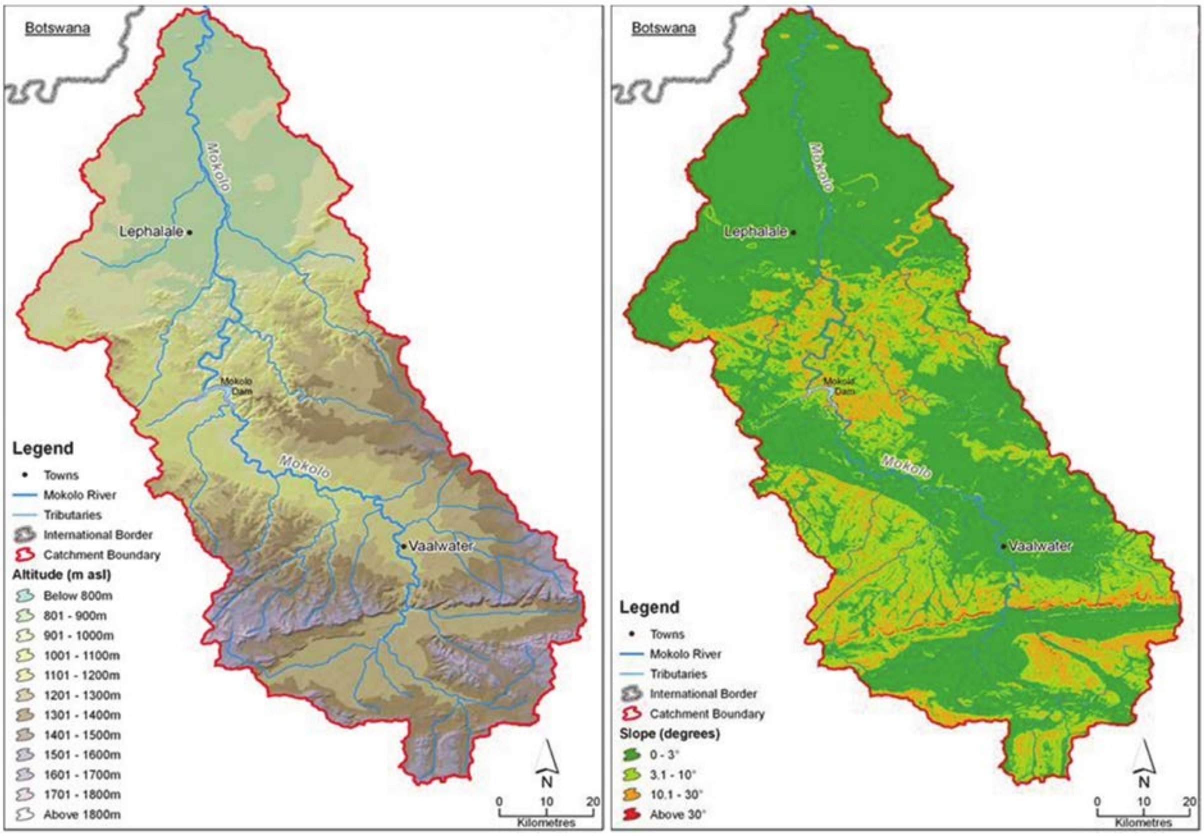

Figure 2-9: The elevation (left) and slope (right) of the Mokolo River catchment (Seaman et al., 2013).

2.3.3. Geological Characteristics

The regional geology of the Mokolo River catchment varies somewhat between the upper and lower regions (Figure 2-10). Most of the upper and middle part of the Mokolo is predominantly metasedimentary rocks of the Waterberg group, consisting mainly of feldspathic sandstones, quartzites, felsites and conglomerates, including parts belonging to the Transvaal Supergroup (River Health Programme, 2006; Oberholster et al., 2010b; Seaman et al., 2013). On the other hand, mudstones, shales and basalts of the coal-bearing Karoo Supergroup make up the lower parts of the Mokolo River catchment towards the Limpopo River (Ashton et al., 2001; Barker et al., 2006). The soils of the upper Mokolo River is characterized by medium to deep sandy-clay loam soils, whilst the moderately deep sandy loam soils are found in the lower Mokolo River catchment (Midgley et al., 1994). The most abundant sulfide mineral found here, is pyrite (Aphane and Vermeulen, 2015).

The Waterberg coalfield in the lower reaches is heavily faulted. The major boundary faults in the Waterberg coalfield is the Zoetfontein (northern boundary), Eenzaamheid (southern boundary), as well as the Daarby fault (north-eastern boundary) (Roux, 2004). The Karoo Supergroup was deposited around 260 - 190 million years ago and during deposition the Zoetfontein fault was tectonically active, but the Eenzaamheid and Daarby faults were not (Fourie et al., 2009). The Karoo sequence contains all the different major formations, namely the Stormberg Group (top), followed by the Beaufort Group, the Ecca Group and the Dwyka Group (bottom) (Wilson and Anhaeusser, 1998). Sediments of the Karoo sequence were deposited on the Waterberg Group south of the Eenzaamheid fault, whilst north of the Zoetfontein fault, they were deposited on Achaean rocks. The Ecca formation is the coal bearing sequence containing the Volksrust formation (with a thickness of ≈60 m) and the Vryheid formation (with a thickness of ≈18 m). That is made up of four coal seams (Roux, 2004; Jeffrey, 2005).

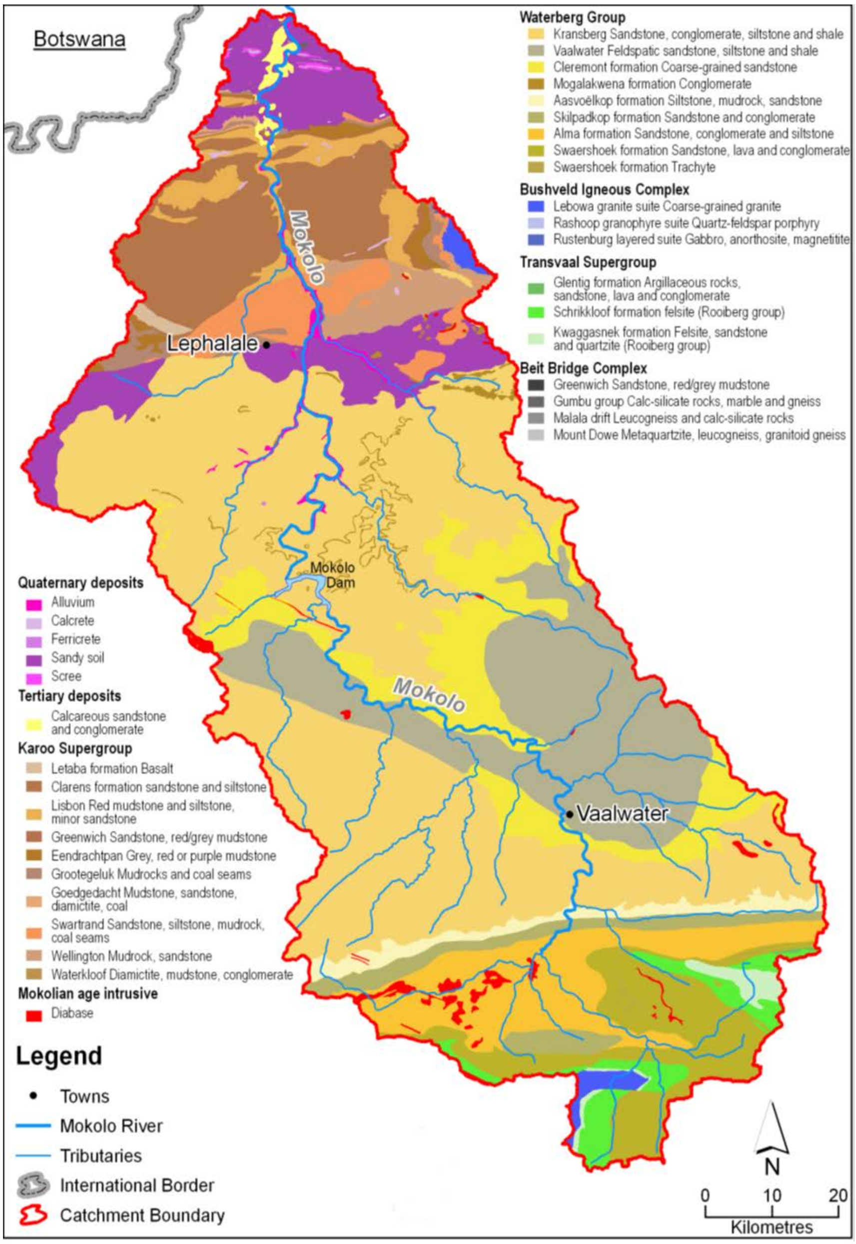

Figure 2-10: The regional geological composition of the Mokolo River catchment (Seaman et al., 2013).

2.3.4. Coal Mining

Coal was discovered in the Waterberg coalfield (Figure 2-11) in 1920 after which the development of the Grootegeluk Colliery started in the 1970s and produced its first tons of low-ash coking coal in 1980 (Wilson and Anhaeusser, 1998). The Waterberg coalfield in the Limpopo Province of South Africa covers an area of roughly 3 520 km^2 (Venter, 2013). An estimated coal reserve of ≈76 billion tons is located in the Waterberg that could potentially support South Africa for 40 - 50 years (Venter, 2013). The Waterberg coalfield is heavily faulted with the Daarby fault, subdividing the coalfield into shallow areas in the west and a deeper part in the north-east (Bester and Vermeulen, 2010).

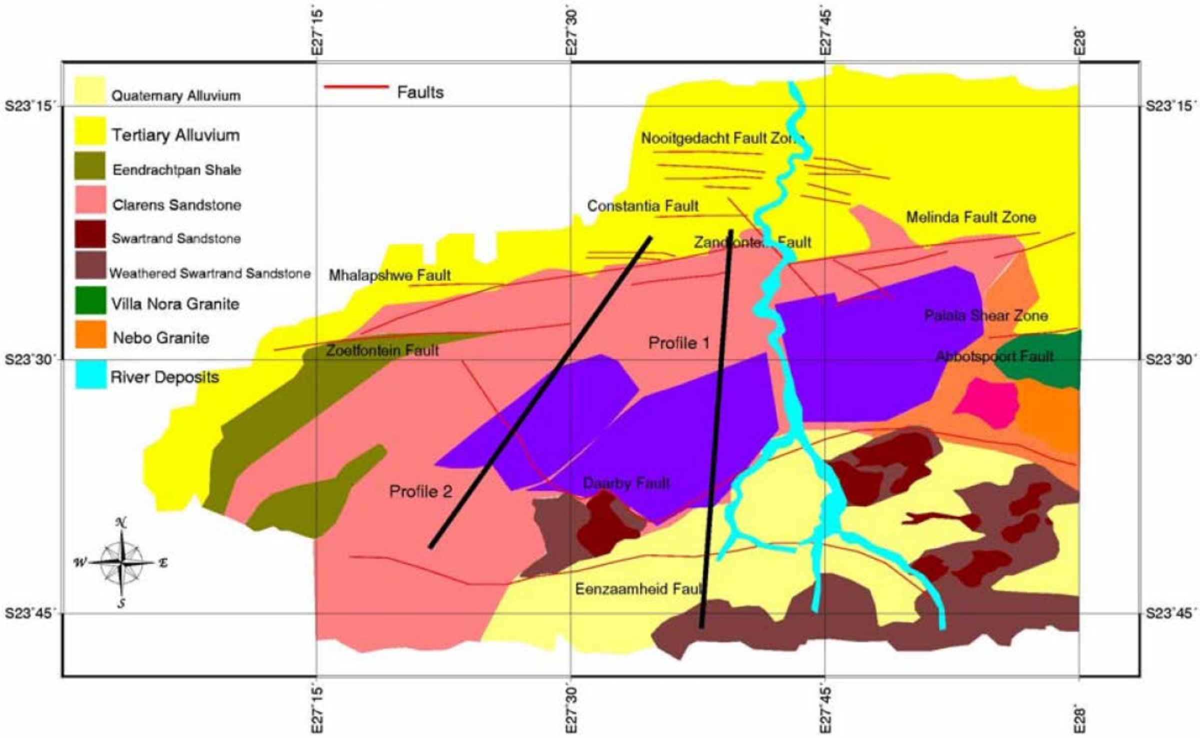

Figure 2-11: A simplified representation of the lithology in the Waterberg coalfield. The purple areas reflect the relative position of the coalfield (Fourie et al., 2009).

The Waterberg coalfield is known to have 11 coal zones (Figure 2-12) that are largely hosted by shales, mudstones, siltstones and sandstones and is ≈115 m thick. Zones 1 - 4 are predominantly dull coal belonging to the Vryheid formation (≈1.5 m - 5.5 m thick), whilst zones 5 - 11 occur in the Grootegeluk formation, with alternating mudstone and thin coal seams that consist of bright coal (Wilson and Anhaeusser, 1998). According to Dreyer (1999), the deepest level to which coal can be economically mined via open cast methods in the Waterberg coalfield is zone 4. However, underground mining operations could potentially exploit deeper coal seams.

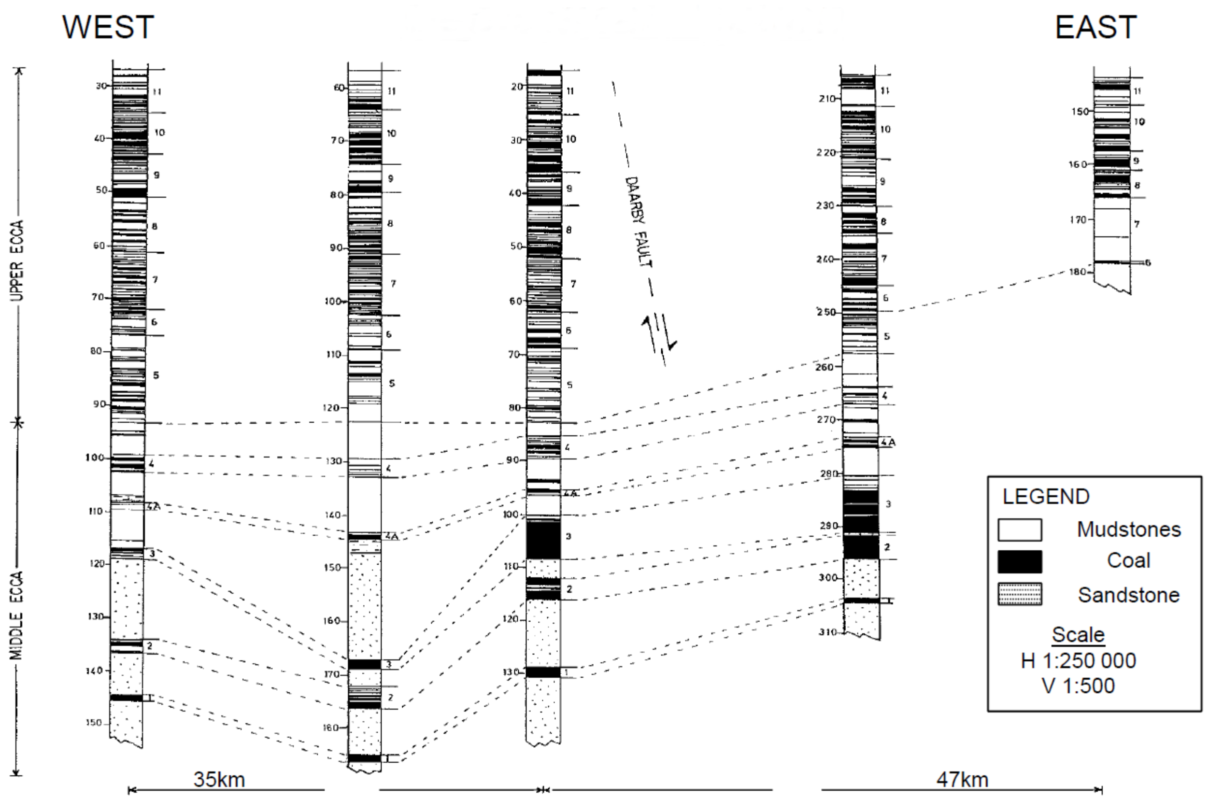

Figure 2-12: The Grootegeluk and Vryheid formations from east to west in the Waterberg coalfield (Dreyer, 1999).

A study by Aphane and Vermeulen (2015) showed that up to half of their samples had a high risk for AMD, whilst another 10% had a medium risk. The remaining samples showed some degree of neutralization. Currently, the only significant mine in the area has been found to have an impact on the nearby water resources (both surface and groundwater) (Vermeulen et al., 2011).

2.3.5. Other Land Uses

In the Mokolo River catchment, various forms of agricultural activities (Figure 2-13) predominate the land use activities in the area (River Health Programme, 2006). These include commercial and small-scale farmers using irrigation and dry-land cultivation (DWAF, 2003). These agricultural practices also use the biggest amount of water (≈87%) to produce products such as tobacco, sorghum, maize, citrus and tropical fruits, sunflower and vegetables, which contribute significantly to the agricultural output of the province (DWA, 2012; Seaman et al., 2013). Currently, game farming occurs on a smaller scale and is mainly used for tourism, although many farmers are converting to game farms due to the uncertainty of water availability in the catchment (Seaman et al., 2013). A small number of towns are present with a number of rural settlements occurring throughout the catchment (Ashton et al., 2001). The main industrial development in the catchment is Eskom's Matimba and Medupi Power Stations. These industries, together with the limited amount of coal mining and domestic water supply, make up the remaining (≈13%) of water use in the catchment

(DWAF, 2008). Sand mining is also taking place in the lower reaches of the river as a result of the nature of the floodplain in this area, with various applications being submitted to expand operations in the future (Seaman et al., 2013). The removal of this fine sand has taken place on a relatively uncontrolled fashion (River Health Programme, 2006) and still appears to be continuing. No noteworthy afforestation is currently taking place in the catchment (DWAF, 2003).

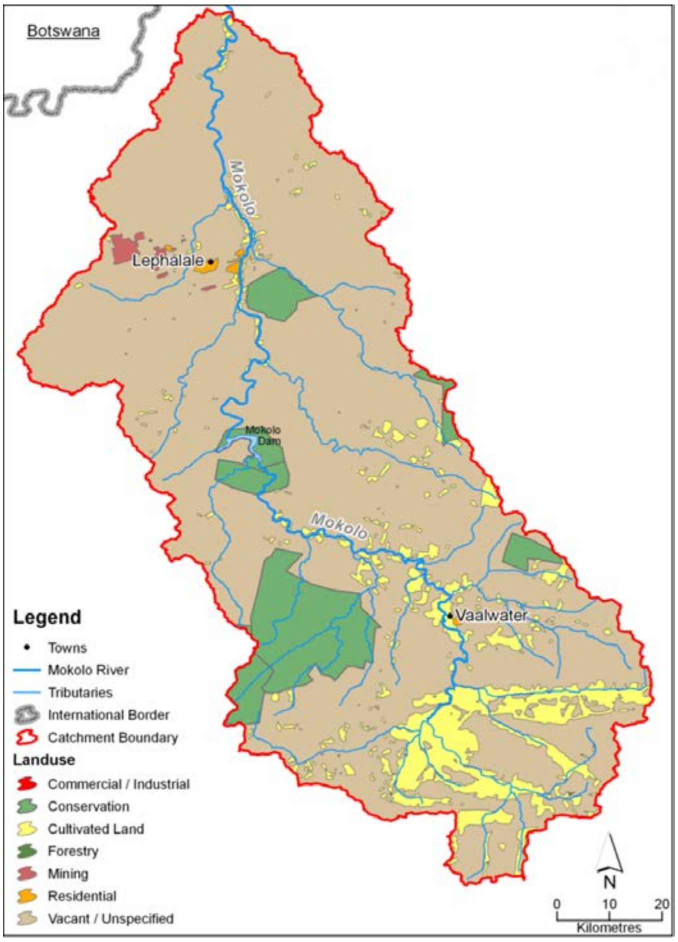

Figure 2-13: A generalist view of the different land use in the Mokolo River catchment (Seaman et al., 2013).

2.3.6. Overall

South Africa needs coal to produce ≈75% of its electricity needs. The bulk of these coal reserves are currently being obtained from the Witbank / Middelburg coalfields, but these reserves are expected to be depleted within the next 10 to 20 years (Prevost, 2007). The Waterberg coalfield is estimated to contain about 40% of South Africa's coal reserves and is thus of strategic importance. The water demand in the Waterberg is expected to increase significantly by 2024 as development in the area expands (Vermeulen, 2010). As a result, water transfers from other basins will become increasingly more important to supply the deficit in the water balance. Acid mine drainage is a real threat in the Waterberg, but the low rainfall in the area may negate some of its risks (Aphane and Vermeulen, 2015). The application of lessons learned from other mining areas is thus of extreme value and applicable for the Waterberg area (Vermeulen and Bester, 2009).

2.4. MONITORING ECOLOGICAL INTEGRITY AND ANTHROPOGENIC IMPACTS

Ecological integrity is the capacity of a biological system to sustain a balanced, integrated and adaptive system with a full range of elements and processes (Innis et al., 2000). Monitoring or assessing the ecological integrity of an aquatic ecosystem is also beneficial, because it allows for an indication of the integrated effects of the combined activities taking place in their respective catchments (Niemi and McDonald, 2004). As it is not practical to monitor each parameter associated with an aquatic ecosystem in detail, certain indicators (abiotic and / or biotic) are used as an indirect indication of the integrity of the ecosystem (State of Rivers Report, 2001).

2.4.1. Abiotic Monitoring

The abiotic characteristics of an aquatic ecosystem may be affected by both physical and chemical parameters (Dabrowski and De Klerk, 2013). The two most noticeable abiotic phases in aquatic ecosystems in which these parameters can be measured, are water and sediment. Although water and sediment may be monitored individually to provide an indication of ecosystem integrity, there are various shortfalls. One of the most important shortfalls is the fact that many pollutants are known to have antagonistic or synergistic effects (Coors and De Meester, 2008). For this reason, monitoring a single phase (thus, either water or sediment), may result in biased information (whether positively or negatively). An important gap in the provision of reliable information using abiotic monitoring, especially in rivers, relates to the frequency of data collections (Walling et al., 1992). Rivers, for example, are characterised by flowing water that drain a specific catchment containing various land surfaces and uses (Chapman, 1996). A single measurement is thus a mere snapshot in space and time that creates the possibility of missing important pollution driven events. Another factor constraining abiotic monitoring programmes is the differentiation between the background values of a specific pollutant and those that were contributed anthropogenically (Bartram and Ballance, 1996). This is an important consideration, because geology and other natural processes may contribute to certain parameter concentrations. However, this is often difficult to take into consideration.

2.4.1.1. *Water Quality*

Water quality is one of the most crucial components in an aquatic ecosystem and has a direct effect on an ecosystem's associated biotic components (Dallas and Day, 2004; Malan et al., 2004). Various physical and chemical parameters which exist in water can influence its quality, namely salts, nutrients, metals, physical variables and toxic substances (Ouyang et al., 2006). An imbalance in these parameters can lead to various degrees of ecosystem deterioration (Lowell et al., 1995). These parameters may also be affected naturally through, for example, biochemical reactions taking place in an ecosystem (Chapman, 1996) and suspended material (Blettler et al., 2015). Thus, when monitoring the surface water of a specific ecosystem, it is important to make a distinction between the dissolved and suspended components (De Klerk et al., 2012). Many water quality monitoring programmes have been developed over time, but most of these consider only one approach to monitoring (e.g., selected chemical endpoints). This type of approach inevitably leads to certain shortcomings.

2.4.1.2. *Sediment Quality*

Aquatic sediments play an important role in the sustainability of aquatic ecosystems (Blettler et al., 2015). The physical particle size of aquatic sediments is able to influence various biological and chemical variables (Hill, 2005). For the past six to seven decades much attention has been focussed on the monitoring of sediment quality (Den Besten et al., 2003). This is mainly due to the ability of these particles to adsorb various compounds leading to an increased deposition of pollutants, which in turn has the ability to affect the overlying waters (Burton Jr., 2002). Therefore, one of the biggest advantages of monitoring aquatic sediments is the fact that they can give an indication of historical pollution (Den Besten et al., 2003). Certain biota live in the sediment and are in constant contact with these particles. Thus, sediment contamination is more important for these communities than dissolved concentrations in surface waters (De Jonge et al., 2010). Due to the fact that sediments usually contain higher levels of pollutants and are less affected by variation, it provides a more stable monitoring platform compared to water (Van Damme et al., 2008).

Overall, it is evident that the abiotic environment alone does not provide sufficient information to anticipate the larger impacts on ecological integrity and therefore require the incorporation of biological endpoints.

2.4.2. Biological Monitoring

Numerous biological assessment tools have been developed for different types of aquatic ecosystems and for various monitoring purposes (Bain et al., 2000; Simon, 2000). A large proportion of these tools are aimed at monitoring the ecological integrity of the respective systems (Davis and Simon, 1995; Barbour, 1997; Resh, 2008). Aquatic organisms, or aquatic biota, have been used as monitoring tool due to the fact that these organisms are constantly exposed to its surrounding environment, e.g. water and sediment (Karr, 1999). Thus, they are able to reflect a long-term integrated state of their respective ecosystems (Hohls, 1996; Chutter, 1998; De la Rey et al., 2004). These tools are based on the assumption that anthropogenic impacts will result in changes in the community structure, abundance or diversity of these biota (Karr and Chu, 1998; USEPA, 2002a; DWAF, 2004d). This helps to overcome some of the shortcomings of traditional water and sediment monitoring tools (Harris and Silveira, 1999). This biomonitoring approach also tends to be more cost effective and rapid due to the numerous chemical endpoints that need to be measured which is often very expensive (USEPA, 2002b). As a result, regulatory authorities are increasingly enforcing / encouraging the use of such monitoring techniques that incorporate biological communities to manage different aquatic resources and monitor ecological integrity (Dickens and Graham, 2002).

With regard to rivers, a number of different biomonitoring methods have been developed and implemented. These monitoring methods may be based on various types of biota, but aquatic invertebrates and freshwater fish are the two groups of organisms that have received the most attention (Whitfield and Elliott, 2002). These organisms occupy different trophic levels and have different characteristics. They therefore reflect different ecosystem levels and each has their own unique (dis)advantages. However, often several groups need to be studied to properly understand the wide range of anthropogenic impacts (Allen et al., 1999). Although biomonitoring tools have great advantages in assessing / monitoring aquatic ecosystems, there are also certain disadvantages. For example, biomonitoring methodologies may require intensive training / specialist expertise (Taylor et al., 2007). Because many of these biomonitoring tools require a strong human element to conduct these assessments, it may allow for subjectivity in the interpretation of the results. Biomonitoring tools are also not able to identify the specific stressor impacting a specific system (Hohls, 1996). Thus, it has been noted that choosing which assemblage to use in a specific study depends on the objectives of the study and the characteristics of the study area (Resh, 2008).

2.4.2.1. *Aquatic Invertebrates*

Aquatic invertebrates are probably the most extensively and commonly used organism to monitor lotic systems (Little et al., 2006; Haase and Nolte, 2008). One of the reasons for this is due to the fact that they make up a large proportion of existing species on earth and therefore are very important for proper ecosystem functioning (Dixon et al., 2002). They are also widely used, because they are adapted to certain physical and chemical conditions (Rinne, 1990; Hillman and Quinn, 2002) and occupy an important link in aquatic food-webs (Yoshimura et al., 2006). These organisms are able to indicate alterations as a result of human-induced disturbances (Rosenberg and Resh, 1993). Aquatic invertebrates are particularly sensitive to organic and various other compounds (Assmuth and Penttilä, 1995; Kucuk, 2008) and they have the ability to integrate and reflect these effects during their lifetime (Ten Brink and Woudstra, 1991; Paisley et al., 2003). Invertebrates are relatively abundant in aquatic ecosystems and occupy almost all available biotopes, with certain species well known to be more sensitive to pollution than other species (USEPA, 1997; Dickens and Graham, 2002; Kasangaki et al., 2006). Two approaches are usually employed to characterize ecosystem conditions using invertebrates. These are either a taxonomic approach (i.e., measuring changes in the richness or diversity of the community) or a functional approach (which focusses on morphological and behavioural changes) (Cummins et al., 2005). Using aquatic invertebrates does, however, have certain disadvantages, such as being severely susceptible to floods (Dickens and Graham, 2002). The sampling capabilities and ease of identification also tend to impact on the reproducibility and validity of the results (Resh, 2008).

2.4.2.2. *Fish*

As with invertebrates, fish are also regarded as sensitive to water quality changes and valuable indicators of aquatic pollution (Hinck et al., 2008). Fish are able to integrate various adverse effects and because they are relatively long-lived, they can provide a long-term record of anthropogenic impacts (Zhou et al., 2008). Fish communities may therefore reflect direct and indirect impacts on the entire aquatic ecosystem (Kleynhans et al., 2007). Furthermore, when compared to invertebrates, fish are easier to identify. They include species from various trophic levels as well as groups that are both sedentary and mobile (Whitfield and Elliott, 2002). Some of the main biomonitoring approaches used that are based on fish, include indicator taxa or guilds; species richness, diversity, and evenness; multivariate methods; and the index of biotic integrity (Kleynhans, 1999). The degree of resolution is one of the big disadvantages when using fish communities, because it is difficult to know whether a species is sensitive to a specific source of pollution only. Also, results may be influenced by the fact that taxa may not be present at a localized site due to, for example zoogeographic barriers and not pollution (Resh, 2008). Sample size, including sampling equipment, may have a major influence on the results (Fausch et al., 1990), whilst the fact that fish are very mobile may result in biased results, due to time of day differences or in an attempt to avoid localized impacts. As a result of this mobile nature of fish, and the fact that many fish species may be severely tolerant to certain chemical and physical impacts, diverse fish communities have been found in systems that are severely impacted (Whitfield and Elliott, 2002).

2.4.3. Biochemical and Molecular Monitoring

The reliability of old-fashioned chemical and biological monitoring is significantly improved when combined with biochemical and molecular assessments (Mahalingam and Fedoroff, 2003; Oberholster et al., 2016). Molecular responses have been widely investigated for a long time under different unimpacted and stressed conditions, resulting in several biomarkers being developed for different aquatic organisms over the years (McIntyre and Pearce, 1980; Livingstone, 1991; Regoli, 1992; Walker et al., 2003). Toxic effects caused by, inter alia, pollution incidences may lead to enzyme inactivation and protein degradation, enhancement of lipid peroxidation processes, deoxyribonucleic acid (DNA) damage and cell death (Winston and Di Giulio, 1991). Therefore, changes in antioxidant content and in the activity of antioxidant enzymes, as well as DNA damage offer useful biomarkers of pollution driven oxidative stress in aquatic ecosystems (Viarengo et al., 1989; Regoli and Principato, 1995; Oberholster et al., 2016). These biomarkers can be used as biomarkers of exposure (i.e., measures of exposure) or biomarkers of effect (i.e., measures of effect of exposure) (Walker et al., 2003).

2.4.3.1. *Reactive Oxygen Species, Antioxidants and Anti-oxidative Enzymes*

Various forms of abiotic or biotic stresses may affect cellular functions and lead to a production of reactive oxygen species (ROS) (Navrot et al., 2007; Li et al., 2009). These stressors may include drought, salinity, metals, xenobiotics, hypoxia and nutrient deficiency (Apel and Hirt, 2004; Mittler, 2006).

Plants and animals are known to employ similar mechanisms to manage the impacts of ROS (Martindale and Holbrook, 2002). Reactive oxygen species are produced by the incomplete reduction of oxygen. It takes place in various cell components, but in animals ROS production mainly takes place in the mitochondria (Kristiansen et al., 2009). The major species of ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Chen and Gibson, 2008). The respective concentrations of these oxygen species are constantly changing until equilibrium is reached (Chen et al., 2009) and can cause extensive oxidative damage (Halliwell, 2006).

The impacts of ROS accumulation are well known, especially their role in disease and ageing (Beal, 2002; Melov, 2002; Perry et al., 2002) and ROS may also play a role in cell signalling (Van Breusegem et al., 2008). The removal of ROS is mediated by various enzymes present in an organism (Nyathi and Baker, 2006; Fritz et al., 2007). Antioxidants and scavenging enzymes are present in cells to maintain ROS levels so as to ensure normal function and growth in an organism (Jaspers and Kangasjärvi, 2010). On the other hand, stressors may down regulate a cell’s antioxidant capacity, thus leading to an accumulation of ROS during periods of stress that have various harmful effects and signalling functions (Møller et al., 2007; Foyer and Noctor, 2009).

Various other redox-active molecules can be formed, namely nitric oxide (NO) and reactive oxylipins, in conjunction with ROS. Although NO and oxylipins are also known to have a strong role in stress signalling, ROS are by far the most commonly studied (Mueller and Berger, 2009). Thus, with ROS known to play a fundamental role in many cellular processes (e.g., programmed cell death), monitoring ROS production and movement have been successfully employed to supplement traditional biomonitoring methods and so support the management of aquatic ecosystems (Meskauskiene et al., 2001; Costet et al., 2002; Wagner et al., 2004).

2.4.3.2. *Genotoxicity*

Biomonitoring of aquatic ecosystems is becoming more and more important due to the progression and development of modern society and even more so where these areas are already compromised (Avishai et al., 2002; Tamie et al., 2005; Obiakor et al., 2010a). To appropriately understand the impact of pollutants in aquatic ecosystems, it is generally acknowledged that genotoxicological assessments need to form part of any monitoring program (Oberholster et al., 2016). This is due to the fact that it allows for the complete assessment of the pollutants present and also reflects the bioavailable contamination (Hund-Rinke et al., 2002). Genotoxic pollution refers to pollutants having the potential to cause mutagenic, teratogenic and / or carcinogenic effects (Badr and El-Dib, 1978; Ali et al., 2008) that ultimately affect the integrity of an organism's genetic material (i.e., DNA) (Smith, 1996). Many substances are known to be genotoxic (Hayashi et al., 1998; Ali et al., 2008), including metals (Tamie et al., 2005; Igwilo et al., 2006) and polycyclic aromatic hydrocarbons (PAHs) (Germain et al., 1993). Their genotoxic potential lies in their ability to form strong covalent bonds with DNA which affect the replication of the genetic material (Hartwell et al., 2004; Luch, 2005). Although genetic impairment is usually associated with these environmentally and / or anthropogenically induced changes, for example physical (e.g., climate change), chemical (e.g., pollution) or biotic (e.g., parasites), it can also be related to impacts that are intrinsic in origin (e.g., genetic drift and / or inbreeding) (Bijlsma et al., 1997).

In genotoxicology, there is a difference between genotoxicity testing and monitoring (Dixon et al., 2002). During testing, the genotoxic potential can be tested using *in vitro* and *in vivo* studies and the results of which may be included into monitoring systems to better understand the effects of metabolism and DNA repair efficiency (Jha et al., 2000). On the other hand, monitoring genotoxicity requires frequent monitoring of selected systems using an ecologically relevant population (Cardis et al., 1996). However, there is a deficiency in the use of suitable model organisms / methods to effectively evaluate and / or monitor the genotoxicity of aquatic ecosystems (Raisuddin and Jha, 2004). Considerable progress has been made to understand genotoxic risks to human health, but the environment and its associated natural populations have received very little attention (Dixon et al., 2002; Jha, 2004). Currently, most of the environmental techniques that have been developed from human studies have been adopted for use with aquatic species (Bolognesi et al., 2004). Thus, the development of appropriate models / techniques is crucial to understand the extent and / or impact of genotoxicity in surface waters, as well as the comparison between anthropogenic and natural sources (Jha et al., 2000).

One of the greatest challenges in genotoxicological studies has always been to determine the link between these molecular changes and ecological consequences (Moore, 2002). Fish and invertebrates (especially higher order invertebrates such as decapods) are exceptional model organisms to study the genotoxic potential of the surface waters of an aquatic ecosystem (Dixon et al., 2002; El-Shehawi et al., 2007), because they are able to respond within a short time to even low concentrations of the toxicants that they are exposed to (Saotome and Hayashi, 2003; De moraes Pantaleão et al., 2006). This type of impact is thought to be mostly irreversible and may hereditarily be passed on to future generations. The end-result in aquatic ecosystems may be, for example, severe consequences for species survival, a significant decline in species diversity and ultimately impairment of ecosystem functioning (Bickham et al., 2000). Higher trophic level organisms (e.g., humans) may also be impacted through biomagnification (Obiakor et al., 2012). Aquatic invertebrates have a unique ability called multi-xenobiotic resistance (MXR) which enables them to withstand multiple xenobiotics in aquatic ecosystems (Kurelec, 1992; Smital and Kurelec, 1998). This ability to survive such multi-stressor environments makes them even better model organisms for genotoxic studies, because they are able to provide better insights into the range of genotoxic impacts under different degrees of pollution (Jha, 2004). With this said, chemosensitizers may inhibit MXR, resulting in invertebrates being more susceptible to genotoxins (Smital and Kurelec, 1998). Overall, invertebrate cells have also been shown to be more sensitive towards genetic damage, compared to mammalian cells (Raisuddin and Jha, 2004).

Through the inclusion of genotoxicological biomarkers in biomonitoring programs unique insights, that are not available using other techniques, can be provided into an aquatic ecosystem, such as the early detection of low level ecosystem degradation (Villela et al., 2006). Such a multilevel monitoring program will thus provide insights into the combined effect of stressors at molecular, population and ecosystem level, and the inclusion of the genotoxic evaluations of the aquatic environment is crucial for taking the promise of sustainable development forward (Lee and Steinert, 2003; Okpokwasili, 2009). This is especially important due to the fact that most toxicological studies usually focus on a single compound activity, but an aquatic ecosystem contains a mixture of toxic substances

(Don-Pedro, 1996; Viaroli et al., 2004). As a result, the behaviour of a specific pollutant, (e.g., metals) may vary from its original single toxic characteristics due to these co-occurring chemicals (Oyewo, 1998; Otitoloju, 2001). Therefore, the use of genetic markers in monitoring programs becomes even more crucial to prevent genotoxic effects such as congenital abnormality, genetic anomalies and diseases (Obiakor et al., 2010b). Lastly, although it is important to study contaminated / impacted sites to understand the true environmental potential of genotoxicants, it is also crucial to evaluate relatively less impacted / pristine sites to generate background and historical control data of such stressors (Royal Society, 1994).

2.5. CONCLUSION

Large volumes of coal have been and are still being mined within the upper Olifants River. These operations have been taking place for decades in this catchment, but these coalfields are approaching depletion in the near future. Since the Mokolo River catchment contains the next largest coal deposits in South Africa, it inevitably faces a similar degree of coal mining in future. Therefore, using a comparative catchment approach investigating a multitude of these endpoints may contribute to the effective management of a relatively pristine catchment (i.e., Mokolo River) that will be exposed to similar anthropogenic influences associated with coal mining (such as the upper Olifants River for example).

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CHAPTER 3: A WATERSHED APPROACH IN IDENTIFYING KEY ABIOTIC ECOSYSTEM DRIVERS IN SUPPORT OF RIVER MANAGEMENT: A UNIQUE CASE STUDY

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Declaration by the candidate

With regard to Chapter 3, the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution
Conceptual design, fieldwork, experimental work, manuscript writing.	70%

The following co-authors have contributed to Chapter 3:

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3.1. ABSTRACT

Since the industrial revolution, the impact of effluents produced by human activities on ecosystems has been a major international environmental concern. This study was aimed at observing the changes in water and sediment qualities at a watershed level of two different river systems facing the same land use practices, but impacted to different degrees. Samples were collected at strategically selected sites within the mainstream of both rivers, the major tributaries draining into them, as well as a major impoundment in each system. A distinct difference between the two different rivers was observed. It was established that certain variables, for example pH, contributed to the differential water and sediment quality signatures in the upper Olifants and Mokolo rivers, having important considerations for the future management of both river ecosystems. Other abiotic factors, such as alkalinity and sulfate levels were also found to be important. The tributaries were found to play an important role in the purification and / or pollution of the mainstream rivers. On the other hand, the present impoundments in the Mokolo River were observed to affect the water and sediment qualities downstream. Overall, through the use of comparative models, it was observed that the upper Olifants River was in a different state than the Mokolo River and the information from this study may aid in the future management of the Mokolo River to prevent a shift to an undesirable state.

3.2. INTRODUCTION

There are a number of land and water use activities in South Africa that are of strategic importance to the country. Activities, including mining, agriculture, power generation and wastewater treatment works (WWTWs) may rely heavily on aquatic ecosystems for receiving treated wastewater. A diverse range of pollutants derived from these activities include metal- and sulfate contamination via acid mine drainage (AMD), chemicals via industrial effluent, as well as nutrient enrichment and salinization emanating from sewage effluent and agricultural activities (Bell et al., 2001; Rodhe et al., 2002; De Villiers and Thiart, 2007; Hobbs et al., 2008; Oberholster et al., 2010). The effects of the above-mentioned activities on aquatic ecosystems and key water and sediment quality variables associated with these systems are very important and can affect the thresholds that govern potential regime shifts within a system (Oberholster et al., 2005). Because of the presence of these types of activities in the catchment of the upper Olifants River in the Mpumalanga Province of South Africa, it has been described as one of the most polluted rivers in southern Africa (Grobler et al., 1994; Hobbs et al., 2008; DWA, 2011). Not surprising, the water and sediment quality of the upper Olifants River has been shown to be severely impacted by a complex blend of pollutants varying throughout the catchment on a temporal and spatial scale (Dabrowski et al., 2015). This mixture of pollutants has recently resulted in various ecological concerns, particularly in Loskop Dam (a large man-made reservoir) where high incidents of diseases like obesity and pansteatitus have occurred in resident aquatic species (fish and crocodiles) (Ashton, 2010; Botha et al., 2011; Truter et al., 2014). This has resulted in mass fish mortalities in Loskop Dam during the last 15 years (especially between 2003 and 2008). These increased disease incidence and mortality events in Loskop Dam paralleled similar patterns in the fish and crocodile populations in the lower Olifants River in the Kruger National Park (Driescher, 2007; Ashton, 2010; Truter et al., 2014; Van Wyk et al., 2014).

The Mokolo River, located in the Limpopo Province of South Africa (north-west of the upper Olifants River catchment), drains into the Limpopo River and is generally recognized as being less impacted by man-made pollution as is the case with the upper Olifants River. However, this river system currently faces the same types of risks that exert progressively more serious adverse impacts on water quality and aquatic ecosystems in the upper Olifants River. These risks include the possible contamination of the receiving water of the Mokolo River through treated and untreated sewage effluent discharges, agricultural runoff and return flows as well as contamination from mining activities. Consequently, the new developments planned for the area (i.e., mining, power generation and associated activities), especially around the town of Lephalale, have the potential to cause widespread adverse impacts on the water and sediment quality of the aquatic systems in the area and may accelerate the rate at which these aquatic ecosystems deteriorate over time (River Health Programme, 2006). This will be compounded by the fact that increased demands for water from this already water stressed basin will add to the pressures on the system and could increase the severity of the anthropogenic impacts on these aquatic ecosystems (Ashton, 2007). Knowledge of key water and sediment quality parameters contributes to the understanding of a specific river system (Oberholster et al., 2010). Therefore, due to the similarities between the upper Olifants River and the Mokolo River, these two catchments make for a very good and unique case study to determine the changes in various abiotic variables under varying impacted conditions. As a result, the aim of the present study was to evaluate the aquatic ecosystems in both the upper Olifants River and Mokolo River catchments so as to identify the key abiotic drivers for these two ecosystems. This information may assist decision makers regarding the upper Olifants River and also future management of the aquatic ecosystems in the Mokolo River catchment in the face of ever increasing land use activities. Internationally, this information may be useful to mitigate anticipated and current coal mining impacts through a better understanding of the water resources currently impacted by coal mining.

3.3. MATERIALS AND METHODS

3.3.1. Site Selection and Description

For this study sites in both the upper Olifants River and the Mokolo River catchments were studied. Due to the upper Olifants River catchment being heavily impacted by coal mining for more than a century, significant impacts have occurred in these aquatic ecosystems over this period (Swanepoel, 1999; Steyn, 2008). Ironically, this provided researchers with a unique opportunity to gain insights into the effects of coal mining on South Africa's water resources to identify those limited controlling factors that regulate a multitude of variables in such systems. In so doing, management and monitoring efforts may be designed more optimally to ensure the sustainability of South Africa's water resources when threatened in this way. With the bulk of the coal reserves in the upper Olifants River catchment nearing depletion (Prevost, 2007), the Waterberg coalfield (containing about 40% of South Africa's remaining coal reserves) is the focus of much attention (Aphane and Vermeulen, 2015). The Mokolo River is pivotal for expansion in this already water-stressed area, with AMD also posing a real threat (DWAF, 2003; DWA, 2012). The application of lessons learned from areas impacted by long-term coal mining, such as the upper Olifants River, is thus of extreme value and applicable for the Mokolo River.

Through a spatial catchment analysis conducted during this study (Figure 3-1 and Figure 3-2) of the upper Olifants River and Mokolo River catchments, these catchments have been found to be comparable in terms of the specific underlying geology (Table S 3-1, Supplementary Material), as well as the type of impacts on water and sediment quality due to similar land use activities (Table S 3-2, Supplementary Material). However, the extent of impact differs since the Mokolo River catchment has less agriculture impacts (mainly game farming), supports only two power stations compared to the 11 associated with the upper Olifants River catchment, and has significantly less mining operations (DWAF, 2004a). The catchment analysis was conducted using ArcGIS and quantified using the National Landcover dataset for South Africa (Van den Berg et al., 2008), taking into consideration the cumulative effect of the upstream impacts on subsequent selected sites further downstream. In terms of the geological catchment analysis, the geological dataset for the Republic of South Africa (Council of Geoscience) was used. For the upper Olifants River the site labels were annotated as OR followed by the specific site number (i.e., OR01), whilst for the Mokolo River the annotation MR was used. For the major tributaries / inflows of these two river systems, ORI and MRI were used, whilst for Loskop Dam in the upper Olifants River and the Mokolo Dam in the Mokolo River, LDAM and MDAM were used, respectively. Furthermore, we limited sampling to sites that were strategically important for certain analyses with the aid of the catchment analysis information.

3.3.2. The Upper Olifants River

The Olifants River originates in the Highveld of the Mpumalanga Province of South Africa and flows in a north-eastern direction to the Kruger National Park and to Mozambique. The Olifants Water Management Area is divided into four sections, namely the upper Olifants, middle Olifants, Steelpoort, as well as the lower Olifants (DWAF, 2004a). For this study we focussed on the upper section of the water management area, above the Loskop Dam, which included both the Wilge and upper Olifants secondary catchments (Figure 3-1 and Figure 3-2). This selection was based on the fact that the upper section of the Olifants River is the most human impacted of the four sections, which includes extensive coal mining activities, approximately 11 power stations, large-scale agricultural activities and multiple WWTWs (DWAF, 2004a). A total of 53 study sites were selected in the upper Olifants River catchment (including Loskop Dam) to be representative of the anthropogenic impacts associated with the upper section of the Olifants River. These sites were monitored seasonally over a period of three years (2011 - 2013) and included different hydrological extremes. From these sites, ten were located along the mainstream Olifants River. To evaluate the impact of different water sources on the mainstream river, 38 sites were selected and sampled on various tributaries of the upper Olifants River and also monitored during this period (including high- and low flow). Nineteen of the latter sites fall within the catchments of the two main tributaries, namely the Wilge River (nine sites) and the Klein-Olifants River (ten sites). The rest of the sites make up smaller rivers or streams that flow directly into the mainstream Olifants River, before the water enters the man-made reservoir, namely Loskop Dam. The water quality information for some of these tributaries, as well as the five sites within the Loskop Dam was obtained from Oberholster et al. (2013a).

3.3.3. The Mokolo River

The Mokolo River, located in the Waterberg region of the Limpopo Province, is an important tributary of the Limpopo River (Figure 3-1 and Figure 3-2) and forms part of the Limpopo Water Management Area, together with the Matlabas, Lephalala, Mogalakwena, Sand, Nzhelele and Nwanedi rivers (DWAF, 2004b). The Mokolo River flows in a north-western direction and reaches the Limpopo River at the border of South Africa and Botswana, thus highlighting the international importance of the Mokolo River. In contrast to the upper Olifants River catchment, the Mokolo River catchment currently has limited coal mining activities, only two power stations, agriculture (which is mostly in the upper reaches), whilst the rest is restricted to game farming (Figure 3-2). In-stream sand-mining operations are quite intensive, but restricted to the lower reaches around the town of Lephalale (River Health Programme, 2006). A total of 32 sites were monitored within the Mokolo River catchment during the course of the study. From these, 12 sites were selected along the mainstream of the Mokolo River and monitored for a period of six years (2008 - 2013) which included both seasonal and hydrological variability. These sites were selected based on the current land use impacts in the area to give a holistic picture of the entire Mokolo River. Furthermore, 17 of these sites were main tributaries flowing into the mainstream Mokolo River and these sites were also monitored during different hydrological extremes (including high- and low flow periods) for a period of three years (2011-2013). Water quality was measured at three sites within the man-made reservoir in the catchment, namely the Mokolo Dam, over a period of two years (2012 - 2013).

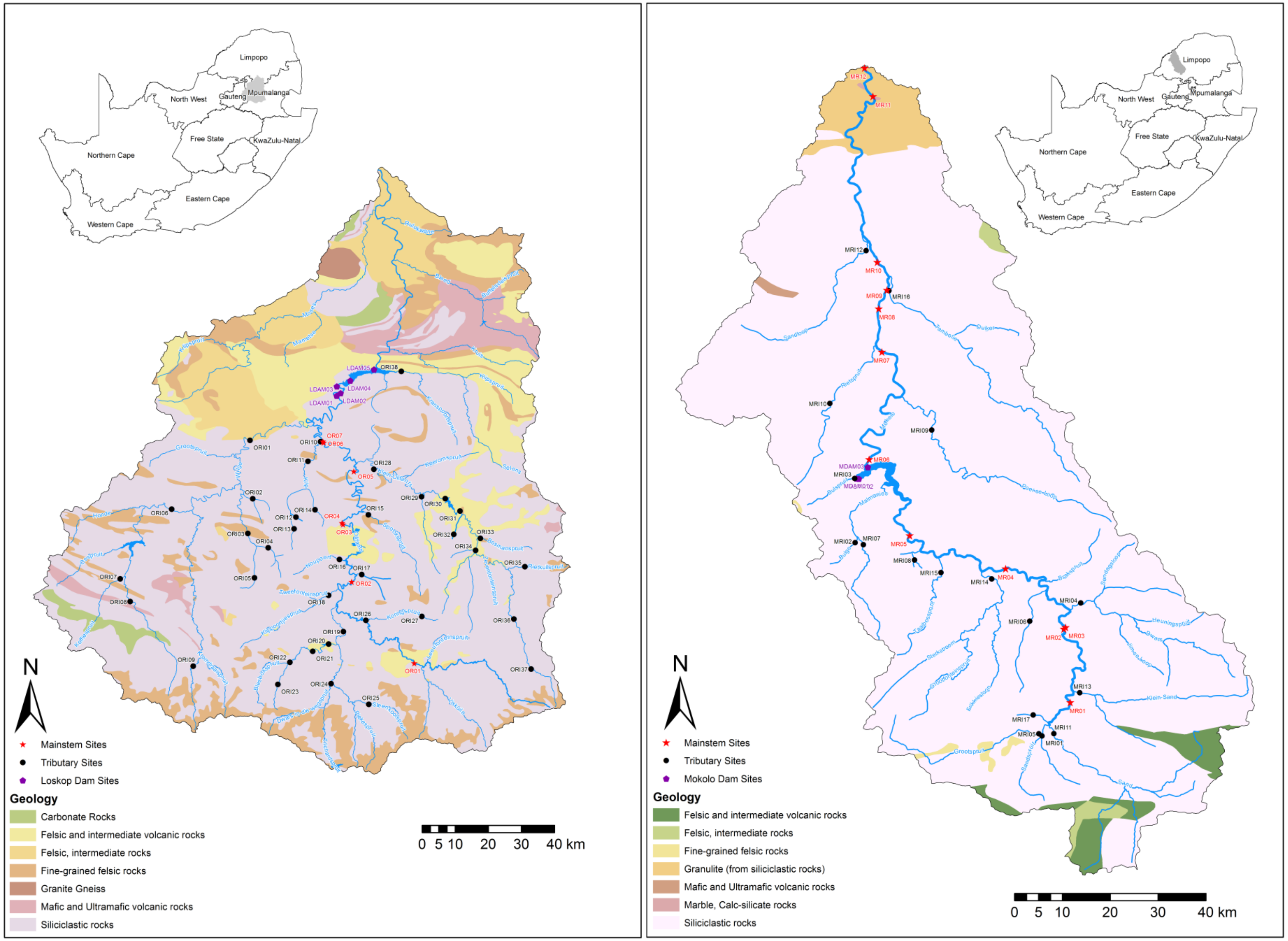

Figure 3-1: The predominant geology type of the upper Olifants River catchment (left) and the Mokolo River catchment (right). The sites used in this study area, either on the mainstream river, the main tributaries, as well as the Loskop Dam and Mokolo Dam are indicated. The mainstream rivers are indicated with a thicker blue line than the other tributaries.

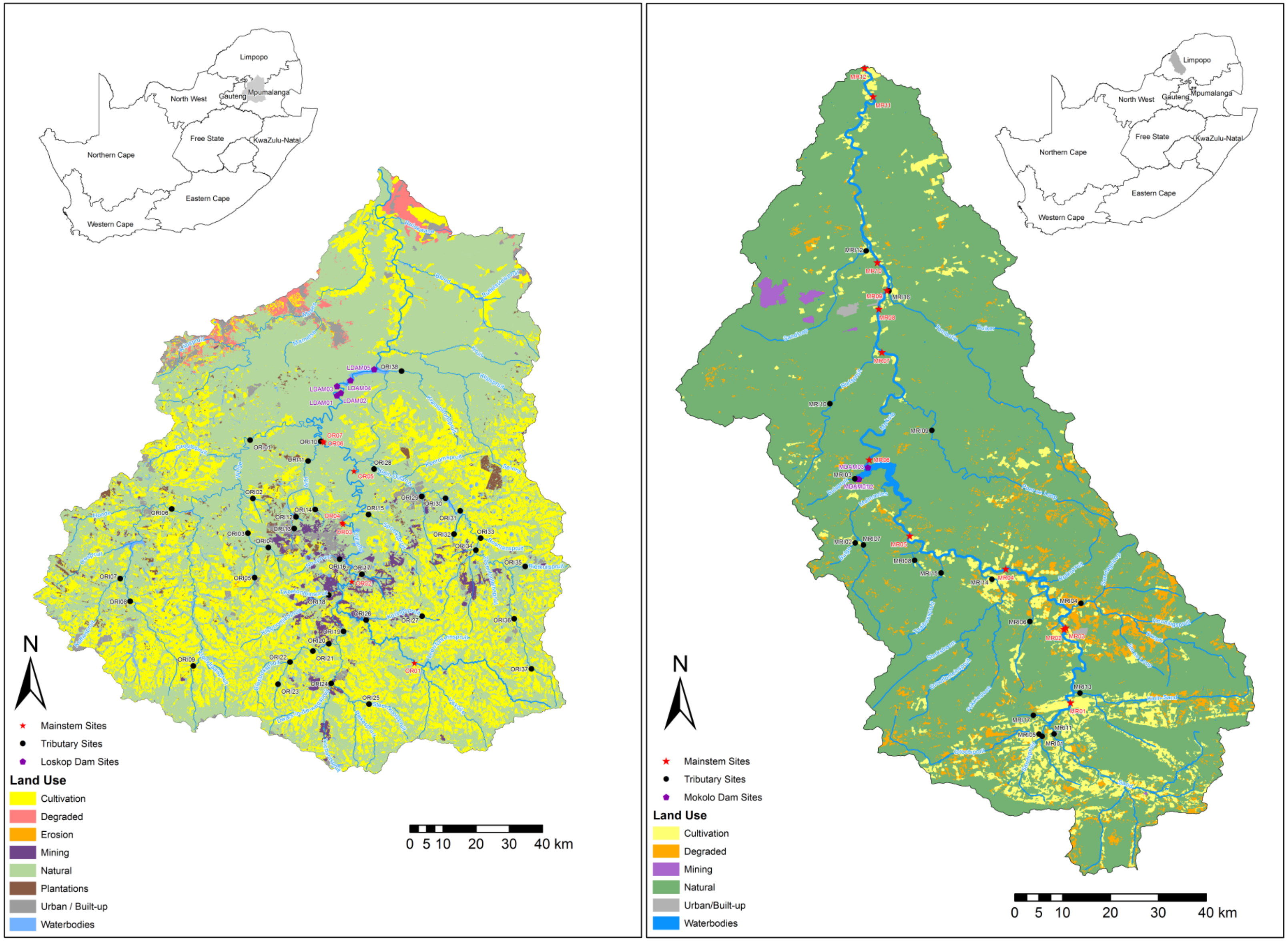

Figure 3-2: The upper Olifants River catchment (left) and the Mokolo River catchment (right). The sites used in this study area, either on the mainstream river, the main tributaries, as well as the Loskop Dam and Mokolo Dam are indicated along with the predominant land use type. The mainstream rivers are indicated with a thicker blue line than the other tributaries.

3.3.4. Water Quality

3.3.4.1. *Field Measurements*

At each sampling site, *in situ* water quality parameters, namely temperature, pH and electrical conductivity, were measured at the water surface using a Thermo 5 Star pH/RDO/Conductivity meter set (Thermo scientific, USA). Water samples were collected at each site for chemical analyses. All water samples were collected in clean, pre-rinsed one-litre polyethylene bottles and kept on ice and in the dark. After returning from the field, the samples were analysed for the subsequent list of parameters.

3.3.4.2. *Laboratory Analysis*

Samples collected from the field were filtered through 0.45 µm filter paper and used for the following analyses: ammonium (NH_4^+), nitrite and nitrate (NO_2^- + NO_3^-), chloride (Cl^-), potassium (K), sodium (Na), magnesium (Mg^{2+}), sulfate (SO_4^{2-}), calcium (Ca^{2+}) and alkalinity (HCO_3^- + CO_3^-). The unfiltered samples collected from field were used to analyse the following: chemical oxygen demand (COD), dissolved organic carbon (DOC), total Kjeldahl nitrogen (TKN) and total phosphate (TP). Total nitrogen (TN) values (expressed as the sum of NH_4^-, NO_2^-, NO_3^- and TKN) were calculated. In-house quality control samples, as well as external proficiency testing samples were used for quality control. Ammonium, NO_2^- + NO_3^- and Cl^- concentrations were analysed according to USEPA (1983) using a LACHAT QuickChem® Flow Injection Analyser. Cations, namely K, Na, Mg^{2+} and Ca^{2+}, as well as anions, namely SO_4^{2-} and TP were analysed using an inductively coupled plasma optical emission spectrometer (ICP-OES) (Thermo scientific, MA, USA). Total Kjeldahl nitrogen was determined according to Kjeldahl (1883) and Clesceri et al. (1998). The alkalinity of the surface waters was determined using a TitraLab® titration workstation (Radiometer Analytical TIM860). The measurement of chlorophyll *a* was conducted according to the method of Sartory and Grobbelaar (1984). Dissolved concentrations of metallic or metallic-like elements, namely aluminium (Al), arsenic (As), cadmium (Cd), iron (Fe), lead (Pb), Nickel (Ni), selenium (Se) and vanadium (V) were analysed within the water column. These elements were chosen to be representative of the fate and transport of metallic and metallic-like elements in the aquatic ecosystems being studied. The concentrations of these various elements were determined using ICP-OES and / or inductively coupled plasma mass spectrometry (ICP-MS). Two quality control methods were used. Firstly, the concentrations of measured anions and cations were summed and compared to the total dissolved salts (TDS) values, calculated from the electrical conductivity value of the sample. Secondly, the ionic balance of the major anions and cations (expressed as milli-equivalents per litre), were compared using the formula of Appelo and Postma (2005). During both analyses, a variation of less than 5% was regarded as acceptable, however, where the difference exceeded 5%, the analysis on the sample was repeated. If the repeated analysis did not improve, the sample was rejected and none of the results were used.

3.3.5. Sediment Quality

3.3.5.1. *Field Measurements*

Sediment core samples (top 10 cm) were collected in triplicate according to described methods (USEPA, 2001), from a pre-selected area at each site at a water depth of approximately 1 m. Sediment samples were transferred into pre-cleaned plastic containers and stored in a freezer (-20°C) until analysis could be performed, in order to prevent the loss of organic matter through digestion by invertebrates and organic decomposition by bacteria.

3.3.5.2. *Laboratory Analysis*

The concentrations of metallic or metallic-like elements (the same suite as tested for the water quality analysis), as well as total phosphorous were determined (Loring and Rantala, 1992) using a partial microwave digestion method utilising a CEM Mars Xpress (CEM corporation). Analyses were carried out using an ICP-OES. Quality control was performed by digesting and analysing a sediment reference material (PACS-2 NCR material) with every batch of 11 samples. The first sample in each batch was duplicated to monitor the reproducibility of the analysis. The sediment samples were also analysed for total organic carbon (TOC) content using a Vario EL Elementar III Elemental Analyser Instrument (Elementar, Germany), whilst quality control was performed using a sediment reference standard (MBSS-2). Reproducibility, as well as quality control (percentage recovery) was determined / monitored for each element included in this study and an acceptable tolerance range was set at <10%. Water soluble salts and nutrients associated with the sediment samples, were analysed according to the method described for the water samples. The method adapted from Plumb Jr. (1981) was used to wet-sieve a homogenised sub-sample of sediment to determine the particle size distribution through a gravimetric method by weighing each of the different fractions and determining the percentage composition by relating each fraction to the total sample size. Results are reported on a dry mass basis and particle sizes categorized into a classification system adapted and modified from Wentworth (1922).

3.3.6. Statistical Analysis

This study focussed on the mainstream upper Olifants River and the Mokolo River. Thus, these results were presented together with the data from the respective dams occurring within these rivers to obtain an understanding of the abiotic drivers in each system. The results from the tributaries were handled separately in order to determine the influence (whether good or bad) of each of these tributaries on the mainstream rivers. Significant differences were determined using an Analysis of Variance (ANOVA) test in combination with the Fisher's LSD post-hoc test (Statistica 12, Statsoft, US). Significance was accepted at probability (P) value equal to or less than 0.05. The normality of the data were evaluated using the Kolmogorov-Smirnov test, the Shapiro-Wilk W test, as well as the Lilliefors test, whilst homogeneity of variance was tested using the Levene's and Brown and Forsythe's tests. Non-parametric data were transformed using boxcox or rank transformations in order to meet the ANOVA assumptions. Multivariate statistical analyses, namely Principal Component Analysis (PCA) were conducted to assess the difference in water quality between the various tributaries within the upper Olifants and Mokolo River catchments. The results obtained were expressed as an ordination pattern on a two-dimensional base, where the placements of the samples reflect similarities and dissimilarities between sampling sites. In this way we were able to determine which of the various tributaries were responsible for the impacts seen in the mainstream rivers. Interpretive diagrams, namely Durov diagram (Durov, 1948), were used to show the nature of the different water qualities at the selected sites. By using Durov diagrams we are able to detect changes in the water quality signatures within the mainstream rivers, as well as the dams occurring in each river system. Ternary comparative models were constructed using the recorded values of specific parameters after being transformed and scaled and plotted on a two dimensional scatterplot using Statistica 12 (Statsoft, US). For the water nutrient enrichment plot TN, TP and chlorophyll *a* were used as indicators, whilst for sediment TOC was used instead of chlorophyll *a*. To determine other potential impacts on sediment, a comparative model was constructed using SO_4^{2-}, Cl^- and TOC.

3.4. RESULTS

3.4.1. Water Quality

Most of the sites selected in the upper Olifants River had a relatively low degree of alkalinity (Figure 3-3A), compared to OR01, OR03 and OR04. In general, a decrease in alkalinity is observed along with an increase in SO_4^{2-} further downstream within the mainstream Olifants River, with sites such as OR07 having low alkalinity levels, yet high SO_4^{2-} concentrations. The pH and TDS levels varied little among all the sites (≈8 and ≈200-400 mg/l, respectively). Only site OR07 had a decrease in pH (≈6), whilst site OR02 had a higher TDS (≈450 mg/l). Comparable water quality signatures were recorded at the downstream sites in the mainstream Olifants River with those noted throughout Loskop Dam. The pH and TDS concentrations recorded in the Mokolo River throughout the study at the different sites did not vary significantly (≈7 and ≈50 mg/l, respectively). The water quality signature of the Mokolo River was mostly dominated by Cl^- and SO_4^{2-} compared to the higher alkalinity and SO_4^{2-} recorded in the water from the upper Olifants River. Site MR05 differed from the rest of the Mokolo River sites in that Cl^- concentrations were found to be much lower than recorded for the other sites, whilst higher SO_4^{2-} levels were noted. The water from the Mokolo River also differed quite distinctly from the water from the Mokolo Dam, especially with regard to alkalinity.

Based on the comparative nutrient enrichment model (Figure 3-3B), our recordings in the upper Olifants River indicated that the suspended chlorophyll *a* concentrations measured at site OR02 (≈75 µg/l), was significantly ($p \leq 0.05$) elevated, when compared to the rest of the sites (≈6.2 µg/l), whilst OR04 clustered separately mainly due to the significant increase in TN (≈14.75 mg/l) recorded at this site. High levels of TN were also observed at four of the five Loskop Dam sites. In the Mokolo River chlorophyll *a* concentrations recorded varied significantly among some sites. Significantly lower concentrations were recorded at sites MR04, MR10 and MR11 (≈1 µg/l) when compared to the other sites, such as MR12 (≈20 µg/l). The concentrations of TN and TP in the mainstream Mokolo River did not vary significantly (≈0.8 mg/l and ≈0.03 mg/l, respectively), whilst the TN and TP concentrations recorded in the Mokolo Dam (≈0.6 mg/l and ≈0.025 mg/l, respectively) were also comparable to the values recorded in the mainstream river.

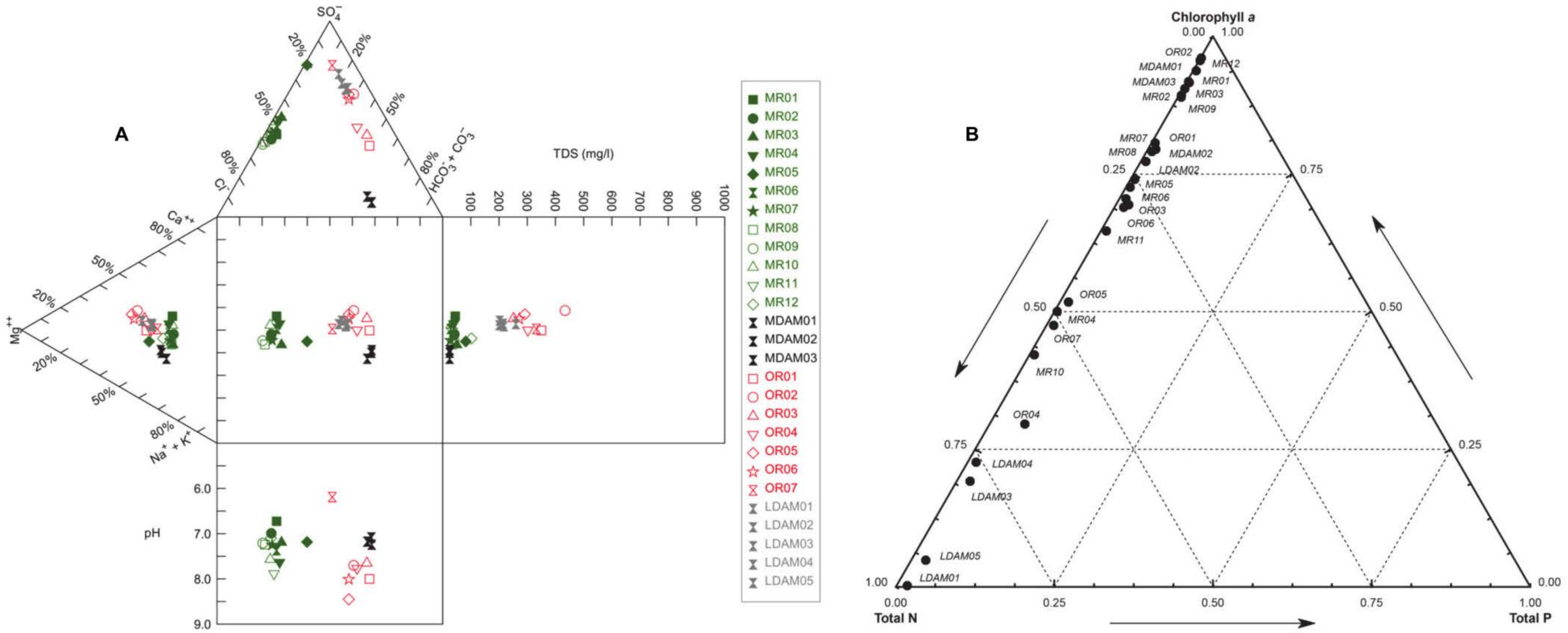

Figure 3-3: The water quality fingerprint including all the selected sites within the mainstream upper Olifants and Mokolo River systems, relating to the composition of the major anions, cations, pH and total dissolved salts (TDS) concentrations, presented as a Durov diagram (A). A comparative nutrient enrichment model (B) of both the upper Olifants and Mokolo River systems constructed using the transformed chlorophyll *a*, total nitrogen and total phosphate concentrations recorded during the study. In these figures OR = Olifants River, LDAM = Loskop Dam, MR = Mokolo River and MDAM = Mokolo Dam.

Results from the PCA (Figure 3-4A) showed that most of the tributaries within the upper Olifants River catchment grouped together, whilst the water quality at sites ORI02, ORI08, ORI12, ORI13 and ORI36 were observed to be dissimilar from the rest. This dissimilarity corresponded to the increase in concentrations of various pollutants (See Figure 3-4A). It is noteworthy that site ORI13, where the low pH values (pH less than 5) corresponded to parts of the Klipspruit tributary, is impacted by AMD, WWTWs and adjacent industries. Higher levels of SO_4^{2-} and lower levels of alkalinity were also measured at these impacted sites, namely ORI02, ORI12 and ORI13. These sites also correlated negatively with pH, confirming acidic conditions, associated with increased sulfate levels. Sites ORI08 and ORI36 correlate strongly ($p < 0.05$) with increasing TP, DOC, COD and Pb values and were mostly K / Cl^- dominated. Several other sites were also associated with certain pollutants, although not as markedly as the rest, for example ORI09, ORI10, ORI14, ORI29 and ORI32 (Figure 3-4A). In addition, a group of tributaries in the upper Olifants River catchment (indicated with the blue encirclement in Figure 3-4A) correlated strongly with an increase in alkalinity.

Water quality variables recorded in the different tributaries of the Mokolo River catchment (Figure 3-4B) of sites MRI01, MRI03, MRI04, MRI11 and MRI12 were found to be dissimilar to the majority of the other tributary sites. Of these sites, MRI11 was observed to be the most pronouncedly impacted in that most of the variables recorded, were significantly increased compared to the rest of the sites (See Figure 3-4B). Site MRI11 also showed signs of eutrophication (increase of chlorophyll *a*, TN and TP). In addition, water collected at the MRI11 site also had a low pH, confirming acidic conditions, and was also associated with an increase in the concentrations of As. On the other hand, sites MRI01, MRI03, MRI04 and MRI12 were mostly associated with an increase in Se, Al, Fe and Ni concentrations (Figure 3-4B). In both the PCA plot of the upper Olifants River tributaries, as well as the PCA plot of the Mokolo River tributaries, the majority of the sites that appear to be the least impacted (indicated with the green encirclement in Figure 3-4A and B) grouped together and were associated with an increase in pH.

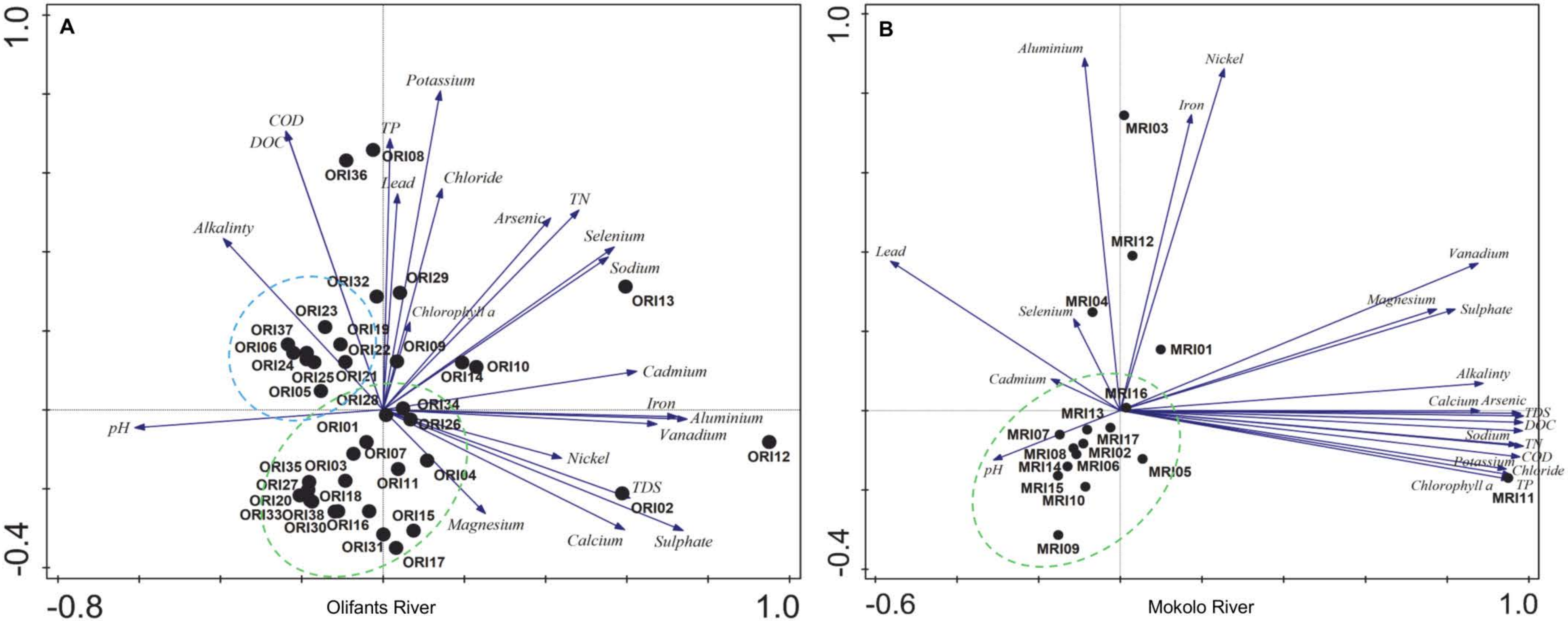

Figure 3-4: Principal Component Analysis (PCA) biplots indicating the (dis)similarities of the main tributaries of both the upper Olifants River (A) and Mokolo River (B), based on the different water quality variables measured at selected sites. The upper Olifants River PCA plot accounts for 23.52% of the variation seen on the 1st axis and 18.7% on the 2nd axis. The Mokolo River PCA plot accounts for 61.99% of the variation seen on the 1st axis and 12.23% on the 2nd axis. The encirclements indicate some of the major groupings found, as discussed above.

The spatial variation of selected dissolved metals determined at selected sites within the mainstream of the upper Olifants River and Mokolo River are presented in Figure 3-5. Within the upper Olifants River, Al concentrations (≈0.8 mg/l) were significantly higher at the most downstream site (OR07). No significant variation was observed with regard to As and Cd, although lower concentrations (relative to other sites) were recorded at sites OR02 and OR07. There was also no significant variation found between the selected sites with regard to Pb and Se concentrations. A significant decrease was found between OR01 and OR02 regarding Fe (≈0.2 mg/l and ≈0.01 mg/l, respectively), whilst significantly higher Ni concentrations were measured at OR07 (≈35 µg/l). Vanadium concentrations (≈7.5 µg/l) were recorded to be significantly variable among sites, with high concentrations determined at OR01, OR04, OR05 and OR06, when compared to OR02 and OR07 (≈1 µg/l).

The analysis of the water samples from the mainstream Mokolo River indicated that Al and Pb did not vary significantly amongst the selected sites (Figure 3-5). Arsenic concentrations were recorded to be significantly higher at MR06 (≈0.6 µg/l) compared to MR07 and MR08 (≈0.2 µg/l). The same trend was observed regarding Cd concentrations at MR06 (≈1.75 µg/l), which was significantly higher than those measured at the rest of the sites (≈0.25 µg/l). Iron concentrations showed variation when comparing the different sites, although concentrations were observed to be higher at MR02, MR06 and MR07 (≈0.3 µg/l) when compared to the rest of the sites (≈0.1 µg/l). Nickel concentrations varied at the different sites with the lowest concentrations found at MR06 and MR07 (≈0.5 µg/l) which differed significantly to the concentrations recorded at certain other sites. Higher Se concentrations were determined at sites MR02, MR03, MR06 and MR07 (≈0.8 µg/l) when compared to the rest of the sites (≈0.3 µg/l), whilst lower V concentrations were observed at sites MR05, MR08 and MR09 (≈0.25 µg/l) when compared to the rest of the sites (≈1.25 µg/l).

When comparing all of the sites in the Mokolo River to the sites in the Olifants River, lower Al concentrations than OR01 and OR07 were observed, but higher than OR02 – OR06. The concentrations of Cd were generally found to be similar although an increase was observed at MR06. On the other hand, Pb and Se concentrations were found to be similar between the two river systems, whilst the Olifants River sites had significantly higher As and V concentrations. The Mokolo River had higher Fe concentrations at the selected sites, whilst the concentrations of Ni were similar, except for OR07, where a significant increase was observed.

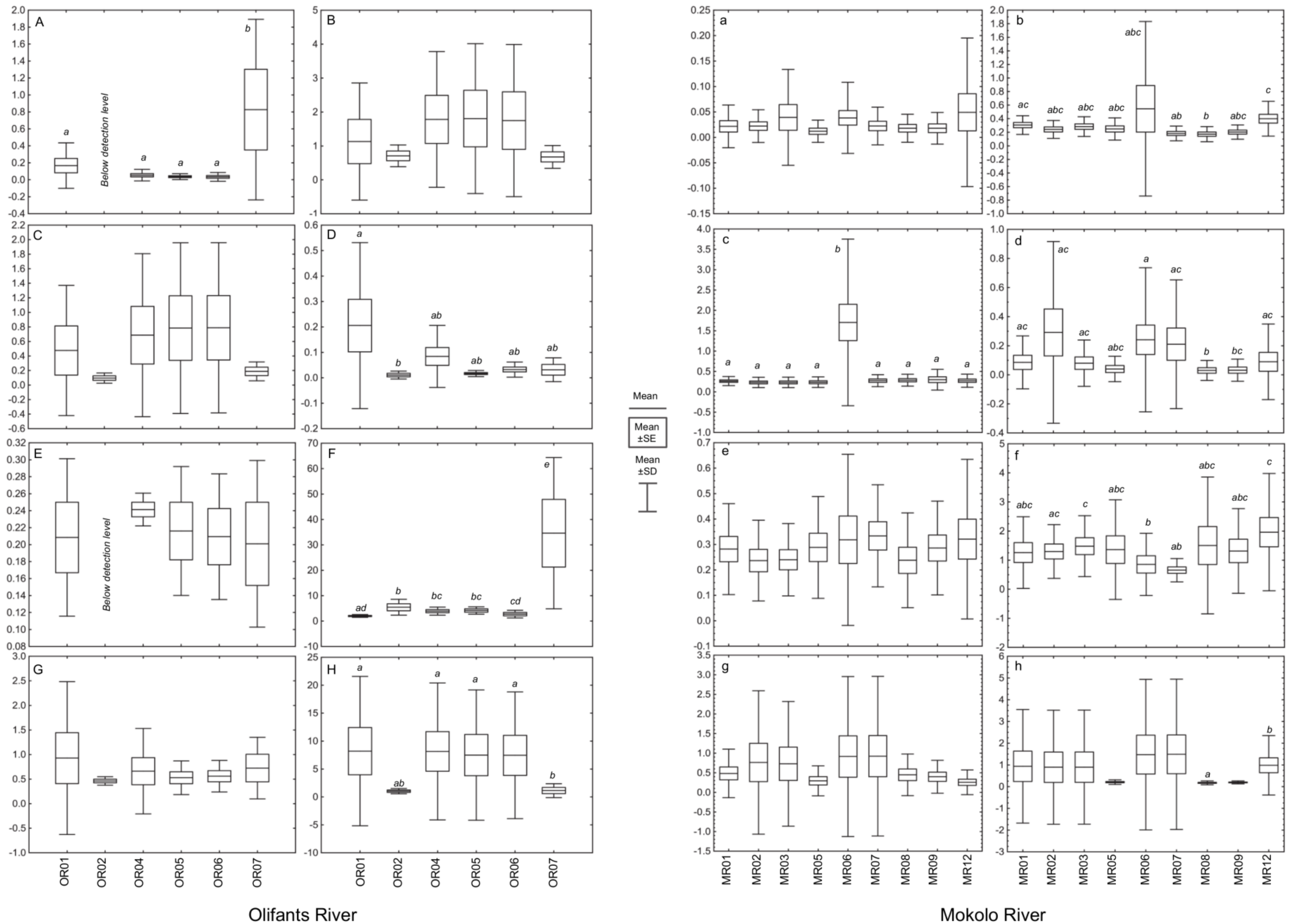

Figure 3-5: The dissolved metal concentrations of (A) = Aluminium, (B) = Arsenic, (C) = Cadmium, (D) = Iron, (E) = Lead, (F) = Nickel, (G) = Selenium and (H) = Vanadium in the surface water of the mainstream rivers. Aluminium and Fe are reported as mg/l, whilst the rest of the metals are reported as µg/l. The graphs with capital letters represent sites selected on the upper Olifants River, whilst those with lower case letters represent sites selected on the Mokolo River. Significant differences between sites are presented by italic letters (the sites with different letters differ significantly from each other).

3.4.2. Sediment Quality

The spatial distribution of the total metals bound to the sediment particles at the selected sites of the two river systems is presented in Figure 3-6. From these results a similar trend can be derived with regard to the spatial distribution of the different metal concentrations in the upper Olifants River in that OR04 and OR07 showed the highest concentrations of Al ($\approx$20 g/kg and $\approx$35 g/kg, respectively), As ($\approx$6 mg/kg and $\approx$2 mg/kg, respectively), Fe ($\approx$20 g/kg and $\approx$30 g/kg, respectively), Pb ($\approx$18 mg/kg and $\approx$20 mg/kg, respectively), Ni ($\approx$15 mg/kg and $\approx$40 mg/kg, respectively) and V ($\approx$60 mg/kg and $\approx$70 mg/kg, respectively) compared to the rest of the sites. Cadmium concentrations did not vary significantly, although a high variation was observed at OR04. Within the Mokolo River, Cd and Se concentrations did not vary significantly at the respective sites ($\approx$0.12 mg/kg and $\approx$1.2 mg/kg, respectively). Aluminium, As, Fe, Pb, Ni and V showed the same trend within the Mokolo River regarding their respective concentrations measured at the various sites. Higher concentrations of these metals were measured at sites MR01, MR02, MR03, MR06 and MR12 compared to the other sites. The highest concentrations of Al were found at MR03 and MR12 ($\approx$8 g/kg and $\approx$11 g/kg, respectively); As at MR06 ($\approx$1.5 mg/kg); Fe at MR12 ($\approx$12 g/kg); Pb at MR03 and MR06 ($\approx$7 mg/kg and $\approx$5 mg/kg, respectively); Ni at MR03 and MR12 ($\approx$7 mg/kg and $\approx$9 mg/kg, respectively); and V at MR03 and MR12 ($\approx$17 mg/kg and $\approx$22 mg/kg, respectively). When comparing these two river systems it was found that in general the upper Olifants River had significantly higher Al, Cd, Pb, As, Ni, Fe and V concentrations in the sediment compared to the Mokolo River.

The results from the sediment analyses (Figure 3-7A) showed that all of the selected sites within both the upper Olifants River and the Mokolo River consisted mainly of fine, medium and coarse sized sand particles which ranged from 0.063 mm to 2 mm. The comparative model between the level of TOC, Cl^- and SO_4^{2-} in the sediment of both river systems resulted in separate clustering of the sites selected in the upper Olifants River to those selected in the Mokolo River (Figure 3-7B). Within the upper Olifants River the Cl^- concentrations did not vary significantly, whilst the SO_4^{2-} concentrations were significantly increased at sites OR04, OR05 and OR06 ($\approx$750 mg/l) compared to concentrations determined at OR01 and OR07 ($\approx$100 mg/l). With regard to the Mokolo River (Figure 3-7B), the Cl^- levels tend to be higher than that of the upper Olifants River and significantly lower SO_4^{2-} concentrations were observed. This resulted in a subgroup of the Mokolo River's sites with higher SO_4^{2-} concentrations recorded at MR02 and MR05 ($\approx$50 mg/l) and lower concentrations at MR07, MR08 and MR09 ($\approx$5 mg/l). The nutrient enrichment plot (Figure 3-7C) also clustered the sites from the upper Olifants River separately from the Mokolo River, especially with regard to OR01, OR04 and OR07 where significantly higher TN concentrations were recorded ($\approx$6 -

13 mg/l), when compared to the other sites in the upper Olifants River (<5 mg/l). The recorded concentrations of TP did not vary significantly, but were the highest at OR06 (≈2 mg/l). The highest concentrations of TP in the Mokolo River were measured at MR05 (≈8 mg/l), which decreased with increasing TOC. On the other hand, TN did not vary significantly throughout the different study sites selected in the Mokolo River (Figure 3-7C).

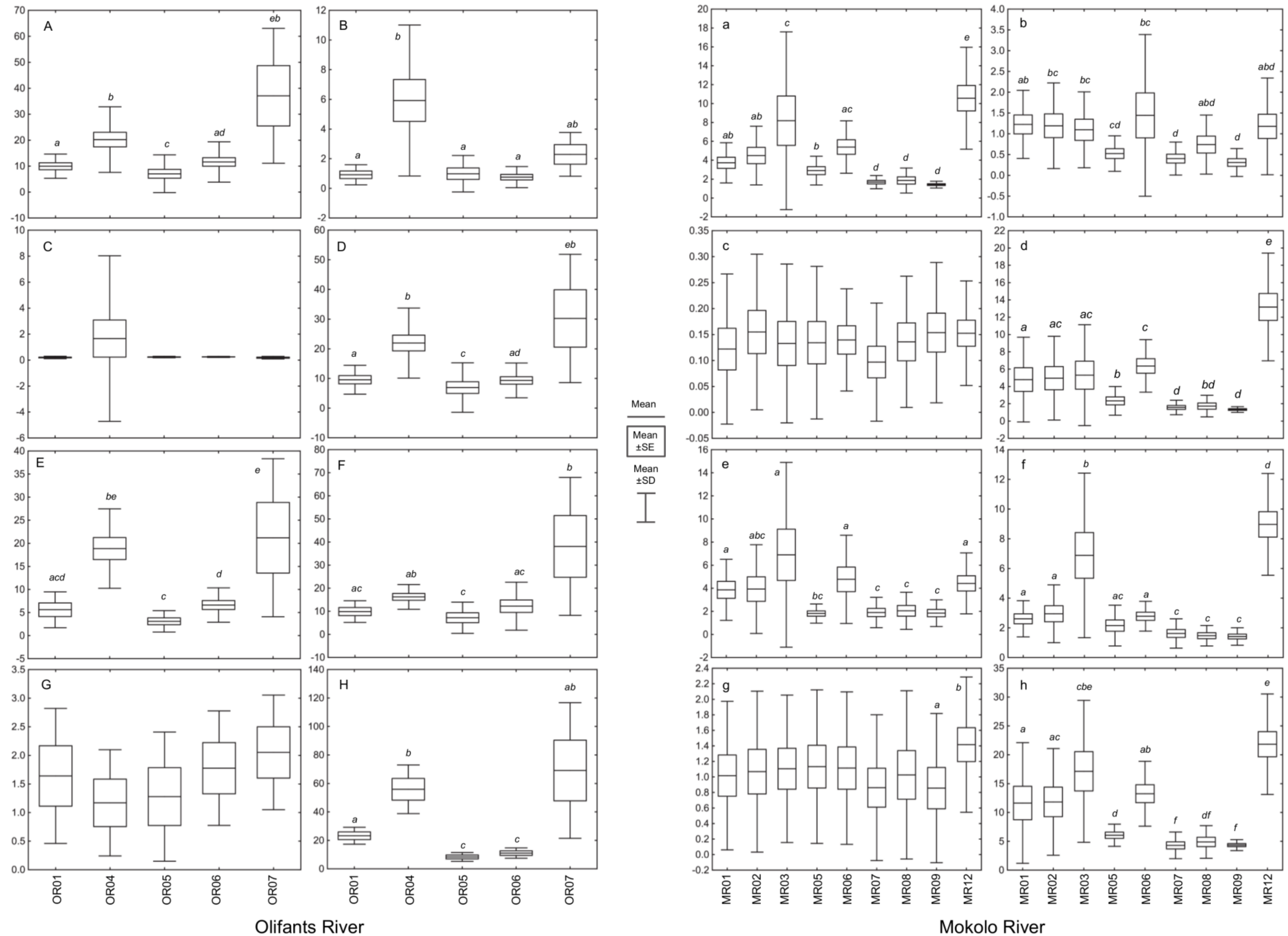

Figure 3-6: The total metal concentrations of (A) = Aluminium, (B) = Arsenic, (C) = Cadmium, (D) = Iron, (E) = Lead, (F) = Nickel, (G) = Selenium and (H) = Vanadium in the bottom sediment of sites selected in the mainstream rivers. Aluminium and Fe are reported as g/kg, whilst the rest of the metals are reported as mg/kg. The graphs with capital letters represent those sites selected on the upper Olifants River, whilst those with lower case letters represent those sites selected on the Mokolo River. Significant differences between sites are presented by italic letters (the sites with different letters differ significantly from each other).

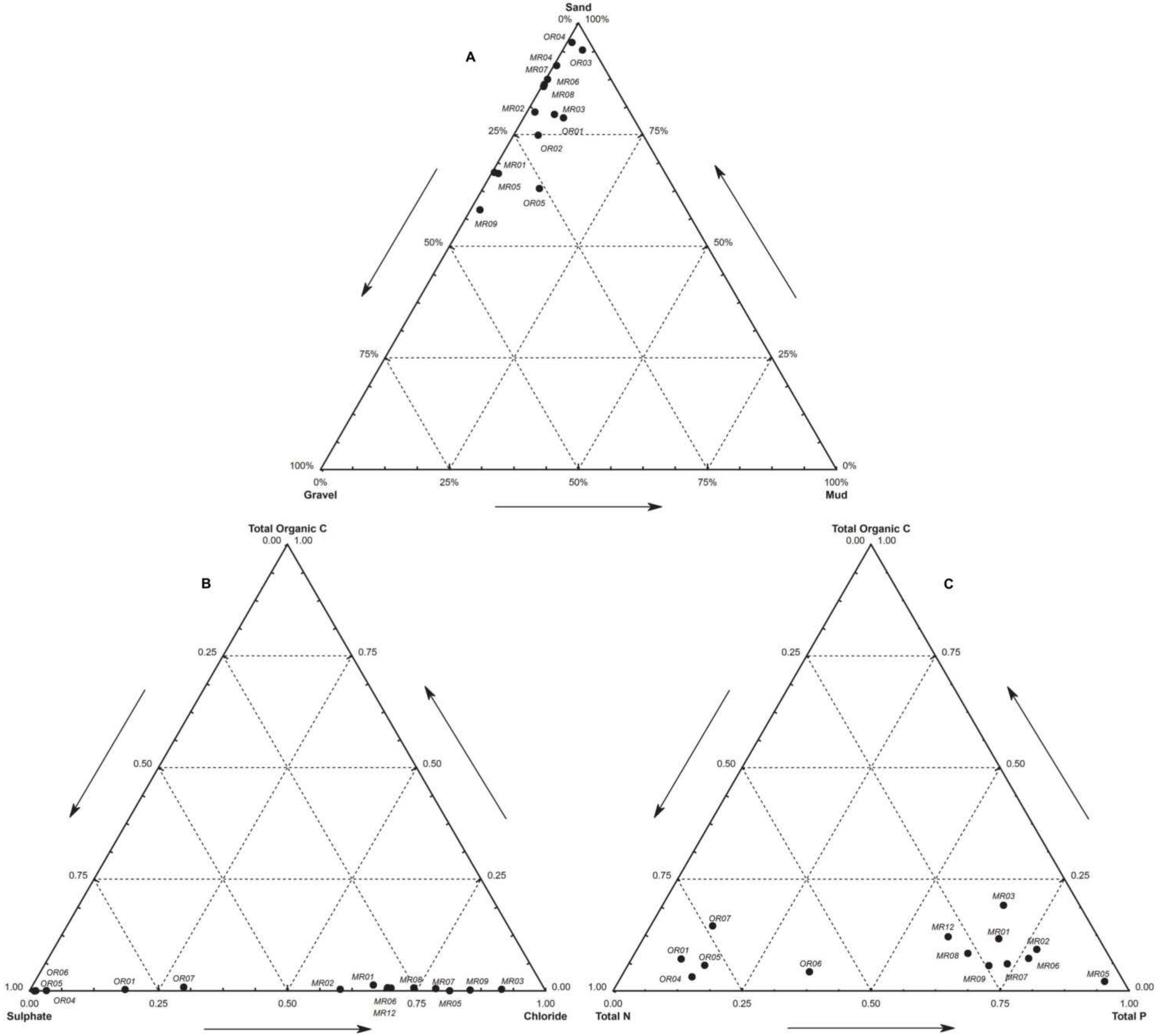

Figure 3-7: Ternary comparative models comparing the results of the sediment sampled at the selected sites on the mainstream upper Olifants River and Mokolo River with regard to particle size (A), sulfate and chloride normalised against total organic carbon (B), as well as nutrient enrichment (total N and P) normalised against total organic carbon (C).

3.5. DISCUSSION

From the current study it was evident that certain water and sediment characteristics varied significantly between the upper Olifants River and the Mokolo River. The input of acidic and polluted water from the associated land use activities were observed to significantly impact the water quality of the upper Olifants River. As a result, it was observed that the alkalinity of the water within the river decreased further downstream, whilst SO_4^{2-} concentrations increased. This is not only an indication of the loss in buffering capacity of the system (Björnsson et al., 2001; Neal, 2001), but also the SO_4^{2-} contamination due to AMD (Sams and Beer, 2000), which corresponds with the observed decrease in pH. This was most evident at the site OR07 and this observation also corresponds with an increase in certain dissolved metal concentrations (i.e., Al and Ni) at OR07. On the other hand, other metals such as As, Cd and V showed decreasing dissolved concentrations at OR07. Considering the layout of the upper Olifants River catchment, the Klipspruit is the only tributary that enters the Olifants River between sites OR06 and OR07 and the probable source of this impact can be found here. The five sites tested on the Klipspruit, namely ORI10 – ORI14, had very acidic water with relatively high concentrations of dissolved metals and nutrients which may be transported into the mainstream Olifants River. This was possibly due to the low pH of the water which has a mobilising effect on metals and also affects the mobilisation of nitrogen (Austin and Lee, 1973; Akcil and Koldas, 2006). This indicated that most of the impact observed at OR07 was as a result of the poor water quality being introduced into the Olifants River from the Klipspruit. From the spatial analysis it was observed that the Klipspruit runs through various impacted areas and as such is impacted by non-functional WWTWs, industries and AMD. As this acidic water of the Klipspruit (pH = ≈4.75) meets the more alkaline water of the Olifants (pH = ≈7.7) most of the metals are precipitated out onto the sediment as hydroxides (Akcil and Koldas, 2006). From our results these include Al, As, Fe, Pb, Ni and V. This is also true for the nutrients which are mobilised and precipitated due to changes in pH resulting in the increase in TN seen in the sediment at OR07, which enhances the eutrophication potential of the receiving waterbody (Austin and Lee, 1973).

Discharge from WWTWs may contain a suite of different pollutants and can contaminate and de-stabilize the receiving waterbodies (Morrison et al., 2001; Cheung et al., 2003; Oberholster et al., 2013b). These pollutants include the transport of high concentrations of nitrogen and phosphorous (the key nutrients that control the trophic status of freshwater systems) into the system, leading to eutrophication (Heathwaite, 1995; Oberholster et al., 2008). This corresponded well with the increase in TN recorded within the water, as well as the increase in metal contamination and SO_4^{2-} in the sediment at OR04. Another main non-point source of TN and TP is agricultural practices (Walmsley, 2000). This is due to the addition of higher concentrations of nutrients during the application of fertilizers than those removed as produce, which ultimately result in the progressive degradation of an aquatic ecosystem (Carpenter et al., 1998). As a result, some of the impacted tributaries may also add nutrients to the Olifants River, for example ORI08, ORI09, ORI29 and ORI36.

Compared to the more pristine Mokolo River the water quality was noticeably different in the upper Olifants River. Increased suspended chlorophyll *a*, TN and TP concentrations, can be used as a proxy to indicate nutrient enrichment (Hellawell, 1986). Thus, from the nutrient enrichment comparison, it was evident that the Mokolo River has not yet undergone a shift to the state which is currently governing the upper Olifants River and as a result the TN concentrations in the Mokolo River did not vary significantly. However, a change in TP was recorded. The difference seen in alkalinity between the Mokolo River and the upper Olifants River is also quite important for future decisions regarding the Mokolo River. This is because this reduced ability of the Mokolo River to withstand sudden changes in pH may make it more susceptible for anthropogenic impacts such as AMD (Björnsson et al., 2001). The spatial changes with regard to metal concentrations in the water and sediment of the Mokolo River were very site specific. Most of the higher concentrations of certain metals, when compared to the other sites, were found at sites MR03, MR06 and MR12. A common denominator that these sites share is that they are all directly downstream of small to medium impoundments (namely MR03 and MR12) or the larger Mokolo Dam (namely MR06). It is known that the chemical signature of water contained within dams can be significantly different from the water entering a dam (McCartney et al., 2001). This was also found to be true in the case of the Mokolo Dam, as observed from the different water quality signatures recorded. This is especially true for the alkalinity and SO_4^{2-} levels. This may be the reason for the increased metal concentrations found in the water and sediment at the sites directly downstream of these impoundments (Solomons and Förstner, 1984; McCartney et al., 2001; Taghinia Hejabi et al., 2010; Dabrowski and De Klerk, 2013). Thus, from our results a very good comparison between the increase in certain dissolved metals (Al, As, Fe and Pb) and those adsorbed to the sediment at MR03, MR06 and MR12, downstream of impoundments can be drawn.

Changes in the concentrations of some of the metals within the sediment at sites MR07, MR08 and MR09 were also observed. These sites were characterised by a sandy substrate with no exposed bedrock or other large material. It is well known that smaller particles have a higher surface / area ratio and therefore are able to bind more to elements or pollutants, for example metals (McCave, 1984; Rubio et al., 2000). The impact of the informal settlements and agricultural activities, for example at sites MR03 and MR05, was evident from the sediment analyses with the increased Cl^- and SO_4^{2-} concentrations which corresponded well with the increase in TN and TP concentrations in the sediment (Zampella et al., 2007;

Boyacioglu and Boyacioglu, 2008). This is especially true for MR05 which is significantly impacted by agricultural activities that may also contribute to the increased SO_4^{2-} concentrations recorded in the surface water at this site (Böhlke, 2002).

The tributary, MRI04, drains from areas of extensive degradation, whilst MRI12 drains from an area used for coal mining activities (Figure 3-2). These activities are known to severely impact water quality (Sams and Beer, 2000; Tiwary, 2001). Site MRI03, on the other hand, is impacted by not only degraded and agricultural land, but also game ranching which is a known contributor to land degradation that can impact receiving water bodies (Smet and Ward, 2005). Of all of the different tributaries studied, MRI11 was found to have the most impacted water quality and is adjacent to informal settlements, as well as intensive agricultural practices. These impacts also apply to MRI01, and this may explain the impacted nature of both sites as informal settlements and agriculture are known to impact water systems through increased nutrients and a variety of other pollutants. One of the mechanisms through which this is taking place is by increasing silt loads (and their associated pollutants) into the system which affects the river (Buermann et al., 1995). Another way is through the waste discharges from informal settlements and the leaching of agricultural soils, both of which are usually associated with metals and nutrients (Jackson et al., 2007; Edokpayi et al., 2014).

Based on what has been observed from the upper Olifants River catchment and put into the context of the Mokolo River catchment, the following considerations appear to be of importance for the future water resource management of the Mokolo River catchment in the face of ever increasing mining, urbanisation, industrialisation and other associated activities. The influence of pH in the Mokolo River will be of paramount concern. Any activity in this catchment that influences this variable may cause this river to shift to an undesirable state. As revealed by the study on the upper Olifants River, most of the water quality impacts and associated ecosystem degradation was as a result of the changes in pH levels brought about by certain land use activities. Therefore, AMD impacts must be avoided at all costs in the Mokolo River. The study of Sams and Beer (2000), where coal mining has taken place in the Allegheny and Monongahela River catchments for more than 200 years, makes for a further good comparison along with the data derived from the upper Olifants River. This study has shown that of the 270 discharges tested, many had a pH less than 3.0 (Williams et al., 1996). This resulted in the Allegheny and Monongahela rivers transporting around 1.2 – 1.3 million tons of sulfate into the Ohio River. Thus, monitoring the sulfate loads will also be of importance since it was seen in the upper Olifants River that the alkalinity levels decreased as sulfate levels increased. This is particularly important since the Mokolo River has a lower buffering capacity compared to the upper Olifants River.

The fact that higher concentrations of Al (compared to the upper Olifants River) are already observed at some of the sites in the Mokolo River is of concern and also emphasizes the need to properly manage this system. Another factor of potential concern is the impact of sand mining within the Mokolo River. These activities can result in serious environmental impacts which may destabilize the system and have the ability to cause serious damage to the physical and biological environment of a river and consequently compromise its water purification ecosystem service (Padmalal et al., 2008). An example of such an impact is the ability of sand mining to re-suspend various types of pollutants (Langer, 2003). This is an important factor to take into consideration because, as seen from the results of this study and discussed earlier, these sediment beds have the ability to improve water quality by removing pollutants from the water through the high surface / area ratio of the particles.

Dams are of strategic importance to many countries and their communities, but if not managed properly, they can pose a serious risk to the users of the dam, as well as to the downstream environment. The enrichment of Loskop Dam with metals and nutrients has had severe environmental effects, which is as a result of the impacts on the upper Olifants River that flows into the Loskop Dam (Dabrowski et al., 2013). The Mokolo Dam needs to be carefully monitored and managed to avoid it sharing the same fate as Loskop Dam, as studies have shown that a threshold limit of 40 µg/l P (winter) and 30 µg/l P (summer) should not be exceeded in the Mokolo Dam (Oberholster et al., 2012). The present study has shown the effect that impoundments such as the Mokolo Dam have downstream with regard to increased metal pollution. Thus, the water quality entering this dam should be monitored and managed to prevent a shift in the system that would negatively affect downstream users. Lastly, the importance of the tributaries in the Mokolo River catchment must not go unrecognized. As can be concluded from the upper Olifants study, impacted tributaries can have a major impact on the water and sediment quality of the mainstream rivers. Likewise, good quality tributaries can be the saving grace for hard working mainstream rivers, resulting in a much needed dilution effect and a safe haven from where biological recruitment can take place. Economic development is important for any country and progress inevitably results in environmental impacts. If one can preserve strategically placed tributaries that support the mainstream river, one could possibly safeguard against the severe environmental decline of the Mokolo River and preserve the biodiversity found within these systems.

3.6. CONCLUSION

From the analysis of the upper Olifants River and Mokolo River catchments it was observed that these two catchments are very comparable. This was found in terms of their underlying geology, as well as the anthropogenic impacts (i.e., land use practices) that they were either subjected to or are at risk of. This provided us with a unique insight into learning from the upper Olifants River so that we could potentially apply this knowledge within the Mokolo River catchment for improved integrated water resource management so as to preserve this ecological infrastructure upon which many rely. Various changes in water and sediment quality were observed when comparing the Mokolo River with the upper Olifants River. From this it was found that pH is a very important variable to monitor and manage within the Mokolo River due to the knock-on effect it may have on, inter alia, metal pollution and nutrient enrichment. The consideration of the alkalinity and sulfate levels within the river system is also of significance. The importance of managing healthy tributaries was also evident through the observation of the impact that an impacted tributary may have on the mainstream river. The role of dams in a river system was highlighted in terms of their impact on water and sediment quality and underpins the importance of proper management of dams in river systems. Overall, it is important to prevent the Mokolo River from becoming as impacted as the upper Olifants River in the near future. Thus, this study provides a valuable opportunity in understanding the changes brought about by similar activities in a more impacted catchment. This is especially true in the light of certain metal concentrations, for example dissolved Al, already being present in higher concentrations at some places within the Mokolo River when compared to the upper Olifants River.

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3.8. SUPPLEMENTARY MATERIAL

Table S 3-1: The percentage (%) of the geological composition of the upper Olifants River and Mokolo River catchments.

Lithology Type	Mokolo River Catchment	Upper Olifants River Catchment
Carbonate rocks	0.00	1.37
Felsic and intermediate volcanic rocks	2.31	11.65
Felsic and intermediate rocks	0.67	8.35
Fine-grained felsic rocks	0.36	11.03
Granulite (from siliciclastic rocks)	3.53	0.53
Mafic and ultramafic volcanic rocks	0.17	4.23
Marble and calc-silicate rocks	0.03	0.00
Siliciclastic rocks	92.93	62.82

Table S 3-2: The predominant land use (%) of the various sites selected in the upper Olifants River and Mokolo River catchments (excluding the reservoir sites).

Mainstream Upper Olifants River						
Site	**Cultivation**	**Degraded**	**Mining**	**Natural**	**Urban**	**Waterbodies**
OR01	46.58	0.07	0.23	51.81	0.03	1.27
OR02	42.53	0.02	5.22	49.39	1.02	1.82
OR03	40.12	0.02	4.96	51.65	1.45	1.8
OR04	40.12	0.02	4.96	51.65	1.45	1.8
OR05	39.72	0.02	5.15	51.87	1.39	1.85
OR06	39.91	0.01	4.04	52.71	1.45	1.88
OR07	39.03	0.01	4.26	53.03	1.82	1.84
Tributaries - Upper Olifants River Catchment						
Site	**Cultivation**	**Degraded**	**Mining**	**Natural**	**Urban**	**Waterbodies**
ORI01	36.08	0.1	0.55	58.47	1.53	3.27
ORI02	31.15	0.3	2.03	64.42	0.88	1.22
ORI03	31.15	0.3	2.03	64.42	0.88	1.22
ORI04	31.15	0.3	2.03	64.42	0.88	1.22
ORI05	31.15	0.3	2.03	64.42	0.88	1.22
ORI06	38.76	0.08	0.5	54.43	2.43	3.8
ORI07	41	0	0.02	51.15	4.26	3.57
ORI08	50.7	0.06	0.18	41.07	2.59	5.4
ORI09	48.33	0.01	0.5	45.99	0.43	4.74
ORI10	19.94	0	4.98	69.11	5.24	0.73
ORI11	23.36	0	8.16	58.76	8.58	1.14
ORI12	23.36	0	8.16	58.76	8.58	1.14
ORI13	23.36	0	8.16	58.76	8.58	1.14
ORI14	23.36	0	8.16	58.76	8.58	1.14
ORI15	33.4	0	8.23	55.29	0.41	2.67
ORI16	22.41	0	15.48	52.56	4.47	5.09
ORI17	22.41	0	15.48	52.56	4.47	5.09
ORI18	45.6	0	13	38.79	0.54	2.08
ORI19	43.1	0.01	3.81	50.68	0.95	1.45
ORI20	45.37	0.04	5.36	46.83	0.5	1.9
ORI21	45.37	0.04	5.36	46.83	0.5	1.9
ORI22	45.37	0.04	5.36	46.83	0.5	1.9
ORI23	45.37	0.04	5.36	46.83	0.5	1.9
ORI24	40.78	0	5.16	50.31	2	1.75
ORI25	43.67	0	0	55.85	0	0.48
ORI26	46.34	0.05	1.51	50.51	0.33	1.26
ORI27	45.83	0	4.27	47.68	0.98	1.24
ORI28	42.79	0	2.54	50.86	1.69	2.11
ORI29	44.34	0.01	3	48.35	1.81	2.5
ORI30	46.15	0.01	2.72	47.71	0.67	2.74
ORI31	46.15	0.01	2.72	47.71	0.67	2.74
ORI32	46.15	0.01	2.72	47.71	0.67	2.74
ORI33	41.39	0.01	3.94	51.49	0.96	2.22
ORI34	41.39	0.01	3.94	51.49	0.96	2.22
ORI35	41.39	0.01	3.94	51.49	0.96	2.22
ORI36	41.35	0.03	0.09	56.26	1.12	1.15
ORI37	41.35	0.03	0.09	56.26	1.12	1.15
ORI38	16.93	0	0	79.65	0.07	3.35

Table S 3-2: Continued.

Mainstream Mokolo River						
Site	**Cultivation**	**Degraded**	**Mining**	**Natural**	**Urban**	**Waterbodies**
MR01	19.42	9.81	0.02	69.45	0.18	1.12
MR02	19.42	9.81	0.02	69.45	0.18	1.12
MR03	19.42	9.81	0.02	69.45	0.18	1.12
MR04	15.09	12.27	0.01	71.67	0.18	0.78
MR05	10.82	9.56	0.01	78.75	0.12	0.75
MR06	8.79	8.43	0.01	82.05	0.09	0.62
MR07	8.79	8.43	0.01	82.05	0.09	0.62
MR08	7.96	7.4	0.02	83.84	0.24	0.53
MR09	7.96	7.4	0.02	83.84	0.24	0.53
MR10	7.16	6.17	0.44	85.59	0.2	0.44
MR11	7.16	6.17	0.44	85.59	0.2	0.44
MR12	7.16	6.17	0.44	85.59	0.2	0.44
Tributaries - Mokolo River Catchment						
Site	**Cultivation**	**Degraded**	**Mining**	**Natural**	**Urban**	**Waterbodies**
MRI01	22.06	12.15	0	64.48	0	1.31
MRI02	3.04	4.56	0	91.45	0	0.96
MRI03	3.04	4.56	0	91.45	0	0.96
MRI04	7.31	16.66	0	75.66	0.17	0.19
MRI05	22.06	12.15	0	64.48	0	1.31
MRI06	7.31	16.66	0	75.66	0.17	0.19
MRI07	3.04	4.56	0	91.45	0	0.96
MRI08	3.04	4.56	0	91.45	0	0.96
MRI09	1.52	4.39	0	93.9	0	0.19
MRI10	1.52	4.39	0	93.9	0	0.19
MRI11	19.7	9.64	0.07	69.45	0.16	0.98
MRI12	4.07	1.44	2.04	92.34	0.04	0.08
MRI13	17.22	8.2	0	73.16	0.33	1.09
MRI14	5.79	12.66	0	81.28	0.11	0.16
MRI15	3.04	4.56	0	91.45	0	0.96
MRI16	3.56	1.9	0.11	93.34	1.03	0.06
MRI17	17.22	8.2	0	73.16	0.33	1.09

CHAPTER 4: A COMPARATIVE STUDY OF THE DYNAMICS AND IMPACT OF METAL POLLUTION IN TWO COAL-RICH CATCHMENTS IN SUPPORT OF IMPROVED RIVER MANAGEMENT

This research chapter has been _submitted_ *to an ISI accredited peer review journal for publication and is currently* _under review_.

Ecotoxicology & Environmental Safety

Declaration by the candidate

With regard to Chapter 4, the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution
Conceptual design, fieldwork, experimental work, manuscript writing.	75%

The following co-authors have contributed to Chapter 4:

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</table>

4.1. ABSTRACT

During the industrial revolution, various toxic metals and metalloids have continuously been introduced into the environment. These pollutants are extensively produced worldwide and accumulate in soils, resulting in global concerns due to their nature of being persistent and able to bioaccumulate. In South Africa, the upper Olifants River catchment has been subjected to intensive coal mining for decades. However, with these coal resources dwindling, the Mokolo River catchment is at the centre of planned coal mining activities in future. The aim of this study was to determine and understand the dynamics relating to metal pollution from both the upper Olifants and Mokolo rivers to support improved river management. The level of metal pollution in the water, sediment and various biota (algae, crabs and fish) were determined in both rivers. The role of dietary uptake of metals was also examined, whilst selected biomarkers were used to determine possible impacts at a biochemical level. The present study showed that the upper Olifants River is heavily impacted by metal pollution compared to the Mokolo River and resulted in increased bioaccumulation of metals. The major exposure route for the freshwater crab, *Potamonautes warreni*, and the freshwater fish, *Tilapia sparrmanii*, was found to be through dietary uptake of contaminated sediment and algae. This resulted in an increase of reactive oxygen species which can be used to monitor the impact of metal pollution on organisms at a biochemical level. The use of *Potamonautes warreni* provided an excellent model to study the future fate, transport and impact of metal pollution. The results from this study highlighted the value of such a unique comparative case study for international riverine management strategies so as to better understand and anticipate the dynamics of metal pollution in the face of increasing land use activities.

4.2. INTRODUCTION

The contamination of aquatic ecosystems through metal pollution has been an important subject for decades and has highlighted the significance of understanding the complexity of bioaccumulation and its impacts, whether physiologically or biochemically (Wang and Rainbow, 2008). Metal pollution in aquatic ecosystems is usually measured by determining the relative concentrations of a specific metal in the water, sediment and biota of a specific aquatic ecosystem (Yuan et al., 2004; Bonanno and Lo Giudice, 2010). The availability of the metals for uptake by biota (bioavailability) is regulated by a variety of physical, chemical and biological characteristics of the water and the sediment (Chowdhury and Blust, 2002; Gray, 2002; Barwick and Maher, 2003; Ruus et al., 2005). The term bioaccumulation refers to the process that causes an increase in the concentration of a specific metal in an organism relative to the concentration of the same metal in the surrounding environment (De Jonge et al., 2010). Bioaccumulation can only occur if a chemical is bioavailable in this surrounding environment, whether within the water or sediment of a particular aquatic ecosystem (DeForest et al., 2007). The release of high concentrations of metals into aquatic ecosystems has the ability to cause the elimination of the most sensitive biota and may result in disrupted ion regulation, a decrease in swimming speed, as well as an overall decline in the growth and condition of fish (Sorensen, 1991; Alsop et al., 1999; Hollis et al., 1999; Bervoets and Blust, 2003). Thus, it can be expected that excessive metal pollution may also result in alterations of biological communities, for example species composition, richness and trophic composition.

Globally, green filamentous macroalgae are used extensively to study metal pollution in freshwater ecosystems due to their ability to accumulate large amounts of metals present at pollution sites, as well as their prolonged existence at such sites (Conti and Cecchetti, 2003; Stengel et al., 2004). Decapoda from the genus *Potamonautes* are strongly associated with the surficial sediments in aquatic systems and are thus easily exposed to metal pollution during feeding, etc. (Dallinger et al., 1987; Van Eeden and Schoonbee, 1991; Burton, 1992). On the other hand, in many aquatic systems fish are considered to be top consumers and consequently any pollutant released into these systems has the potential to accumulate in the fish and in so doing represent a potential risk (Dallinger et al., 1987). Metal pollution has particular significance in ecotoxicology, because metals have the ability to be highly persistent, they are naturally bioaccumulated and their toxicity potential is affected by other environmental parameters (Dyer and Belanger, 1999; Bennett et al., 2004; DeForest et al., 2007; Öztürk et al., 2009; De Klerk et al., 2013). Anthropogenic activities, for example mining, often result in high metal concentrations in water and sediment and can therefore have a severe impact on river biota (Hoiland et al., 1994; Bonzongo et al., 1996; Vinyard, 1996).

In South Africa, one of the most polluted rivers is the Olifants River (Grobler et al., 1994; Oberholster et al., 2010). This is as a result of mining, industrial and other anthropogenic activities which have been taking place for decades in the catchment of this river (Driescher, 2007; Oberholster et al., 2010). This is understandable, because this area is underlain with the Witbank coalfield, one of the largest coalfields in South Africa, but which is now nearing depletion. This system is severely degraded and is increasingly being impacted by a range of pollutants, including metals. The extent of pollution in the modern day Olifants River may severely stress aquatic organisms, if not the whole ecosystem (Kotze et al., 1999). On the other hand, the coal reserves in the Waterberg region in the Limpopo Province of South Africa are the only other large coal resource available for future mining in South Africa (Bester and Vermeulen, 2010). The Mokolo River in the Waterberg is at the centre of new development aspirations due to the fact that it has a greater exploitation potential, compared to the other rivers in the area (DWAF, 2003). It is thus crucial to properly understand the potential dynamics of metal pollution in the aquatic systems of the Waterberg area to prevent the same deleterious effects as can be seen in the Olifants system (Kotze et al., 1999). This is especially important since the Waterberg region is subject to low rainfall and limited surface-water resources (Bester and Vermeulen, 2010). Thus, the aim of this study was to conduct a comparative study on the the fate, transport and impact of metal pollution taking place in the upper Olifants and Mokolo River systems. Through this, potentially useful insights can be gained for the protection of aquatic systems, such as the Mokolo River, due to increasing land use activities globally to meet the demands of a growing population. Internationally, this information may also aid in planning for future practices, pollution protection and impact mitigation of other countries, for example Botswana and Mozambique.

4.3. MATERIALS AND METHODS

4.3.1. Study Area and Sampling Design

For this study the upper Olifants River catchment (Mpumalanga Province) and the Mokolo River catchment (Waterberg area, Limpopo Province) were chosen. Both of these systems were found to be very similar in terms of geology, land use and comparable in terms of abiotic ecosystem drivers (De Klerk et al., 2016). These two river systems also have an international importance, with downstream implications for countries such as Botswana and Mozambique (Ashton and Dabrowski, 2011; De Klerk et al., 2016). A total of four sites were selected to be representative of the upper Olifants River, whilst nine sites were identified for assessing the Mokolo River (Table 4-1). Study sites were chosen to be representative of the respective rivers and included all possible influences (receiving waters) reaching the respective catchments. The information from the various chemical and biochemical analyses at respective sites was collated to obtain a reliable overall measurement of the selected variables or impression of the status of the respective rivers. These sites were sampled seasonally for a period of two years (2012 -2013) in the Olifants River and three years (2011 – 2013) in the Mokolo River.

Water samples were collected in pre-cleaned 1 L polyethylene containers and sampled approximately 10 cm below the water surface as close to the centre of the river as possible. Sediment samples were collected using shallow sediment cores (50 mm diameter) to obtain a sample (n = 3) of the upper 10 cm layer from a predefined area which is at a water depth of approximately 1 m. The sediment samples were transferred into plastic bags. The water and sediment samples were placed on ice and stored frozen (-20°C) until analysis in the laboratory. Green filamentous macroalgae were collected at each sampling site and stored in pre-cleaned containers (Oberholster et al., 2012). Approximately 100 g algae (wet weight) were collected within a 50 m stretch and kept at 4°C until analysis. Freshwater crabs (*Potamonautes warreni*) were collected at the different sites by using baited crab traps, whilst fish (*Tilapia sparrmanii*) were collected using an electro-fishing unit (SAMUS 725-M, SAMUS Special Electronics) in a ≈ 50 m stretch of river.

The crab and fish specimens were captured, euthanized and used in an ethical manner to ensure the least amount of suffering. The South African National Standard for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008), Gardner (1997), ANZCCART (2001), Hajek et al. (2009) and Yue (2009) guidelines were used as reference to ensure compliance. The authors affirm that all sampling and analysis were conducted in such a manner that it adhered to the Animals Protection Act (Act 71 of 1962) of South Africa.

Table 4-1: Location and brief description of the study sites selected for the bioaccumulation studies.

Site Number	Latitude	Longitude	Site Description
Mokolo River			
Site 1	28.104342	-24.427303	Downstream of agricultural practices, as well as non-point source pollution from informal settlements.
Site 2	28.092386	-24.289449	Upstream of sewage stabilization ponds which regularly overflow into the river.
Site 3	28.095493	-24.286274	Downstream of sewage stabilization ponds which regularly overflow into the river.
Site 4	27.802295	-24.113663	Downstream of a big conglomeration of agricultural practices and upstream of the Mokolo Dam.
Site 5	27.726264	-23.970681	Directly downstream of the Mokolo Dam.
Site 6	27.750041	-23.768693	Upstream of extensive sand mining operations.
Site 7	27.744158	-23.687369	Downstream of extensive sand mining operations and upstream of the town of Lephalale.
Site 8	27.759659	-23.652237	Downstream of the town of Lephalale (locals report of regular sewage inputs into the Mokolo River).
Site 9	27.717571	-23.234908	Most downstream site to monitor the final water quality characteristics and inputs into the Limpopo River.
Upper Olifants River			
Site 1	29.462332	-26.222566	Surrounded by farms and mainly impacted by agricultural practices and erosion.
Site 2	29.266414	-25.841445	Downstream of sewage treatment works, thus mainly impacted by treated and partially treated sewage effluent.
Site 3	29.29821	-25.701216	Located on a game farm, receiving cumulative water quality impacts from upstream agricultural activities.
Site 4	29.21623	-25.623028	Located in a nature reserve, receiving cumulative water quality impacts from upstream activities.

4.3.2. Water and Sediment Characterisation

Water samples were analysed for their chemical oxygen demand (COD) according to Pitwell (1983), dissolved organic carbon concentration (DOC), using a Vario EL Elementar III Elemental Analyser Instrument (Elementar, Germany), as well as the concentration of total suspended solids (TSS). The pH and total dissolved salts (TDS) measurements were measured *in situ* at each site using a Thermo 5 Star pH/RDO/Conductivity meter set (Thermo Scientific, USA). In order to assess the buffering capacity of the respective surface waters, alkalinity was measured using a TitraLab® titration workstation (Radiometer Analytical TIM860). Water samples were analysed in triplicate against known standards and calibration was carried out using matrix matched calibration standards. During the analysis selected samples in each batch were also duplicated to further determine the quality of the analysis (i.e., reproducibility). A reproducibility tolerance range of <10% was deemed acceptable.

For the sediment samples, the organic matter content was determined through Loss of Ignition (LoI) by incinerating dry sediment at 550°C. Acid Volatile Sulfides (AVS) were extracted from wet sediment using a titrimetric method (USEPA, 1991) and calculated using Equations 1 and 2, whilst the remainder of the extract was analysed by inductively coupled plasma optical emission spectrometry (ICP-OES) for the simultaneously extracted metals (SEM) of aluminium (Al), copper (Cu), vanadium (V) and zinc (Zn) to determine the ratio between the SEM:AVS.

Equation (1):

$$mgS^{2-}/L = \{[(ml\ I_2 \times I_2\ Normality) – (ml\ Na_2S_2O_3 \times Na_2S_2O_3\ Normality)] \times 16000\} / ml\ sample$$

Equation (2):

$$mgS^{2-}/kg = (mgS^{2-}/L \times g\ original\ sample\ mass) / 100\ ml$$

4.3.3. Biological Characterisation

Green filamentous algae: In the present study, algal samples (namely *Oedogonium* sp. and *Spirogyra* sp.) collected at each site were identified according to Janse van Vuuren et al. (2006). Prior to metal analysis the algae were washed to remove impurities, suspended sediment and any adsorbed metals by using the method described in Pawlik-Skowrońska (2001).

Crabs: Muscle tissues (≈10 g wet mass) as well as the hepatopancreas of the crabs were removed and snap frozen in liquid nitrogen for biochemical analysis, whilst the remaining tissue samples were frozen at -20ºC for subsequent metal analysis. Each crab was weighed, sexed and measured prior to analysis (Steenkamp et al., 1993; Sanders et al., 1998; Reinecke et al., 2003; Thawley et al., 2004). The stomach was also carefully removed and preserved using 10% neutral buffered formalin for subsequent evaluation according to Oberholster et al. (2012). No significant size and gender effects have been reported with regard to metal concentrations previously determined in *P. warreni* (Kotze et al., 1999; Sanders et al., 1999; Soucek et al., 2002). Thus, random samples of different sizes, masses and genders were used to ensure that the variability is distributed evenly. Special attention was given to ensure that all organisms used were in a similar moulting period (Sanders et al., 1998).

Fish: Samples of *T. sparrmanii* were weighed, sexed and measured before dissection. Muscle tissue (≈10 g wet mass) and the liver were removed and snap frozen in liquid nitrogen for subsequent biochemical analyses. The remainder of the muscle tissue was kept at -20°C until metal analysis could be conducted. Thereafter, the stomach was carefully removed, preserved and analysed as described above for the crabs. Random samples of different sizes, masses and genders of *T. sparrmanii* were also used to ensure that variability is distributed evenly.

4.3.4. Metal Analysis

Dissolved concentrations of Al, Cu, V and Zn in the water samples were determined using ICP-OES and / or inductively coupled plasma mass spectrometry (ICP-MS) analyses (Thermo Scientific). Quality control was conducted for the water sample analysis by analysing the samples in triplicate against known standards for the selected metals, whilst calibration was carried out using matrix matched calibration standards.

The concentrations of these metals in the sediment samples were determined using a modified partial microwave digestion method according to Loring and Rantala (1992). The emphasis was on the potential environmental toxicity instead of geochemical content, therefore the hydrofluoric acid (HF) was replaced with nitric acid (HNO_3), perchloric acid ($HClO_3$) and hydrogen peroxide (H_2O_2). Quality control was performed by digesting and analysing sediment reference samples (PACS-2 NCR material) with every batch of 11 samples.

The metal concentrations in the biota were determined following a microwave digestion method using HNO_3, $HClO_3$ and H_2O_2. Analyses were carried out using ICP-OES analysis. Quality control was performed by digesting and analysing a suitable reference material for metal analysis, namely TORT-2 NCR material (lobster hepatopancreas) with every batch of 11 samples. For the water, sediment and biota determinations, the first sample in each batch being analysed was duplicated to monitor the percentage recovery. A limit of <10% variance in the percentage recovery was deemed acceptable.

4.3.5. Biochemical Analysis

Selected endpoints were chosen to determine the increase in reactive oxygen species (ROS), namely H_2O_2, superoxide dismutase (SOD), thiobarbituric acid reactive substances (TBARS) and protein carbonyl derivatives. The hepatopancreas and liver tissue of both *P. warreni* and *T. sparrmanii*, respectively, were screened for TBARS as this is the first detoxifying pathway in organisms. Muscle tissue was used to screen for SOD and protein carbonyl derivatives to assess the biochemical impact of the sequestered metals on the respective organisms from each river. These analyses were done using the SOD activity assay kit (STA-340), the TBARS assay kit (STA-330), as well as the protein carbonyl enzyme-linked immunosorbent assay (ELISA) kit (STA-310) from Cell Biolabs Incorporated. The algal samples were used to measure H_2O_2 as a general oxidative stress marker using the OxiSelectTM hydrogen peroxide assay kit (Colorimetric) (STA-343). Sample preparation and analysis were conducted at 4°C. The Bradford method (Bradford, 1976) was used to determine the protein concentrations of each sample. At least three biological samples per site were used per sampling trip of which each was analysed in triplicate (thus, n=9), although often the biological sample size was much higher.

4.3.6. Statistical Analysis

Significant differences were determined using Analysis of Variance (ANOVA) in combination with the Fisher's LSD post-hoc test (Statistica 12, Statsoft, US). Normality and homogeneity of variance were also considered and tested. Significance was accepted at probability (p) values equal to or less than 0.05. Pearson's chi-squared test was used to test for significant differences between categorical data.

The bioaccumulation factor (BAF) was taken as the ratio between the concentration of a chemical in an organism and the concentration of that same chemical in the environment (DeForest et al., 2007). The relationship between the BAF of a specific metal and the concentration found in the environment (water or sediment) was then determined using linear regression analysis (log:log scale) according to DeForest et al. (2007). This type of BAF analysis is important to allow for comparison between the two river systems, as well as between metals. Using the regression data, it also allows for the determination of the degree of bioaccumulation in an organism of a specific metal proportionate to its exposure concentration.

4.4. RESULTS

4.4.1. Water and Sediment Characterisation

In general, the upper Olifants River had higher concentrations of Al, Cu, V and Zn when compared to the concentrations found within the Mokolo River (Figure 4-1; Table S 4-1, Supplementary Material). The dissolved metal concentrations of Cu, V and Zn were all significantly ($p \leq 0.05$) higher in the upper Olifants River than in the Mokolo River. In contrast, the Al concentrations recorded in the surface waters of the Mokolo River (≈20 µg/l) were significantly ($p < 0.05$) higher than that of the upper Olifants River (≈13 µg/l). On the other hand, the Mokolo River had significantly ($p = 0.0003$) less Al (≈4 541 mg/kg) in the sediment when compared to the upper Olifants River (≈10 538 mg/kg). The same trend was observed for Cu, V and Zn concentrations in the sediment.

The additional water and sediment quality parameters measured revealed that the upper Olifants River had higher levels of TSS, COD, DOC, TDS, LoI, as well as a higher SEM:AVS ratio (Table S 4-1, Supplementary Material). Overall, the pH levels measured within these two systems remained comparable (Figure 4-1).

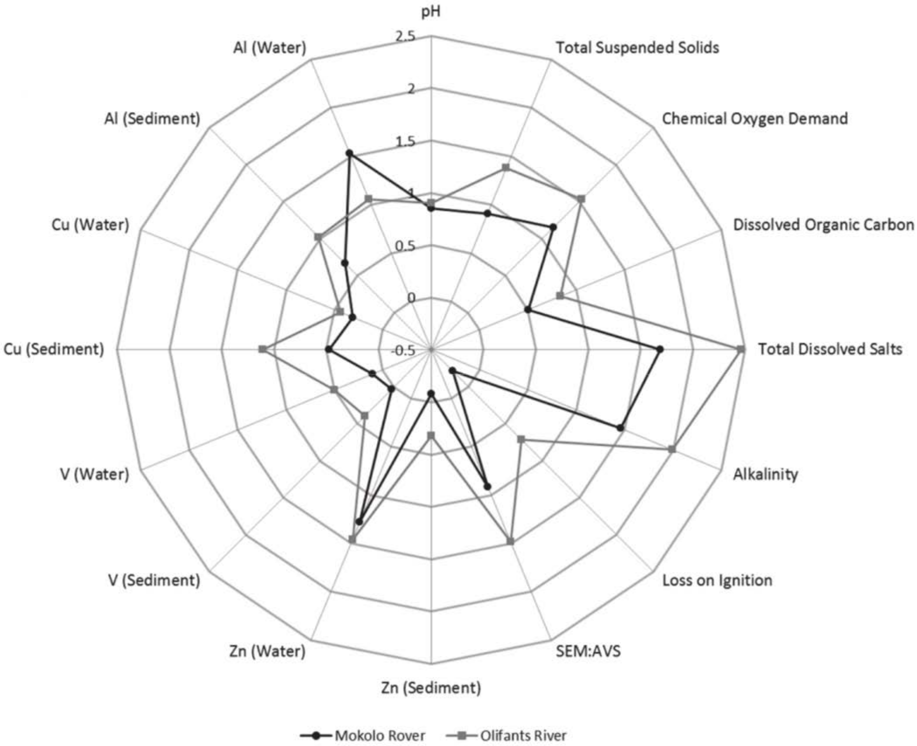

Figure 4-1: A radar graph used as a comparative model to compare the two rivers in terms of metal pollution in the surface water and sediment, as well as additional water and sediment quality parameters. The values of the different endpoints were standardized using a log transformation.

4.4.1.1. *Metal Log-linear Relationships*

The correlation between the sediment and water concentrations in the Mokolo River for the four metals tested was positive (Figure 4-2), although Cu only had a slight positive slope (Slope = 0.005) (Table S 4-2, Supplementary Material). Only the relationship of Zn in the water and sediment was found to be significant (p = 0.018). On the other hand, the relationship between the water and sediment in the upper Olifants River with regard to Al and V had a slight negative slope (≈-0.056 and ≈-0.094, respectively). The relationship of the other two metals, namely Cu and Zn, showed a positive slope (≈0.068 and ≈0.175, respectively). None of the correlations of the upper Olifants River samples were found to be significant (p > 0.05) (Table S 4-2, Supplementary Material).

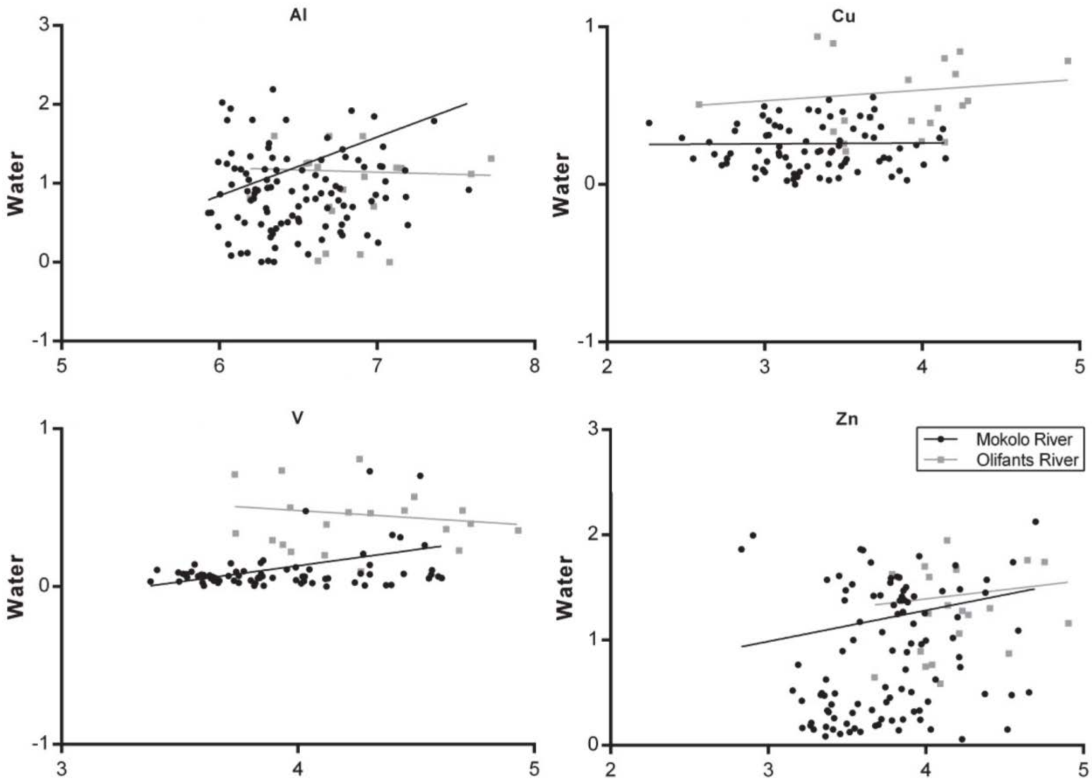

Figure 4-2: The log-linear relationship (log:log scale) between the concentrations of Al, Cu, V and Zn measured in the water and sediment.

4.4.2. Biological Characterisation

4.4.2.1. *Tissue Metal Concentrations*

It was recorded that the metal concentrations found in the different organisms (expressed on a dry weight basis) were generally higher in the upper Olifants River (except for the Al concentrations in algae) (Table S 4-1, Supplementary Material). With regard to algal samples, the concentrations of Cu, V and Zn were found to be significantly ($p < 0.05$) higher in the upper Olifants River (≈4.5, ≈1.3 and ≈9.96 mg/kg, respectively) as opposed to the Mokolo River (≈0.56, ≈0.62 and ≈1.42 mg/kg, respectively) (Table S 4-1, Supplementary Material). On the other hand, the concentrations of Al found in the algal samples were higher in the Mokolo compared to the upper Olifants River (≈1014 and ≈660 mg/kg, respectively). In crab tissues (muscle) obtained from the upper Olifants River, only Cu and Zn were significantly (≈35.83 and ≈145.95 mg/kg, respectively) higher compared to those in the Mokolo River (≈13.95 and ≈0.05 mg/kg, respectively). Analyses of fish tissue (muscle) showed that Al, Cu, V and Zn were all significantly higher in fish collected from the upper Olifants River when compared to fish collected from the Mokolo River ($p \leq 0.05$) (Table S 4-1, Supplementary Material). It is noteworthy that in both river systems, the highest concentrations of Cu and Zn were often found in the crab and fish specimens, rather than the sediment (Table S 4-1, Supplementary Material). The crab tissue had significantly ($p <$

0.05) higher Al, Cu and Zn than the fish tissue, more profoundly so in the organisms collected from the upper Olifants River.

4.4.2.2. *Oxidative Stress*

The expression of the different ROS species (H_2O_2, SOD, TBARS and protein carbonyl) measured in organismal tissue collected from the two rivers varied significantly among the different biotic groups (Figure 4-3; Table S 4-1, Supplementary Material). The expression of H_2O_2 in the algal samples did not vary significantly among the two rivers. The crabs, on the other hand, had increased levels of SOD and TBARS in the upper Olifants River, when compared to the Mokolo River (≈3.5 and ≈9.4 fold, respectively). In contrast, the fish samples of the Mokolo River showed an increase in both SOD and protein carbonyl (≈6.1 and ≈61 fold, respectively), compared to fish collected from the upper Olifants River (Table S 4-1, Supplementary Material). The results from the TBARS assay showed that the fish from the upper Olifants River had increased levels when compared to the Mokolo River (≈0.5 fold) in the fish tissue.

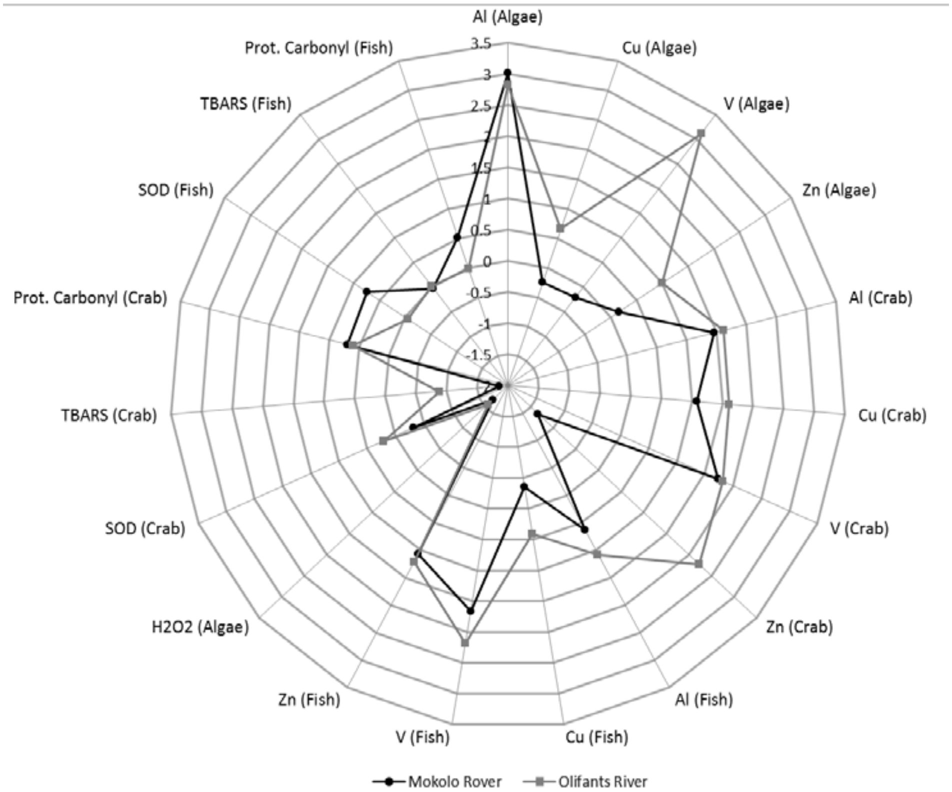

Figure 4-3: A radar graph used as a comparative model to compare the level of metal pollution in the biological samples collected from the two rivers and their respective level of oxidative stress using various biomarkers. The values of the different endpoints were standardized using a log transformation.

4.4.2.3. *Stomach (Diet) Analyses*

The analysis of the stomach contents of the crab and fish specimens revealed a high contribution of Bacillariophyceae, Chlorophyceae, as well as sediment and plant material (Table 4-2). Dinophyceae, Cyanophyceae, Cladocera, Cyclopoida and Rotifera were found to be seldom present within the stomachs of the crabs and fish from both river systems. No significant differences ($p < 0.05$) were found between the Mokolo and upper Olifants rivers with regard to the composition of the stomach contents recorded from the crab and fish samples.

Table 4-2: The percentage composition of the stomach contents of the crab and fish samples collected from the various sites for both the Mokolo River and upper Olifants River.

Stomach Content	Mokolo River	Olifants River	Mokolo River	Olifants River
	Crab		Fish	
Bacillariophyceae	7.57	10.85	19.65	14
Dinophyceae	0.44	2.58	0.97	2.7
Chlorophyceae	19.25	36.94	20.24	33.7
Cyanophyceae	2.92	3.55	3.17	7.03
Cladocera	0.02	0.15	0.47	0.83
Cyclopoida	0.04	0.27	0.14	1.27
Rotifera	0.2	0.45	0.97	2.23
Sediment	13.05	11.55	11.17	6.43
Vegetation	27.79	15.55	14.23	3.07

4.4.2.4. *Bioaccumulation Factor (BAF)*

When using water as exposure route for the algae (Figure 4-4A), crabs (Figure 4-4B) and fish (Figure 4-4C) a significant inverse relationship was observed in the BAF of Al in both the Mokolo River and upper Olifants River. However, the relationships in the Mokolo River were found to be less inverse (namely, a more positive slope) than that of the upper Olifants River (Table S 4-2, Supplementary Material). The relationship of the BAF of Cu for crabs and fish was found to be more positive (slope = $\approx$-1.1) in the upper Olifants River than that of the Mokolo River (slope = $\approx$-2.4). A strong positive correlation was observed for the BAF of Cu in algae (slope = $\approx$2.663) from the upper Olifants River. The inverse relationship between the BAF of Zn for all three organisms versus water also showed a significant negative inverse relationship ($p \leq 0.05$), although the slope from the upper Olifants River was more positive (slope = $\approx$-0.6) than that of the Mokolo River (slope = $\approx$-1.3). (Table S 4-2, Supplementary Material). The BAF of V for all three organisms tested showed no clear correlation (Figure 4-4) in the Mokolo River, whilst in the upper Olifants River some correlation in relation to the water concentrations was observed (Figure 4-4).

Using sediment as the exposure route for crabs (Figure 4-5A) and fish (Figure 4-5B), it was observed that a significant inverse relationship existed between the BAF for the different metals tested for both the crab and fish specimens versus the sediment concentrations for both river systems (Table S 4-2, Supplementary Material). When comparing the slope of the inverse relationships between the BAF of crabs and fish and the sediment concentrations of the metals, it was found that the upper Olifants River generally showed a more positive relationship for Al, Cu and Zn compared to the Mokolo River. However, for V, the relationship was more positive in the Mokolo River (Table S 4-2, Supplementary Material).

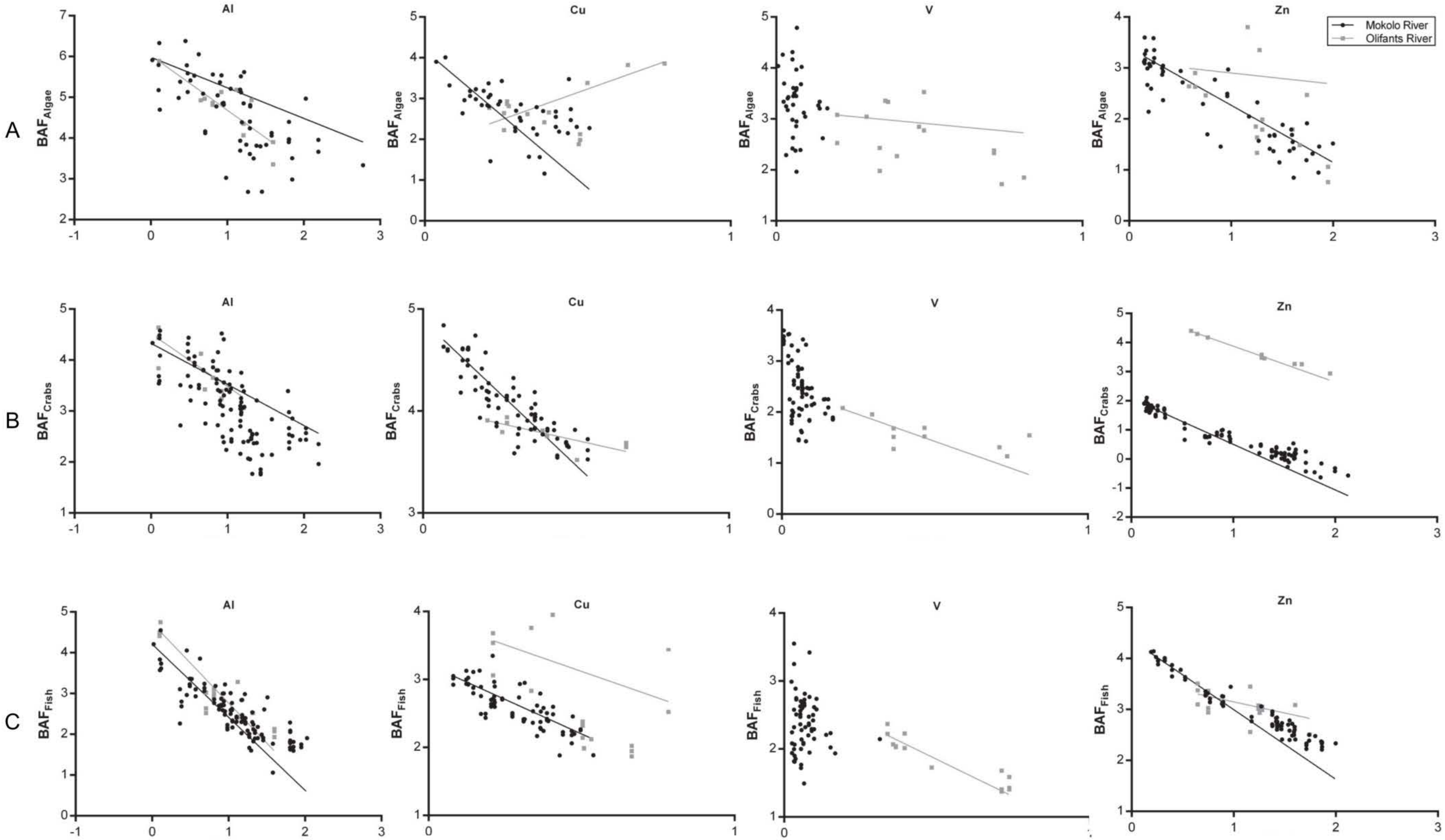

Figure 4-4: The log-linear relationship (log:log scale) between the bioaccumulation factor (BAF) of Al, Cu, V and Zn of the algae (A), crabs (B) and fish (C) compared to the water concentrations.

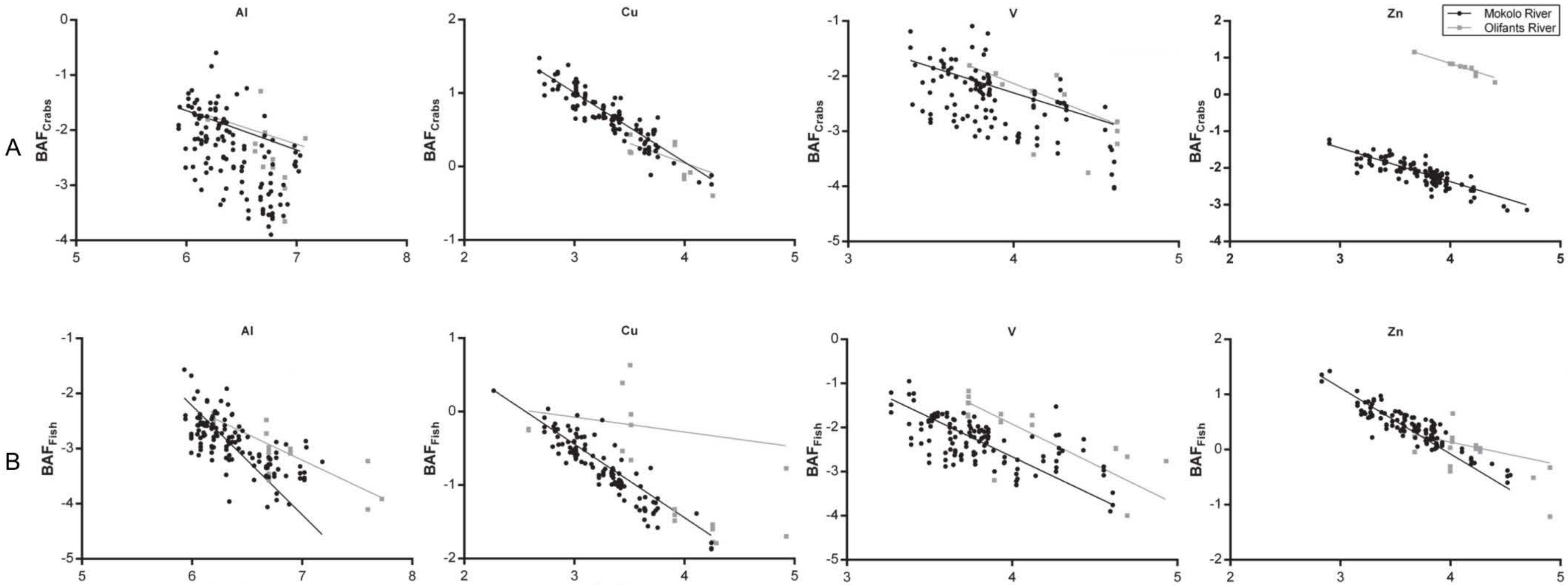

Figure 4-5: The log-linear relationship (log:log scale) between the bioaccumulation factor (BAF) of Al, Cu, V and Zn of the crabs (A) and fish (B) compared to the sediment concentrations.

4.5. DISCUSSION

Metal pollution is known to pose a serious threat to the sustainability of aquatic ecosystems, including the survival of organisms inhabiting such systems (Bervoets et al., 2005). With the Mokolo River in the Waterberg area of South Africa facing an increasing threat to metal pollution through changes in land use activities, an interesting comparison can be made with the upper Olifants River, which is known to have been severely impacted for an extensive period by similar land use activities. The current investigation comparatively explored the fate and transport of selected metals within these two river systems and its associated impacts on oxidative pathways in aquatic algae, invertebrate and vertebrate specimens using various biomarkers. From the analysis of the metal content in both the abiotic phase (water and sediment), as well as the biotic phase (algae, crabs and fish tissue), it was evident that the upper Olifants River was considerably more contaminated than the Mokolo River. These results confirm previous studies on the Olifants River and highlight the degree of metal pollution present in the system due to more than a century of coal mining (Kotze et al., 1999; Oberholster et al., 2013).

4.5.1. Abiotic Characterisation

Metal contamination has generally been thought to only be problematic in acidic waters and not neutral waters, because of its low solubility in neutral waters. But studies are beginning to suggest otherwise (Soucek et al., 2002) and the current study is also in line with this relatively new suggestion, as both rivers had near neutral pH levels (pH between 7-8) and high metal concentrations. In addition to this, the increased TSS and DOC levels in the surface water of the upper Olifants River add to its ability to carry metals in the water column as they offer strong binding sites for metals (De Klerk, 2010; De Klerk et al., 2012; Johnson et al., 2012). Also, the high SEM:AVS ratios measured in the sediment of the upper Olifants River (De Jonge et al., 2009) along with the high organic content (%LoI) increases its ability to accumulate metals (Berkman et al., 1986). These factors may lead to the higher level of metals observed in both the water and sediment of the upper Olifants River compared to the Mokolo River. The widespread agricultural practices in catchment of the Mokolo River may have resulted in the increase in Al concentrations within the Mokolo River. This may be due to the use of lime to condition the soil, which may contain large amounts of Al (Poléo et al., 1994; Oberholster et al., 2013). Thus, the recorded levels of Al in the Mokolo River were not only higher than in the upper Olifants River, but also exceeded the trigger value of 55 µg/l (pH corrected value to reflect the current study conditions) determined by Australian and New Zealand Environment and Conservation Council and Agriculture and Resource Management Council of Australia and New Zealand (2000).

The positive correlation between the water and sediment concentrations of Cu and Zn in the upper Olifants River may indicate that the long-term inputs of metals into the system have caused the sediment to become a source of metal pollution in the upper Olifants River (Dabrowski and De Klerk, 2013). On the other hand, the weak relationship observed for Al and V may indicate that various industries are the main drivers behind the pollution of these metals. In the upper Olifants River catchment, one of the main drivers of Al contamination was also found to be agricultural related (Oberholster et al., 2013), whilst V smelters may contribute to the high concentrations of V recorded in the water and sediment (≈1.88 µg/l and ≈24.63 mg/kg, respectively). The higher COD levels recorded in the upper Olifants River, compared to the Mokolo River, corroborate the suggestion of excessive anthropogenic inputs of contaminants into the upper Olifants River. The fact that all of the metals tested in the surface water of the Mokolo River had a positive correlation with the underlying sediment concentration, suggests a potentially strong geological origin for the current metal concentrations. This may aggravate the impact of agricultural practices on water quality, for example Al concentrations, as cultivation may also lead to increased runoff and water / sediment contact.

In the upper Olifants River, the availability and toxicity of metal pollution may also be affected by the higher degree of salinity and alkalinity recorded in the system compared to the Mokolo River. For example, Na, Ca and Cl^- are known to chelate metals, thus decreasing its availability to aquatic biota (Zitko and Carson, 1976; Wright, 1977, 1995; Everall et al., 1989; Spry and Wood, 1989; Sanders et al., 1998; Thawley et al., 2004). Thus, since the Mokolo River had a significantly lower salinity and alkalinity (De Klerk et al., 2016), the Mokolo River could be much more sensitive to metal pollution compared to the upper Olifants River. But even with an increased buffering capacity and decreased toxicity due to the high salinity levels, the longevity and severity of the pollution in the upper Olifants River still resulted in impacts being observed on the different aquatic biota studied.

4.5.2. Biological Characterisation

Metal-mediated pollution can either directly or indirectly lead to an excessive generation of ROS species linked to oxidative stress and also has the ability to affect the rate of peroxidation. This may result in membrane damage (Gutteridge, 1995), as well as protein and nucleic acid oxidation, leading to protein dysfunction, impaired DNA repair, mutagenesis and carcinogenesis (Ercal et al., 2001). Green filamentous macroalgae are known to absorb large amounts of metals (Kleinmann, 1990; Das et al., 2009; Lee and Chang, 2011), whilst algal biomass is also affected by metal precipitates (Oberholster et al., 2010, 2013). The stronger correlation of Cu, V and Zn, found in the algal samples related to the dissolved metal concentration of the upper Olifants River compared to the Mokolo River confirms these reports. The accumulation of high concentrations of these metals in the algae from the upper Olifants River may be due to reduced metal elimination pathways (Oberholster et al., 2012). In spite of the relatively high metal concentrations, the H_2O_2 expression in algae from the upper Olifants River did not vary significantly from those measured in the Mokolo River. The higher concentration of Al found in the algae collected from the Mokolo River may suggest that algae accumulate Al at a rate proportional to or above exposure concentrations (DeForest et al., 2007). Thus, in this study the importance of considering the degree of exposure to truly understand metal bioaccumulation can also be seen.

The uptake of metals by aquatic organisms like crabs and fish could potentially occur through several pathways, for example the gills or body surface or through the ingestion of food and other particles (Jennings and Rainbow, 1979). This was highlighted through the degree of metal accumulation recorded in both the crab and fish samples during this study. This was further emphasized through the fact that the highest observed Cu and Zn concentrations of all the different abiotic and biotic samples tested were generally found in the crab and fish tissue. The more positive relationship of Cu and Zn concentrations in both crabs and fish with the overlying water in the upper Olifants River, compared to the Mokolo River further indicates the impact of the metal pollution in the upper Olifants River. This is in agreement with the water quality trigger values set by Australian and New Zealand Environment and Conservation Council and Agriculture and Resource Management Council of Australia and New Zealand (2000) for Cu (hardness corrected to reflect the current study) and Zn (1.4 and 8 µg/l, respectively) that are both severely exceeded in the upper Olifants River, namely 2.9 and 25.91 µg/l, respectively. The higher dissolved Al concentrations in the Mokolo River may explain the stronger relationship observed in the crab and fish samples compared to the upper Olifants River and suggests that Al is being bioaccumulated and transferred through the food web in the Mokolo River. The absence of a clear relationship with regard to the BAF of V in relation to the dissolved water concentration in all the different biological samples tested from the Mokolo River, whilst a noticeable trend was observed in the upper Olifants River, supports the fact that dissolved V concentrations were almost six times higher in the upper Olifants River compared to the Mokolo River.

The stomach content analysis showed that the filamentous green algae (Chlorophyceae) and sediment particles made up a substantial contribution of the stomach content of both the crabs and the fish examined from both river systems. As a result, these organisms may be exposed not only to the overlying dissolved metal concentrations in the water column, but also through the mobilization of metals from the algae and sediment particles during digestion. This poses a severe risk, because as the results from this study have shown, the metal concentrations of the algae and sediment exceeded that of the overlying water by far.

By comparing the slopes of the regression analyses of the BAF for the crabs and fish relative to water to those relative to sediment, it appears that sediment and most probably also algae are large contributing factors to the bioaccumulation of Al, Cu, V and Zn in *P. warreni* and *T. sparrmanii* through dietary intake. This is in agreement with Lee et al. (2000) who stated that the dietary uptake of metals best explain the relationship between bioaccumulation and the sediment concentrations. This may have resulted in the V uptake in the Mokolo River at a rate higher than that of the upper Olifants River via the sediment. On the other hand, Al, Cu and Zn from the upper Olifants River samples, in accordance with the abiotic results, had higher bioaccumulation in the crab and fish tissue via the sediment. Thus, this study highlights the importance of considering dietary metal uptake when assessing the transport, fate and impact of metal pollution (Dallinger et al., 1987; Kraal et al., 1995; Langevoord et al., 1995).

Potamonautes warreni have similar target areas than *T. sparrmanii* where it can be affected by metal pollution, for example gills. Studies have shown that the greatest concentration of metals is found on the gills of *P. warreni* (Schuwerack et al., 2001). In this study, *P. warreni* accumulated higher metal concentrations compared to *T. sparrmanii*, especially Al and Cu. A study by Colvocoresses and Lynch (1975) found that Zn is well regulated in crustaceans with excess metals stored in the hepatopancreas, which leads to an increase in lipid peroxidation, thus increasing the levels of TBARS seen in the upper Olifants River, compared to the Mokolo River. According to Sanders et al. (1999), a decapod's ability to regulate metals depends on the difference between the rate of metal uptake and excretion. One of the main excretory mechanisms of crustaceans is moulting, which does not occur frequently (Giesy et al., 1980). Metal bound metallothionein may also regulate metal pollution at a cellular level within *P. warreni* (Engel and Brouwer, 1987). Thus, in a relatively unpolluted river such as the Mokolo River, there may be a balance between uptake and excretion, whilst in metal polluted rivers (such as the upper Olifants River) the rate of uptake is probably higher than the rate of excretion which can lead to bioaccumulation. It therefore seems likely that Zn concentrations in the Mokolo River is still effectively regulated by *P. warreni*, whilst in the upper Olifants River this threshold has been exceeded, leading to the extremely high metal concentrations being bioaccumulated. As with Al and Cu, these metals are also not well regulated in *P. warreni*, thus resulting in the degree of accumulation seen in this study (Steenkamp et al., 1993). These muscle tissue concentrations (e.g., Cu) are higher than that of the surrounding environment (i.e., water and sediment) as well as what is recorded in possible dietary sources (i.e., algae). This may have led to the increase in SOD recorded in the upper Olifants River compared to the Mokolo River (Amici et al., 1989; McKersie et al., 1993). Comparerd to previous studies, the Al concentrations found in the freshwater crab of both rivers (≈ 30 – 40 mg/kg) were lower than what was found by Van Stormbroek (2007) who recorded Al concentrations in a *Potamonautes* sp. of 100 – 200 mg/kg in the Lourens River in the Western Cape. A similar trend was observed when comparing Cu and Zn concentration to previous studies. Significantly higher Cu concentrations were found in *P. warreni* by Van Eeden and Schoonbee (1991), as well as Van Strombroek (2007), namely ≈ 70 mg/kg and ≈ 40 mg/kg respectively. On the other hand, the Zn concetrations (≈ 60 mg/kg) reported by Van Stormbroek (2007) and ≈ 102 mg/kg reported by both Sanders et al. (1999) and Van Eeden and Schoonbee (1991) were also significantly higher.

The increased SOD and protein carbonyl derivatives within fish samples of the Mokolo River, compared to the upper Olifants River, may be due to the high dissolved concentrations of Al recorded in the Mokolo River. Aluminium toxicity is known to affect ion regulation in fish (Gensemer and Playle, 1999), whilst other studies suggest that Al toxicity to fish is through the transformation of Al^{3+} to polymers / colloids by hydrolysis with increased pH. This disruption could lead to suffocation when the polymers precipitate on the gills (Neville and Campbell, 1988; Exley et al., 1996). As these metals are taken up into the blood stream where the levels of antioxidants (e.g., SOD) are increased (McKersie et al., 1993), the oxidative cleavage of proteins results in the formation of protein carbonyl derivatives (Amici et al., 1989). The TBARS in the fish, produced during lipid peroxidation (Antczak et al., 1997) was not significantly different in the Mokolo River compared to the upper Olifants River, most probably due to catecholamines (or other hormones) responsible for inducing adaptive changes in liver tissue (Klee et al., 1990; Pickering, 1993; Wepener et al., 2001). The low degree of biomarker response in *T. sparrmanii* in the upper Olifants River could also possibly suggest a degree of acclimation of *T. sparrmanii* due to the long-term chronic exposure to its polluted environment (Walters et al., 2016).

4.6. CONCLUSION

Although both river systems are in essence unique, studies have found the Mokolo River and upper Olifants River to be comparable. From the current study it was observed that although the Mokolo River is much less impacted than the upper Olifants River, there are already some causes for concern. There is thus a need to devise proper monitoring strategies for this river with proper metal focussed indicators, especially in the light of increasing land use activities anticipated for this area. Compared to the highly impacted and disturbed upper Olifants River, certain metals (for example Al) are already present in higher concentrations in the surface water of the Mokolo River and appears to be bioaccumulated to a higher degree in the algal tissue. This is especially significant in the Mokolo River due to the lower alkalinity in this system, which may make it more sensitive to increased anthropogenic inputs of metals compared to the upper Olifants River. As can be seen from the results of the upper Olifants River, industries play a major role in driving metal pollution.

Since the industrial boom is yet to happen in the Mokolo River catchment, current activities such as sand mining operations in the lower Mokolo River may pose a threat to the sustainability of this river. Instead of directly increasing metal pollution (as in the case of smelters in the upper Olifants River), sand mining may affect the Mokolo River's ability to sequester and deal with metal pollution in future. Based on the results of this comparative study, it is therefore important to take into account variables such as alkalinity and salinity of surface waters when planning for future practices to safeguard against metal pollution and mitigate its potential impact. The freshwater crab, *P. warreni*, has been found to be a good model organism to monitor or understand the dynamics of metal pollution due to its ability to bioaccumulate high concentrations of metals and because it responds indicatively at biochemical level. It is also crucial to not only normalize metal pollution levels recorded in biota against exposure concentrations, but also to include dietary analysis in monitoring programmes. Biomarkers within *P. warreni* have also been proven to be useful indicators to monitor the impact of metal pollution. Overall, this was a unique opportunity to conduct a novel catchment scale comparative study that has international implications for riverine management.

4.7. REFERENCES

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4.8. SUPPLEMENTARY MATERIAL

Table S 4-1: The median measurements (± standard deviation) of the different variables measured. The number of replicates is indicated by the n value.

Sample	Variable	Unit	Mokolo River	Olifants River
Water	Al	µg/l	20.04 ± 61.37 (n = 105)	13.15 ± 12.76 (n = 20)
	Cu	µg/l	0.81 ± 0.63 (n = 81)	2.90 ± 2.18 (n = 20)
	V	µg/l	0.33 ± 0.71 (n = 76)	1.88 ± 1.37 (n = 20)
	Zn	µg/l	16.13 ± 22.93 (n = 101)	25.91 ± 23.31 (n = 20)
	pH	-log[H+]	7.08 ± 0.44 (n = 117)	8.02 ± 0.55 (n = 20)
	DOC	mg/l	3.20 ± 1.68 (n = 117)	6.79 ± 1.73 (n = 20)
	TSS	mg/l	8.14 ± 8.66 (n = 107)	23.91 ± 15.91 (n = 20)
	COD	mg/l	14.31 ± 12.83 (n = 117)	34.29 ± 22.53 (n = 20)
	TDS	mg/l	48.76 ± 47.88 (n = 117)	291.00 ± 85.58 (n = 19)
	Alkalinity	mg/l $CaCO_3$	29.19 ± 24.38 (n = 117)	98.08 ± 40.45 (n = 18)
Sediment	Al	mg/kg	4541.04 ± 5003.5 (n = 120)	10538.91 ± 12837.18 (n = 20)
	Cu	mg/kg	2.89 ± 3.08 (n = 116)	12.77 ± 17.82 (n = 20)
	V	mg/kg	10.71 ± 9.34 (n = 120)	24.63 ± 21.2 (n = 20)
	Zn	mg/kg	8.17 ± 9.05 (n = 120)	20.93 ± 19.11 (n = 20)
	LoI	%	0.61 ± 0.81 (n = 111)	5.20 ± 7.01 (n = 20)
	SEM:AVS	mmol/kg	8.23 ± 4.15 (n = 78)	30.29 ± 15.55 (n = 20)
Algae	Al	mg/kg	1014.58 ± 1616.15 (n = 66)	660.82 ± 570.08 (n = 16)
	Cu	mg/kg	0.56 ± 0.76 (n = 66)	4.51 ± 10.48 (n = 16)
	V	mg/kg	0.62 ± 1.31 (n = 66)	1.32 ± 1.7 (n = 16)
	Zn	mg/kg	1.42 ± 1.26 (n = 66)	9.96 ± 22.59 (n = 16)
	H_2O_2	mU/ml/mg protein	2.15E-02 ± 2.56E-02 (n = 108)	2.77E-02 ± 3.45E-02 (n = 48)
Crabs	Al	mg/kg	30.62 ± 61.59 (n = 127)	40.93 ± 58.81 (n = 12)
	Cu	mg/kg	13.95 ± 26.3 (n = 127)	35.83 ± 85.1 (n = 12)
	V	mg/kg	0.06 ± 0.09 (n = 127)	0.07 ± 0.04 (n = 12)
	Zn	mg/kg	0.05 ± 0.1 (n = 127)	145.95 ± 310 (n = 12)
	SOD	Units/mg protein	4.70E-04 ± 1.41E-03 (n = 216)	1.63E-03 ± 2.13E-03 (n = 144)
	TBARS	µM/mg protein	1.37E-02 ± 2.65E-02 (n = 216)	1.29E-01 ± 1.29E-01 (n = 96)
	Protein carbonyl	nmol/mg protein	3.90E+00 ± 1.65DE+00 (n = 648)	3.01E+00 ± 1.61E+00 (n = 432)
Fish	Al	mg/kg	4.68 ± 4.74 (n = 126)	14.08 ± 26.89 (n = 18)
	Cu	mg/kg	0.42 ± 0.57 (n = 126)	2.61 ± 4.19 (n = 18)
	V	mg/kg	0.04 ± 0.04 (n = 126)	0.17 ± 0.09 (n = 18)
	Zn	mg/kg	11.89 ± 4.66 (n = 126)	17.32 ± 10.63 (n = 18)
	SOD	Units/mg protein	5.48E-03 ± 4.34E-03 (n = 216)	8.85E-04 ± 6.81E-04 (n = 108)
	TBARS	µM/mg protein	-5.58E-02 ± -1.04E-01 (n = 216)	3.00E-02 ± 1.70E-02 (n = 72)
	Protein carbonyl	nmol/mg protein	2.22E+00 ± 1.98E+00 (n = 648)	-3.63E-02 ± 8.96E-01 (n = 324)

Table S 4-2: The regression coefficients for the various log-linear (log:log scale) relationship analyses conducted (BAF = Bioaccumulation factor; MR = Mokolo River; OR = Olifants River). Significant *p*-values (<0.05) are indicated with bold font.

Metal	River	BAF Algae vs. Water					BAF Crabs vs. Water					BAF Fish vs. Water				
		Slope	Intercept	R^2	n	*p*-Value	Slope	Intercept	R^2	n	*p*-Value	Slope	Intercept	R^2	n	*p*-Value
Al	MR	-0.750	5.984	0.283	59	**< 0.0001**	-0.807	4.329	0.371	108	**< 0.0001**	-1.792	4.210	0.449	106	**< 0.0001**
	OR	-1.344	6.028	0.939	12	**0.0215**	-1.036	4.518	0.573	9	**0.0003**	-1.961	4.728	0.846	17	**< 0.0001**
Cu	MR	-6.325	4.176	0.650	43	**< 0.0001**	-2.843	4.888	0.729	78	**< 0.0001**	-2.033	3.215	0.495	75	**< 0.0001**
	OR	2.663	1.817	0.794	16	0.5907	-0.668	4.046	0.684	9	**0.0119**	-1.563	3.901	0.205	18	**0.0089**
V	MR	-2.916	3.924	0.016	37	0.1864	-17.910	3.533	0.527	72	**0.0002**	-3.840	2.835	0.032	62	0.8267
	OR	-0.571	3.189	0.064	15	0.1124	-2.112	2.482	0.707	10	0.0879	-2.192	2.942	0.767	15	**< 0.0001**
Zn	MR	-1.121	3.390	0.522	52	**< 0.0001**	-1.567	2.070	0.879	100	**< 0.0001**	-1.366	4.361	0.958	77	**< 0.0001**
	OR	-0.218	3.119	0.016	16	**0.0072**	-1.235	5.111	0.996	10	**< 0.0001**	-0.436	3.585	0.303	18	**0.0355**
Metal	**River**	**Water vs. Sediment**					**BAF Crab vs. Sediment**					**BAF Fish vs. Sediment**				
		Slope	**Intercept**	R^2	**n**	***p*-Value**	**Slope**	**Intercept**	R^2	**n**	***p*-Value**	**Slope**	**Intercept**	R^2	**n**	***p*-Value**
Al	MR	0.743	-3.615	0.054	105	0.488	-0.713	2.634	0.053	125	**< 0.0001**	-1.978	9.647	0.246	126	**< 0.0001**
	OR	-0.056	1.532	0.002	20	0.701	-0.648	2.280	0.073	12	**0.0474**	-0.944	3.403	0.705	17	**< 0.0001**
Cu	MR	0.005	0.242	0.000	80	0.954	-0.946	3.844	0.737	112	**< 0.0001**	-1.000	2.559	0.780	108	**< 0.0001**
	OR	0.068	0.327	0.015	20	0.540	-0.534	2.187	0.435	9	**0.0205**	-0.203	0.533	0.059	18	**0.0001**
V	MR	0.204	-6836.000	0.094	76	0.266	-0.942	1.467	0.130	117	**< 0.0001**	-1.784	4.476	0.355	123	**< 0.0001**
	OR	-0.094	0.858	0.026	20	0.945	-1.186	2.612	0.710	12	**0.0062**	-1.836	5.423	0.605	18	**0.0002**
Zn	MR	0.296	0.101	0.022	101	**0.018**	-0.915	1.291	0.685	124	**< 0.0001**	-1.198	4.714	0.740	123	**< 0.0001**
	OR	0.175	0.691	0.025	20	0.298	-0.958	4.678	0.972	10	**< 0.0001**	-0.419	1.812	0.192	18	0.0596

CHAPTER 5: AN EVALUATION OF THE USE OF ECOTOXICOGENOMICAL ENDPOINTS, USING THE FRESHWATER CRAB, *POTAMONAUTES WARRENI*

This research chapter has been provisionally accepted for publication in an ISI accredited peer review journal.

Ecological Indicators

Declaration by the candidate

With regard to Chapter 5, the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution
Conceptual design, fieldwork, experimental work, manuscript writing.	75%

The following co-authors have contributed to Chapter 5:

Name	Email address and institutional affiliation	Nature of contribution	Extent of contribution
Prof A-M Botha	ambo@sun.ac.za Department of Genetics, Stellenbosch University	Conceptual design, experimental work, manuscript editing.	25%
Prof JH van Wyk	jhvw@sun.ac.za Department of Botany and Zoology, Stellenbosch University	Conceptual design, manuscript editing.	
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5.1. ABSTRACT

Although traditional chemical monitoring provides useful information regarding the toxicity and water quality of natural water resources, these provide little information with regard to the complex interaction between pollutants and aquatic biota and ultimately the resulting impacts on ecosystem health. This is one of the important challenges that aquatic ecotoxicologists face, as it is difficult to understand the state of a specific aquatic ecosystem and the biota inhabiting it, without biological assessment information. Few studies have the opportunity to be able to comparatively assess the current and potential future impacts of coal mining through the use of two different river systems impacted by similar land use activities, yet different degrees of coal mining. Such information is vital for improved water resource management and sustainable development. The toxicity of the surface water in both the upper Olifants River (impacted) and Mokolo River (less impacted) was assessed. Various toxicogenomical endpoints were assessed using a fresh water crab (*Potamonautes warreni*) as model organism. These endpoints included antioxidative enzyme activity, protein measurements and gene expression through RT-qPCR analysis. From the various endpoints used, the use of p53, advanced oxidation protein products and protein content proved to be useful in indicating the chronic impacts associated with the upper Olifants River. The results obtained from the use of *Potamonautes warreni* also indicated that this freshwater decapod can be successfully used to identify both acute and chronic aquatic ecosystem impacts. This information can assist in substantially improving the management and sustainable development of the Mokolo River in the near future by screening for these selected biomarkers as early warning indicators.

5.2. INTRODUCTION

Fresh water is needed to sustain life and is crucial for social and economic development (DWAF, 2004), however, South Africa ranks amongst the 30 most water scarce countries in the world (Aphane and Vermeulen, 2015). This water-stressed situation is further complicated through the extensive pollution of many of its limited water resources (CSIR, 2010) through land use activities that include mining operations, agricultural practices, industrial activities, urbanization and wastewater treatment works (DWAF, 2004; DEAT, 2006; CSIR, 2010). These activities have the potential to cause impacts on water resources through either point- or non-point pollution. The Olifants River is recognized as one of the most impacted rivers in South Africa (Grobler et al., 1994; Oberholster et al., 2013b) and has been subjected to large-scale coal mining for many decades (Driescher, 2007; McCartney and Arranz, 2007; Hobbs et al., 2008). This has resulted in large amounts of acid mine drainange (AMD) being discharged as sulfide-bearing material is exposed to oxygen and water (Akcil and Koldas, 2006). In the upper Olifants River catchment the Witbank, Highveld and Ermelo coalfields produce ≈81% of South Africa's coal needs that support ≈48% of the country's total power generation. This catchment thus represents the largest active coal mining area in the country (Tshwete et al., 2006; DMR, 2009). It is therefore no surprise that the Olifants River has seen recent (2008 – 2009) ecological tragedies such as the mass mortalities of, inter alia, fish, birds and crocodiles (Driescher, 2007; Myburgh and Botha, 2009; Ashton, 2010; Ferreira and Pienaar, 2011).

South Africa has roughly the ninth largest coal reserves in the world and as the Witbank / Middelburg coalfields are nearing depletion, plans exist to start the exploitation of the near virgin coalfields in the Waterberg region, estimated to represent ≈40% of South Africa's remaining coal reserves (Aphane and Vermeulen, 2015). The Mokolo River catchment, one of the larger and most important tributaries in the Waterberg (Jacobsen and Kleynhans, 1993; Schachtschneider and Reinecke, 2014), is therefore expected to face the greatest risk from current and planned activities in the area as it has larger quantities of surface water available (DWAF, 2003; DWA, 2012; Tshikolomo et al., 2013). Not surprisingly, the Mokolo River has recently been classified as an endangered system (LEDET, 2012). In light of the largely unexploited coal reserves, it is anticipated that an increase in mining and ancillary activities will affect both water quantity and quality in the area (Bester and Vermeulen, 2010). With the upper Olifants River catchment being an active coal mining area for more than a century, with little to no regulation at times (McCartney and Arranz, 2007), a relatively rare and unique scenario has presented itself to study these long-term effects so as to potentially improve management decisions in future mining areas.

Ecotoxicogenomics, defined as the assessment of changes in gene and protein expressions, associated with exposure to pollutants (Jha, 2004; Snape et al., 2004), allows for an improved understanding of the sub-lethal impacts of pollutants (Poynton et al., 2007; Oberholster et al., 2016). If the link between pollutant exposure and biological response is not understood, it becomes a difficult task to bridge the gap in predicting responses to pollutants in aquatic ecosystems, the knock-on effects from one trophic level to another, as well as the influences of duration on exposure impacts (Jha, 2004; Kim et al., 2010). These approaches are hugely beneficial when dealing with a complex mixture of pollutants from different land use activities, as this is usually the situation observed in natural water resources occurring in a multi-use environment (Oberholster et al., 2016). This type of information could assist in developing early warning systems and in so doing enhance integrated water resource management systems and sustainable development, as well as reduce environmental risks (Snell et al., 2003; Moore et al., 2004).

Given the long-term impact of coal mines on the environment, the upper Olifants River allows for obtaining information on multiple variables associated with long-term effects of coal mining on water resources. This study, therefore, aims to assess biochemical and molecular endpoints so as to evaluate the genotoxicological impacts of the surface water on the biota in both catchments. Through this, we hope to contribute to the understanding of the controlling factors that regulate a multitude of variables in an impacted environment (i.e., the upper Olifants River) so as to improve the sustainable development of water resources in less impacted areas facing an uncertain future (i.e., the Mokolo River). Few studies have the opportunity to be able to conduct such a comparative study to anticipate the likely impacts of future land use changes by learning from a relevant (but impacted) example. This type of information is essential, because both the rivers are of international importance and the information from this study can therefore influence integrated water resource management.

5.3. MATERIALS AND METHODS

5.3.1. Study Area and Sampling Design

The study areas selected for this study, namely the upper Olifants and Mokolo rivers, have been proven to be comparable (De Klerk et al., 2016) and the different sites in each area are presented in Figure 5-1 and described in Table 5-1. This study was designed to conduct seasonal sampling over multiple years within both the Mokolo River and the upper Olifants River. This was to ensure that representative samples were obtained under various hydrological extremes. Study sites were selected to represent the different variations within each catchment (namely type of impact, degree of impact, habitat composition, etc.) based on available spatial information and an initial survey of each river system (Table 5-1) so as to obtain a realistic overall representation.

Table 5-1: The Global Positioning System (GPS) coordinates of the different sites selected in the upper Olifants River (OR) and Mokolo River (MR) with a short description of each site.

Sites	Latitude	Longitude	Description and Land Use Activities
OR 1	26.222566°	29.462332°	Mainly surrounded and impacted upon by agriculture.
OR 2	25.841445°	29.266414°	Located in an urban area and mostly impacted by untreated and partially treated sewage effluent.
OR 3	25.701216°	29.29821°	Located on a private game farm. Mainly receives the cumulative effect from activities further upstream.
OR 4	25.623028°	29.21623°	Located in a reserve. Mainly receives the cumulative effect from activities further upstream.
MR 1	28.092386°	24.289449°	Located upstream of the town of Vaalwater and downstream of agricultural activities.
MR 2	28.095493°	24.286274°	Located downstream of the town of Vaalwater, as well as a sewage stabilisation pond that periodically overflows.
MR 3	27.802295°	24.113663°	Located further downstream below a massive conglomeration of agricultural activities.
MR 4	27.726264°	23.970681°	Directly downstream of the Mokolo Dam on a privately owned game farm.
MR 5	27.744158°	23.687369°	Located upstream of the town of Lephalale.
MR 6	27.759659°	23.652237°	Located downstream of the town of Lephalale.

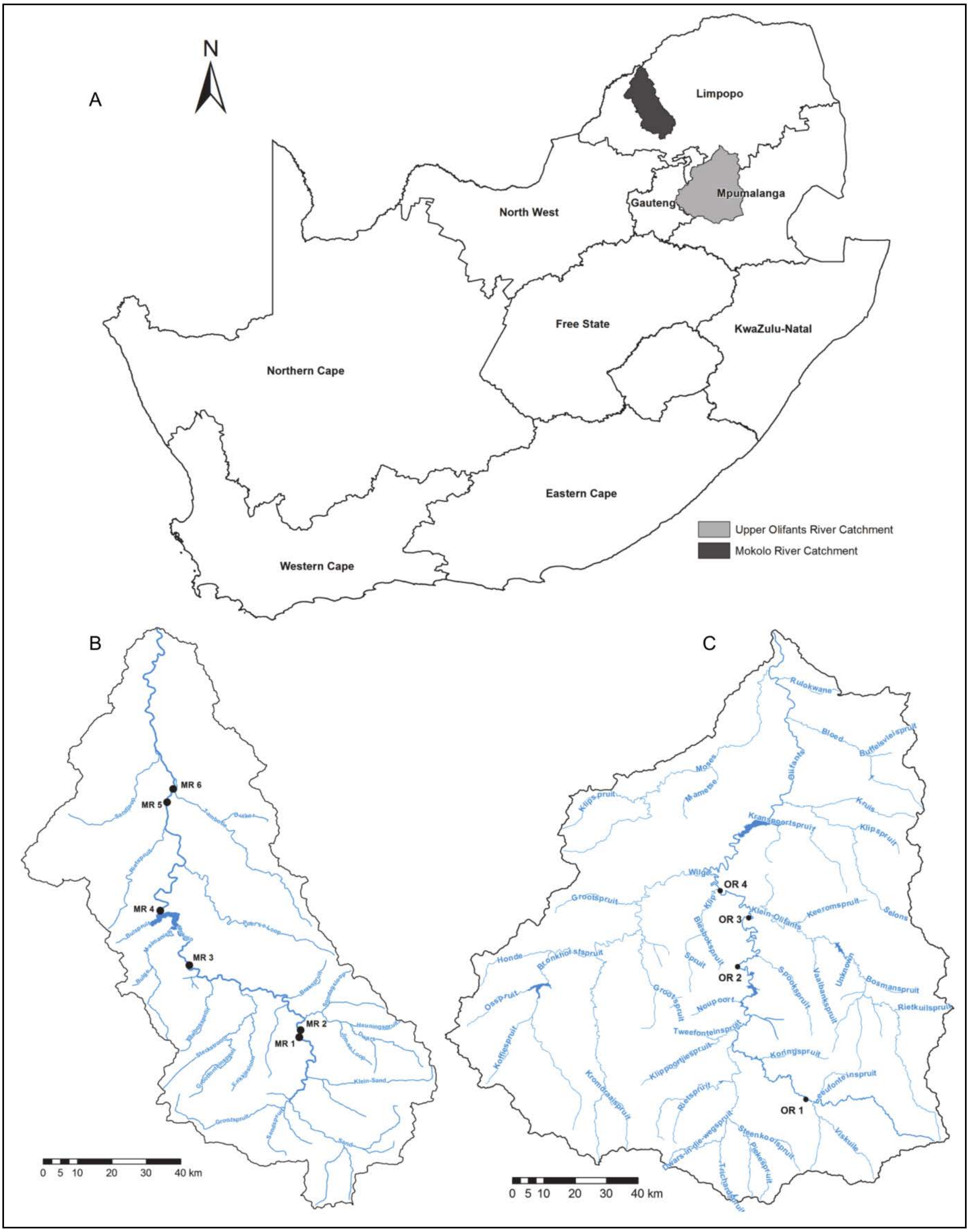

Figure 5-1: The location of the two study areas in South Africa is presented in (A), with the detailed location of the different study sites in the Mokolo River indicated in (B) and the upper Olifants River in (C).

5.3.2. Water Quality

Using a Thermo 5 Star pH/RDO/Conductivity meter set (Thermo scientific, USA), *in situ* water quality parameters such as temperature, pH and electrical conductivity were measured at each site during each field visit. Surface water samples (1 L each) were collected at each site during each field visit and kept at 4°C with no exposure to sunlight until analysis could be performed. Upon returning from each field visit, the water samples were analysed for various constituents according to APHA, AWWA and WEF (2012). The method of Sartory and Grobbelaar (1984) was used to determine the chlorophyll *a* content. Quality control was conducted by comparing the sum of the measured anion and cation concentrations to the total dissolved salts (TDS) values. The quality of the analysis was also checked by using the ionic balance method of Appelo and Postma (2005). In all cases only a variation of less than 5% was regarded as acceptable.

5.3.3. Potamonautes warreni

Freshwater crabs (*Potamonautes warreni*) were sampled at all sites during all sampling trips in both river systems using baited crab traps. A minimum of three specimens per site were used per seasonal sampling trip for each of the rivers, although this minimum was often exceeded. Approximately 100 g of muscle tissue, as well as the entire hepatopancreas, were collected and snap frozen in liquid nitrogen. On return to the laboratory, the samples were stored at -80°C until analyses were conducted. Although no significant correlation relating to changes in size and gender in *P. warreni* specimens were reported (Kotze et al., 1999; Sanders et al., 1999; Soucek et al., 2002), each crab was weighed, sexed and measured prior to dissection. To ensure that the variability is distributed evenly, specimens of different sizes, masses and genders that were all in a similar stage of moulting were randomly selected (Sanders et al., 1998). Crab specimens were captured, handled and used in an ethical manner. The South African National Standard for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008), ANZCCART (2001), Gardner (1997), Hajek et al. (2009) and Yue (2009) guidelines were used as reference to ensure compliance. The authors affirm that all sampling and analyses were conducted in such a manner that it adheres to the Animals Protection Act (Act 71 of 1962) of South Africa.

5.3.4. Oxidative Stress Indicators

Oxidative stress was assessed through the analysis of selected reactive oxygen species (ROS) using the muscle and hepatopancreas tissue of *P. warreni*. The total peroxidation activity in *P. warreni* was measured using the guaiacol test (George, 1953) and expressed as µmol tetraguaiacol.min^{-1}.mg^{-1} protein. Total glutathione (GSSG/GSH) and advanced oxidation protein products (AOPP) were measured using the relevant OxiSelect^{TM} kits from Cell Biolabs Incorporated (STA-312 and STA-318, respectively) and analysed according to the manufacturer's protocols. Total glutathione and AOPP activity were expressed as µM.mg^{-1} protein.

The tissue samples (muscle and hepatopancreas) were homogenized in phosphate saline buffer (pH 7.5) at 4°C, where after samples were centrifuged at 4°C for 15 minutes. The resultant supernatant was removed and stored on ice for enzymatic analysis. Both total peroxidation and AOPP were measured in the muscle tissue, whilst GSSG/GSH was measured in the hepatopancreas tissue. The analyses, including all preparations, were conducted at 4°C. Both biological (n > 3) and technical (n > 3) repeats were conducted from each seasonal sampling campaign (n > 9). Enzyme activity was expressed relative to the amount of tissue analysed (mg protein).

5.3.5. Protein Determinations

As an additional line of evidence to elucidate the anthropogenic impact on *P. warreni*, an assessment was done on the total protein content of the muscle and hepatopancreas tissues according to Walters et al. (2016). This was carried out using the Bradford method (Bradford, 1976) using bovine serum albumin (Bio-Rad Laboratories, USA) as standard.

5.3.6. Genotoxicological Assessment

5.3.6.1. ***Total RNA Isolation and cDNA Synthesis***

Muscle tissue from the *P. warreni* specimens was used to isolate total RNA. Total RNA was isolated using Qiashredder columns, as well as the RNeasy mini kit according to the manufacturer’s protocols (Qiagen). The quality of the extracted RNA was evaluated using agarose gel electrophoresis and Nanodrop analysis (Thermo Scientific, USA). Afterwards, each RNA sample was DNase I (Sigma, ZA) treated. Complimentary DNA (cDNA) synthesis (single-stranded) was conducted on all RNA samples using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's protocol.

5.3.6.2. ***Gene Expression***

The relative expression of messenger RNA (mRNA) of isocitrate dehydrogenase 1 (IDH-1), catalase (CAT) and tumor protein p53 (p53) was assessed using real-time RT-qPCR with 18S-RNA as reference gene. The sequences of the of primer pairs for IDH-1, CAT and p53 genes are listed in Table 5-2. All three these target genes were selected due to their roles in various pathways, for example glucose metabolism (e.g., IDH-1) and apoptosis (e.g., p53), as well as their ability to indicate anthropogenic impacts (e.g., CAT) that can be associated with coal mining related pollutants such as metal pollution (Campisi et al., 2001; Torres et al., 2002; Dimitrov et al., 2015).

Table 5-2: Primer sequences in the 5' to 3' direction selected for RT-PCR analysis.

Target	Primer Sequence	Tm (°C)	RT-qPCR Efficiency (%)
18S rDNA	F: ATTGGAGGGCAAGTCTGGTG R: TGCGCCTGAGATCCAACTAC	57.2	99.5
Isocitrate dehydrogenase 1 (IDH-1)	F: GGAATGTGAAGATTGAGGC R: AATGATGCCCTTGAAGATA	49.7	97.4
Tumor protein p53	F: CGTAGCGATTCTGACGATGC R: TGTATGCGCCAGCTATCCTG	56.5	94.9
Catalase (CAT)	F: CAACACTCCCATCTTCTTCATCAGG R: TGTTGTTTCTGGACGCAGGGTGAT	59.1	101.8

5.3.6.3. *RT-qPCR Analysis*

The PCR reactions were conducted using 20 µl reactions consisting of 3 µl cDNA template (30 ng cDNA per reaction), 10 µl Luminaris Color Hi Green qPCR Master Mix (Thermo Scientific), 0.6 µl of each primer and nuclease-free water. A standard PCR program for all primers was used consisting of an enzyme activation step (95°C; 10 minutes), denaturing = 40 cycles (95°C; 15 seconds), annealing (57.5°C; 30 seconds) and elongation (72°C; 30 seconds). An internal non-template control (cDNA = 0 ng) and a five-point two-fold serial dilution of cDNA, transcribed from the RNA from a *P. warreni* specimen from a reference location, were included in every PCR plate. All samples and controls were conducted in triplicate (n = 3) and melting curve analysis was used to assess the amplicon quality. The Pfaffl method (Pfaffl, 2001) was used to quantify the gene expression compared to the 18S-RNA normalizer and *P. warreni* specimens from a reference location. Each primer pair during each PCR programme was assessed to determine the amplification efficiencies. The Grubbs' test (Burns et al., 2005) was used to identify outliers per treatment group that were subsequently removed.

5.3.7. Statistical Analysis

The various sets of data (including the biomarker data) were checked for normality using the Kolmogorov-Smirnov test, the Shapiro-Wilk W test, as well as the Lilliefors test. Homogeneity of variance was evaluated using Levene's test and Brown & Forsythe's test. An Analysis of Variance (ANOVA) test was conducted to determine differences between the different data sets where applicable (parametric). This included using Tukey's unequal N Spjotoll-Stoline corrected HSD post-hoc test. Non-parametric data were analysed using Kruskal-Wallis ANOVA in combination with a multiple comparison of mean ranks post-hoc test. In order to conform to the assumptions of an ANOVA, the gene expression data were firstly rank transformed after which analysis was conducted using the previously mentioned tests. The statistical significance of results was established using a probability value of $p < 0.05$. All statistical analyses were performed using Statistica 12 (Statsoft Inc., USA), whilst the multivariate statistical analysis, namely the Principal Component Analysis (PCA), was conducted using CANOCO version 5.1 (Microcomputer Power, USA). During the PCA analysis all the data, including the oxidative stress biomarker data, were centered and standardized using the built-in treatment option of CANOCO version 5.1 (Microcomputer Power, USA).

5.4. RESULTS

5.4.1. Water Quality

Noticeable differences were observed between the Mokolo River and upper Olifants River with regard to the chemical parameters analysed in the surface water of both river systems (Table 5-3). In terms of the nutrient enrichment status the selected parameters (NH_4^+, NO_3^- +NO_2^-, TKN and PO_4^{3-}) were relatively similar at the various sites in the two river systems, with certain sites having increased concentrations, for example PO_4^{3-} and NH_4^+ at OR 2. This trend is supported by the concentration of suspended chlorophyll *a*, with MR 1, MR 2, OR 1 and OR 2 having relatively higher concentrations (9.44, 9.18, 8.32 and 5.74 µg/l, respectively) than the other sites in the two rivers. The metal pollution levels in these two systems differed, with significantly higher levels of dissolved Cu and V (≈2.9 and ≈1.88 µg/l, respectively) in the upper Olifants River, whilst the Mokolo River had higher concentrations of Fe and Mn (≈43.8 and ≈47.48 µg/l, respectively).

The sites from the upper Olifants River generally had significantly ($H_{1,98}$ = 43.02; p < 0.01) higher alkalinities (≈93.2 mg/l) than the Mokolo River (≈25.4 mg/l), as well as significantly ($F_{1,96}$ = 64.86; p < 0.01) higher *in situ* pH levels (≈8.02 and ≈7.14, respectively). Significantly ($H_{1,98}$ = 44.64; p < 0.01) higher electrical conductivity values were measured in the upper Olifants River (≈600.1 µS/cm) compared to the Mokolo River (≈94.78 µS/cm). This corresponds to the significantly (p < 0.01) higher concentrations of Na, K, Ca, Mg^{2+}, Cl^- and SO_4^{2-} measured in the water samples of the upper Olifants River. Relative to the Mokolo River, significantly ($H_{1,91}$ = 22.93; p < 0.01) higher levels of TSS (≈7.12 and ≈23.91 mg/l, respectively) as well as significantly ($H_{1,98}$ = 38.35; p < 0.01) higher dissolved organic carbon (DOC) (≈3.17 and ≈6.79 mg/l, respectively) were recorded in the upper Olifants River. The chemical oxygen demand (COD) levels measured in both rivers were significantly ($H_{1,98}$ = 13.80; p < 0.01) higher at most of the upper Olifants River sites (≈34.29 mg/l) than those of the Mokolo River (≈14.38 mg/l), thus supporting the trends of the various chemical pollutants seen in this study.

Table 5-3: The mean chemical concentrations of the selected parameters from the various sites in the Mokolo River (MR) and upper Olifants River (OR).

Variable	Unit	OR 1	OR 2	OR 3	OR 4	MR 1	MR 2	MR 3	MR 4	MR 5	MR 6
Alkalinity	as $CaCO_3$ mg/l	122.35	112.43	72.01	66.01	30.50	34.96	35.71	17.37	15.65	18.19
Aluminium	µg/l	12.87	10.17	23.10	6.46	13.71	14.55	11.98	5.87	19.27	21.05
Ammonium (NH_4^+)	mg/l	0.08	6.56	0.16	0.13	0.07	0.10	0.13	0.14	0.07	0.06
Arsenic	µg/l	0.50	0.72	0.53	0.45	0.24	0.29	0.25	0.20	0.16	0.19
Cadmium (Cd)	µg/l	0.12	0.09	0.10	0.10	0.20	0.20	0.21	0.26	0.36	0.37
Calcium (Ca)	mg/l	36.29	31.69	35.16	29.66	4.84	5.17	5.95	2.50	2.65	4.14
Chemical Oxygen Demand (COD)	mg/l	12.47	57.04	33.72	33.93	12.90	13.37	17.49	13.99	13.85	14.69
Chloride (Cl^-)	mg/l	20.77	26.56	17.40	18.62	7.56	9.56	13.18	5.11	8.60	12.44
Chlorophyll *a*	µg/l	8.32	5.74	2.21	12.18	9.44	9.18	3.04	2.10	2.45	4.95
Copper (Cu)	µg/l	2.25	4.09	2.60	2.66	0.68	0.86	0.77	0.46	1.23	0.79
Dissolved Organic Carbon (DOC)	mg/l	5.81	9.22	6.20	5.92	3.40	3.77	4.17	2.58	2.47	2.61
Dissolved Oxygen	%	100.56	68.80	141.72	104.96	100.91	103.41	93.75	101.77	105.80	105.05
Electrical Conductivity	µS/cm	743.80	619.80	534.00	502.60	85.69	101.42	174.18	53.57	70.90	82.92
Iron (Fe)	µg/l	11.65	22.91	14.81	13.84	65.95	75.37	39.73	17.60	29.85	34.31
Magnesium (Mg^{2+})	mg/l	27.39	19.51	26.44	22.17	2.96	3.18	7.44	1.71	1.89	2.95
Manganese (Mn)	µg/l	4.20	37.89	18.73	2.40	19.20	62.65	23.87	7.27	123.80	48.08
Nickel (Ni)	µg/l	2.02	3.35	4.16	2.74	1.38	1.58	1.56	0.90	1.88	1.40
Nitrate+Nitrite (NO_3^-+NO_2^-)	mg/l	0.04	0.93	0.66	2.44	0.17	0.14	0.25	0.05	0.02	0.02
Ortho-Phosphate (PO_4^{3-})	mg/l	0.06	1.40	0.07	0.30	0.03	0.03	0.03	0.04	0.02	0.03
pH	-log[H^+]	8.16	7.47	8.62	7.81	6.92	7.17	7.20	7.10	7.23	7.20
Potassium (K)	mg/l	4.43	8.09	5.26	6.75	3.74	4.01	2.13	0.83	0.96	1.48
Selenium (Se)	µg/l	0.37	0.37	0.44	0.49	0.24	0.29	0.26	0.25	0.43	0.37
Sodium (Na)	mg/l	37.65	36.41	23.91	23.62	7.36	9.76	13.97	4.42	6.40	9.13
Sulfate (SO_4^{2-})	mg/l	133.14	101.91	145.65	113.95	2.59	2.87	24.15	1.85	2.77	3.78
Total Kjeldahl Nitrogen (TKN)	mg/l	0.76	8.97	1.13	0.95	0.61	0.73	0.76	0.51	0.53	0.50
Total Suspended Solids (TSS)	mg/l	42.14	25.92	15.09	12.48	8.14	9.64	8.69	4.06	5.40	6.81
Vanadium (V)	µg/l	2.47	1.36	1.78	1.90	0.16	0.18	0.18	0.11	0.14	0.16
Zinc (Zn)	µg/l	19.76	29.81	31.25	22.81	14.80	18.50	10.28	13.07	23.05	12.73

5.4.2. Oxidative Stress

Differences in the degree of oxidative stress were evident between the *P. warreni* specimens from the upper Olifants River compared to the Mokolo River (Figure 5-2). Total glutathione levels measured in the hepatopancreas samples were generally higher in the *P. warreni* samples obtained from the Mokolo River (≈0.72 µM.mg^{-1} protein), but statistically significant differences ($F_{9,70}$ = 3.35; p = 0.001) were only found between site MR 5 with the highest total glutathione levels (0.98 µM.mg^{-1} protein) and OR 2 and OR 3 in the upper Olifants River (0.21 and 0.29 µM.mg^{-1} protein, respectively), where the lowest levels were recorded.

In the muscle tissue samples, peroxidase activity varied significantly ($F_{9,110}$ = 15.32; p < 0.01) between most of the Mokolo River sites and the sites in the upper Olifants River, indicating increased concentrations in the Mokolo River (≈1.84 x 10^{-5} µmol tetraguaiacol.min^{-1}.mg^{-1} protein). The concentrations of AOPP varied significantly ($F_{9,422}$ = 10.65; p < 0.01) among most sites with the AOPP levels being less upregulated at three of the Mokolo River sites (MR 1, MR 2 and MR 4). All of the sites in the upper Olifants River had increased (positive) AOPP levels (≈0.48 µM.mg^{-1} protein).

5.4.3. Total Protein Assessments

The total protein assessment (wet mass corrected) indicated significantly ($H_{9,1080}$ = 566.59; p < 0.01) lower muscle protein levels in the *P. warreni* specimens from the upper Olifants River (Figure 5-3). Protein levels varied from ≈240.48 µg.ml^{-1} in the Mokolo River to ≈133.97 µg.ml^{-1} in the upper Olifants River. The same trend was observed with regard to the protein levels measured in the hepatopancreas samples of *P. warreni* in both rivers. The hepatopancreas protein levels of *P. warreni* samples from the upper Olifants River (≈31.87 µg.ml^{-1}) differed significantly ($H_{9,648}$ = 543.96; p < 0.01) from the concentrations measured in the specimens from the Mokolo River (≈140.27 µg.ml^{-1}).

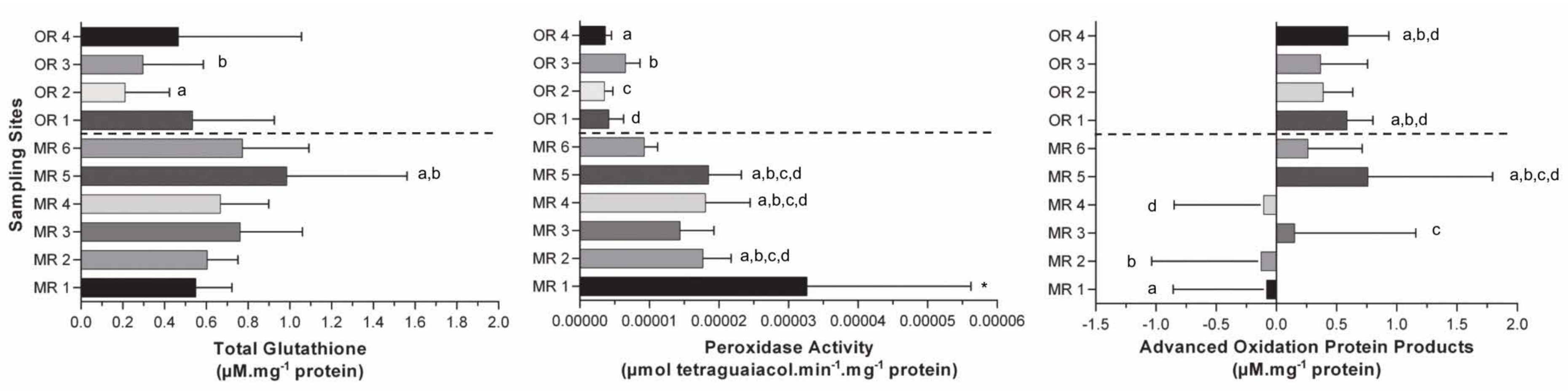

Figure 5-2: Oxidative stress indicators in the natural populations of the freshwater crab, *Potamonautes warreni*. The mean (± standard deviation) levels of total glutathione, peroxidase activity and advanced oxidation protein products measured from the different sites in the Mokolo River (MR) and upper Olifants River (OR) are presented. Similar alphabetic letters show where statistically significant differences (p < 0.05) were found (this does not apply to sites with multiple superscripts). The * denotes a significant difference to all other sites.

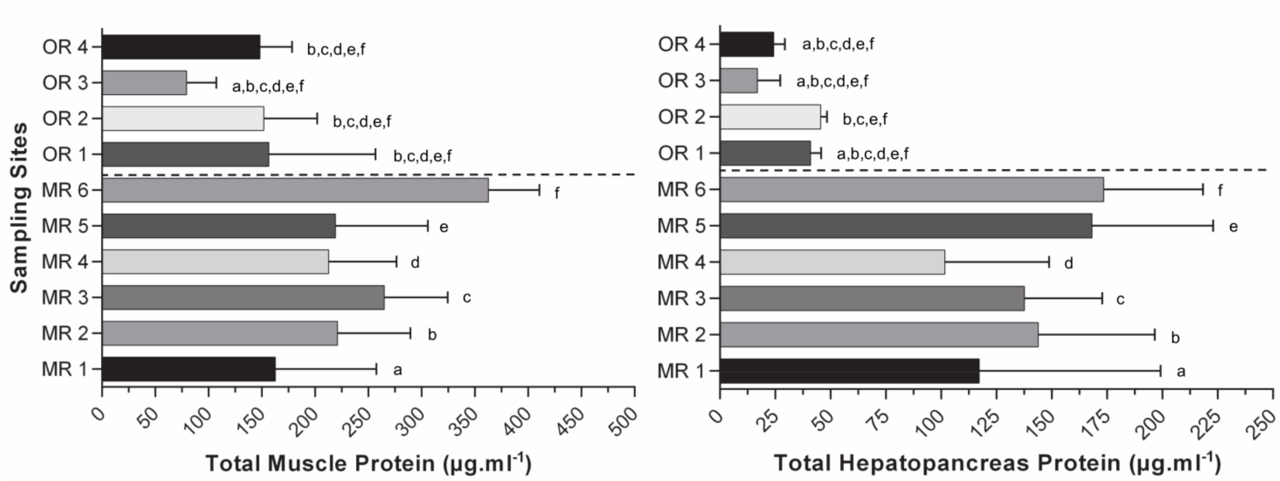

Figure 5-3: The mean (± standard deviation) total protein concentrations in the muscle and hepatopancreas tissue of natural populations of *Potamonautes warreni* obtained from the selected sites in the Mokolo River (MR) and upper Olifants River (OR). Concentrations were corrected for differences in wet tissue mass. Similar alphabetic letters signify where statistically significant differences (p < 0.05) were found (this does not apply to sites with multiple superscripts).

5.4.4. Gene Expression

Through the evaluation of the relative mRNA expression of the selected genes (Figure 5-4) it was recorded that the expression of p53 was not upregulated at any of the Mokolo River sites compared to the control. However, a significant upregulation ($F_{9,80} = 17.34$; $p < 0.01$) was found in the *P. warreni* specimens from the upper Olifants River relative to the Mokolo River samples. Isocitrate dehydrogenase 1 (IDH-1) expression was also recorded to significantly differ between the two river systems ($F_{9,56} = 12.38$; $p < 0.01$) with *P. warreni* specimens from the Mokolo River being upregulated instead of the upper Olifants River (except for OR 2). The *P. warreni* samples from the Mokolo River also had upregulated CAT expression at four of the six sites, which differed significantly ($F_{9,51} = 8.23$; $p < 0.01$) from the *P. warreni* samples of the upper Olifants River where no upregulation was found (except for OR 2).

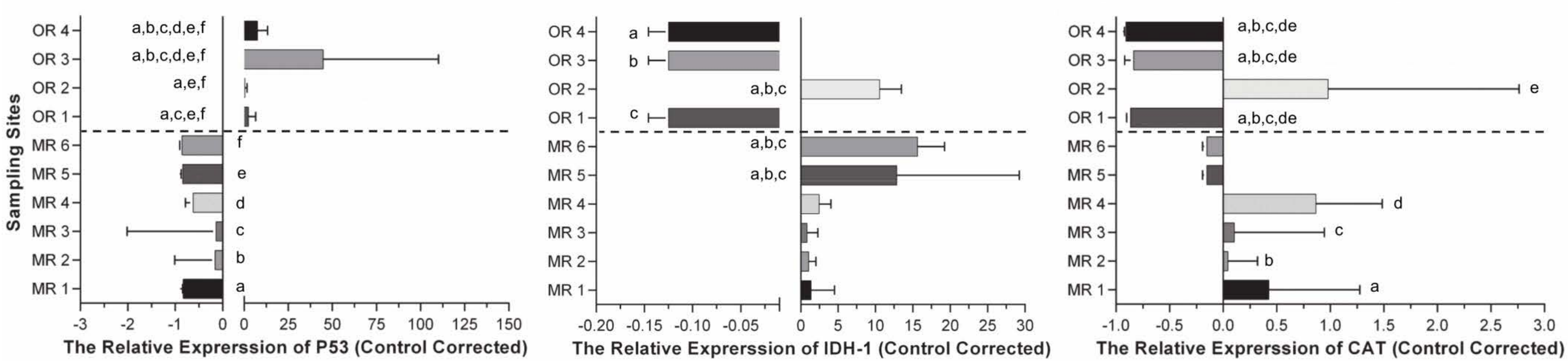

Figure 5-4: Differential gene expression: The mean (± standard deviation) relative expression of tumor protein (p53), isocitrate dehydrogenase (IDH-1) and catalase (CAT) in natural populations of *Potamonautes warreni* obtained from the different sites in the Mokolo River (MR) and upper Olifants River (OR). Similar alphabetic letters signify where statistically significant differences ($p < 0.05$) were found (this does not apply to sites with multiple superscripts).

5.4.5. Multivariate Integration

It was observed that there is a distinct difference (in groupings) between the upper Olifants River and the Mokolo River (Figure 5-5). Most of the chemical determinants were strongly correlated with the sites in the upper Olifants River where OR 1, OR 3 and OR 4 grouped together (red encirclement) and OR 2 grouped separately. It was observed that OR 1, OR 3 and OR 4 were strongly associated with AOPP and p53, whilst OR 2 was associated with NH_4^+, TKN and PO_4^{3-}.

On the other hand, all six sites in the Mokolo River grouped together (green encirclement) and were strongly correlated with an increase in the biological parameters, namely CAT, IDH-1, muscle protein, hepatopancreas protein, peroxidase activity and total glutathione. The main chemical determinants associated with the Mokolo River sites were Fe, Mn and Cd.

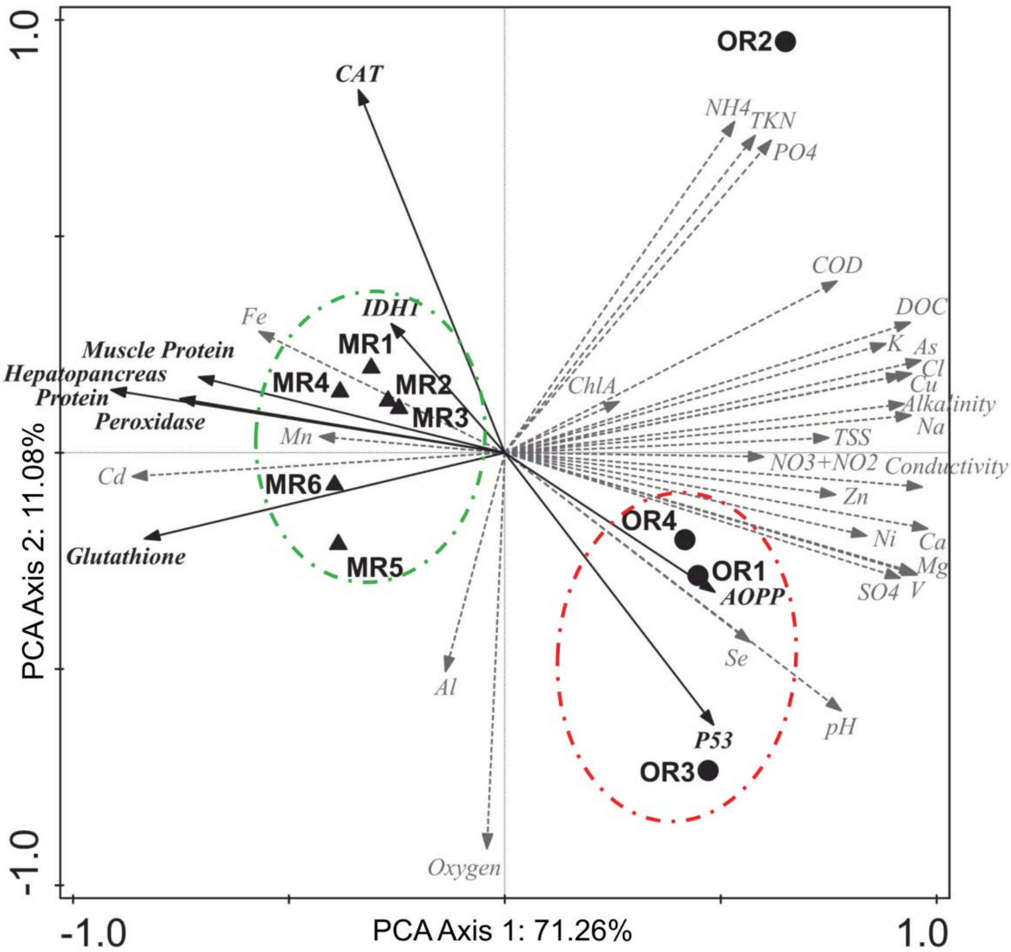

Figure 5-5: A Principal Component Analysis (PCA) bi-plot indicating the association between the respective sites in the Mokolo River (MR) and upper Olifants River (OR) with regard to changes in the water quality parameters and biological responses. The biological response data, namely enzyme activity, protein content and gene expression, were treated as supplementary variables in the analysis.

5.5. DISCUSSION

With plans to expand to the Waterberg coalfield underlying the Mokolo River (Venter, 2013), this comparative study endeavoured to add to the knowledge base generated from the upper Olifants River so as to pre-empt any possible deleterious effects in the Mokolo River. During this study it was observed that in terms of nutrient enrichment the two river systems were similarly impacted, which may be attributed to the degree of sewage and agricultural impacts currently occurring in both catchments (Ashton and Dabrowski, 2011; Seaman et al., 2013; De Klerk et al., 2016). However, the degree of mining associated pollution, such as metal pollution, is far worse in the upper Olifants River and has significant implications for the Mokolo River.

5.5.1. Water Quality

Good water quality in natural water resources is a national priority and is of critical importance, for example to ensure ecological integrity, ecosystem health, biodiversity conservation and human health (Kleynhans, 1999; Dickens and Graham, 2002; Amis et al., 2007). Due to a variety of factors, both the upper Olifants and Mokolo rivers had been differently impacted by coal mining over the last 100 years, resulting in the significant differences in water quality related to coal mining (i.e, AMD) impacts. The breakdown of sulfide minerals during AMD is known to significantly increase the electrical conductivity levels and concentrations of various minerals in receiving water bodies (Akcil and Koldas, 2006), as observed in the upper Olifants River during this study. These results are also in comparison to the findings from other studies on the upper Olifants River (Oberholster et al., 2013a; Dabrowski et al., 2015).

Since the upper Olifants and Mokolo River catchments are known to have relatively similar geology (De Klerk et al., 2016), the noted differences in concentrations of certain metals (i.e., Cu and V) recorded in the upper Olifants River may be specifically associated with AMD (DeNicola and Stapleton, 2002). Although the upper Olifants River generally had higher pH levels than the Mokolo River, the increase in alkalinity (also known as buffering capacity) may contribute to reducing the impacts of the acidic water flowing from tributaries, such as the Klipspruit, and entering the mainstem Olifants River (Dabrowski et al., 2015). The pollutants from the AMD may precipitate from the acidic water and contribute to the contamination of the Olifants River (Austin and Lee, 1973; Akcil and Koldas, 2006). In the Mokolo River, the negative impact that impoundments may have on the water quality of this river has been observed (De Klerk et al., 2016). These structures may therefore contribute to the higher concentration of dissolved metals, including Fe and Mn, in the Mokolo River, whilst agriculture may also be important (Jackson et al., 2007; Edokpayi et al., 2014). Although Fe and Mn concentrations were higher in the Mokolo River than in the upper

Olifants River, these concentrations are relatively low, falling within the South African water quality guideline limits of <0.1 mg/l (DWAF, 1996a, 1996b).

Despite the fact that nutrient levels appeared similar in both river systems, concentrations in both rivers far exceeded the target water quality guideline levels relating to NH_4^+ (<0.007 mg/l) and PO_4^{3-} (<0.005 mg/l) set by DWAF (1996a). The NO_3^-+NO_2^- guideline level (DWAF, 1996a) was only exceeded (>0.5 mg/l) at OR 2 and OR 4. Nutrient contamination in the upper Olifants River may be compounded by improper functioning or dysfunctional sewage treatment plants (Oberholster et al., 2013b), resulting in nutrient enrichment that significantly exceeds guideline limits for various N and P specific parameters (DWAF, 1996a). On the other hand, agricultural return flows contribute to the nutrient loading at a wider catchment level (Dabrowski and De Klerk, 2013). These nutrient enrichment trends were supported by the findings from the chlorophyll *a* assessment, which indirectly indicate nutrient enrichment (Paisley et al., 2003). A similar trend was observed in the Mokolo River at MR 1 and MR 2, where an increase in nutrients and chlorophyll *a* was recorded. The upper reaches of the Mokolo River may be affected by the sewage wastewater and discharges from informal settlements in the area, as well as agricultural activities (Seaman et al., 2013; De Klerk et al., 2016). This may therefore be further exacerbated by an increase in coal mining activities which may result in an increase in urbanization (i.e., sewage treatment plants), agriculture, etc. The measurement of a water resource's COD is well-known as an indicator of water quality (Morrison et al., 2001), Therefore, the COD results recorded during this study support the overall findings of a higher level of pollution in the upper Olifants River, relative to the Mokolo River, and based on the COD concentrations the upper Olifants River is classified as a class 5 in terms of maintenance of aquatic life in freshwater systems (UNECE, 1994).

5.5.2. Oxidative Stress Indicators

The production of antioxidative enzymes is one of the main detoxifying pathways in decapod crustaceans, such as *P. warreni* (Walters et al., 2016). This may lead to an increase in ROS, such as GSSG/GSH, peroxidase and AOPP (Oberholster et al., 2016). Glutathione can be induced by low levels of oxidative stress (Stein et al., 1992; Zhu et al., 2011), whilst peroxidases are essential for the enzymatic removal of stress-induced toxic oxygen species such as H_2O_2 (Velikova et al., 2000).

Although the water quality results indicated that the Mokolo River was less polluted than the upper Olifants River, the glutathione and peroxidase data indicate higher levels of stress in the Mokolo River. As mentioned, these ROS species are known for their ability to resist toxic effects caused by oxidative stress and as such act as indicators of pollutant exposure (Zhang et al., 2004). On the other hand, the over-production of ROS in response to constant exposure may in itself lead to significant oxidative damage, including a loss in compensatory mechanisms. This ultimately leads to a suppression / decrease in antioxidant enzyme activity / concentrations (Zhu et al., 2011). This may account for the decrease of both GSSG/GSH and peroxidase levels at the upper Olifants River sites (Zhu et al., 2008). Thus, the usefulness of GSSG/GSH and peroxidase to accurately reflect long-term anthropogenic impacts is doubtful. These ROS may, however, be useful to act as an early warning indicator for unpolluted / short-term pollution events, as Zhang et al. (2004) also points out.

In contrast, AOPP, which are well known as uremic toxins that may accumulate due to renal failure (Witko-Sarsat et al., 1996, 2003), was found to be elevated in *P. warreni* from the upper Olifants River sites. These toxins have cytotoxic properties and are useful *in vivo* markers of protein damage (Heinecke et al., 1993). This may be especially useful in hepatopancreas tissue, as this organ has been found to be a more sensitive organ to pollution than for example gills (Hao et al., 2009; Walters et al., 2016). Thus, the increase in AOPP may indicate organ failure or other extreme levels of impacts in *P. warreni* from the upper Olifants River that far exceeds short-term oxidative stress.

5.5.3. Protein Assessment

Proteins are a key component in almost all biological processes and are very sensitive to pollution, especially metal contamination (Jacobs et al., 1977; Voet et al., 2001). The reduced protein content observed in the muscle and hepatopancreas tissue of the *P. warreni* samples from the upper Olifants River (compared to the Mokolo RIver) may suggest impacts at cellular level (Qin et al., 2012). This is because it has been well recorded that protein concentrations may be impacted by pollution in various ways, for example through impacts on the synthesis capacity of the endoplasmic reticulum, increased catabolic activity by lysosomal enzymes, increased proteolysis and increased detoxification in the hepatopancreas (Jacobs et al., 1977; Murty and Devi, 1982; Chaudhary et al., 1989; Elumalai and Balasubramanian, 1999; Kumar, 2004). In the present study, variation in total protein also corresponds with and supports the AOPP results, suggesting a reduction / change in protein involvement in a range of biological processes associated with stress caused by the pollution in the upper Olifants River (Vijayavel and Balasubramanian, 2006; Singaram et al., 2013). Furthermore, these results are also in line with water quality results recoded during this study.

5.5.4. Gene Expression

The upregulation of the CAT gene is indicative of pollution (such as metal pollution), leading to stress caused by an overproduction of H_2O_2 (Torres et al., 2002). The upregulation of CAT expression may initially be caused by exposure to pollutants, but has subsequently shown to decrease due to chronic exposure (Reid and MacFarlane, 2003). Thus, during chronic exposure the upkeep of antioxidative enzymes to counteract for example metal pollution (e.g., Fe and Al) results in a significant energy cost that is difficult to maintain (Hendry and Brocklebank, 1985; Cakmak and Horst, 1991; Belcheva et al., 2011). This may explain the expression profiles observed in the present study resulting in a suppression of the CAT gene relative to the control in the upper Olifants River. This is supported by the profiles observed for the IDH-1. The involvement of the IDH-1 gene, as the primary producer of NADPH in most tissues (Xu et al., 2004) and the fact that it is known to be strongly associated with the cytoplasm, peroxisome and endoplasmic reticulum (Fu et al., 2010; Guo et al., 2011) suggest that a suppression of IDH-1 may impact protein synthesis (Ernst et al., 1978). This may explain the reduction in protein content observed at the upper Olifants River sites. The expression profiles of both CAT and IDH-1 therefore further supports the results from the, peroxidase and GSSG/GSH assessment in the present study.

The p53 gene is important for tumor suppression that prevents metastasis (Campisi et al., 2001). According to Lundberg et al. (2000), significant impacts on the p53 pathway may affect its ability to control senescence. Important substances that may cause a disruption in the p53 pathway include various metals (Rosenstock and Cullen, 1994; Glover-Kerkvliet, 1995; Nriagu, 1996). Both p53 and AOPP showed a similar increasing trend towards the upper Olifants River, thus indicating a strong association with molecular pathways involved in cellular stress response, DNA repair systems and low protein content. These findings are in accordance with other studies (i.e., Oberholster et al., 2016) that found an upregulation in the senescence associated genes during exposure experiments of water from the upper Olifants River.

Overall, from the multivariate PCA the poor water quality of the upper Olifants River was evident and had a strong association with AOPP and p53, as well as the decrease in protein content. On the other hand, the Mokolo River correlated with an increase in GSSG/GSH, peroxidase, CAT and IDH-1. From the PCA it can be seen that these biochemical and molecular responses may be associated with increased metal concentrations, for example Fe, Mn, Cd and Al. From this multivariate comparative analysis it can be seen that these biochemical and molecular markers need to be properly monitored for sufficient management of the Mokolo River to prevent the type of impact on biota currently seen in the upper Olifants River.

5.6. CONCLUSION

The use of various biochemical and molecular endpoints resulted in a better understanding of the impacts of pollutants on a long-term coal mining impacted aquatic ecosystem such as the upper Olifants River. The results of this study therefore have significant implications for the future management of rivers such as the Mokolo River that are potentially facing increased coal mining in future. Cellular level information proved to be more specific in determining the state of a river, as well as the degree of impact on biota, compared to traditional chemical analyses. This is especially significant, since it can be seen that although not in the same state as the upper Olifants River, the Mokolo River is already impacted to a degree resulting in impacts on biota. The use of enzymatic activity and mRNA expression associated with relevant pathways can be successfully used as early warning systems to improve water resource management. This may also aid to not only prevent a similar degree of metal pollution as seen in the upper Olifants River, but also to improve the nutrient enrichment currently observed in the Mokolo River. The use of protein content assessments in *P. warreni* as bioindicators is also very promising and may significantly assist in the identification of anthropogenic impacts of aquatic biota and promote sustainable development in the Mokolo River catchment. Finally, the molecular evidence obtained from *P. warreni* through this study indicated that these freshwater decapods are suitable genetic model organisms through their ability to integrate and reflect environmental stress at various degrees of severity.

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CHAPTER 6: A COMPARATIVE ANALYSIS OF INVERTEBRATE AND FISH COMMUNITY STRUCTURES FROM THE MOKOLO AND UPPER OLIFANTS RIVERS (SOUTH AFRICA) USING MULTIPLE LINES OF EVIDENCE

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Journal of Freshwater Ecology

Declaration by the candidate

With regard to Chapter 6, the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution
Conceptual design, fieldwork, experimental work, manuscript writing.	75%

The following co-authors have contributed to Chapter 6:

Name	Email address and Institutional affiliation	Nature of contribution	Extent of contribution
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6.1. ABSTRACT

Significant increases in land use activities, such as coal mining, may have serious implications for aquatic communities. The upper Olifants River represents such a system where long-term coal mining has taken place. In light of the potential future expansion in coal mining activities, the relative pristine Mokolo River faces similar impacts. In order to add value to management plans to limit the likely ecological impacts associated with changing land use, the aim of this study was to use multiple endpoints in a comparative biotic community study of the upper Olifants and Mokolo rivers. The use of aquatic invertebrates proved to be more useful to characterize the ecosystem integrity, detect turnover and determine links to possible contaminants. The use of a sensitivity calculation method for the invertebrate and fish communities, as well as a habitat availability index, proved to be valuable in determining biodiversity changes related to water quality and / or habitat availability impacts. This was further confirmed using univariate approaches to assess the invertebrate and fish communities. The data obtained from the bivariate assessment confirmed the impact of certain abiotic variables on the fauna from both rivers, whilst the multivariate analysis provided a good integrated summary of the changes in biodiversity associated with variation in water quality. The impact of poor water qualities at certain sites co-varied with changes observed using biochemical endpoints, which were often more sensitive than the taxonomical or functional assessments of the communities. This approach proved to be useful as an early warning system to detect coal mining impacts on biological communities.

6.2. INTRODUCTION

A multitude of factors determine the health of a river ecosystem, but because it is not practical to measure every single factor in detail, certain ecological indicator groups (biotic or abiotic), that are representative of the larger ecosystem, are often used in ecological assessments (Davis and Simon, 1995; Weigel et al., 2002; Kasangaki et al., 2006; De Klerk and Wepener, 2011, 2013). It is important to assess the ecological integrity of a river, because it is a direct reflection of the combined effects of all the activities occurring in the catchment that they drain (Hynes, 1975; Allanson et al., 1990; Huntley, 1990; Allanson, 1995; Davies and Day, 1998; Dallas and Day, 2004). It has been shown that the ecological integrity of a river can be determined using the species composition of a biotic community (Hohls, 1996; Dallas and Day, 2004), and as such, indirectly indicate the health of an ecosystem (Chutter, 1998; De la Rey et al., 2004). The use of biological assessments greatly enhances the chemical and physical monitoring of freshwater systems, because it provides a direct measurement of the health of the ecosystem as biota integrate all the different factors impacting on them (Davis and Simon, 1995; Barbour, 1997; Resh, 2008). This is because chemical monitoring cannot indicate human impacts such as habitat degradation and destruction, which may influence biological integrity (Roux et al., 1993).

Aquatic invertebrate communities are an important group to use in the integrated assessment of water quality (Karr, 1991; Metcalfe-Smith, 1994) and as indicators of river condition (Plafkin et al., 1989; Rosenberg and Resh, 1993; Marchant et al., 1997), because some species are more sensitive to pollution than other species (USEPA, 1997). Aquatic invertebrates are also ideal for use in biological assessments, because they are abundant in aquatic environments and constantly exposed to the surrounding water. Thus, aquatic invertebrates are immediately vulnerable to any decline in water quality (Kasangaki et al., 2006). They are also known to be easy to identify, have largely sedentary habits (which is ideal for assessing site-specific impacts), have rapid life cycles and are relatively visible to the naked eye (Dickens and Graham, 2002). Aquatic vertebrates, such as fish, are also regarded as sensitive to water quality changes and therefore considered to be valuable indicators of aquatic pollution (Wester et al., 2002; Hinck et al., 2008). This is because, as with invertebrates, they are also able to integrate environmental variability at different spatial scales (Oberdorff et al., 2002). As a result, an assessment of fish species composition, richness and abundance is consistent with river degradation (Oberdorff and Hughes, 1992; Rosenberg and Resh, 1993). Thus, to effectively protect and restore water resources, biological measures (for example invertebrates and fish) need to be incorporated with chemical and physical measures to properly understand the health or integrity of rivers (Oberdorff and Hughes, 1992).

A number of land use activities, such as mining, can impact on the integrity of a river system (Bell et al., 2001; De Villiers, 2007; Oberholster et al., 2010). On the other hand, these activities are also of strategic importance for countries such as South Africa and as such pro-active monitoring is needed to ensure the sustainable use of aquatic resources. The upper Olifants River in the Mpumalanga Province of South Africa is one of the most polluted rivers in Southern Africa (Grobler et al., 1994; Hobbs et al., 2008; Ashton and Dabrowski, 2011; DWA, 2011; Dabrowski and De Klerk, 2013; Oberholster et al., 2013). As this river system has been subjected to coal mining for more than 100 years (Hobbs et al., 2008), a clear understanding of anthropogenic impacts on this river and its associated temporal and spatial changes in faunal composition is important. This provides a unique opportunity to gain sufficient ecological information relating to faunal composition changes subjected to coal mining impacts. The lack of such information is problematic for the proper management of aquatic systems in South Africa in the face of future economic growth and development (Van Vuuren, 2009). The Waterberg region in South Africa is one such region which is at the centre of increased development. As South Africa's coalfields within the catchment of the upper Olifants River are slowly nearing depletion, activities within the Waterberg are increasing as to further exploit the virgin coal reserves in the area. These activities are expected to occur mostly within the Mokolo River catchment, placing the Mokolo River at risk of increased anthropogenic activities. The Mokolo River catchment is located in the northern parts of South Africa, an area which is characterised by high temperatures and low rainfall and which may be more sensitive to pollutants than the upper Olifants River (De Klerk et al., 2016). With this in mind, the potential impact of anthropogenic activities in the Mokolo River may be even more pronounced than in the upper Olifants River.

The present study used this unique opportunity to comparatively study the invertebrate and fish fauna of the upper Olifants River (historically impacted) and Mokolo River (relatively pristine) in order to understand the implications of future coal mining activities, to assist with future management of this river. This is especially important since both river systems have international implications for neighbouring countries. Secondly, this study evaluated a range of endpoints to be used as early warning signs of negative impacts on the integrity of biotic communities that may be used internationally to assess and monitor the impact of coal mining.

6.3. MATERIALS AND METHODS

6.3.1. Study Areas and Study Design

Within the upper Olifants River, four different sites were selected, whilst in the Mokolo River nine sites were selected (Figure 6-1). The selected sites were identified to be representative of the river system. Samples were collected seasonally over a period of two years (2012 – 2013) in the upper Olifants and three years (2011 – 2013) in the Mokolo River to account for seasonality and different hydrological extremes. At each site, selected *in situ* water quality parameters were measured, whilst water samples were collected for standard chemical analysis of a suite of different variables. Changes in faunal composition of these rivers were determined by focussing on invertebrate and fish populations, as well as biochemical analysis to determine biotic impacts at a cellular level. A Habitat Availability Index (HAI) was developed based on site-specific habitat assessments. The HAI was derived by compiling a reference list of potential habitat types for invertebrates and fish from literature and then assessing each site for the availability of these habitats (MPCA, 1992; Kleynhans, 1999; Dickens and Graham, 2002). The site assessments included scoring the availability of these habitats (on a scale from 0 = none to 5 = very abundant) and the HAI value (expressed as percentage) derived through the sum of each habitat score divided by the total value. The HAI value represents standardised scores which allow for comparison between different rivers. For the sites in the upper Olifants River, site names were abbreviated as OR (i.e., OR01), whilst for the Mokolo River the abbreviation MR was used (i.e., MR01).

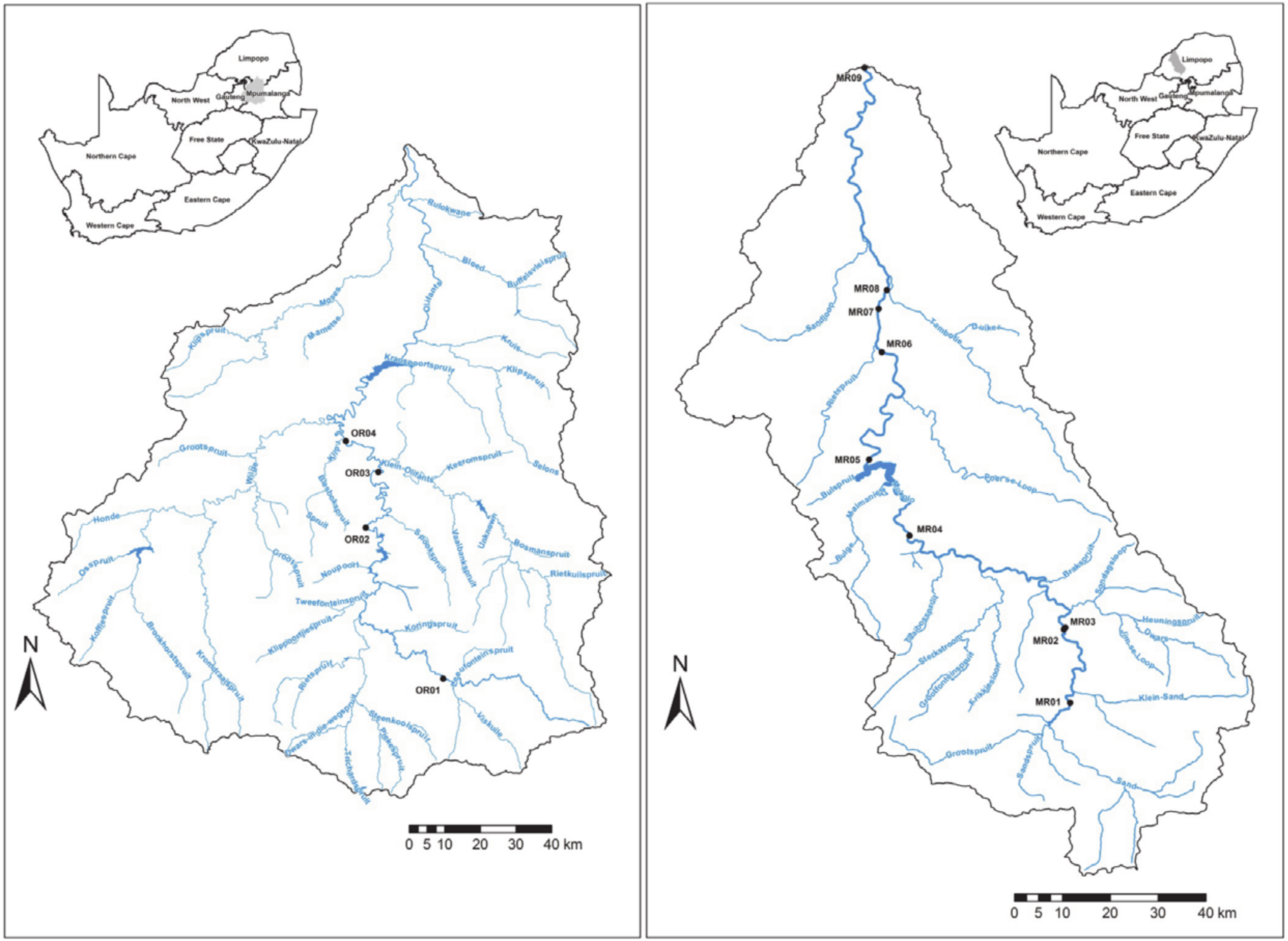

Figure 6-1: The study area, upper Olifants River (left) reflecting the four study sites (OR01 – OR04) and the Mokolo River (right) reflecting the nine study sites (MR01 – MR09).

6.3.2. Ecological Integrity Assessments

6.3.2.1. *Water Quality*

In situ water quality measurements, namely temperature, pH and electrical conductivity, were measured using a Thermo 5 Star pH/RDO/Conductivity meter (Thermo Scientific, USA). Water samples were collected at each site and analysed for a suite of different parameters using standard procedures (APHA, AWWA, WEF, 2012). Throughout the analyses various quality control and quality assurance protocols were followed, including determining the ionic balance in each sample, using matrix matched calibration standards and measuring reproducibility by duplicating the first sample of each batch. Random forest analyses were used to determine the importance of the results of the different water quality variables tested in terms of their impacts on the invertebrate and fish community (R-project v3.2.2). Only those variables deemed to significantly contribute the most in terms of explaining the variation were retained and presented in this study. These variables are pH, arsenic (As), iron (Fe), zinc (Zn), sulfate (SO_4^{2-}), alkalinity (as $CaCO_3$), dissolved organic carbon (DOC), total phosphate (Total_P) and total nitrogen (Total_N).

6.3.2.2. *Aquatic Invertebrates*

In South Africa, the South African Scoring System Version 5 (SASS5) is a rapid assessment based on aquatic invertebrates which assesses the ecological integrity of a section of a river (Dickens and Graham, 2002). Because this methodology was designed for low to moderate flow hydrology, it is not well suited for high flow hydrology typically encountered during the summer rainy seasons. For the purposes of the present study, all available aquatic macroinvertebrate biotopes were identified (Dickens and Graham, 2002) and the use of a sweep net method, instead of the SASS5 methodology, was used to sample aquatic macroinvertebrates at the identified biotopes (De Klerk and Wepener, 2013). With this approach, invertebrate species can be sampled very efficiently (Turner and Trexler, 1997) across seasons and without being bound to time limits, as is the case with SASS5 (Dickens and Graham, 2002). Aquatic macroinvertebrate samples were preserved in 10% neutral buffered formalin, with added Rose Bengal stain (De Klerk and Wepener, 2013), for subsequent identification and enumeration in the laboratory.

Generally, there are two approaches when using invertebrates, namely a taxonomic approach (i.e., a measurement of invertebrate richness or diversity) or a functional approach (based on morphological and behavioural characteristics), of which both can be used to characterize ecosystem conditions (Cummins et al., 2005). For the taxonomic assessment, invertebrate samples were identified using guides to the freshwater invertebrates of southern Africa (Day et al., 1999, 2001a, 2001b, 2002; Day and De Moor, 2002a, 2002b; De Moor et al., 2003a, 2003b; Stals and De Moor, 2007), afterwhich the Shannon diversity index (H′) and

Margalef's index (d) were calculated as described later on. For the functional assessment feeding groups were assigned according to Cummins et al. (2005) and Merritt et al. (2002). The various invertebrates identified at the selected sites were subsequently classified according to the following classes: shredders, scrapers, filtering collectors (FC), gathering collectors (GC), predators and other (unknown), using a modified ten-point matrix system (Yoshimura et al., 2006).

6.3.2.3. *Fish*

Quantitative fish sampling was undertaken to determine the current fish assemblages at each sampling site. A section of each river (≈50 m) was sampled at each site using an electro-fishing unit (SAMUS 725-M, SAMUS Special Electronics). Sampling took place for a period of approximately 20 to 30 minutes, whilst the operator of the electro-fishing unit was accompanied by two samplers (on both sides of the operator), who collected the stunned fish using dip nets. Sampling commenced downstream of the selected river section, working upstream, sampling all available habitats. The collected fish were mainly collected in buckets (filled with aerated water) until identification and enumeration, where after they were released back into the river at the site of collection.

6.3.3. Reference Lists and Sensitivity Indicator Values

Lists of invertebrate and fish species expected to occur at each site (reference list) were compiled using various databases (Kleynhans et al., 2007). These databases are compiled based on the expert opinion of specialists, as well as reference lists for the specific eco-regions in South Africa. An adapted species list was then compiled by aggregating the species physically sampled at each site, together with the species that were expected to occur there, based on the presence of suitable habitat and historical records. Sensitivity indicator values were derived for invertebrates and fish at each site to provide an overall indication of the sensitivity (intolerance or preference) of different biotic communities to specific conditions (for example water quality or flow). Aquatic invertebrate sensitivity values were based on the scores assigned to certain taxa by Dickens and Graham (2002). The sensitivity values for fish were assigned between 0 and 5 for both water quality (WQS) and flow condition (FCS) sensitivities. These specific indicator values were combined to obtain a total sensitivity score (out of 10) for the species present at each site according to Equation 1 (Roux, 1999; Kleynhans, 2003).

Equation (1):

$$FSS\ (\%)_{\text{per species}} = (WQS + FCS / 10)100$$

The total invertebrate (ISS) and fish (FSS) sensitivity scores for each site were calculated by adding the sensitivity values of all the respective invertebrate and fish species together to obtain a total sensitivity value for invertebrates and fish at each site, respectively.

6.3.4. Oxidative Stress

As a general indicator of cellular impacts on invertebrate and fish populations in both rivers, the tissue concentration of the reactive oxygen species (ROS), namely hydrogen peroxide, was determined. The freshwater crab (*Potamonautes warreni*) and the banded tilapia (*Tilapia sparrmanii*), were chosen as indicator organisms to represent the general invertebrate and fish populations. These organisms were captured, euthanized and used in an ethical manner. To ensure compliance, the South African National Standard for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008), Gardner (1997), ANZCCART (2001), Hajek et al. (2009) and Yue (2009) guidelines were used. The Animals Protection Act (Act 71 of 1962) of South Africa was adhered to at all times.

The biotic samples were snap frozen in liquid nitrogen and stored at -80°C until analysis could be performed. Muscle tissue obtained from these organisms was used to estimate the hydrogen peroxide concentrations using the OxiSelect^{TM} Hydrogen Peroxide Assay Kit (Colorimetric) (STA-343). All preparations and analyses were carried out at 4°C. Protein concentrations were determined using the Bradford method (Bradford, 1976). The ROS assays were conducted using triplicate biological samples per site (n = 3) with each sample analysed in triplicate, thus the whole experiment was repeated nine times (n = 9) for each site during each sampling trip. Lastly, within the Mokolo River we had to limit the number of sites due to difficulty in obtaining a sufficient number of organisms. Furthermore, a sufficient number of *T. sparrmanii* could not be found at one site in the upper Olifants catchment (i.e., OR01). The remaining sites were still deemed sufficient to retain a catchment perspective.

6.3.5. Statistical Analysis

A series of statistical analysis were applied in order to elucidate spatial changes in the invertebrate and fish community structures. Univariate statistical analyses (Primer v6) included Shannon diversity index (H′) and Margalef's index (d). These were calculated according to Equations 2 and 3 obtained from Shannon (1948) and Margalef (1951), respectively:

Equation (2): $H' = -SUM(Pi*Log(Pi))$

Equation (3): $d = (S-1)/Log(N)$

Because of the difficulty to interpret differences in H′ score due to the incorporation of different variables, the species richness component in the form of the Margalef's index was included to supplement the scores obtained from the Shannon diversity index (Ludwig and Reynolds, 1988). Bivariate statistical analysis (R-project v3.2.2) was carried out using Pearson product-moment correlation coefficient to assess the linear correlation (dependence) between the different variables using two separate variables at a time. Multivariate statistical analyses (Canoco v5) were used to assess relationships between the invertebrate and fish community structures and the water quality variables identified through the Random Forests analysis. For this purpose redundancy analysis (RDA) was used as a method to extract and summarise variation in a set of response variables (species data) to be explained by a set of explanatory variables (environmental variables). The values used were the best-fit data estimated from multiple linear regressions between each response variable and a second matrix of environmental data. This enabled us to determine the relationship between invertebrate and fish community structures and selected environmental variables. To determine significance, an Analysis of Variance (ANOVA) test was used in combination with the Fisher's LSD post-hoc test (Statistica 12, Statsoft, US). The Kolmogorov-Smirnov test, the Shapiro-Wilk W test, as well as the Lilliefors test were used to evaluate the normality of the data. Levene's and Brown and Forsythe's tests were used to test the homogeneity of variance. A probability (P) value equal to or less than 0.05 was deemed as significant (Wild and Seber, 1999). The correlation strength was determined according to Evans (1996).

6.4. RESULTS

6.4.1. Habitat Availability and Sensitivity Indicators

6.4.1.1. *Aquatic Invertebrates*

In the upper Olifants River, a significant decrease in the total sensitivity of the invertebrate population at Site OR02 (ISS = 53) compared to the other upper Olifants River sites (ISS > 100) was observed even though the habitat availability remained similar to the rest of the sites (IHAI = ≈64%) (Table 6-1). The community composition of all the sites in the upper Olifants River was found to consist mainly of gathering collectors, scrapers and predators (a cumulative proportion of ≈81%). The invertebrate functional assessment highlighted a significant increase in predatory invertebrates at sites OR02 and OR03, compared to sites OR01 and OR04.

Relatively high sensitivity scores for the invertebrate populations were also observed within the upper reaches of the Mokolo River (ISS = ≈165) (Table 6-1), with a decrease observed at MR06 to MR08 (ISS = ≈80). This decrease corresponded with the decrease in habitat availability observed at these sites (IHAI = ≈33%) compared to the upstream sites (IHAI = ≈72%). The invertebrate functional assessment in the Mokolo River showed that the community composition at the various sites also consisted mainly of gathering collectors, scrapers and predators (a cumulative proportion of ≈79%). At site MR09 a shift in this community was observed through a decrease in the proportion gathering collectors (19.41%) compared to the general trend in the Mokolo River of ≈35.5% gathering collectors). Besides the site specific changes identified above, the overall degree of habitat availability and total sensitivity of the invertebrate community were similar in the upper Olifants and Mokolo rivers.

6.4.1.2. *Fish*

The sensitivity scores for the fish communities in the upper Olifants River generally increased following a downstream gradient (FSS of OR01 = 50 and FSS of OR04 = 64.2). This corresponded with an increase in habitat availability downstream (FHAI of OR01 = 60% and FHAI of OR04 = 82%) (Table 6-1).

No observable trend was found between the spatial changes in the FSS of the Mokolo River (FSS = ≈88), whilst the FHAI remained similar throughout (FHAI = ≈63%). However, the sensitivity of the fish populations observed in the Mokolo River (FSS = ≈87.93) tend to be higher than those in the upper Olifants River (FSS = ≈56.5). Nevertheless, the habitat availability in the Mokolo River (FHAI = ≈62.62) was generally less than that which was found in the upper Olifants River (FHAI = ≈74%).

Table 6-1: A summary of the invertebrate functional analysis, the results for the habitat assessments for the invertebrate and fish communities, as well as the total sensitivity scores obtained for the invertebrate and fish populations in the respective rivers.

Sites		Invertebrate Functional Feeding Groups (%)						Invertebrate Habitat Assessment Index (IHAI)	Invertebrate Sensitivity Score (ISS)	Fish Habitat Assessment Index (FHAI)	Fish Sensitivity Score (FSS)
		Shredders	Scrapers	Filtering Collectors	Gathering Collectors	Predators	Other				
Olifants River	OR01	5.06	30.23	19.51	33.76	8.47	2.97	60.00	117	60.00	50.00
	OR02	6.98	22.66	12.80	27.07	30.45	0.04	62.22	53	70.00	57.40
	OR03	1.88	18.39	8.01	33.73	36.63	1.36	64.44	165	83.33	54.30
	OR04	6.09	30.08	10.94	41.35	11.01	0.53	68.89	192	82.00	64.20
Mokolo River	MR01	13.27	24.25	14.40	31.09	14.91	2.08	75.56	153	56.12	88.40
	MR02	8.62	21.29	14.41	32.82	22.55	0.30	71.11	160	73.33	70.20
	MR03	9.19	26.30	11.01	30.54	22.39	0.58	73.33	174	50.67	89.50
	MR04	11.84	25.51	23.87	22.13	15.90	0.75	66.67	174	69.33	80.50
	MR05	16.73	29.68	10.44	27.34	15.81	0.00	64.44	110	77.50	96.20
	MR06	3.22	19.50	0.92	54.94	20.66	0.77	31.11	84	50.00	86.40
	MR07	5.67	23.48	5.67	38.43	26.75	0.00	35.56	101	60.00	104.80
	MR08	3.43	30.45	5.45	47.12	13.04	0.50	33.33	62	56.67	86.50
	MR09	9.87	26.02	13.24	19.41	28.96	2.50	71.11	120	70.00	88.90

6.4.2. Univariate Diversity and Richness Variation

6.4.2.1. *Aquatic Invertebrates*

The results presented in Figure 6-2A show that in the upper Olifants River at sites OR03 and OR04 the invertebrate diversity and richness increased (H′ = ≈2.1 and d = ≈4.1, respectively). On the other hand, the lowest invertebrate diversity and richness was observed at OR02 (H′ = 1.2 and d = 1.9, respectively). The invertebrate population of the Mokolo River generally decreased in diversity and richness at sites MR05 - MR08 (H′ = ≈2.1 and d = ≈3.7, respectively) (Figure 6-2B). The highest level of invertebrate diversity and richness was found in the upper reaches of the Mokolo River at MR01 – MR04 (H′ = ≈2.43 and d = ≈4.64, respectively). These results obtained from the univariate analyses for both the upper Olifants and Mokolo rivers corresponded with the results obtained from the sensitivity assessment (Table 6-1).

6.4.2.2. *Fish*

In the upper Olifants River, no real trend could be observed with regard to the diversity and richness of the fish populations at the different sites. However, the highest standard deviation was found at OR01 and OR03 (Figure 6-2A). The diversity and richness of the fish populations in the Mokolo River generally followed an increasing trend downstream from MR01 to MR09. The highest fish diversity and richness were found at MR04 to MR08 (H′ = ≈1.66 and d = ≈2.08, respectively), with the lowest being observed at MR01 to MR03 (H′ = ≈1.47 and d = ≈1.5, respectively) (Figure 6-2B). Overall, the diversity and richness values seen in the Mokolo River were significantly higher than those in the upper Olifants River which corresponded to the same trend observed in (Table 6-1) with regard to the FSS values.

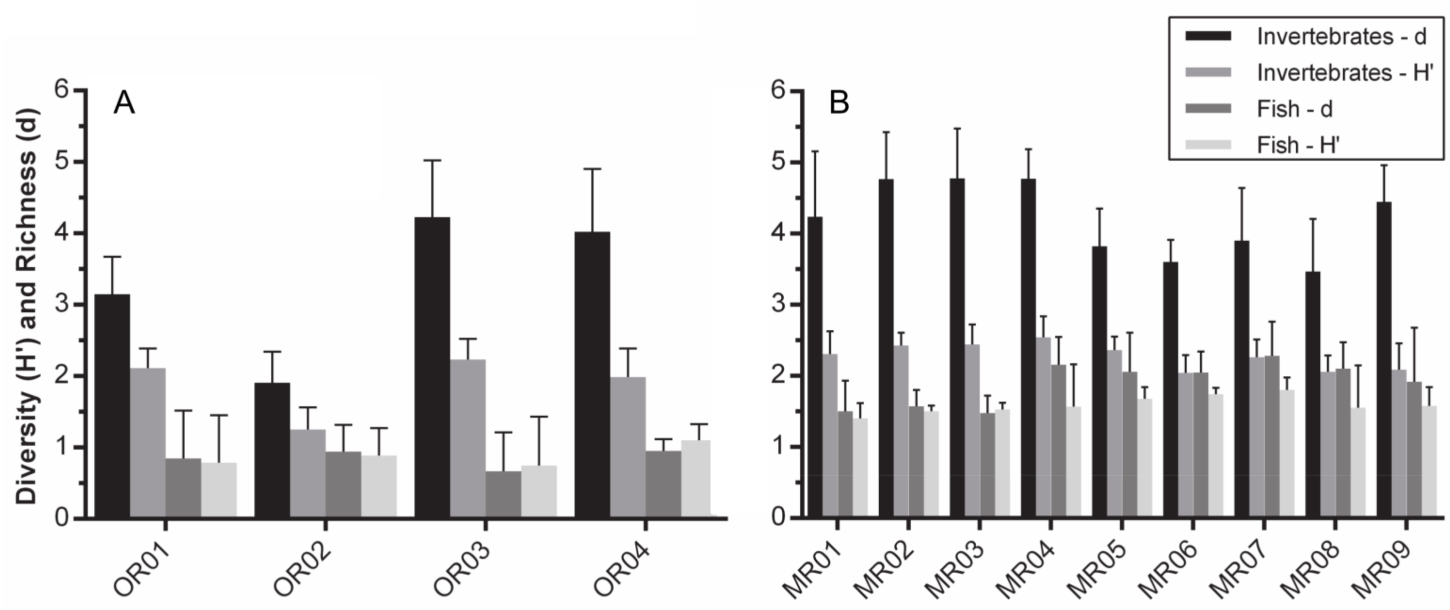

Figure 6-2: The Shannon's diversity (H') and Margalef's richness (d) index results for the invertebrates and fish of the upper Olifants River (A) and Mokolo River (B).

6.4.3. Bivariate Correlations

6.4.3.1. *Water Quality*

In the upper Olifants River most of the significant correlations were related with either DOC or Total_P as each of these variables had significant correlations with five of the eight other variables tested (Figure 6-3). The level of pH had a strong negative correlation with DOC (r = -0.50 and p = 0.051), Total_P (r = -0.58 and p = 0.018) and Total_N (r = -0.52 and p = 0.04). A relationship was also observed between DOC, Total_P and Total_N which was strongly positive (r > ≈0.7 and p < ≈0.01). Arsenic concentration was also observed to be correlated positively with DOC (r = 0.46 and p = 0.077) and Total_P (r = 0.43 and p = 0.096).

In the Mokolo River (Figure 6-4), DOC and alkalinity was observed to be the pivotal variables as both these variables had a significant correlation (p < 0.05) with seven of the eight other variables tested. As with the upper Olifants River, pH was also found to be negatively correlated, albeit weakly, with DOC (r = -0.29 and p < 0.01). Arsenic and SO_4^{2-} correlated with six and five of the other eight variables, respectively.

6.4.3.2. *Aquatic Invertebrates*

In the upper Olifants River (Figure 6-3) it was observed that the invertebrate community (H′) had moderate to strong negative correlations with As (r = -0.51 and p = 0.043), DOC (r = -0.58 and p = 0.019), Total_N (r = -0.73 and p < 0.01) and Total_P (r = -0.61 and p = 0.013). At the same time a moderate positive correlation was observed with pH (r = 0.48 and p = 0.061).

The invertebrate community (H′) in the Mokolo River had significant positive correlations, albeit relatively weak, with Fe (r = 0.22 and p = 0.049) and Total_N concentrations (r = 0.21 and p = 0.061) (Figure 6-4). On the other hand, significant correlations were found with alkalinity (r = 0.39 and p < 0.01) and DOC (r = 0.33 and p < 0.01).

6.4.3.3. *Fish*

In the upper Olifants River (Figure 6-3), the fish community (H′) had a significantly negative correlation of a moderate nature with Zn (r = -0.52 and p = 0.04) and SO_4^{2-} (r = -0.53 and p = 0.034), whilst a moderate positive correlation existed with As (r = 0.41 and p = 0.11), albeit non-significant.

On the other hand, the fish community of the Mokolo River (Figure 6-4) showed significant negative correlations of a relatively weak nature with As (r = -0.24 and p = 0.028), alkalinity (r = -0.23 and p = 0.037) and Total_N (r = -0.19 and p = 0.09).

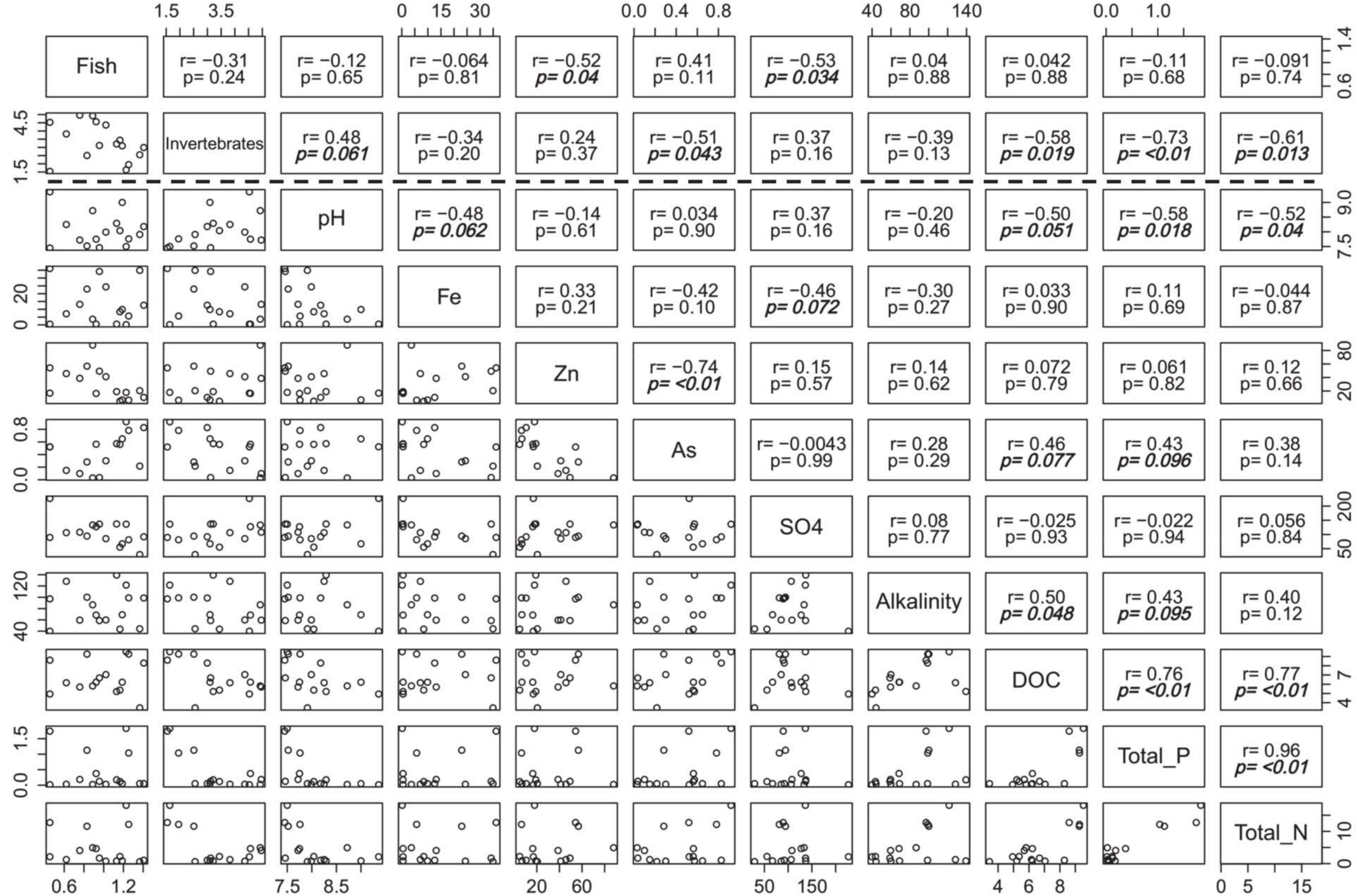

Figure 6-3: The correlations between the invertebrate and fish communities (using H′ values) with the environmental variables selected through the Random Forest analysis in the upper Olifants River. The significant correlations (Wild and Seber, 1999) are indicated in bold italic font. The dotted line separates the abiotic and biotic variables.

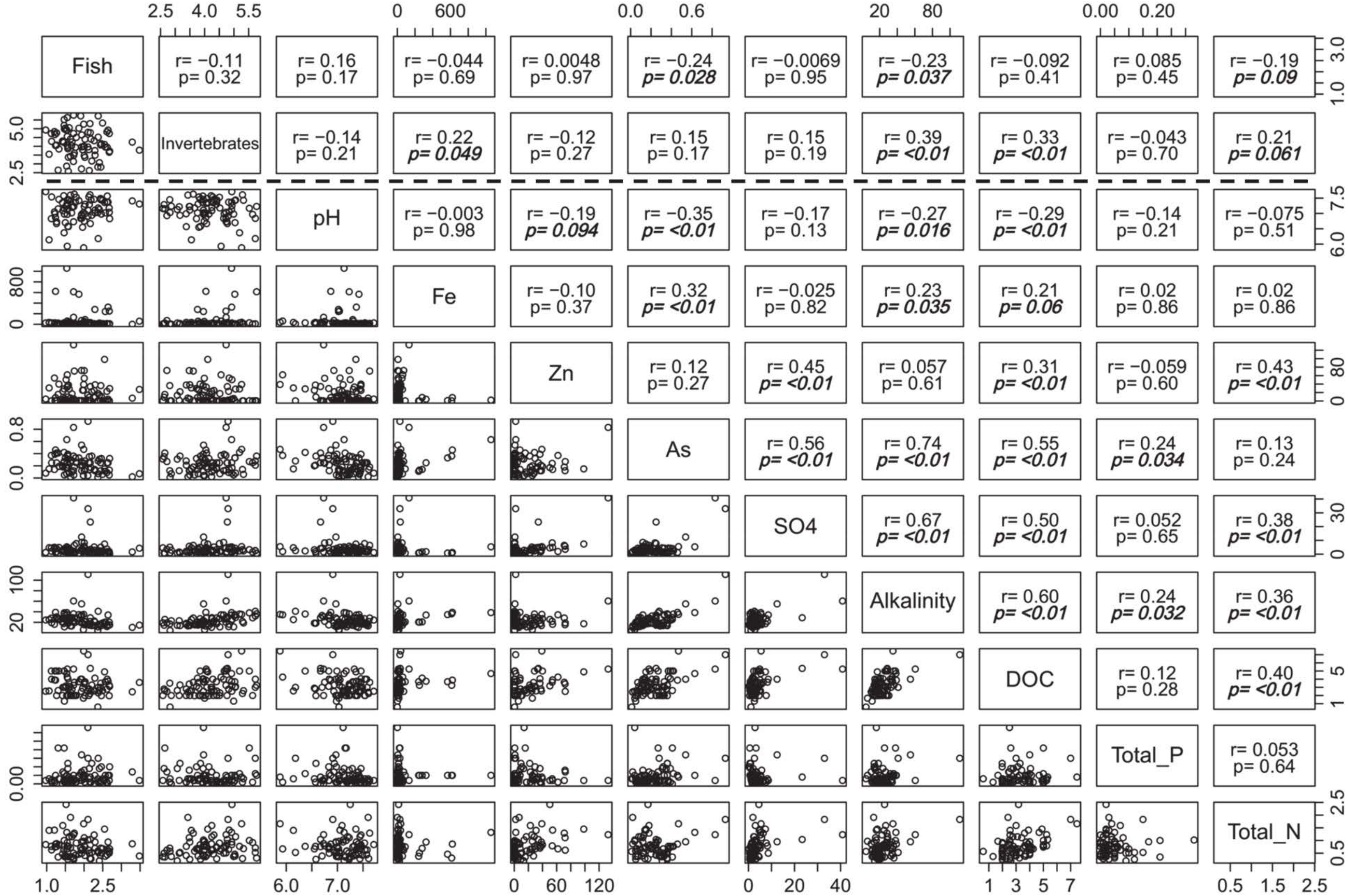

Figure 6-4: The correlations between the invertebrate and fish communities (using H′ values) with the environmental variables selected through the Random Forest analysis in the Mokolo River. The significant correlations (Wild and Seber, 1999) are indicated in bold italic font. The dotted line separates the abiotic and biotic variables.

6.4.4. Multivariate Relationships

6.4.4.1. *Aquatic Invertebrates*

Within the upper Olifants River it was observed that sites OR03 and OR04 were very similar (blue encirclement) based on the taxonomic assemblage of the invertebrates and the overlying water quality variables found at these sites (Figure 6-5A). The RDA furthermore indicated that the water quality signature is dominated by a strong correlated increase in sulfates and pH at these two sites. These sites were also characterized by much more sensitive aquatic invertebrates, such as Perlidae and Heptageniidae. On the other hand, sites OR01 and OR02 were dissimilar from the other sites with OR02 being significantly influenced by an increase in Total_N, Total_P and DOC, whilst an increase in Fe and As was also observed. Not only can a distinct decrease in invertebrate diversity be seen at site OR02 (which corresponds with Figure 6-2A), but this site was also characterized by less sensitive aquatic invertebrates, namely Oligochaeta, Chironomidae, Physidae, Psychodidae and Hirudinea.

The RDA for the Mokolo River grouped sites MR01 – MR04 together (purple encirclement), as well as sites MR06 – MR08 (green encirclement) based on their invertebrate assemblages and the overlying water quality (Figure 6-5B). It was observed that sites MR01 – MR04 showed an increase in invertebrate taxa (corresponding to Figure 6-2B), compared to MR06 – MR08 that were not strongly associated with an increase in invertebrate taxa. Site MR09, whilst having a relatively high diversity of invertebrates, also appears to be the locality where an increase in all of the different water quality variables was noted.

6.4.4.2. *Fish*

The RDA using fish assemblages with the overlying water quality showed a similar trend as found with the use of aquatic invertebrates with regard to the grouping of the sites and the driving water quality variables in the upper Olifants River (Figure 6-6A). Site OR02 were also mainly dominated by exotic fish species such as *Gambusia affinis* and *Micropterus salmoides*.

Using the fish assemblage data, a similar grouping was observed than with the use of the invertebrate data in the Mokolo River (Figure 6-6B), although MR04 and MR05 now showed a stronger similarity with MR09 where there were less fish taxa observed. The fish found at these three sites mainly included *Clarias gariepinus*, *Oreochromis mossambicus* and *Labeo molybdinus*. The highest diversity of fish was associated with MR06 - MR08, whilst MR01 - MR03 showed low diversities (which corresponds to Figure 6-2B).

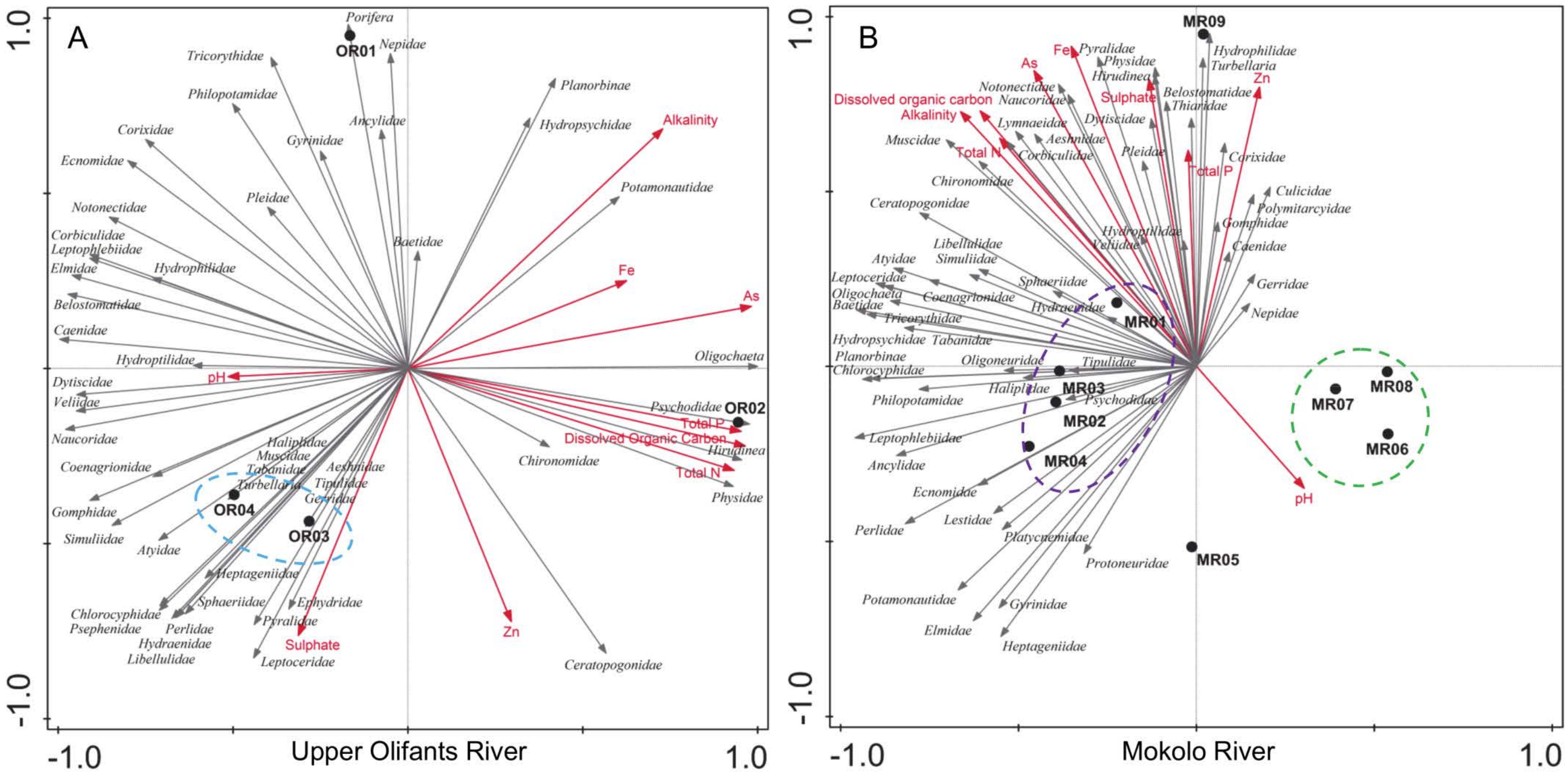

Figure 6-5: The redundancy analysis (RDA) for the upper Olifants River (A) and Mokolo River (B) using invertebrate taxa as the response variable, whilst overlain with the selected water quality variables as the explanatory variables. Encirclements indicate sites that were similar, whilst red arrows represent abiotic variables and black arrows represent invertebrate taxa.

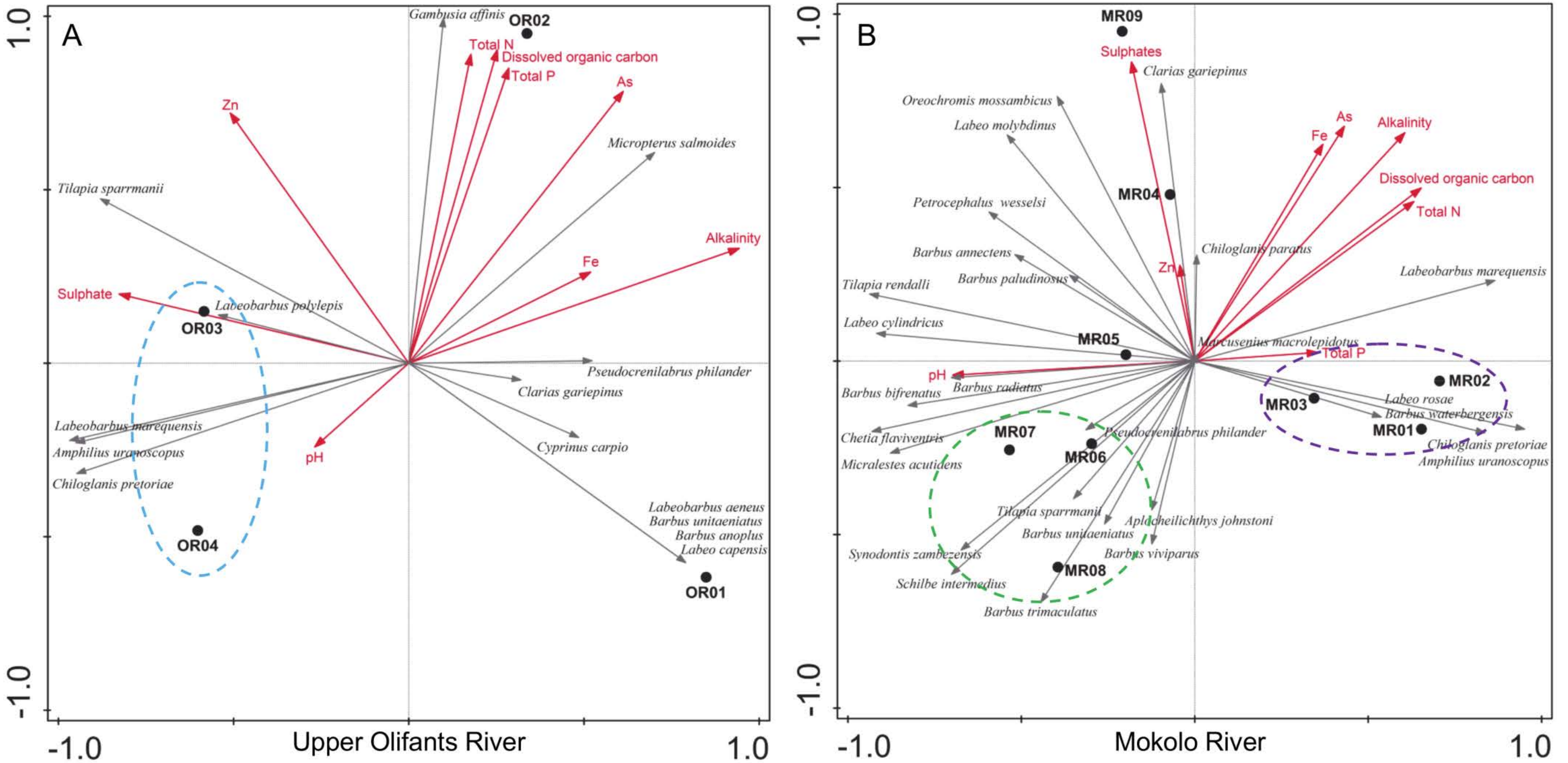

Figure 6-6: The redundancy analysis (RDA) for the upper Olifants River (A) and Mokolo River (B) using fish taxa as the response variable, whilst overlain with the selected water quality variables as the explanatory variables. Encirclements indicate sites that were similar, whilst red arrows represent abiotic variables and black arrows represent fish taxa.

From Table 6-2 it can be seen that a high proportion of the variation in both cases (invertebrates and fish) from both river systems was explained using the redundancy analysis and graphical representation of it on a two-dimensional plot.

Table 6-2: The Eigen values obtained from the redundancy analysis that was performed in both rivers using invertebrate and fish population data.

Site	Axis 1	Axis 2	Axis 3
Upper Olifants River Invertebrates	0.5804	0.2744	0.1452
Upper Olifants River Fish	0.6069	0.2723	0.1208
Mokolo River Invertebrates	0.4496	0.2646	0.1069
Mokolo River Fish	0.4794	0.1659	0.1124

6.4.5. Oxidative Stress

The results from the ROS analysis of the muscle tissue of *P. warreni* and *T. sparrmanii* in the upper Olifants River (Figure 6-7A) corresponded to the diversity and richness trends observed of the invertebrate and fish communities (Figure 6-2A). There was a significant increase in ROS levels at OR03 in *P. warreni* (6.5×10^{-6} mU/ml/mg protein). On the other hand, a decrease in ROS levels was seen at OR03 in *T. sparrmanii* (5.4×10^{-4} mU/ml/mg protein).

In general, the ROS concentrations of *P. warreni* in the Mokolo River decreased following a downstream gradient (MR02 = 2.5×10^{-5} mU/ml/ mg protein and MR08 = 8.8×10^{-6} mU/ml/mg protein). A similar (but less distinct) trend was also observed for *T. sparrmanii* (MR02 = 0.9×10^{-2} mU/ml/mg protein and MR08 = 0.2×10^{-2} mU/ml/mg protein) in the Mokolo River (Figure 6-7B). Two sites, namely MR02 and MR07 (*P. warreni*) and MR02 and MR03 (*T. sparrmanii*) showed the most elevated ROS levels in the Mokolo River.

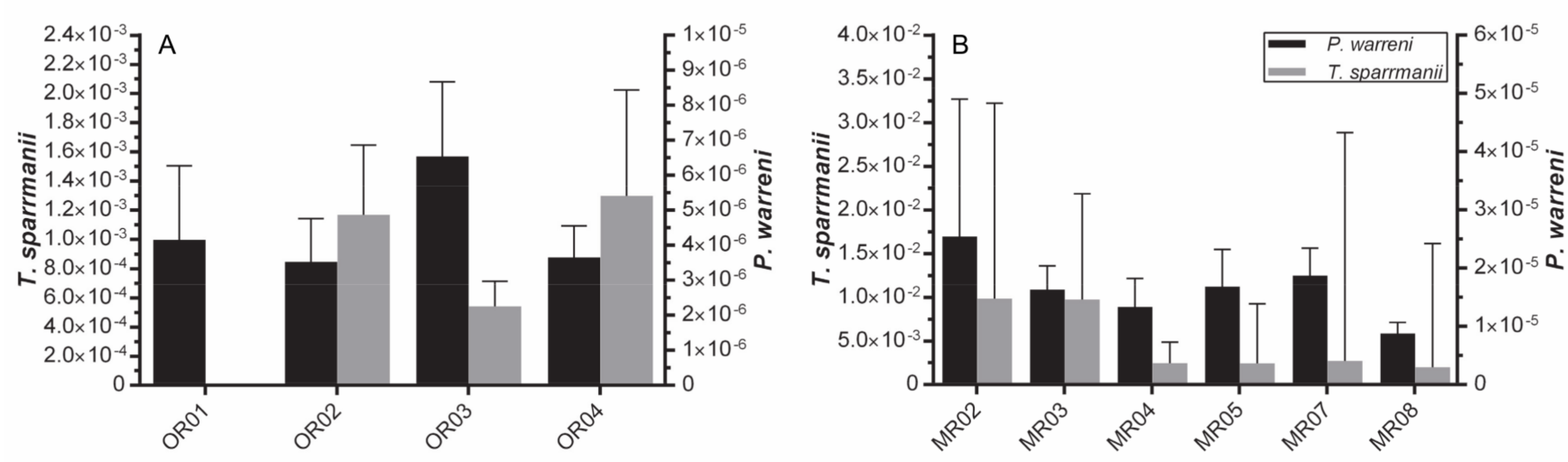

Figure 6-7: The Reactive Oxygen Species (ROS) analysis using hydrogen peroxide (mU/ml per mg protein) measured within the muscle tissue of *Potamonautes warreni* and *Tilapia sparrmanii* from the upper Olifants River (A) and Mokolo River (B). No *Tilapia sparrmanii* samples could be collected from OR01.

6.5. DISCUSSION

In South Africa the upper Olifants River is known to be severely impacted by various land use activities (Bollmohr et al., 2008; Ashton and Dabrowski, 2011; Dabrowski and De Klerk, 2013; Oberholster et al., 2013). On the other hand the Mokolo River is less impacted. However, an envisaged shift in coal mining activities into this catchment in the near future may dramatically change this situation. In order to prevent significant degradation of the Mokolo River in the face of increasing and changing land use activities, a comparative approach may facilitate improved proactive management practices.

The fact that DOC and Total_P correlated strongly with several water quality parameters highlighted their important role in the upper Olifants River. This may be due to the fact that DOC is well known to alter the nutrient cycling process and is also an important factor to take into account when trying to elucidate the toxicity of various contaminants (Williamson et al., 1999). Site OR02 had the worst water quality due to variables such as Total_P which is characteristic of anthropogenic impacts (Oberholster et al., 2013). The negative correlation observed between these variables and pH was the main abiotic driver behind the spatial changes observed between the sites, for example OR03 and OR04 that have a strong relationship with increasing pH.

Similar to the upper Olifants River, DOC was also found to be an important variable in the Mokolo River. The fact that alkalinity correlated with almost all of the other variables supports previous findings in the Mokolo River with regard to its importance, as this river generally has low alkalinity levels (De Klerk et al., 2016). The significant association between MR09 and an increase in most of the water quality parameters (i.e., Total_P, Zn, SO_4^{2-}, etc.), with the exception of pH, highlighted the poor water quality at this site. On the other hand, the strong relationship recorded at MR06, MR07 and MR08 with increased pH levels, is an important consideration, because a negative correlation between pH and DOC was found to exist. Increases in Total_N and Total_P were found to be more confined to the upper reaches of the Mokolo River at MR01, MR02 and MR03 where agricultural practices predominate (Seaman et al., 2013; De Klerk et al., 2016).

6.5.1. Aquatic Invertebrates

6.5.1.1. *Upper Olifants River*

Changes in the invertebrate community of the upper Olifants River (which consisted mainly of scrapers, predators and gathering collectors) were found to be mainly independent of habitat availability. Therefore, changes in the invertebrate diversity / richness and functionality suggested to be related mostly to the decrease in water quality. The negative correlation between the aquatic invertebrate community in the upper Olifants River and abiotic drives such as DOC, Total_N and Total_P was confirmed using the various analyses. As a result, a variety of impacts was observed, such as a decrease in the invertebrate sensitivity score, as well as diversity and richness at certain sites (e.g., OR02). It is known that DOC has the ability to bind to contaminants and alter their toxicity, as well as affect nutrient availability (Herndl et al., 1993; Welsh et al., 1993; Williamson et al., 1999). Usually this will result in a decrease in the potential toxicity, but from the results of the present study it seems more likely that the DOC (possible bound with various contaminants) increased the impact on the biota. On the other hand, high levels of phosphorous and nitrogen may lead to a decline in sensitive invertebrate taxa, resulting in a dominance of tolerant taxa (Paisley et al., 2003). Generally, the process of excessive nutrient enrichment is difficult to reverse and is therefore regarded as pollution when these levels are increased by anthropogenic activities (Schmitz, 1996).

The positive relationship of the invertebrate fauna with pH confirmed that an acidic environment may be detrimental to a balanced invertebrate community composition (Burton and Allan, 1986). This may explain the increase in invertebrate diversity / richness, as well as overall sensitivity of the community, as sites OR03 and OR04 were dominated by pollution sensitive taxa such as Perlidae and Heptageniidae (Dickens and Graham, 2002). The decrease in diversity / richness and sensitivity experienced at OR02 was evident through the domination of taxa related to pollution, namely Oligochaeta, Chironomidae, Physidae, Psychodidae and Hirudinea (Clarke and Warwick, 1998; Weigel et al., 2002). This was accompanied by a shift in the functional assemblage at this site resulting in an increase in the number of predators, which may also be a further indication of anthropogenic impacts (Palmer et al., 1996).

Although a relatively high diversity / richness of invertebrates was observed at sites OR03 and OR04, these communities may still be stressed due to the historical anthropogenic impacts of coal mining in the catchment. These impacts may also be linked with increased runoff (and their associated pollutants) into a water body from a degraded catchment (Ellis, 1936; Hellawell, 1986), as well as the discharge of acid mine drainage (AMD) (Williams et al., 1996; Sams and Beer, 2000). It has also been recorded that the toxic potential of wastewater may persist for long distances in rivers (Oberholster et al., 2013) and the toxic potential may only be realised further downstream. Either way, the impacts observed at OR03 and OR04 through the increase in ROS (hydrogen peroxide) can lead to molecular damage and appears to be positively correlated with the increase in SO_4^{2-} at OR03 and OR04, which is an indication of the impact of AMD (Antczak et al., 1997; Herbig and Helmann, 2001).

6.5.1.2. *Mokolo River*

In contrast to the upper Olifants River, a positive correlation existed in the Mokolo River between the invertebrate population and DOC, as well as Total_N. This could be explained by the fact that the Mokolo River is less polluted and thus the DOC in the Mokolo River is not associated with high levels of pollutants which may be detrimental to invertebrates. The high organic content in such a scenario may therefore be beneficial in increasing food availability through the stimulation of primary producers and mitigating toxicity (De Haan, 1993; Lindell and Rai, 1994; Williamson et al., 1999). On the other hand, a lower degree of nutrient enrichment may be beneficial to invertebrates (Malmqvist, 2002). The positive relationship with alkalinity confirms the importance of well buffered water for the invertebrates and supports the earlier findings with regard to pH levels and its impact on the invertebrate community (Wren and Stephenson, 1991). This correlates to the findings of this study that a higher diversity / richness of invertebrates was found in the upper reaches of the Mokolo River (MR01 - MR04) where an increase in nutrients was recorded. It was noteworthy how these four sites differed from the invertebrate assemblages in the lower reaches (MR06 – MR08), where a decrease in invertebrate sensitivity and diversity was recorded. These changes may be related to changes in habitat availability as observed during this study (Hohls, 1996).

Similar to the upper Olifants River, the aquatic invertebrate community of the Mokolo River also comprised mainly of gathering collectors, scrapers and predators and these assemblages were affected at MR09. This was mainly driven by the increase in gathering collectors, although the diversity / richness index values were not affected. The decrease in water quality recorded at MR09 may be negatively affecting the invertebrate community at this site. This is mainly due to the fact that the first changes observed in an invertebrate community will relate to species replacing one another, rather than a reduction in diversity / richness (Crowns et al., 1992; Clements, 1994). Interestingly, a decreasing trend was observed in the Mokolo River with regard to ROS levels in *P. warreni,* which may suggest that although a higher diversity / richness was recorded in the upper reaches, a higher level of oxidative stress may be experienced (higher ROS activity). This could be linked to current increases in land use activities (mainly agricultural) associated with the upper reaches compared to the lower Mokolo River reaches (Seaman et al., 2013; De Klerk et al., 2016). This may result in an impact that can only be observed in subtle non-lethal stress pathways on a chronic scale, rather than acute.

When comparing the less impacted Mokolo River to the upper Olifants River, it was observed that the invertebrate population sensitivities were very similar to that of the upper Olifants River. However, the Mokolo River did have higher invertebrate diversities than the upper

Olifants River, which is an important consideration for the future management of the Mokolo River.

6.5.2. Fish

6.5.2.1. *Upper Olifants River*

The negative correlation found between the fish diversity and SO_4^{2-} and Zn confirmed previous studies reporting increased SO_4^{2-} and Zn to be toxic to fish (DWAF, 1996; Elphick et al., 2011). The decrease in the number of fish species recorded at site OR03 during this study, along with increases in SO_4^{2-} and Zn, further highlights this association. An increase in *G. affinis* numbers, which is known to be tolerant to poor water quality (Cech Jr et al., 1985), supports the findings from OR02 that the dominance of such species resulted in a decrease in fish diversity (Warwick, 2001). Although no clear trend with regard to changing diversities in the fish assemblages along the river could be observed, the sensitivity of the fish community, however, did increase downstream as habitat availability increased. This also confirms the link between habitat availability and biotic integrity in the upper Olifants River. The variation in ROS activity in fish tissues did not provide any clear inter-site patterns, however, the fish (*T. sparrmanii)* collected from site OR03 had the lowest ROS levels recorded for fish in the system. This is in contrast to the invertebrate data using *P. warreni*, where the highest ROS levels were recorded at the same site.

These contradictory results may be due to two possible reasons, firstly because invertebrates do not disperse as greatly as fish and remain largely sedentary or display high site fidelity (Dickens and Graham, 2002). Therefore, invertebrates may be better site specific indicators reflecting impacts brought about by land use activities (Ten Brink and Woudstra, 1991). Secondly, due to fish (especially species belonging to Chichlidae) generally having a longer life expectancy than invertebrates (for example freshwater crabs), some degree of acclimation may take place in these organisms in response to chronic exposure (Day et al., 2001b; Skelton, 2001; Walters et al., 2016). Overall, the comparative data for fish and invertebrates obtained from this study has shown that by using invertebrates and fish a similar trend can be obtained. However, combined with the biochemical data, it generally suggests that invertebrates may be useful to provide accurate information relating to chronic and acute exposure to pollutants in a coal mining impacted system.

6.5.2.2. *Mokolo River*

The negative correlations of fish with abiotic variables such as alkalinity and Total_N suggest that the fish population in the Mokolo River is sensitive to increases in these variables. Alkalinity is known to affect the chronic and acute toxicity of metals (DWAF, 1996) and due to the fact that the Mokolo River has low alkalinity levels, it may explain why they are sensitive to alkalinity changes (De Klerk et al., 2016). In contrast to the invertebrate fauna, the negative impact of nutrient enrichment on the fish fauna may have resulted in the difference in fish diversity observed at the upstream sites (MR01 – MR03) compared to the rest. Again, this may be linked to current agricultural practices taking place (Seaman et al., 2013). The increased fish diversity / richness downstream at MR04 – MR08 may also be linked to the natural increase in organic material that takes place in the lower reaches of a river (Dodds and Welch, 2000). However, at the most downstream site (MR09) the poor water quality recorded during this study at this site may be as a result of its proximity to the Limpopo River, which is known for poor water quality and may therefore affect the fish assemblages (Ashton, 2007). The ROS analysis of the fish, as with the invertebrates, indicated a decreasing trend following a downstream gradient. This may be linked to the degree of agriculture taking place in the upper reaches, compared to the lower reaches.

In general, the sensitivity of the fish populations in the Mokolo River was much higher than the sensitivity of the populations in the upper Olifants River. In the Mokolo River the fish fauna also appear to be less affected by the habitat availability, as the Mokolo River largely had a lower habitat availability compared to the upper Olifants River. This is yet another important consideration for the future management of the Mokolo River.

6.6. CONCLUSION

Through this study various techniques and statistical approaches were utilized to characterize invertebrate and fish fauna in both the upper Olifants and Mokolo rivers. In so doing multiple lines of evidence were generated to comparatively study these catchments for an improved understanding of the impacts on biotic integrity in a catchment subjected to long-term coal mining. This information may aid in not only understanding the current situation in a river system less impacted by coal mining at the moment, but also to better understand which end-points to monitor once the degree of coal mining increases. Form these results it was evident that the impact of nutrients and other associated pollutants (i.e., SO_4^{2-} and metals) in the upper Olifants River was very important. Therefore, the contamination of the Mokolo River needs to be properly managed, especially through AMD, to conserve biotic integrity. This is especially true, as the Mokolo River is possibly more susceptible than the upper Olifants River due to a reduced buffering capacity (as indicated by the lower alkalinity levels) and the correlation between these faunal groups and alkalinity, for example fish. This may have deleterious consequences further up the food chain and may result in an impact on the faunal community, for example the increase in gathering collectors already being observed at MR09. The use of biochemical endpoints proved to be valuable to provide a measure of early detection towards environmental impacts that could be used to mitigate substantial impacts. This is especially true if using an invertebrate model, such as *P.*

warreni, compared to fish. Moreover, it is recommended to include a community assemblage approach when monitoring the Mokolo River, as well as multivariate analyses, which provided a good overall summary of the results obtained through the univariate and bivariate analyses. Due to the uniqueness of such a case study and the fact that both these rivers have an international importance, this study may contribute to international water management practices. In so doing, the ecological and biotic integrity of rivers such as the Mokolo River may be conserved, so as not to share the same fate as the upper Olifants River.

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CHAPTER 7: THE EFFECT OF REHABILITATION MEASURES ON ECOLOGICAL INFRASTRUCTURE IN RESPONSE TO ACID MINE DRAINAGE FROM COAL MINING

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Declaration by the candidate

With regard to Chapter 7, the nature and scope of my contribution were as follows:

Nature of contribution	**Extent of contribution**
Conceptual design, fieldwork, experimental work, manuscript writing.	70%

The following co-authors have contributed to Chapter 7:

Name	**Email address and Institutional affiliation**	**Nature of contribution**	**Extent of contribution**
Prof A-M Botha	ambo@sun.ac.za Department of Genetics, Stellenbosch University	Conceptual design, manuscript editing.	30%
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Mrs LM Schaefer	lschaefer@csir.co.za Natural Resources and the Environment, CSIR	Experimental work.	

7.1. ABSTRACT

Water treatment is an important ecosystem service in natural systems and wetlands are well-known to have increased functionality in this regard. The rehabilitation of productive wetlands plays an important role in improving this functionality in degraded wetlands, but it is not well-known to what extent these activities improve acid mine drainage impacted waters. The aim of this study was to evaluate the significance of the effect of such interventions on the ecological functioning of a test wetland affected by acid mine drainage. A degraded wetland influenced by acid mine drainage in the Mpumalanga Province of South Africa was identified for the case study. This site was rehabilitated using a variety of hard (e.g., weirs / dams) and soft (e.g., earth berms) structures and examined to determine whether its ecological functioning (i.e., the improvement of water quality) and biotic community structures have improved. From the results it was evident that a substantial improvement in water quality occurred below the rehabilitated area, even though the wetland still continued to receive acid mine drainage. This was observed through the decrease in metal pollution in conjunction with an increase in pH and alkalinity levels. This resulted in increased productivity, reduced toxicity (embryotoxicity and teratogenicity), as well as changes in the biotic community structures which were a reflection of a less polluted environment. The study has shown that the rehabilitation of ecological infrastructure can be used to mitigate the effect of coal mining related pollution such as acid mine drainage. In the face of ever increasing land use activities that occur globally to meet the demands of growing populations, this information can be useful to mitigate negative residual influences resulting from these activities, e.g. acid mine drainage.

7.2. INTRODUCTION

Ecological infrastructure refers to naturally-functioning ecosystems (e.g., wetlands) that generate and deliver valuable services to people. It is the nature-based equivalent of built or hard infrastructure and can be just as important for providing services and underpinning socio-economic development (SANBI, 2014). It is well-known that anthropogenic activities can have adverse effects on aquatic ecosystems (Carpenter et al., 1998). The effects of activities such as opencast coal mining that result in a decrease in ecological services provided by these systems, have also been well-documented (Boyer and Wratten, 2010). Both direct and indirect ecosystem services are tangible and typically linked at a broader landscape or catchment scale, such as regulating various ecological processes which contribute to the integrity of ecosystems and a healthy environment (Kotze et al., 2009a; Macfarlane et al., 2014). The upper Olifants River in the Mpumalanga Province of South Africa is a good example of an aquatic ecosystem negatively influenced by, inter alia, intensive coal mining activities (Hobbs et al., 2008). As a result of these anthropogenic activities, it has been described as one of the most polluted rivers in southern Africa, especially due to the effects of acid mine drainage (AMD) (Grobler et al., 1994).

The numerous land use activities taking place in the upper Olifants River catchment rely heavily on ecological services produced by this river and the surrounding wetlands (Dabrowski and De Klerk, 2013). Wetlands provide more ecosystem services per hectare than any other ecosystem, being sites of intense biogeochemical activity that play an important role in improving water quality (Lischeid et al., 2007; Tauchnitz et al., 2010). However, by virtue of their positions in the landscape and relationship to drainage networks, wetlands are frequently affected by coal mining activities, especially opencast methods (Hobbs et al., 2008; Ochieng et al., 2010; Silva et al., 2011). These effects will be ongoing, since coal is a strategic resource in South Africa and will continue to be mined extensively to support the country's development (Hall, 2013). At the same time, however, regulatory authorities and the public now have a greater, more informed understanding of the range of economic, social, ecological and hydrological costs of wetland loss and degradation.

The benefits of investing in the maintenance and rehabilitation of ecological infrastructure are also becoming more evident as research in this domain continues (Fennessy and Mitsch, 1989; Baker et al., 1991; SANBI, 2014). Globally, several different approaches have been used for the rehabilitation of wetlands (Pfadenhauer and Grootjans, 1999; Russel, 2009). The rehabilitation of wetlands focuses on improving wetland ecosystem structure and function, thereby improving the ecological integrity of the system (McKenna Jr., 2003; Porter and Nairn, 2008). This methodology incorporates the physical, chemical and biological components of a wetland ecosystem at different trophic levels. At present, the Highveld and Witbank coalfields in the catchment of the Olifants River are the source of more than 80% of South Africa's total coal outputs (Hancox and Götz, 2014). However, these coalfields are nearing depletion and as a result, the relocation of operations to other parts of the country, such as the Waterberg coalfields in the Limpopo Province of South Africa, is being planned for the near future. A geological assessment has shown that the Waterberg has the potential to generate AMD and proper mitigation strategies should thus be in place if the need arises (Bester and Vermeulen, 2010).

The capacity of a biological system to sustain an integrated and adaptive system is known as ecological integrity. Maintaining the ecological integrity allows for the sustainability of the full range of elements and processes occurring within such a system (Innis et al., 2000). Monitoring the ecological integrity of ecological infrastructure is beneficial as it allows for an indication of the integrated effect of the activities taking place in a specific catchment (Niemi and McDonald, 2004). As it is not practical to monitor each parameter associated with an aquatic ecosystem in detail, certain indicators (biotic and / or abiotic) are used as an indirect indication of the integrity of the ecosystem (State of Rivers Report, 2001; Wolkersdorfer, 2012). Microorganisms (e.g., bacteria) play a vital role in the biogeochemical recycling of wetlands and are very important for the removal of pollutants (Gutknecht et al., 2006; Faulwetter et al., 2009). Many species are capable of functioning under both aerobic and anaerobic conditions and respond to changing environmental conditions (Kirchman, 2002; King et al., 2013). They can therefore be ideally used as indicators of the parameters affecting wetland functioning, like low pH (Tian and Hua, 2010; Bueche et al., 2013).

On the other hand, algae are primary producers and therefore at the base of most aquatic food webs (McCormick and Cairns Jr., 1994). They have been used as water quality indicators in various biomonitoring tools (Taylor et al., 2007), providing useful time-integrated water quality information, as they can respond rapidly to water quality changes (Bate et al., 2004), especially since many algal species have specific ecological, chemical and physical preferences (Bellinger and Sigee, 2010). Monitoring the teratogenic potential and embryotoxicity of surface waters has also been widely implemented to monitor the effects of pollutants in aquatic ecosystems (Tietge et al., 2000). This is especially important to evaluate the developmental toxicity linked to AMD (Dawson et al., 1985) and therefore provides useful information to the quality and risk of ecological infrastructure (Hoke and Ankley, 2005).

As coal mining will continue in South Africa, there is a need to determine whether wetland rehabilitation can improve degraded water qualities received from AMD and as such improve the ecological infrastructure. The objective of this study was thus to evaluate the outcome of wetland rehabilitation methods on the water quality and ecological integrity of the aquatic ecosystem influenced by coal mining activities. The outcomes of this research is of global importance since the sustainable development of our natural resources are crucial to a growing population, especially when considering the exploitation of the virgin coalfields in the Waterberg region of South Africa. It will also improve the ability to manage already exploited areas, such as the Olifants River basin, and aid its recovery. Thus, the aims for this study were to: (a) determine the degree of improvement in selected water and sediment quality parameters; (b) determine the effects of the rehabilitation efforts on the ecological integrity through the evaluation of the changes observed in the bacterial and algal assemblages; and (c) determine the changes in the embryotoxicity and teratogenicity potential of the surface waters which can be linked to human health.

7.3. MATERIALS AND METHODS

7.3.1. Study Area

The Grootspruit Wetland (25° 54' 25.93" S; 29° 3' 12.09" E, WGS84) is a tributary of the Zaalklapspruit Wetland system and is situated in the upper Olifants River catchment of the Mpumalanga Province of South Africa (Figure 7-1). The portion of wetland selected for rehabilitation has been affected by historical cultivation and artificial drainage resulting in channel incision, as well as high concentrated flows (>0.5 m/sec) through the wetland. These concentrated flows reduced the ability of the wetland to filter and clean the water flowing through it. In addition, the wetland receives AMD from an upstream coal mining operation. Study sites were strategically selected above and below the conglomeration of interventions in the rehabilitation area (described below), along with a suitable reference site higher up in the catchment. The objective with the site selection was to obtain an understanding of the difference in water quality entering and exiting the rehabilitated area. A general overview of ecosystem characteristics of the upstream, downstream and reference sites were determined according to Kotze et al. (2009a) and Oberholster et al. (2014a) and is presented in Table S 7-1, Supplementary Material. The study area falls within a summer rainfall region (thus receiving rain mostly from November to February). Samples were collected during this period, before and after rehabilitation, in order to avoid the influence of seasonal dynamics. Three sampling trips were undertaken during this period at the selected sites prior to rehabilitation (2012 / 2013) and, once rehabilitation was completed, three post-rehabilitation sampling trips were additionally conducted (2013 / 2014). The results obtained from the two sites upstream and the two sites downstream were pooled to give an overall picture of the conditions of the upstream and downstream areas both before and after rehabilitation. The results from the reference, upstream and downstream sites before and after rehabilitation are referred to as follows: Ref-Pre, Ref-Post, Down-Pre, Down-Post, Up-Pre and Up-Post.

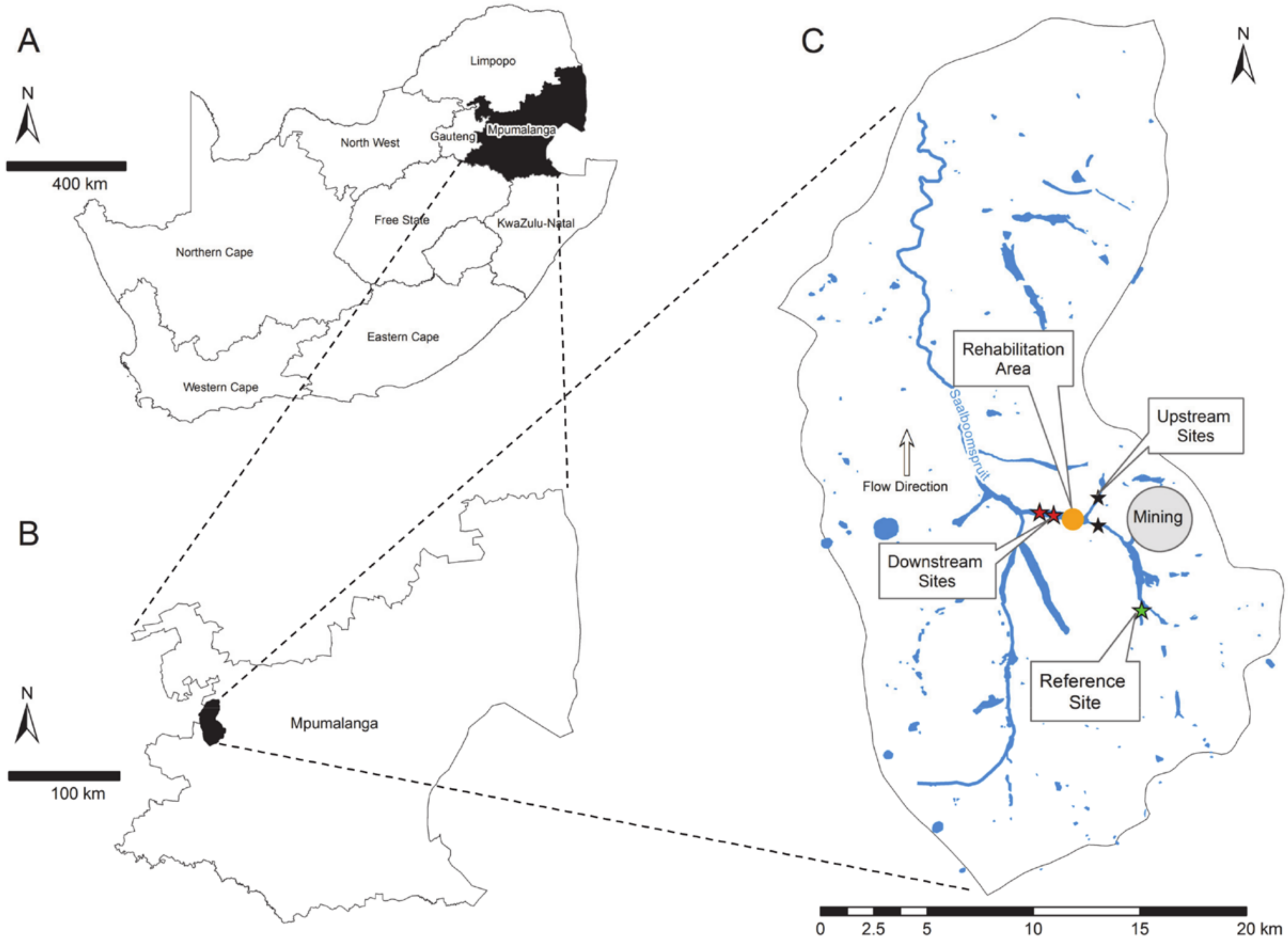

Figure 7-1: Study area in the Mpumalanga Province of South Africa (A), within the B20G catchment (B). Location of the respective sampling sites, the rehabilitation area, as well as the mining area is presented (C).

7.3.2. Rehabilitation Interventions

The Grootspruit Wetland is a naturally un-channelled valley bottom wetland system (≈140 ha). Based on the fact that the main aim of the rehabilitation project was to investigate the ability of rehabilitation interventions to enhance the water quality treatment function of a system receiving AMD, 13 different intervention points were identified for effective rehabilitation (Figure 7-2A). These areas were identified to be strategic areas in the wetland to potentially improve the overall functioning. At each of these sites various activities took place to address different rehabilitation objectives. A detailed list of the different types of interventions, their purpose, etc. is recorded in Table S 7-2, Supplementary Material. The wetland was rehabilitated, inter alia, by using a combination of hard (concrete) and soft (earth berm) man-made structures (e.g., Figure 7-2B, C) to slow down the velocity of the water (<0.2 m/sec) through the eroded channel of the wetland and to redirect it away from the erosion channel with the aid of earth berms. In doing this, it was expected that the water flow would be expanded laterally towards the drained wetland area adjacent to the erosion channel, thereby reinstating the dried wetland. As a result, it was anticipated that the amount of sediment infiltration would increase, which in turn would increase the contact time between water and sediment. Therefore, the wetland rehabilitation could potentially improve biologically mediated processes which strongly affect the water chemistry, e.g. resulting in the removal of contaminants from the water column.

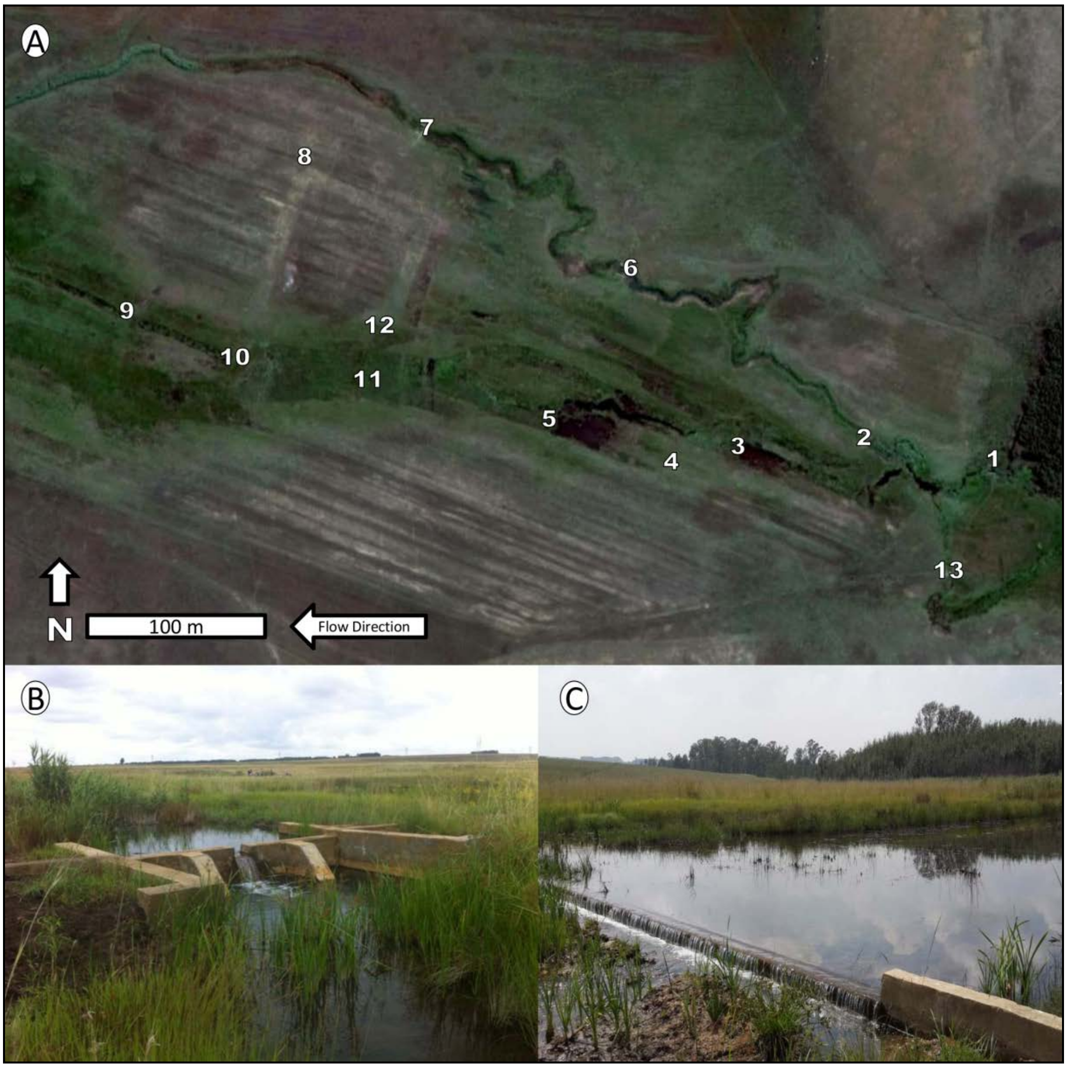

Figure 7-2: Respective locations of the 13 rehabilitation interventions (A), including two examples of these interventions, namely intervention number 2 which is a concrete weir (B) and intervention number 3 which is a low concrete wall (C).

7.3.3. Water and Sediment Sampling

Monitoring changes in water and sediment quality is an efficient means of determining the change that rehabilitation efforts are making in a wetland. Various parameters in the water and sediment phase of the wetland were analyzed to establish the level of change (improvement), if any, after the rehabilitation has taken place. The *in situ* water quality parameters, namely pH and total dissolved salts (TDS conversion factor of 0.64) were measured using a Thermo Five Star handheld water quality meter (Thermo Scientific, USA). Water samples were collected in 1 L bottles and kept on ice according to de Klerk et al. (2012). Sediment core samples (top 10 cm) were collected according to USEPA (2001). Sediment samples were transferred into pre-cleaned plastic containers and stored in a freezer at -20 °C until analysis could be performed, in order to prevent the loss of organic matter through digestion by invertebrates and organic decomposition by bacteria. Both water and sediment samples were collected in triplicate and on each sample three technical repeats were conducted (n = 9).

7.3.3.1. *Chemical Determinations*

The water samples were filtered using 0.45 µm cellulose nitrate filter paper and analysed for the following major ions and metals: K, Na, Ca, Mg^{2+}, SO_4^{2-}, Cl^-, Al, Fe, Mn, Ni and Zn using an inductively coupled plasma optical emission spectrometer (ICP-OES) (Thermo ICap 6500) and / or inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500cx). For quality control purposes, matrix matched reference standards were analysed in parallel, as well as calculating the ionic balance using the formula of Appelo and Postma (2005). The alkalinity of the surface waters was measured with a TitraLab® titration workstation (Radiometer Analytical TIM860). In addition, the chemical oxygen demand (COD) levels of the water samples were analysed according to the method of Pitwell (1983). For the determination of chlorophyll *a*, the protocol according to Sartory and Grobbelaar (1984) was used. The concentrations of the selected metals in the bottom sediment (same as for the water samples) were analysed using a partial microwave digestion method utilising a CEM Mars Xpress (CEM Corporation) in conjunction with ICP-OES (Thermo ICap 6500). A sediment reference material (PACS-2 NCR material) was used for quality control. The data median and data spread are indicated in Figure 7-4 for both water and sediment samples. The sediments were also analysed for total organic carbon (TOC) content using a Vario EL Elementar III Elemental Analyser Instrument (Elementar, Germany) and using a sediment reference standard (MBSS-2) for quality control. The particle size distribution was determined according to a method adapted from Plumb Jr. (1981) and categorized according Wentworth (1922). During the analysis of the water and sediment samples, the first sample in each batch was duplicated to monitor the reproducibility of the analysis. The acceptable tolerance range for the reproducibility as well as quality control for each element was set at <10% and was achieved.

7.3.4. Bacterial Diversity

Roche 454 pyrosequencing (Roche 454 Life Sciences) was applied to evaluate bacterial diversity. This was done by collecting water (2 L) from the sites. These samples were filtered through 0.45 µm cellulose nitrate filters. DNA was extracted from the remaining cell debris by gently scraping the debris from the filter and resuspending it in 2 mL of 1 × phosphate buffered saline (PBS) (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), pH 7.4. The suspension in 1 × PBS was centrifuged at 13 000 rpm (rotations per minute) to pellet the cells and the supernatant was removed. A DNeasy blood and tissue kit (Qiagen) was then used to extract DNA following the manufacturer's protocol. The universal 16S rRNA primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') (Weisburg et al., 1991) and 518R (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al., 1993) were used to amplify the V1, V2 and V3 hypervariable regions of the gene. Sequencing was carried out on a 454 GS FLX titanium sequencing platform (Roche 454 Life Sciences). Using the sequencing data, BLAST® (Basic

Local Alignment Search Tool, National Center for Biotechnology Information (NCBI)) searches were carried out using the 16S ribosomal RNA sequences (bacteria and archaeal) database. The bacteria identified were subsequently assigned to their known bacterial phyla.

7.3.5. Algal Assemblages

At each site, the presence of epilithic filamentous algae (algae growing on gravel, stones and bedrock) was sampled, identified, assessed and reported separately in Oberholster et al. (2016). This was done by isolating a substrate surface area of 5 cm in diameter for sampling using a syringe extended with a tygon tube (Hauer and Lamberti, 2011). The algal abundance in the samples was evaluated by counting the presence of each species (as cells in a filament or equal number of individual cells). All algae were identified using a compound microscope at 1 250 times magnification according to Taylor et al. (2007) and Janse van Vuuren (2006). The samples were sedimented in an algal chamber and were analysed using the strip-count method (APHA, AWWA, WEF, 2012). The detailed information and list of algal species found are reported in Oberholster et al. (2016); this study only makes use of this information as an additional line of evidence to identify biodiversity improvement.

7.3.6. Teratogenic Potential and Embryotoxicity – Xenopus laevis

The Frog Embryo Teratogenicity Assay *Xenopus* (FETAX) technique was applied according to the standard of the American Society for Testing and Materials (ASTM) (1998), with slight modifications, to evaluate the teratogenic potential and embryotoxicity of surface water collected pre- and post-rehabilitation. Undiluted water samples from the various sites were used and the results were compared to Oberholster et al. (2014b) to determine the effect of the rehabilitation measures on the teratogenic potential and embryotoxicity of the surface waters. Adult female and male *Xenopus laevis* were primed with 50 IU (International Units) and 100 IU human chorionic gonadotropin (hCG), respectively and maintained until breeding glands were prominent on the fore-limbs of males and cloacal labia red and swollen in females. Clasping and spawning were subsequently induced using boosting injections of 300 IU and 500 IU of hCG in male and female frogs, respectively, and housed overnight in 15 L tanks containing FETAX solution (625 mg/l NaCl, 96 mg/l $NaHCO_3$, 30 mg/l KCl, 15 mg/l $CaCl_2$, 60 mg/l $CaSO_4.2H_2O$, 75 mg/l $MgSO_4$). Fertilized eggs were de-jellied by gentle swirling in a 2% w/v l-cystein solution (Sigma) (pH 8.1) after which viable larvae were identified according to Nieuwkoop and Faber (1994) at stages 8 – 11 using a stereo microscope and assigned to exposure vessels. For this study, two replicates, each containing 25 individuals, were used and the test medium was replaced daily. Four negative control replicates, each containing 25 larvae in FETAX solution, were also included in the study as reference. The experiment was performed subject to a 14:10 light:dark cycle at 24 ± 2 °C. The larvae were monitored daily for mortalities, which were confirmed using a stereomicroscope or tactile stimuli, and deceased individuals were immediately removed. All animals were euthanized after a 96 h exposure using 0.1% benzocaine (Heynes Mathew, Ltd.) and transferred to buffered 10% formalin. Developmental malformations were classified according to Bantle et al. (1994) under Leica EZ4 and ES2 stereo microscopes and photographed. Teratogenicity was expressed as malformation incidence (%). Moreover, growth inhibition was determined by comparing the total lengths of exposed individuals to that of the control group. The developmental stages of tadpoles were classified according to Nieuwkoop and Faber (1994). Animal husbandry, treatment and handling were performed according to the South African National Standard: The Care and Use of Animals for Scientific Purposes (SANS 10386:200X). All breeding and general maintenance procedures, as well as the experimental protocol, were approved by the Animal Ethics Committee of the Stellenbosch University (Approval No. SU-ACUM 12-00013 and SU-ACUM 12-00014).

7.3.7. Statistical Analysis

All the data obtained during the present study were evaluated for normality using the Kolmogorov-Smirnov test, the Shapiro-Wilk W test, as well as the Lilliefors test. Levene's, Brown and Forsythe's tests were used to assess the homogeneity of variance. Statistical differences between the pre- and post-rehabilitation conditions were evaluated using an Analysis of Variance (ANOVA) test in combination with a Tukey's unequal N Spjotoll-Stoline corrected HSD *post hoc* test. In the event of non-parametric data being obtained, a Kruskal-Wallis ANOVA test in combination with a multiple comparison of mean ranks *post hoc* test was used (Statistica 12, Statsoft, US). Statistical significance levels were determined using a probability value of $p < 0.05$. A Durov diagram was used to evaluate changes in the water qualities at the selected sites before and after rehabilitation (Durov, 1948). The median values of the water and sediment variables measured, as well as respective detection limits, are presented in Table S 7-3, Supplementary Material. Where measurements were below detection, the commonly used substitution of below detection limit data by half of the detection limit value was used (Singh and Nocerino, 2002). A Shannon diversity index (H') (Shannon, 1948), was used to determine the diversity of the bacteria and algae at the different sites before and after rehabilitation.

7.4. RESULTS

7.4.1. Water and Sediment Characterisation

The pH and alkalinity (expressed as HCO_3^- + CO_3^{2-}) of the downstream sites, 5.30 and 3 mg/l, respectively, increased significantly ($p < 0.05$) after rehabilitation to 7.6 and 22 mg/l, respectively (Table S 7-3, Supplementary Material). The pH for the water is thus within the optimal range for healthy aquatic ecosystems after rehabilitation (DWAF, 1996), whilst the alkalinity concentrations are more comparable to that of the reference site (≈27 mg/l). The alkalinity upstream remained low throughout the study period (<0.5 mg/l). The TDS and SO_4^{2-} concentrations decreased significantly (p < 0.05) downstream after rehabilitation (≈610 mg/l and ≈600 mg/l, respectively) when compared to the concentrations before rehabilitation values (≈940 mg/l and ≈980 mg/l, respectively). The Durov diagram (Figure 7-3A) shows that the water quality signature of both the downstream and upstream sites corresponded to a "Ca_2^+/SO_4^-" type, whilst the reference site corresponded to "$Na^++K^+/HCO_3^-+CO_3^{2-}$" type. The COD levels also decreased significantly downstream (from ≈11.5 mg/l to ≈4.25 mg/l) whilst the upstream levels remained similar (≈7 mg/l) (Figure 7-3C). From the sediment characterisation it was evident that all of the sites consisted mainly of sand (0.063 – 2 mm), whilst some decrease in the percentage sand at the upstream sites before rehabilitation was observed (Figure 7-3B). The TOC levels in the sediment were found to decrease at all of the sites during the second phase of sampling (Figure 7-3D).

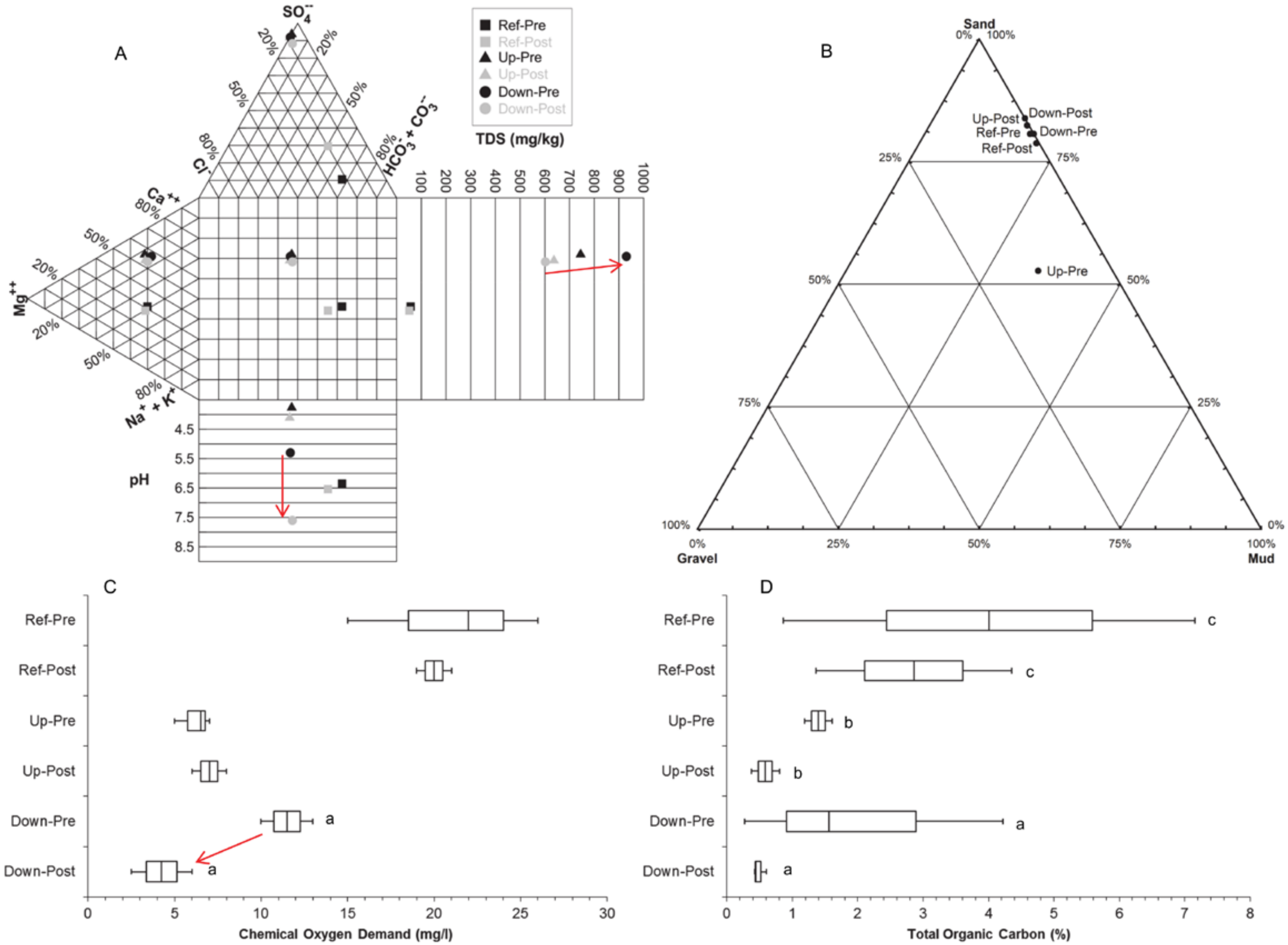

Figure 7-3: Recorded water and sediment quality variables at the different sites before and after rehabilitation: (A) main ions, pH and total dissolved salts (TDS) presented in a Durov diagram; (B) percentage gravel, sand and mud in the sediment; (C) chemical oxygen demand in the water; and (D) total organic carbon in the sediment. The box and whisker plots indicate the median line inside the box, whilst the whiskers are set at 1.5 times above and below the third and first quartile. Similar superscripts indicate significant differences. Arrows indicate the major changes observed at the downstream sites after rehabilitation.

The metals primarily associated with influences from AMD are Al, Fe, Mn, Ni and Zn. The concentrations of these metals before and after rehabilitation in both the water and sediment are shown in (Figure 7-4). The concentrations for most metals measured in the water from the reference site were very low. The Al concentration in the water decreased significantly from ≈890 µg/l to below the detection limit (<5 µg/l) after rehabilitation at the downstream site (thus, a decrease of ≈99%). The rest of the metals selected also decreased significantly, namely Fe ≈76% (from ≈29.5 µg/l to 7 µg/l), Mn ≈96% (from ≈7 000 µg/l to ≈250 µg/l), Ni ≈97% (from ≈210 µg/l to ≈6 µg/l) and Zn ≈99% (from ≈380 µg/l to ≈5 µg/l).

On the other hand, the opposite trend was observed for the metals measured in the sediment, showing significant ($p < 0.05$) increases after rehabilitation. Aluminium increased with ≈49% (from ≈17 000 mg/kg to ≈25 290 mg/kg), Fe ≈254% (from ≈11 000 mg/kg to ≈39 000 mg/kg), Mn ≈767% (from ≈67 mg/kg to ≈581 mg/kg), Ni ≈160% (from ≈10 mg/kg to ≈26 mg/kg) and Zn ≈233% (from ≈13.5 mg/kg to ≈45 mg/kg).

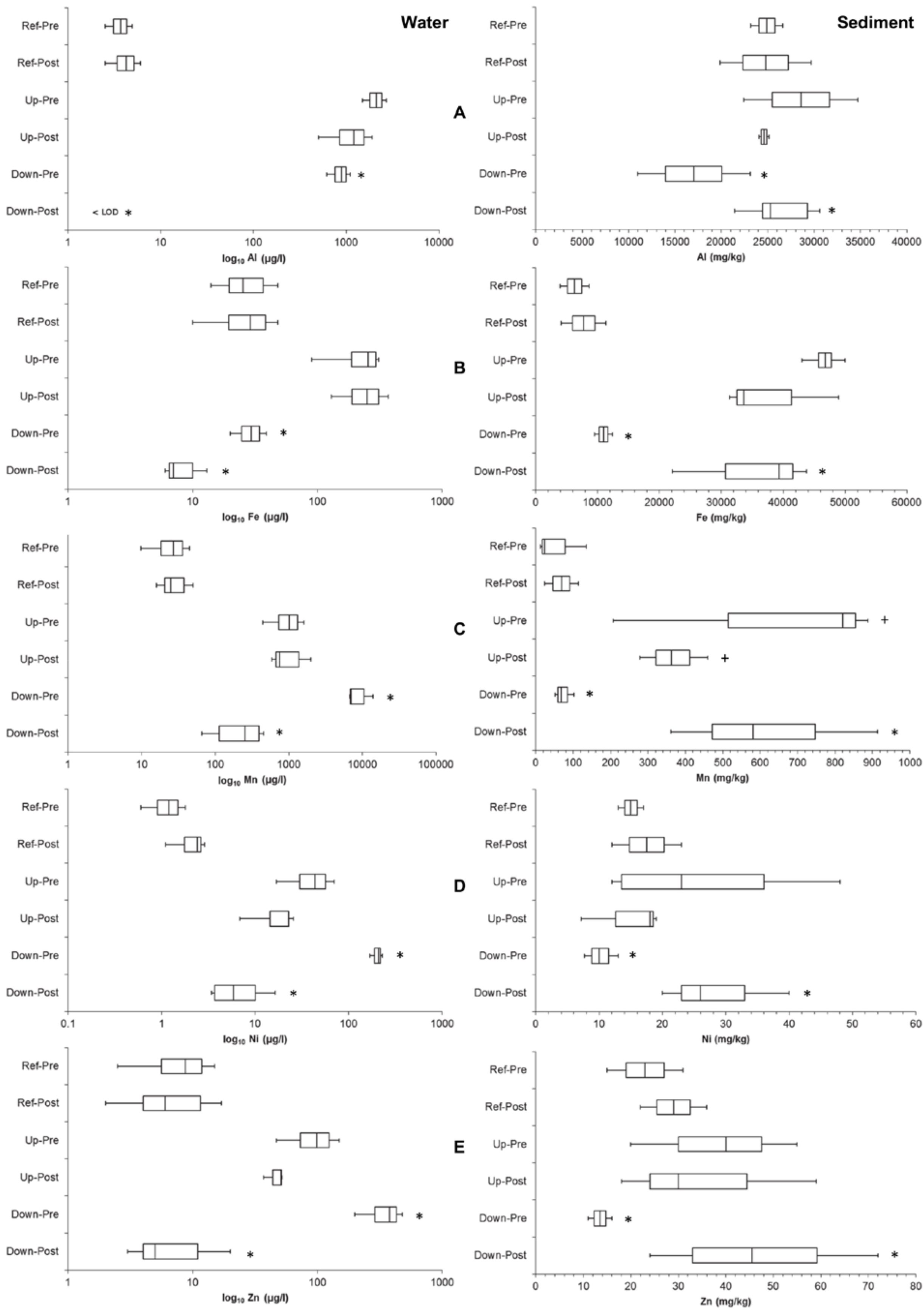

Figure 7-4: Box and whisker plots of the metal concentrations (A: Al B: Fe; C: Mn; D: Ni and E: Zn) in the water (left column, logarithmic scale) and sediment (right column) at the different sites before and after rehabilitation. The box and whisker plots indicate the median line inside the box, whilst the whiskers are set at 1.5 times above and below the third and first quartile. Similar symbols indicate significant differences.

7.4.2. Bacterial Consortium and Freshwater Algae

At the downstream site the most abundant described bacteria were mainly from the phylum Proteobacteria (66.83%) prior to rehabilitation (Table 7-1). Bacteria belonging to the phylum Firmicutes, Bacteriodetes and Actinobacteria were also present with relatively low contributions. After rehabilitation, bacteria from the phylum Firmicutes increased significantly ($p < 0.05$) to 37.29%. The relative contribution by the other bacterial groups also changed, namely Proteobacteria (43.80%), Actinobacteria (2.55%) and Bacteriodetes (4.15%). There was also a small occurrence of bacteria from the phylum Planktomycetes (0.17%) and Verrucomicrobia (0.08%) at the downstream sites after rehabilitation. These findings corresponded to the changes in the calculated bacterial diversity at the downstream sites which increased after rehabilitation (from ≈0.7 H′ to ≈1.1 H′), whilst the diversity at the upstream sites remained similar (≈1 H′) (Figure 7-5A). The high variance obtained in the H′ values from the reference site before rehabilitation, may affect the statistical significance of the changes observed at the downstream sites. However, the similar H′ scores recorded at the upstream sites before and after rehabilitation, with slight variance, can be used to substantiate the increase in bacterial diversity observed at the downstream sites directly below after rehabilitation.

According to Oberholster et al. (2016), the green filamentous algae *Mougeotia* cf. *laevis* and the diatoms *Craticula buderi* and *Nitzschia clausii* were found to be dominant at the downstream sites before rehabilitation. After rehabilitation the downstream sites became dominated by *Klebsormidium rivulare* (kutzing) as well as the diatoms *Tabellaria flocculosa* (roth) and *Nitzschia nana* (grunow). This was related to the changes in the calculated algal diversity which increased from ≈1.7 H′ to ≈2 H′ (Figure 7-5B). After rehabilitation the chlorophyll *a* concentrations at the downstream sites also increased from ≈2 µg/l to ≈6 µg/l (Figure 7-5C).

Table 7-1: The relative presentation (% composition) of the various bacterial communities identified using Roche 454 pyrosequencing at the selected sites. ND = Not detected.

Community	Ref-Pre	Ref-Post	Up-Pre	Up-Post	Down-Pre	Down-Post
Firmicutes	0.12	35.57	3.34	51.66	0.25	37.29
Proteobacteria	64.13	46.24	48.83	29.59	66.83	43.8
Bacteriodetes	ND	4.21	0.41	0.86	0.25	4.15
Cyanobacteria	ND	1.83	0.11	0.32	0.12	ND
Actinobacteria	1.64	0.54	3.55	7.02	0.64	2.55
Deinococcus-Thermus	ND	ND	0.71	0.37	ND	ND
Acidibacteria	ND	ND	ND	0.04	ND	ND
Verrucomicrobia	ND	0.27	ND	0.08	ND	0.08
Planctomycete	ND	0.13	ND	ND	ND	0.17
Chloroflexi	ND	ND	ND	0.07	ND	ND
Gemmatimonadetes	ND	ND	ND	ND	ND	ND
Fusobacteria	ND	ND	0.59	ND	0.89	ND
Tenericutes	1.14	ND	2.01	ND	ND	ND
Aquificae	ND	ND	ND	0.91	ND	ND

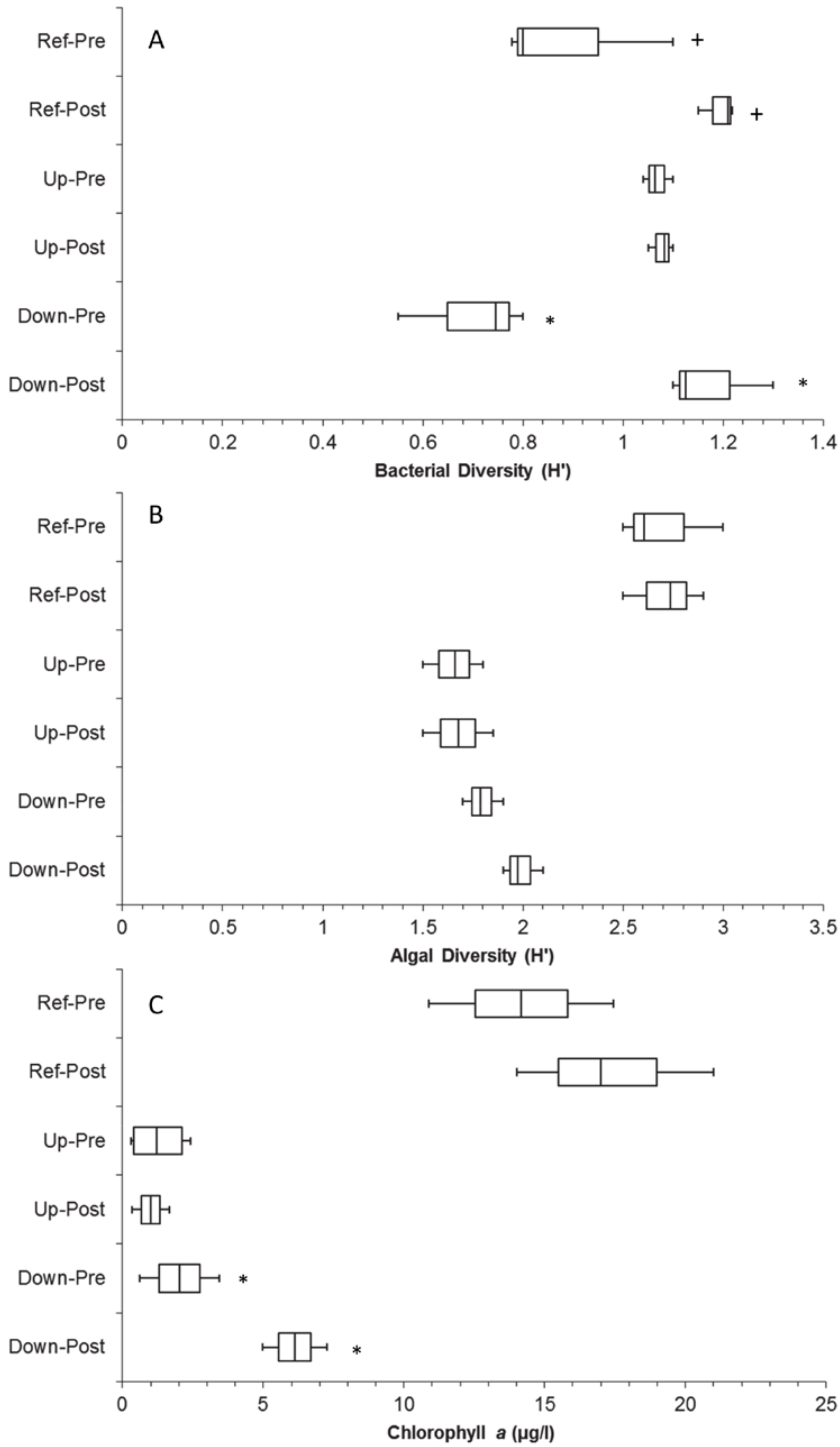

Figure 7-5: Shannon diversity index values of the bacterial consortium (A) and algal community (B), as well as chlorophyll *a* concentrations (C) at the different sites before and after rehabilitation. The box and whisker plots indicate the median line inside the box, whilst the whiskers are set at 1.5 times above and below the third and first quartile. Similar symbols indicate significant differences.

7.4.3. Teratogenic Potency and Tadpole Embryotoxicity

According to Oberholster et al. (2014b), the incidence of malformations before rehabilitation was higher at the upstream site (up to 89%), than the downstream site (≈30%) (Figure 7-6A). However, after rehabilitation this study observed lower frequencies of malformations for both the upstream and downstream sites (Figure 7-6A). In contrast to the results from before rehabilitation, the proportions of malformed individuals were lower at the downstream site (≈7.5%) compared to the negative control after rehabilitation. A significant decrease in the incidence of malformations was also seen after rehabilitation at the downstream sites compared to upstream (≈187% decrease), whilst this was not the case before rehabilitation. No significant difference was observed using the tadpole length measurements among the different treatment groups (Figure 7-6B). After rehabilitation, a high proportion of larvae exposed to water collected upstream from the rehabilitated area suffered from malformations in the digestive tract (Figure 7-6C). Malformations in the developing tadpoles observed in this study included thoracic oedema, gut malformation (i.e., improper gut coiling), axial deformity and abdominal oedema. The incidence of the gut malformations (which included improper coiling of the gut, abdominal oedema and diverted gut) were lower compared to before rehabilitation in tadpoles exposed to downstream water after rehabilitation.

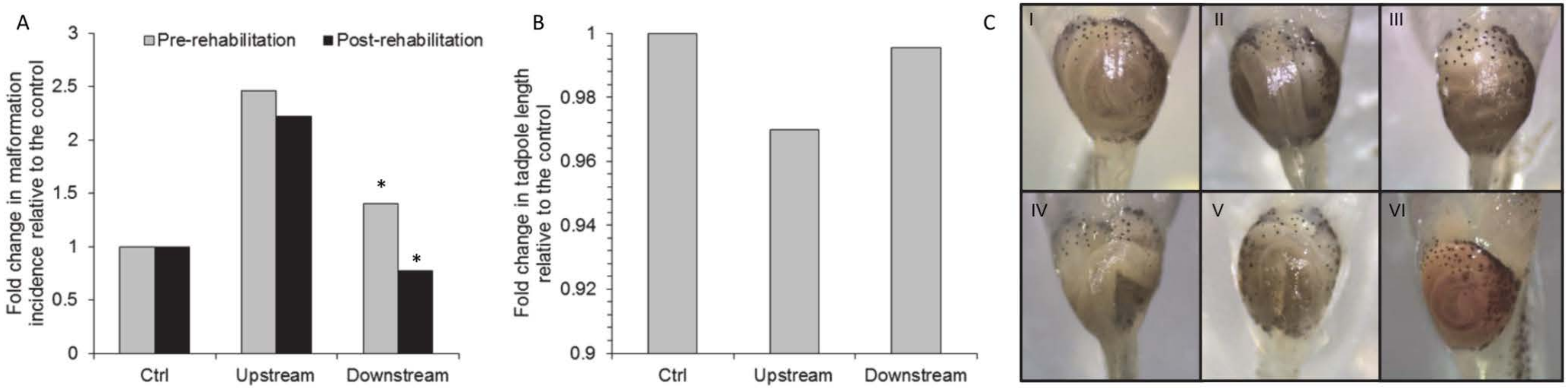

Figure 7-6: Incidence of malformations in *Xenopus laevis* larvae exposed to water from the different sites before and after rehabilitation (A); changes in tadpole length after rehabilitation (B); and macroscopic imagery of the gastro-intestinal tracts (C) of a normally developed tadpole (I) exposed to FETAX solution (negative control); abnormally developed gastro intestinal tracts (i.e., improper coiling) in tadpoles exposed to surface water collected upstream of the rehabilitated area (II – V); and a tadpole exposed to surface water collected at the downstream site after rehabilitation (VI). Asterisks indicate significant differences.

7.5. DISCUSSION

During the rehabilitation of ecological infrastructure, measures are taken to return an affected system to a similar condition prior to the influence that took place and to prevent any further degradation (Tanner, 2007; Kotze et al., 2009b). This is very important so as to restore ecosystem structure, function, biotic composition and associated ecosystem services (Cowden et al., 2014). Water quality enhancement is a very important ecosystem service provided by wetlands which may have a substantial effect further downstream (Kotze et al., 2009a). This is especially true where systems are affected by AMD and where rehabilitation could potentially play a substantial role in reducing the effect of polluted water downstream.

7.5.1. Water and Sediment Characterisation

The assessment of water quality is essential to determine the outcomes of ecological rehabilitation. The rehabilitation efforts evaluated during the present study allowed for metal removal and neutralization as well as increased alkalinity. Thus, it was observed that the rehabilitation had a positive effect on the water quality downstream from the rehabilitated area. This was further evident from the observed reduction in metal concentrations (namely Al, Fe, Mn, Ni and Zn) in the water column which may be linked to the observed increase in pH as well as alkalinity. For example, Al decreases in solubility at a pH level above 5 (Nordstrom and Ball, 1986). On the other hand, the precipitation of Fe is more complex and relies on pH levels, redox potential and the presence of potential ligands such as sulfate and carbonate anions in the water (Konhauser and Ferris, 1996). A reducing environment and the presence of suitable bacteria are also essential for the removal of ferric (Fe^{3+}) and ferrous (Fe^{2+}) iron (Hedin et al., 1994; Robbins and Norden, 1994), whilst the decrease in Mn may be as a result of co-precipitation (Hem, 1963; Soylak, 2007). The present study area (the Grootspruit Wetland) however, consists of anaerobic regions as well as oxygen-rich overland surface water flow, which could have contributed to the significant reduction in metals after rehabilitation (Hedin et al., 1994).

The significant decrease of Ni and Zn in the water column after rehabilitation is probably due to the formation of highly insoluble sulfide compounds (Ettner, 1999; Smith et al., 2001). During the reduction of sulfate through sulfate reducing bacterial activity, hydrogen sulfide is formed which reacts with the metals to form the highly insoluble compounds (Stumm and Morgan, 1996). These insoluble compounds then precipitate onto the bottom sediment and will remain stable within the wetland as long as the saturated and / or anaerobic conditions are maintained. The results from the sediment analysis confirmed this and showed significant increases in all of the metal concentrations in the sediment after rehabilitation, which further supports the observed improvement in water quality downstream. Long-term monitoring is needed to ensure the rate of metal accumulation in the sediment does not exceed the natural threshold of the system. In the present study the decrease in the COD levels, which is known as a useful indicator of water quality in streams (Kang et al., 1999; Sánchez et al., 2007), downstream after rehabilitation, further confirmed the general improvement in water quality after rehabilitation. The fact that COD measures the equivalent portion to that of the organic material in an environment susceptible to oxidation by a strong chemical oxidant makes it a reliable water quality indicator (Morrison et al., 2001). Along with COD, the increase in chlorophyll *a*, a proxy for the productivity of the system, also supports an improvement in water quality, suggesting increased primary productivity which can be related to improved ecological integrity (Hellawell, 1986; Paisley et al., 2003).

7.5.2. Bacteria and Algae

Microbial communities play a vital role in cycling, transforming and removing of pollutants in water (Faulwetter et al., 2009). This water treatment function is affected when changes in the microbial communities take place that affect the diversity of the population. Pyrosequencing allowed us to observe these changes as it allows for high throughput sequencing of a large number of partial mixed 16S rRNA sequences in parallel (Claesson et al., 2009). The lowest number of reads (469 on average) was obtained from the upstream sites, which is closest to the mining influence, indicating low bacterial diversity. This corresponded strongly with the low water pH levels. Many of the bacteria present at both the upstream and downstream sites prior to rehabilitation were found to have the ability to survive under extreme conditions in harsh environments (Tian and Hua, 2010; Bueche et al., 2013).

A comparison of the results from the downstream sites before and after rehabilitation indicated a shift in the community structure towards an increase in diversity which was associated with an increase in abundance of bacteria from the phylum Firmicutes. This was in contrast to bacteria belonging to the phylum Proteobacteria which had the largest representation prior to rehabilitation. Proteobacteria are the largest and phenotypically the most diverse phylogenetic lineage and several representatives are ecologically important as they play key roles in the carbon, sulfur and nitrogen cycles (Kersters et al., 2006). The shift from a minimal presence of Firmicutes at the downstream sites to a large representation after rehabilitation is reiterated in a study by Pankratov et al. (2011) which showed that Firmicutes are known to be key players in cellulose degradation in neutral habitats. This further highlights the water quality improvement downstream after rehabilitation. Species from the phylums Bacteriodetes and Actinobacteria also increased significantly at the downstream sites after rehabilitation and the community composition at these sites resembled that of the reference site. As the members from these bacterial phyla are key players in biopolymer degradation (Kirchman, 2002; King et al., 2013), these bacteria may provide the foundation for healthy wetland functioning.

The distinct community shift observed after rehabilitation at the downstream sites with regard to the replacement of the dominant tolerant diatoms, *Craticula buderi* and *Nitzschia clausii*, with *Tabellaria flocculosa* (Roth) is an indication of oligotrophic circumneutral waters (Taylor et al., 2007). In addition, the presence of *Nitzschia nana* at the downstream site after rehabilitation also confirmed a moderately polluted environment according to Oberholster et al. (2016). Therefore, the information gained from the algal assessment is in line with the other results from this study and highlights the improvement in water quality observed at the downstream sites after rehabilitation.

The increase in algal diversity at the downstream sites after rehabilitation, corresponded to the general improvement in water quality parameters (increase in pH and alkalinity as well as decreases in metal pollution and COD) and also corresponded to the general increase in primary production (as indicated through the chlorophyll *a* levels). This is because algae are often affected by a low pH (usually 5.5 and lower), as this is usually the window during which inorganic carbon, which is essential for photosynthesis, rapidly starts to become depleted (Rao, 1989; Solimini et al., 2006). This may also explain the changes in the algal assemblages, because a reduction in inorganic carbon, along with the increase in the availability of metals associated with AMD (i.e., Al), have the ability to stimulate the proliferation of certain filamentous green algae (Charles, 1991; Turner et al., 1991; Verb and Vis, 2005). The resulting proliferation / domination of filamentous algae may therefore hinder the growth of other algal species (Oberholster et al., 2013). The results from the algal analysis therefore provide an additional line of evidence highlighting the improvement in ecological integrity downstream of the rehabilitated area.

7.5.3. Teratogenic Potency and Embryotoxicity

The FETAX assay is a well-established biological assay used to detect the teratogenic potential (i.e., drive towards malformation) of chemicals or surface water samples (Hoke and Ankley, 2005). The proportions of malformed individuals were lower at the downstream site after rehabilitation than the negative control treatment, suggesting that the wetland rehabilitation strategy improved the quality of water in terms of the embryotoxicity and teratogenic potential. This is in contrast to the pre-rehabilitation study at the same locality where a higher teratogenic potency than the negative control was found, thus providing further evidence that the wetland rehabilitation strategy that was employed, improved surface water quality. Previous studies have shown gut malformations in association with metal exposure (Plowman et al., 1994; Bacchetta et al., 2012; Peltzer et al., 2013), which may explain the high proportion of gut malformations and abdominal oedema observed at the upstream sites. As a result, the improved water quality obtained from the rehabilitation efforts, which resulted in the reduction of metal pollution, may therefore explain the decrease in the degree of malformations observed at the downstream sites after rehabilitation. Effects observed using FETAX have been shown to correlate highly with those observed in mammalian model organisms and the information generated will thus also be valuable in environmental risk assessments relating to other species (Hoke and Ankley, 2005; Leconte and Mouche, 2013).

7.5.4. Overall

The efficiency of wetlands depends on several mechanisms such as flow rate, rate of removal by filtration (via plant roots), sedimentation, etc. (Perkins and Hunter, 2000).

Adjusting the flow patterns through the wetland to create more diffuse patterns of flow is important for the treatment of polluted water, because it increases the effectiveness of the assimilative capacity of such a system (Kotze et al., 2009b). This is because the height of the water table, depth from the surface, distance from plant roots, etc. result in a complex relationship between anaerobic and aerobic conditions, which lead to a variety of processes occurring in wetlands (Gutknecht et al., 2006). Therefore, the formation of more diffuse patterns of flow through the use of mechanical rehabilitation is one of the main contributors to the positive response seen during this study. This also promotes an increase in wetland vegetation which obstructs the flow of water, allowing for more effective sedimentation (Younger et al., 2002; Davies et al., 2003). Furthermore, wetland vegetation is able to accumulate and store various pollutants such as nutrients and metals, prevent erosion through soil stabilization, etc. (Sheoran and Sheoran, 2006). Oxygen transported to the roots and rhizomes is transferred to the rhizosphere and can also stimulate microbial growth that further aids in water treatment (Brix, 1997).

Through the different assessments done during this study it was observed that a statistically significant improvement in the AMD impacted surface water was observed after rehabilitation. This corresponded well with an improvement in both the bacterial and algal community structures, resulting in increased diversities as a result of the improved water quality. The positive effect of the rehabilitation measures was further evident through the reduction in the embryotoxicity and teratogenic potential of the surface waters. From these results it was evident that the rehabilitation measures had a positive influence on the ecological integrity of the wetland system, resulting in improved ecosystem services that significantly reduce downstream effects. The rehabilitated wetland is expected to continue to remove metals from the water and increase the pH of the surface water as long as the sulfate reducing bacteria remain active (Gutknecht et al., 2006; Koschorreck, 2008; Das et al., 2009). Long-term monitoring is now needed to determine the assimilative capacity of such a rehabilitated system that continually receives AMD.

7.6. CONCLUSION

The AMD influenced wetland used during this case study showed a positive response to the mechanical rehabilitation efforts within a very short period of time. The water quality downstream of the rehabilitated area improved significantly after rehabilitation through a significant decrease in the filtered metal concentrations. Through the rehabilitation of this wetland, we have found that the species diversity of both the bacteria and algal assemblages increased after rehabilitation and corresponded with an increase in primary productivity. This further highlights the improved functioning of this ecosystem and / or ecological integrity after rehabilitation. The reduction of the embryotoxicity and teratogenic potential of the surface waters after rehabilitation also corroborates the positive effect of the rehabilitation measures. From the results of this case study it can be seen that there is significant potential for the use of rehabilitation interventions to improve not only the ecological integrity of an AMD impacted system, but also the ecosystem services (i.e., water treatment) of such a system, which has far reaching benefits for downstream users. Thus, these results are of global importance to ensure the sustainable development of water resources that need to be able to cope with and recover from stresses and shocks, and to maintain or enhance its capabilities and assets so as not to undermine the natural resource base. This is of particular concern for South Africa where the potential of negative influences of AMD in other regions (for example the Waterberg area, which is known as a water scarce region) have been confirmed. Therefore, mitigating water quality problems via rehabilitation holds a promise for the future sustainability of this area.

7.7. REFERENCES

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7.8. SUPPLEMENTARY MATERIAL

Table S 7-1: An overview of the general ecosystem characteristics at the reference, upstream and downstream sites.

Parameter	Classification	Ref-Pre	Ref-Post	Up-Pre	Up-Post	Down-Pre	Down-Post
Main Land Use		Mainly Natural		Farming, Dams		Livestock Grazing, Historic Cultivation	
Slope	**Low / Moderate / High**	Low		Low		Low	
Erosion Control Ability	**Poor / Moderate / Good**	Good		Poor		Poor	Good
Vegetation Establishment	**Poor / Moderate / Good**	Good		Moderate		Poor	Good
Sediment Type	**Mud / Sand / Gravel**	Mud / Sand		Mud / Sand		Mud / Sand	Mud / Sand
Sediment Trapping Ability	**Poor / Moderate / Good**	Good		Poor		Poor	Good
Bank Stability	**Poor / Moderate / Good**	Good		Poor		Poor	Moderate
Degree of Wetland Vegetation Coverage	**Poor / Moderate / Good**	Good		Poor		Poor	Moderate
Stream Velocity	**Slow / Moderate / Fast**	Slow		Fast		Fast	Slow
Stream Flow Regulation	**Poor / Moderate / Good**	Good		Poor		Poor	Good

Table S 7-2: The type of structure and rehabilitation objective of the various rehabilitation interventions used during this study.

Intervention Number	Structure Type	Rehabilitation Objective	Intervention Dimensions
1	Concrete Weir	Neutralise the erosion taking place upstream.	Structure Length = 13.8 m, Width = 3.8 m, Height = 2.72 m.
2	Concrete Weir	Increase the water level and encourage water distribution into adjacent areas.	Structure Length = 11 m, Width = 5.2 m, Height = 2.82 m.
3	Low Concrete Wall and Levelling	Encourage diffuse water flow and stimulate the growth of wetland vegetation.	Structure Length = 28 m, Width = 0.7 m, Height = 0.92 m.
4	Earthworks: Levelling	Encourage diffuse water flow and stimulate the growth of wetland vegetation.	Area Affected: Length = 70 m, Width = 18 m.
5	Low Concrete Wall	Encourage diffuse water flow and stimulate the growth of wetland vegetation.	Structure Length = 29.5 m, Width = 0.7 m, Height = 0.92 m.
6	Concrete Weir	Neutralise erosion taking place and enhance water distribution into adjacent areas.	Structure Length = 11 m, Width = 6.8 m, Height = 3.42 m.
7	Concrete Weir	Neutralise main channel erosion and enhance water distribution.	Structure Length = 11 m, Width = 6.8 m, Height = 3.42 m.
8	Earthworks: Distribution Berm	Neutralise ridge and furrow cultivated areas and encourage diffuse water flows.	Structure Length = 100 m, Width = 8 m, Height = 0.25 m.
9	Earthworks: Berm	Neutralise secondary channel formation and encourage water distribution across adjacent areas, whilst maintaining flows downstream using pipes through the berms.	Structure Length = 10.5 m, Width = 0.4 m, Height = 0.8 m.
10	Earthworks: Berm	Encourage diffuse water flow and stimulate the growth of wetland vegetation.	Structure Length = 7.3 m, Width = 0.4 m, Height = 0.8 m.
11	Earthworks: Berm	Encourage diffuse water flow and stimulate the growth of wetland vegetation.	Structure Length = 31 m, Width = 0.7 m, Height = 0.92 m.
12	Earthworks: Distribution Berm	Neutralise ridge and furrow cultivated areas and encourage diffuse water flows.	Structure Length = 73 m, Width = 8 m, Height = 0.25 m.
13	Concrete Weir	Neutralise secondary channel formation and encourage water distribution across adjacent areas.	Structure Length = 9 m, Width = 4.3 m, Height = 2.2 m.

Table S 7-3: The median measurements of the various water and sediment parameters measured at the selected sites.

Parameter	Unit	Detection Limit	Ref-Pre	Ref-Post	Up-Pre	Up-Post	Down-Pre	Down-Post
	Water							
pH	-log[H+]	Range from 0.5 - 14	6.35	6.53	3.76	4.10	5.30	7.60
TDS	mg/l	5.00	58.33	53.00	755.40	645.70	942.30	610.30
Chl *a*	µg/l	1.00	14.17	17.15	1.20	1.01	2.02	6.11
K	mg/l	0.10	2.67	3.17	7.17	7.34	12.33	9.70
Na	mg/l	0.10	5.13	6.80	28.17	30.76	66.67	49.33
Ca	mg/l	0.10	4.70	5.40	130.80	106.80	242.70	153.30
Mg	mg/l	0.10	2.73	3.55	44.42	38.00	71.33	52.00
SO_4	mg/l	0.10	4.15	15.50	710.70	621.70	976.00	599.30
Cl	mg/l	0.50	6.37	7.70	34.67	40.67	60.00	42.67
Alkalinity	mg/l	0.50	27.33	27.50	0.30	0.25	3.00	22.00
COD	mg/l	5.00	22.00	20.00	6.50	7.00	11.50	4.25
Al	µg/l	5.00	3.70	4.25	2100.00	1200.00	890.00	<5
Fe	µg/l	5.00	25.32	29.00	255.00	250.00	29.50	7.00
Mn	µg/l	0.50	27.14	24.90	1020.00	745.00	7000.00	251.85
Zn	µg/l	2.00	8.75	6.00	98.50	51.00	380.00	5.00
Ni	µg/l	0.50	1.20	2.40	43.50	22.70	210.00	5.90
Parameter	**Unit**	**Detection Limit**	**Ref-Pre**	**Ref-Post**	**Up-Pre**	**Up-Post**	**Down-Pre**	**Down-Post**
	Sediment							
Gravel	%	0.50	0.67	0.50	13.17	0.00	0.00	0.33
Mud	%	0.50	18.67	20.70	34.00	16.14	19.33	17.29
Sand	%	0.50	80.67	78.80	52.67	83.86	80.67	82.37
TOC	%	0.001	4.01	2.86	1.40	0.59	1.56	0.42
Al	mg/kg	50.00	24902.50	24771.50	28570.00	24599.00	17020.00	25290.00
Fe	mg/kg	50.00	6259.50	7718.50	46826.00	33650.00	10934.50	39330.00
Mn	mg/kg	0.50	23.00	68.50	821.00	363.00	67.00	581.00
Zn	mg/kg	0.50	23.00	29.00	40.00	30.00	13.50	45.50
Ni	mg/kg	0.50	15.00	17.50	23.00	18.00	10.23	26.00

CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS

8.1. SUMMARY OF FINDINGS

Through this study it was attempted to contribute to a better understanding of the controlling factors that regulate a multitude of variables in a coal mining impacted environment (i.e., the upper Olifants River). Through the use of this information an attempt was made to expand on our ability to better understand the potential future impacts in a less impacted area facing an increase in coal mining and associated activities (i.e., the Mokolo River) through the use of scientific data generated in both the upper Olifants and Mokolo rivers. The information from this study therefore goes a long way in contributing to the sustainable development of water resources linked to coal mining. Furthermore, the significance of this study was expanded by testing a possible tool to mitigate the coal mining linked impacts associated with acid mine drainage (AMD) by conducting a detailed case study. Very few studies have such an opportunity whererby a comparative study can be conducted to anticipate the likely impacts of future land use changes by learning from a relevant (but impacted) example. This is even more noteworthy since both the rivers used during this study are of international importance. Therefore, the information from this study may influence international riverine management strategies, linked to coal minining impacts, so that these water resources may be able to cope with and recover from stresses and shocks, as well as maintain or enhance its capabilities.

The following conclusions were made in answer to the objectives / questions that were set for this study to address the overall aim. Firstly, both catchments are comparable in terms of not only various environmental aspects (e.g., geology), but also the land use impacts that they are and / or were either subjected to or are at risk of. From this study it was also evident that with regard to nutrient enrichment for example, the upper Olifants and Mokolo rivers were similarly impacted. These impacts may be related to the degree of agriculture and wastewater impacts in both catchments. A distinct difference was, however, observed with regard to the impact of coal mining in these two catchments. This provided us with a relatively rare and unique opportunity to gain insight into a coal mining impacted catchment (i.e., the upper Olifants River) so that we could potentially apply this knowledge, along with the detailed baseline information gained through this study, within the Mokolo River catchment for improved water management.

From the various changes in water and sediment quality observed when comparing the Mokolo River with that of the upper Olifants River, certain abiotic ecosystem drivers, such as pH, alkalinity and sulfate, were found to be of importance. It is therefore essential that these parameters be properly monitored within the Mokolo River during future expansions in the coal mining industry. The importance of proper management and monitoring of major tributaries was also shown, whilst the possible downstream effects of dams on water and sediment quality put traditional thoughts of the impact of these structures into a new perspective.

Certain metals (e.g., aluminium) have been found to be in high concentrations in the Mokolo River, whilst vanadium has been recorded to be bioaccumulated to a higher degree than in the upper Olifants River. Since the Mokolo River has been found to have "softer" water (low alkalinity), it makes the bioaccumulation trends more worrisome as the Mokolo River is therefore more sensitive to increased metal pollution compared to the upper Olifants River. Thus, sand mining operations in the lower Mokolo River have been found to pose a threat to the sustainability of the Mokolo River, because it jeopardises its natural ability to sequester metal pollution. The freshwater crab, *Potamonautes warreni*, compared to the freshwater teleost, *Tilapia sparmanii*, has also been found to be a good model organism for monitoring metal pollution in the Mokolo River. *Potamonautes warreni* is able to accumulate high concentrations of metals and respond indicatively.

Various cellular and molecular endpoints (including total protein determiniations) provided a better understanding of the impacts of pollutants on a chronically (i.e, the upper Olifants River) or acutely impacted (i.e., the Mokolo River) aquatic ecosystem. This information proved more specific in determining the state of the Mokolo and upper Olifants rivers, as well as the degree of impact on the biota. The use of molecular information (genes, enzymes and proteins) can thus be successfully used to improve water resource management by acting as early warning systems. In so doing it may assist in preventing the Mokolo River from sharing the same fate as the upper Olifants River. The results from this study also substantiated the suitability of *P. warreni* as model organism for molecular / genotoxicological monitoring.

The multiple lines of evidence developed during the use of several techniques and statistical approaches to characterize the aquatic invertebrate and fish communities proved useful in understanding the impact of coal mining on biotic integrity. The impacts of nutrients and other pollutants (i.e., metals) on biotic integrity were also substantiated and therefore the generation and discharge of AMD needs to be properly managed to conserve biotic integrity. Overall, the the Mokolo River has a much more sensitive fish population than the Olifants River which does not appear to be significantly influenced by habitat availability. Biochemical endpoints also proved more useful in potentially detecting impacts on biotic assemblages and may assist in preventing substantial impacts on aquatic communities. The information generated using aquatic invertebrate community structures proved more informative and useful than those gained from using the natural fish assemblages.

The rehabilitation case study conducted during this study showed a positive response in dealing with the AMD impacted water within a very short period of time. Downstream of the rehabilitated area the water quality improved significantly, whilst the biotic integrity of the system also improved after rehabilitation and corresponded with an increase in primary productivity. Furthermore, the level of embryotoxicity and teratogenic potential of the system's surface water improved due to the rehabilitation efforts. Overall, the functioning of this ecosystem and / or ecological integrity significantly improved after rehabilitation. After the completion of this case study it was evident that there is significant scope for the potential use / implementation of rehabilitation interventions to not only mitigate the potential future pollution issues in the Mokolo River catchment (e.g., AMD), but also to improve the current ecosystem services (i.e., water treatment) of such a system.

8.2. FUTURE RESEARCH AND RECOMMENDATIONS

It is recommended that certain "hot spot" tributaries be identified that are irreplaceable in terms of supporting the functioning of the Mokolo River. The quality of the water entering and leaving the Mokolo Dam also needs to be monitored more intensively and more regularly as more pressure will be put on this dam due to increased water requirements. The sewage treatment facilities, especially those located around the towns of Vaalwater and Lephalale, need to function optimally at all times. This study has shown the sensitivity of the Mokolo River towards nutrient enrichment and can therefore not afford a scenario such as currently taking place in the upper Olifants River. These facilities need to be upgraded timeously to ensure that it is capable of supporting the influx of people in future.

Current and future sand mining operations need to be closely monitored and managed. This study has shown the impact that sand has on reducing pollution levels. New approaches / methodologies should be used if sand mining is to be continued in the Mokolo River or limits should be implemented on what is allowed to be mined, so as to ensure that the water quality for downstream users is not jeopardised.

It is recommended that biomonitoring programs focus on biotic community assemblages, rather than diversity changes only, to sustain the ecological and biotic integrity of the Mokolo River. This is because pollution driven impacts usually result in the replacement of sensitive species, instead of an overall reduction in diversity.

Future studies should consider further research on the use of invertebrate sentinel organisms, such as *P. warreni*. The development of suitable cell lines, anti-bodies, etc. will be invaluable for the future advancement of aquatic monitoring methods and techniques.

It is recommended that the potential use of artificial, drifting and storm water wetlands be tested in the Mokolo River catchment. This may be of particular concern, because the Mokolo Dam has been shown to significantly affect the downstream water and sediment qualities. Drifting wetlands may provide some assistance if deployed in the Mokolo Dam. The Mokolo River, especially downstream, is prone to flooding due to regular release. Storm water wetlands may aid in reducing the impact of the pollutants flushed during these periods. Lastly, artificial wetlands may be useful as a final treatment step to reduce the impact of wastewater discharge from industries or wastewater treatment works, for example.