The use of *Clarias gariepinus* and associated helminthic parasites as bio-indicators of metal pollution in a subtropical ecosystem

RS Pienaar

orcid.org 0000-0001-9166-3915

Dissertation accepted in fulfilment of the requirements for the degree *Masters of Science in Environmental Sciences* at the North-West University

Supervisor: Prof NJ Smit
Co-supervisor: Dr RJL Gerber

Graduation May 2020
24140430

Acknowledgements:

I would like to extend my sincere gratitude to and acknowledge the following people and organisations for their valuable contributions during this project:

- To my supervisors, Prof. Nico Smit and Dr. Ruan Gerber for all your guidance, patience, support and assistance in the field and in the lab as well as all the time spent reviewing and providing guidance and direction for my dissertation. I would also like to thank you for assisting me with my Project topic.
- To the National Research Foundation (NRF) for the financial assistance with this project (NRF project IFR170210222411 grant 109352, NJ Smit, PI). Opinions expressed and conclusions arrived at are those of the author and are not necessarily those of the NRF.
- To the Water Research Commission (WRC) and GreenMatter for their financial support with a personal study bursary.
- To Ezemvelo KZN Wildlife for allowing us to sample within the Ndumo Game Reserve, to Catharine Hanekom, Regional Ecologist of uMkhanyakude; Bongani Gumede, Section Ranger of Ndumo Game Reserve; and Andile Mhlongo, Conservation Manager of Ndumo Game Reserve. To all other staff at Ndumo Game Reserve for their friendly smiles and always being willing to help with any type of problem.
- To the North-West University (NWU) and the Water Research Group (WRG) for the use of their equipment and facilities for the project.
- To all my fellow postgraduates at the WRG, with a special mention of Dr. Lizaan de Necker, Divan van Rooyen, Hannes Erasmus, Anja Greyling, Marelize Labuschagne and Chantelle Pretorius for their assistance in the field and lab as well as guidance with statistics and the creation of my map.
- To Renier Posthumus for the proof reading and the necessary editorial changes.
- A special thanks to Timmy for keeping me company and listening to me during all the early mornings and late nights at the office.
- To my family (Pappa, Mamma, Sussie en Boetie), the most important people in my life, thank you for all your love and support during the study and especially the period of the write-up.
- Lastly, I would like to thank God for giving me the strength to complete my MSc.

Summary:

Metals are naturally occurring elements that play an important role in the functioning of organisms. However, imbalances in metal concentrations in the environment are of great concern due to their potential detrimental nature. Since the start of the Anthropocene, humans have increased their impact on the environment through anthropogenic activities such as mining, over-exploitation, deforestation, gas emission and pollution. Metals are non-biodegradable and thus will bioaccumulate in organisms that are exposed to metals for extended periods of time. Metals can also biomagnify to higher levels in the food chain, with top predators potentially having the highest levels of metal concentrations. The accumulation of metals along the food chain can have serious health implications for humans consuming these organisms. For example, lead can cause renal failure, mercury and cadmium can cause kidney problems, whilst zinc and copper can lead to nephritis.

Aquatic ecosystems are under constant threat of metal pollution because of runoff into these ecosystems. Lotic and lentic ecosystems act as a sink for metals with metals being adsorbed in the sediment and building up to high concentrations. Metals in aquatic ecosystems are readily available for organisms to accumulate. It is therefore of the utmost importance to detect metal pollution in aquatic ecosystems as early as possible. Scientists use a tool called biomonitoring to assess environmental exposures to synthetic or natural chemicals by testing individual organisms' body fluids or tissue samples, i.e. using these organisms as bio-indicators. The technique uses the knowledge that chemicals leave a footprint within cells after exposure. By measuring the mark after exposure, the amount of chemicals entering the organisms from the environment can be indicated. Biomonitoring is therefore a very important tool to assess pollution levels in the environment.

The bio-indicator approach involves the measurement of the response of an organism from molecular to community levels. For the purposes of this study, *Clarias gariepinus* and its associated endohelminth parasites were used as bio-indicators of metals in three different aquatic systems located in the Ndumo Game Reserve (NGR). The three aquatic systems consist of a freshwater lotic system: The Usuthu River, which flows along the northern border of NGR, a freshwater lentic system: Shokwe Pan and a saline lentic system: Nyamithi Pan. *Clarias gariepinus* is an omnivorous and bottom-dwelling fish that has a long life-span and is therefore a good species to use as a bio-indicator of metal pollution. Parasites are found in all ecosystems on Earth and can be present at high abundances. Parasites feed on their hosts and are therefore classified as occupying a higher level in the food chain than their hosts. *Clarias gariepinus* in NGR

is infected with five different endohelminth parasites, two of which are cestodes and three nematodes. The two cestode species are the *Proteocephalus* sp. and *Tetracampos ciliotheca* located in the intestine of their hosts. The three nematode species are the *Contracaecum* sp. located in the body cavity of their host, *Procamallanus pseudolaeviconchus* located in the stomach of their hosts and *Paracamallanus cyathopgharynx* located in the rectum of their hosts. For this reason, the study chose *C. gariepinus* and its associated helminth parasites.

The metals tested for in the study were the essential metals (Fe, Co, Ni, Zn and Cu) and the non-essential metals (Al, Mn, As, Cd, Pb, Se and Ag) and the biological responses tested for were cellular energy allocation (CEA), metallothioneins (MTs) and reduced glutathione (GSH). In order to determine the metal concentrations of the different samples, the sediment, host tissues and parasites were freeze dried and microwave digested in 2.5 mL nitric acid and 7.5 mL 32% hydrochloric acid and the water samples were acidified with nitric acid, whereafter the metal concentrations were measured using standard inductively coupled plasm mass spectrometry. Univariate statistics were analysed using GraphPad Prism®7 software, where homogeneity was tested using the Shapiro-Wilk test, one-way analysis of variance (ANOVA) with Tukey post-hoc analysis or a Kruskal-Wallis followed with Dunn's multiple comparisons to determine significance. Multivariate statistics were analysed using SPSS version 18 (PAWS Statistics, IBM, USA) to perform a Discriminant Function Analysis (DFA) and Canoco version 5 (Ter Braak & Smilauer, 2012) was used to perform a redundancy analysis (RDA).

The results showed that the freshwater lentic system had the highest levels of metals in the sediment whilst the saline lentic system had the highest levels of metals in the water. The freshwater lotic system had the lowest levels of metals in the environment. The results also showed that the NGR was close to a natural state with no visible human impact on the aquatic systems, and the metal concentrations were below that of other impacted areas in South Africa. After the environmental concentrations were measured, the metal concentrations in the muscle and liver tissues of *C. gariepinus* were compared to the metal concentrations in the environment. The study found that *C. gariepinus* at the three different sites accumulated metals to a higher level than present in the environment and they can therefore be used as a bio-indicators of metals in natural ecosystems. The catfish in the freshwater lentic systems accumulated metals to higher levels than the catfish in the saline lentic and freshwater lotic systems. The catfish in the saline lentic system showed the lowest amounts of accumulation. After the bioaccumulation of *C. gariepinus* was determined its endohelminth parasites were evaluated as bio-indicators of metals.

The study found that the five endohelminth parasites had higher concentrations of all the metals than their host muscle tissues and higher than most of the liver tissues. The study therefore found that endohelminth parasites could be used as bio-indicators of metal pollution and could even be better indicators than their hosts by accumulating metals to higher levels than their hosts. The different aquatic systems played a role in the accumulation of metals in the parasites with the Nyamithi catfish parasites accumulating the least amount of metals. Of the five helminth parasites, the cestode species *Tetracampos ciliotheca* had the highest bioaccumulation rates while the body cavity nematode *Contracaecum* sp. had the lowest bioaccumulation. The study thus established that NGR was in a natural state, that *C. gariepinus* accumulated environmental metal concentrations to higher levels in their tissues and that endohelminth parasites accumulated metals to even higher levels. These findings could then be used to determine the effect of metals on the biological response of both the *C. gariepinus* as well as the endohelminth parasites in their respective environments.

The study found that there was a spatial difference in the biological response of *C. gariepinus* to the accumulation of metals, with catfish in the lotic system having significantly higher concentrations of metallothioneins than the catfish at the two lentic sites. The study also found that the endohelminth parasites, *Contracaecum* sp., had lower concentrations of metallothioneins (MT) than their hosts, but significantly higher levels of Glutathione reductase (GSH) than their hosts. These findings indicate that *Contracaecum* sp. does not have the same ability as their hosts to detoxify and remove metals from their systems. The biomarker responses indicate that an increase in metal concentrations will have a negative impact on their hosts with a decrease in energy. The study found that parasites had an effect on the biomarker response of their hosts to metal concentrations in that the catfish with the least number of parasites had the highest concentrations of MTs and the catfish with the highest number of parasites had the lowest concentration of MTs. This is because the parasites help to remove metals from their hosts by biomagnification, and therefore their host does not have to waste energy in removing the metals. But the study found that the catfish with the highest number of parasites had the lowest available energy because of the parasites feeding off the host. It is thus a “catch-22” situation where an increase in parasites helps to deal with the effect of metals on their hosts but at a cost to their hosts’ energy budget.

Key words: bioaccumulation, bio-indicator, biomagnification, biomonitoring, biomarkers, Cestoda, *Clarias gariepinus*, host-parasite relationship, metallothioneins, Nematoda.

Table of Contents

List of Figures:

List of Tables:

List of abbreviations:

ANOVA	Analysis of variance
BAF	Bioaccumulation factor
CCME	Canadian Council of Ministers of the Environment
CEA	Cellular energy allocation
CRM	Certified reference material
DFA	Discriminate function analysis
DWAF	Department of Water Affairs and Forestry
Ea	Energy available
Ec	Energy consumption
FFG	Functional feeding groups
GSH	Reduced glutathione
HCl	Hydrochloric acid
HNO_3	Nitric acid
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
KNP	Kruger National Park
MT	Metallothionein
NGR	Ndumo Game Reserve
NWU-AnimCare Committee	Ethics Committee on Animal Care, Health and Safety in Research
RCC	River continuum concept
RDA	Redundancy analysis
SQG	Sediment quality guidelines
SEM	Standard error of the mean
TWQR	Target water quality range
WHO	World Health Organization

1. General introduction

1.1. Background

1.1.1. Metal pollution in aquatic ecosystems

Water is the most important and widely distributed resource on Earth and is needed by all living organisms (UNESCO, 1998). Aquatic ecosystems provide important functions such as purification and nutrient cycling as well as providing drinking water and habitats for fish and other aquatic organisms (Heathwaite, 2010). Water can be seen as a renewable resource, but it is not unlimited and is vulnerable to stressors (Clarke, 2013, Loucks & Van Beek, 2017). Although humans are dependent on water to survive, humans tend to have a negative impact on water bodies. In 2002 the chemist and Nobel-Prize winner, Paul Crutzen (cited by Steffen *et al.*, 2007), suggested that we have moved from the Holocene and entered the Anthropocene. The Anthropocene is a time characterised by the geophysical force societies have on the environment and was first apparent in the 1800s at the start of the industrial revolution (Steffen *et al.*, 2007).

Humans impact on the environment in many ways, such as through over-exploitation, deforestation, gas emission and pollution (Vörösmarty *et al.*, 2010, Chenoweth *et al.*, 2014). A by-product of most anthropogenic activities are metal elements that are released into aquatic environments and can subsequently have detrimental effects on the environment (Islam *et al.*, 2015, Palma *et al.*, 2015). Metals are introduced into aquatic ecosystems through either point or non-point source pollution. Point source pollution is pollution that flows into the waterbody at a single point that can result from mines releasing metal waste into aquatic ecosystems (Demirak *et al.*, 2006). Non-point source pollution is where polluted waters flow into a waterbody at multiple sites, e.g. through metal deposition. However, metals are found naturally and can be classified into two main categories, namely essential and non-essential metals. Essential metals are those metals that have a role in the functioning of enzymes in living organisms. The term non-essential metals is used for metals that have no role in the functioning of living organisms and can be detrimental even at trace levels (Miikue-Yobe & Ibara, 2019).

Although some metals are essential for life, the accumulation of metals is of great concern for the health of organisms because high concentrations of even essential metals have the potential to be toxic to an organism (Gupta *et al.*, 2009). According to (Tilzer & Khondker, 1993), metals are non-biodegradable and can bioaccumulate in organisms that are exposed for a sufficient period of time and have the potential to biomagnify throughout the food chain. Venugopal and Luckey (1978) state that the presence of metals in organisms found in food can have serious health implications for the human population. For example, lead can cause renal failure, mercury and cadmium can cause kidney problems, whilst zinc and copper can lead to nephritis (Venugopal & Luckey, 1978). Metal pollution is of great concern for aquatic ecosystems because of their ability to build-up in the sediment and their negative impact on aquatic biota, local communities and water quality (Demirak *et al.*, 2006). It is therefore of utmost importance to detect metal pollution in water bodies as early as possible.

1.1.2. Biomonitoring of ecosystems

Biomonitoring is a tool used to assess the environmental exposure to synthetic or natural chemicals by using individual organism's body fluids or tissue samples (Zhou *et al.*, 2008, Li *et al.*, 2010, Gray *et al.*, 2014). The basis of this technique is to use the knowledge that chemicals leave a footprint after exposure (Zhou *et al.*, 2008). By measuring the mark left by chemicals, it can indicate the amount of synthetic or natural chemicals entering organisms in ecosystems (Zhou *et al.*, 2008). Biomonitoring is thus an important tool to assess if an ecosystem is polluted or not and is commonly used globally. According to Li *et al.* (2010), biomonitoring is used to observe the impact of external stressors on ecosystems over time and space.

Currently there are multiple biomonitoring tools employed for aquatic ecosystems, which include bio-indicators, diversity indices, biotic indices, multimetric approaches, multivariate approaches and functional approaches (Li *et al.*, 2010). Biomonitoring helps to provide robust and sensitive insights into the responses of an assemblage to natural and anthropogenic stressors (Karr, 1999, Barbour & Yoder, 2000). Multivariate approaches are statistical analyses that help predict site-specific fauna patterns when no environmental stress is present, and then compare the observed fauna with the expected fauna (Li *et al.*, 2010).

Functional approaches are divided into two groups: firstly, functional feeding groups and secondly, multiple biological traits. Functional feeding groups (FFG) are one of the most important components of the river continuum concept (RCC) (Vannote *et al.*, 1980) and is used to assess processes at ecosystem-level for rivers and wetlands (Bady *et al.*, 2005). Biological traits are closely related to the habitat characteristics as well as the functions of species (biological and ecological) and can thus give insight into the function and structure of a biocoenosis (Haybach *et al.*, 2004). Biological traits include but are not limited to size, lifecycle, food and feeding habits and reproduction of organisms (Marchini *et al.*, 2008).

1.1.3. Bio-indicators of metal contamination in aquatic ecosystems

As mentioned previously, the use of bio-indicators is an important tool in biomonitoring, particularly for metal contamination. Currently the health of aquatic ecosystems is assessed by using bio-indicators (Markert *et al.*, 2003, Li *et al.*, 2010, Kuklina *et al.*, 2013). According to Pracheil *et al.* (2016), the bio-indicator approach is a bioassessment method that makes use of aquatic organisms as early warning and stress indicators in aquatic ecosystems. The bio-indicator approach involves measuring the response of an organism from molecular to community levels (Adams & Greeley, 2000). Metal concentrations in tissues of organisms indicate past exposure to metals through water and food sources (Terra *et al.*, 2008).

All organisms can be used as bio-indicators, but depending on their lifecycle, they may have different functions as bio-indicators. Organisms with longer lifespans can show long-term exposures (McGeoch & Chown, 1998, Markert *et al.*, 2003). Organisms with short lifespans show short-term exposures and animals such as top predators are indicators for long-term exposure. Bio-indication is also dependent on the interaction between organisms and their environments (Markert, 2007). The different pathways along which organisms are exposed to metals play a major role in the effectiveness of an organism as a bio-indicator (Markert, 2007). Organisms, such as invertebrates, which live in the sediment of aquatic ecosystems, filter their food from the sediment, thus absorbing higher concentrations of metals through feeding (Ahmad *et al.*, 2010).

Metals accumulate in different concentrations across different aquatic organisms; similarly these metals accumulate in different concentrations across different organs within a specific organism (Karadede & Ünlü, 2000, Jezierska & Witeska, 2006, Vinodhini & Narayanan, 2008). In some instances, the metal concentration will be at higher levels in the liver than, for example, in the muscle tissue of the host, since the liver uses metallothionein which binds to the metals and is important for the detoxification of metals (Sures *et al.*, 1997, Vinodhini & Narayanan, 2008). This accumulation can alter the levels of biochemical parameters in fish (Vinodhini & Narayanan, 2008).

Liver tissue is used for biomarker responses of fish, thus a comparison between metal concentration and biomarker response can be made. Biomarkers are cellular or biochemical responses to chemical or anthropogenic stress in an organism that can be measured (Strimbu & Tavel, 2010). Metal accumulates differently in different organs of organisms. The gills of fish are the point of entry for metals and thus indicate short-term exposures, the liver and kidneys are used to detoxify metals and will thus also reflect short-term exposures (Vinodhini & Narayanan, 2008). When metals are not removed from the organism the metals will accumulate in the muscle tissue of the fish, thus reflecting long-term accumulation (Vinodhini & Narayanan, 2008).

It is important to know the metal concentration in the muscle tissue of fish because humans consume the muscle tissue as food source, thus humans will be exposed to metals through consumption of fish (Jezierska & Witeska, 2006, Agah *et al.*, 2009). The concentration of metals in fish tissue can reflect either past exposure to metals through food sources or the water source that the organisms are exposed to but it can also be both (Birungi *et al.*, 2007).

This study focuses on *Clarias gariepinus* and its Helminth parasite fauna as bio-indicators of metal exposure in an aquatic ecosystem. *Clarias gariepinus* is a commercially important fish species that serves as a major food source in third world countries (Farombi *et al.*, 2007). *Clarias gariepinus* is omnivorous and feeds on different trophic levels, from plants (producers) to insects (grazers and predators) to fish (predators) and even sometimes birds (predators) (Gamal *et al.*, 2012). They are a bottom-dwelling species that come into contact with the sediment in lakes and rivers. While the fish swim along the sediment it stirs up the soil that releases metals back into the water column (Calmano *et al.*, 1990, Thornton *et al.*, 1995). *Clarias gariepinus* has a long lifespan and can live up to 15 years and more (Booth *et al.*, 2010).

All the above-mentioned factors contribute to make this organism a suitable bio-indicator species to investigate for metal exposure (Crafford & Avenant-Oldewage, 2010). *Clarias gariepinus* is known to have high infection rates with parasites especially helminth parasites (Akinsanya & Otubanjo, 2006). Parasites are found in all ecosystems on the planet and can be present in high abundances in these ecosystems. Parasites generally feed on their host organisms, be it plants or major predators, and as a result occupy higher levels on the food chain than their hosts (Omacini *et al.*, 2001, Shaw & Hochberg, 2001, Lafferty *et al.*, 2006, Chen *et al.*, 2008). According to de Buron *et al.* (2009), the feeding habits of helminth parasites can reflect the relationship of their hosts to their environment. Helminth parasites typically live in the gut of their hosts and cannot build up the required fatty acids and as a result they must absorb the necessary nutrients needed from their hosts (Bailey & Fairbairn, 1968, de Buron *et al.*, 2009). The aforementioned characteristics make parasites suitable for being good bio-indicators of anthropogenic pollution in the environment (Sures, 2001). The United States' Committee on Biological Markers of the National Research Council (NRC, 1987) divided the biomarker responses of bio-indicators into three classes, namely biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility. This study only focuses on the first two classes.

1.1.3.1. Biomarkers of exposure

Biomarkers of exposure is the measurement of the accumulation of pollution inside an organism (bio-indicator) (Van der Oost *et al.*, 2003). Biomarkers of exposure are used to document if an organism or a population were exposed to a foreign substance and provide a connection between exposure and internal dosimetry (Van der Oost *et al.*, 2003). Bioaccumulation of a foreign substance is considered to be a biomarker of exposure (Van der Oost *et al.*, 2003).

1.1.3.2. Biomarkers of effect

A biomarker of effect is the possible health impairment or disease caused by pollution when looking at measurable physiological, biochemical or other changes in the host organism (Van der Oost *et al.*, 2003). Van der Oost *et al.* (2003) state that biomarkers of effect are used to document alterations of effects in the health of organisms due to external exposure to a foreign substance. Environmental stressors, e.g. metal pollution directly affect organisms at molecular and cellular levels (Downs *et al.*, 2001). Many bioaccumulation studies fail to link a certain pollutant exposure to the biological effects that it can elicit in an organism (Wepener *et al.*, 2011). Biomarkers can be used as early-warning signals to evaluate the effect of environmental changes on organisms at a cellular level.

Van der Oost *et al.* (2003) state that a biomarker is a biological response to pollution that can be evaluated by changes at a cellular level that cannot be detected when looking at an organism as a whole. According to Shugart *et al.* (1992), a biomarker is the measurement of cellular modifications caused by the accumulation of pollution in the fluids, cells or tissue of the host species. In environmental science biomarkers can be used as sensitive indicators of pollution entering species and the distribution of the toxicants through the cells of the organisms (Shugart *et al.*, 1992). In this study we will be focusing on three effect biomarkers, namely metallothionein (MT), cellular energy allocation (CEA), and reduced glutathione (GSH).

Metallothioneins are non-enzymatic proteins that bind to particular metals (Amiard *et al.*, 2006). The chemical behaviour of the protein is dominated by their thiol group and it is normally saturated by multiple atoms (Amiard *et al.*, 2006). Although the function of MTs is still under debate, they play a double role in organisms. The metal-binding capability of the protein firstly plays a role in the homeostatic regulation of essential metals as they act as stores for the metals (Roesijadi & Fowler, 1991). Secondly they play a role in the detoxification of non-essential trace metals gives organisms a higher tolerance against metal pollution (Ritterhoff & Zauke, 1998). An increase in MTs in organisms can be used as a biomarker for metal pollution in an environment.

When organisms are exposed to pollution, such as metals, the organism will lose available energy (Smolders *et al.*, 2004, Wepener *et al.*, 2011). Organisms use energy to detoxify and remove metals from their systems (Moolman *et al.*, 2007). To determine the total amount of energy organisms have available for functions such as growth and reproduction, cellular energy allocation (CEA) is determined (Smolders *et al.*, 2004). Reduced glutathione (GSH) is an important biomarker due to GSH's function of maintaining redox potentials as well as viability in cells of organisms (Atli & Canli, 2007). The tripeptide is mainly present in the cells of an organism and is glutathione (L-y-glutamyl-cysteinyl-glycine) in its reduced form and functions as a nucleophile (Atli & Canli, 2007).

1.2. Hypotheses, aims and objectives

This study was done in the aquatic ecosystems of Ndumo Game Reserve (NGR) which includes the Usuthu River system flowing through the reserve as well as two pans, namely Nyamithi Pan and Shokwe Pan, located inside the reserve. Previous studies conducted in NGR found that *C. gariepinus* was present in all the major water bodies (Smit *et al.*, 2016). These studies also found that all the sites had helminth parasite infections of the fish with some of the sites having higher infection rates than the others (Smit *et al.*, 2016). Due to the high numbers of *C. gariepinus* and high infection rates, it is possible to test the effectiveness of the host and its helminth parasites as bio-indicators. Three different aquatic ecosystems are used to assess the effectiveness of the organisms as bio-indicators in lentic and lotic systems as well as fresh and saline water.

In the study two different lentic systems will be investigated, one being a temporary freshwater pan called Shokwe Pan, and the second being a permanent saline pan called Nyamithi Pan. The lotic system investigated is the Usuthu River on the border of South Africa and Mozambique. The assessment of different aquatic habitats allows for a comparison of the effectiveness of the selected bio-indicators and to study the potential host-parasite interactions to metal exposure.

1.2.1. Hypotheses

To evaluate the efficacy of *C. gariepinus* and its endohelminth parasite fauna as bio-indicators of metals in different aquatic systems as well as to establish host-parasite interactions to metal the following hypotheses have been set for this study:

Hypothesis 1: Because lentic systems function as natural sinks for metals, they will have higher concentrations of metals than lotic systems. The concentrations of metals present in all sites of the study will be below the international guidelines for target water quality as well as international sediment quality guidelines (addressed in Chapter 2).

Hypothesis 2: The bioaccumulation of metals in *C. gariepinus* at all three sites will reflect the environmental conditions of the sites, with both the lentic systems (fresh and saline) having higher bioaccumulation factors than the lotic system. *Clarias gariepinus* will also bioaccumulate the non-essential metals to higher levels than the essential metals (addressed in Chapter 3).

Hypothesis 3: The parasites will prove to be better bio-indicators of both essential and non-essential metals than their hosts due to the bioconcentration and biomagnification of the metals in the parasites (addressed in Chapter 3 and 4).

Hypothesis 4: The parasites in the intestines will have higher accumulation rates of metals than the *Contracaecum* sp. in the body cavity due to the higher concentration of metals in the intestinal tract (addressed in Chapter 4).

Hypothesis 5: There will be a gradient of metal accumulation in the nematode parasites with the highest being in the parasites in the stomach due to the low pH making metals there more bioavailable than in the rectum of the host (addressed in Chapter 4).

Hypothesis 6: An increase in endohelminth parasite numbers will alleviate the body burdens of metals in the hosts thereby resulting in less stress experienced by the hosts to detoxify and remove metals from their organs (addressed in Chapter 5).

1.2.2. Aims and objectives

The first aim of the study is to determine the metal concentration in the three different aquatic ecosystems.

The second aim is to evaluate if *C. gariepinus* and their associated helminth parasites indicate the same metal concentration trends as the environments they live in.

The final aim of the study is to evaluate the effect of parasites on their hosts by looking at biological responses occurring in the hosts due to metal exposure and parasite infection.

To ensure that these aims are achieved, the following objectives were set:

- Determine the concentration of different metals in the environment by analysing water and sediment samples.
- Determine the current status of the aquatic ecosystems of the protected area, namely Ndumo Game Reserve, located in South Africa.
- Determine the concentration of metals in the host muscle and liver tissues by using standard Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis.
- Determine and compare the concentration of metals in the different helminth parasites to the concentration in their hosts.
- Use fish energetics as biomarkers to determine if there are differences between infected and uninfected catfish.
- Determine detoxification of metals through the measurement of the concentrations of metallothioneins (MTs) and reduced glutathione (GSH).

1.2.3. Chapter layout

- **Chapter 1:** Provides the general background needed to understand the importance of the study and gives an overview of why *C. gariepinus* was chosen as a bio-indicator for metal pollution. The chapter gives a brief overview of previous research on the use of parasites as bio-indicators and the effect of parasites on their hosts in terms of metal pollution.

- **Chapter 2:** Introduces the selected study area as well as the three sites selected for the study. The chapter provides the current environmental concentrations of metals present in the selected study area and compares these concentrations with guidelines and other aquatic ecosystems in South Africa.

- **Chapter 3:** Compares the concentration of metals found in the host tissue with the reported environmental concentration. By making use of biomagnification factors and statistical analysis, the chapter provides evidence that *C. gariepinus* is a good bio-indicator for metal pollution and that it reflects its environment.

- **Chapter 4:** Determines if parasites reflect the metal concentrations of their hosts and thus determines the success of helminth parasites as bio-indicators of metal pollution. The chapter gives a comparison of bioaccumulation of metals in parasites located in different parts of their hosts. It compares bioaccumulation by parasites at different locations in their hosts to determine if feeding styles affect bioaccumulation and to determine if parasites reflect the metal concentrations of their hosts.

- **Chapter 5:** Investigates the effect of metal concentration on the biomarker responses of the host as well as its parasites by comparing energetics, metallothioneins (MTs) and reduced glutathione (GSH) detoxification rates of the different organisms. The chapter also investigates the effect of parasites on their hosts regarding metal accumulation.

- **Chapter 6:** Provides a brief summary of the results obtained and the conclusions drawn as well as recommendations for future studies.

1.3. Potential impact

This study will contribute to the general knowledge of parasite-host interactions by researching the possibility of parasites removing metals from their hosts. It opens new possibilities of using helminth parasites as bio-indicators of metal pollution that can assist with future biomonitoring of aquatic ecosystems. It will also contribute to the conservation of aquatic systems in NGR by providing the reserve management with recommendations for their specific aquatic systems. The results of this study were presented at the Southern African Society of Aquatic Sciences Conference in July 2019.

2. Current environmental metal concentrations of the selected study area

2.1. Introduction

Floodplain rivers are one of the most dynamic ecosystems on Earth (Power *et al.*, 1995). As rivers ebb and flow, the floodplains are continually connected and disconnected from rivers (Power *et al.*, 1995). According to Power *et al.* (1995), these fluctuations in resources and habitats play a major role in the functioning of a floodplain. Floodplains are formed when rivers or streams undergo a flooding event and the water breaches the river channel onto the flat area surrounding the river (Dykaar & Wigington, 2000, Grams & Schmidt, 2002, Jain *et al.*, 2008, Damm & Hagedorn, 2010). A floodplain consists of two parts, the first being the river (floodway) and the second the surrounding area (flood fringe). The flood fringe consists of the area between the floodway and the bluff lines of the area. Bluff lines, also known as valley walls, are the areas where valley floors undergo an increase in elevation (Stephenson, 2002, Lord, 2011, Prawiranegara, 2014). Floodplains are formed through two major natural processes namely erosion and aggradation. Erosion is the process where the movement of water displaces parts of the earth's surface resulting in a loss of root depth, nutrients and water imbalances (Yang *et al.*, 2003). Aggradation is defined as the deposition of sediment from the river onto the surrounding areas (Babault *et al.*, 2005). Floodplains are ecosystems that provide services such as water purification, building products as well as a food source for humans (King *et al.*, 2009). Therefore, floodplains are important ecosystems that need to be protected.

Globally, floodplains cover more than 2 x 10^6 km^2 of the earth's surface, but this number is declining drastically and floodplains are one of the most threatened ecosystems (Erwin, 2009). The degradation of floodplains is linked with a decline in freshwater biodiversity due to habitat alteration, pollution, flow regulation and invasive species. Dudgeon *et al.* (2006) classified the threats to freshwater biodiversity according to five major categories: overexploitation, water pollution, habitat degradation, species invasion and flow modification. Over the past decades, the influence of human activity on the environment has increased drastically and therefore the monitoring of water bodies is important (Bartram & Ballance, 1996). These facts indicate the importance of studying water bodies such as rivers, dams, lakes and pans to ensure that these water bodies are protected from pollution. Because of these threats there is an urgent need to preserve existing floodplains as a resource (Erwin, 2009).

The Pongola River Floodplain (PRF) is situated on the Makhathini Flats in the northern part of KwaZulu-Natal on the border with Mozambique (Brown *et al.*, 2018). The floodplain is approximately 13000 ha and starts at the Lower Phongolo River (floodway). The Phongolo River is divided into an upper and lower part due to the construction of the Pongolapoort Dam in 1973 (Dube *et al.*, 2015). According to Dube *et al.* (2015), the floodplain extends to the confluence with the Usuthu River at Ndumo Game Reserve (NGR). The floodplain contains around 65 named and 25 unnamed pans, all with different roles and importance for the ecosystem (Brown *et al.*, 2018).

Since the construction of the Pongolapoort Dam the sustainability of the floodplain is being questioned (Heeg & Breen, 1982). Annual flood releases have been made since the construction of the dam until October 2012 at the end of the dry season to help with the recession of floodplain agriculture and to inundate the floodplain in NGR (Dube *et al.*, 2015, Brown *et al.*, 2018). The floodplain plays an important role for people living in the area who mainly use it for agriculture, a food source and grazing habitat for domestic animals (Dube *et al.*, 2015). Historically, the main source of water for the floodplain and pans of NGR emanated from the Phongolo River; however, since the construction of the dam and cessation of controlled floods, the Usuthu River has been the source of the majority of water in the NGR pans (Anderson, 2009, Brown *et al.*, 2018).

The Usuthu River starts in Amsterdam, Mpumalanga, South Africa and flows in an easterly direction through the Kingdom of Swaziland and into the Indian Ocean at Maputo Bay in Mozambique (Nunes *et al.*, 2017). The river forms the border between South Africa and Mozambique and the 20 km northern border of NGR (Calverley & Downs, 2014). The confluence of the Usuthu- and Phongolo rivers is situated in the NGR and forms the Maputo River (Romano, 1964). The Usuthu River sub-basin has a few tributaries in South Africa such as the Lusushwana, Ngwempisi, Mkhondo and Mpuluzi and has a 16690 km^2 catchment area (Kramer, 2003). Most water from the upper catchment is used for forest plantations, urban and rural water requirements as well as irrigation (Kramer, 2003). In Swaziland, water from the Usuthu River is used mostly for human settlements and the irrigation of sugarcane and citrus plantations (Kramer, 2003). Kramer (2003) states that the small population of people in the settlements next to the river will likely have no significant water abstractions, but in dry seasons irrigation water allocations are increased and can dry the river out completely. Anthropogenic activities such as coal mining and forestry in the tributaries pose threats to the aquatic ecosystem of the Usuthu River.

Pollution from different sources flows into aquatic ecosystems through runoff (Kim & Kannan, 2007, Lucke & Nichols, 2015). These pollutants can have major effects on organisms using the water (Trombulak & Frissell, 2000, Belfiore & Anderson, 2001, Livingstone, 2003, Valavanidis *et al.*, 2006). Natural aquatic ecosystems have the ability to remove such pollutants out of the water through different processes (Ostroumov, 1998, Kivaisi, 2001, Heberer, 2002, Akcil & Koldas, 2006). These processes are critical to maintain a good quality of water to support aquatic organisms and sustain life. Wetlands can reduce pollutants such as organic matter, inorganic matter, pathogens and trace organics in water into harmless by-products (Kivaisi, 2001, Heberer, 2002, Kadlec & Wallace, 2008). The reduction in pollution of water in wetlands is caused by complex mechanisms such as sedimentation, filtration, microbial interactions, chemical precipitation and uptake by vegetation (Kadlec & Wallace, 2008). Unfortunately, these natural processes can only reduce a certain amount of pollution whereafter the pollution agents such as metals will enrich the aquatic ecosystems (Cheng *et al.*, 2002, Rai, 2008, Förstner & Wittmann, 2012).

Although metals are naturally present in ecosystems, trace metals such as cadmium, copper, manganese and many others are known to accumulate in the sediments of aquatic ecosystems (Cheng *et al.*, 2002, Rai, 2008, Förstner & Wittmann, 2012). Sediment acts as a sink for pollutants that precipitate from the water column therefore lowering the level of pollution in the water (Sutherland, 2000, Yi *et al.*, 2011, Jiang *et al.*, 2018). The metals adsorbed in the sediment particles are considered as conservative pollutants but can be released back into the environment through disturbances (Yi *et al.*, 2011, Zhang *et al.*, 2017, Vedolin *et al.*, 2018). According to Yu *et al.* (2001), sediment will act as carrier of pollutants and can thus be classified as a source of pollution.

There are several metal-binding fractions in aquatic ecosystems. According to Gunn *et al.* (1988), the metal-binding fractions are influenced by the pH, ionic strength, biological and chemical redox reactions, redox potential and complexation reaction in the water column. The two most important components of sediment for metal partitioning are Fe oxides and organic matter (Marcıas-Zamora *et al.*, 1999). An important phase in regulating the binding behaviour of metals as well as their bioavailability is the oxidisable phase (Yu *et al.*, 2001). Under the perfect conditions metals will accumulate to dangerous levels in aquatic ecosystems (Yi *et al.*, 2011).

The hypothesis for this chapter is that because lentic systems function as natural sinks for metals, they will have higher concentrations of metals than the lotic system. Ndumo Game Reserve is not located close to any major sources of pollution and thus the concentration of metals present in all sites of the study will be below the international guidelines for target water quality as well as international sediment quality guidelines. The aim for this chapter is to determine the environmental quality of the ecosystems at the three selected sites, with the objective of measuring metals in the major aquatic components, namely the water and sediment.

2.2. Materials and methods

2.2.1. Study area

Ndumo Game Reserve is a 10000 ha reserve situated in the northern part of KwaZulu-Natal on the border of South Africa and Mozambique, east of Swaziland. Both the northern and eastern boundaries of the park are natural river boundaries with the Usuthu River forming the northern boundary and the Phongolo River the eastern boundary. The reserve was proclaimed as a conservation site in 1924 because of the large numbers of hippopotami (*Hippopotamus amphibious*) and is the only protected area on the PRF. According to Combrink (2004), NGR also has the third highest population of Nile Crocodile in South Africa. The wetlands in NGR hold unique biological biodiversity and is an accredited Ramsar sites (Wetlands of International Importance) of South Africa because of the high biodiversity of bird species present (DEAT, 1996.).

Ndumo Game Reserve hosts many Red Data species, which include two Red Data fish species, 10 Red Data reptile species and four Red Data herbivore species. Most of these species are dependent on the floodplain pans of NGR for food sources as well as habitat and shelter (DEAT, 1996.). Ndumo Game Reserve is the conservation area with the highest biodiversity of bird life in South Africa with 430 species recorded (Stuart & Stuart, 2018). According to Heeg and Breen (1982), 120 of these species are dependent on wetlands for survival, and 19 of them are classified as Red Data water birds. Heeg and Breen (1982) found that the PRF houses the only South

African breeding colony of white pelicans. The floodplain also hosts one of only three breeding areas for the open-billed stork.

2.2.2. Site selection

Three sites were selected in Ndumo Game Reserve (Fig. 2.1). One of them is a lotic system and the other two are lentic systems, of which one is a freshwater and the other a saline system. The first site is the Usuthu River, the second site is a temporary freshwater floodplain pan named Shokwe Pan which is fed by the Usuthu River, and the third site is the largest permanent saline pan named Nyamithi Pan. The site selection was based on the presence of both the host as well as parasite infection at the sites (Smit *et al.*, 2016, Svitin *et al.*, 2019). Previous studies found different parasite infection rates at the three sites (Svitin *et al.*, 2019). The three sites were also chosen because they are all different aquatic ecosystems.

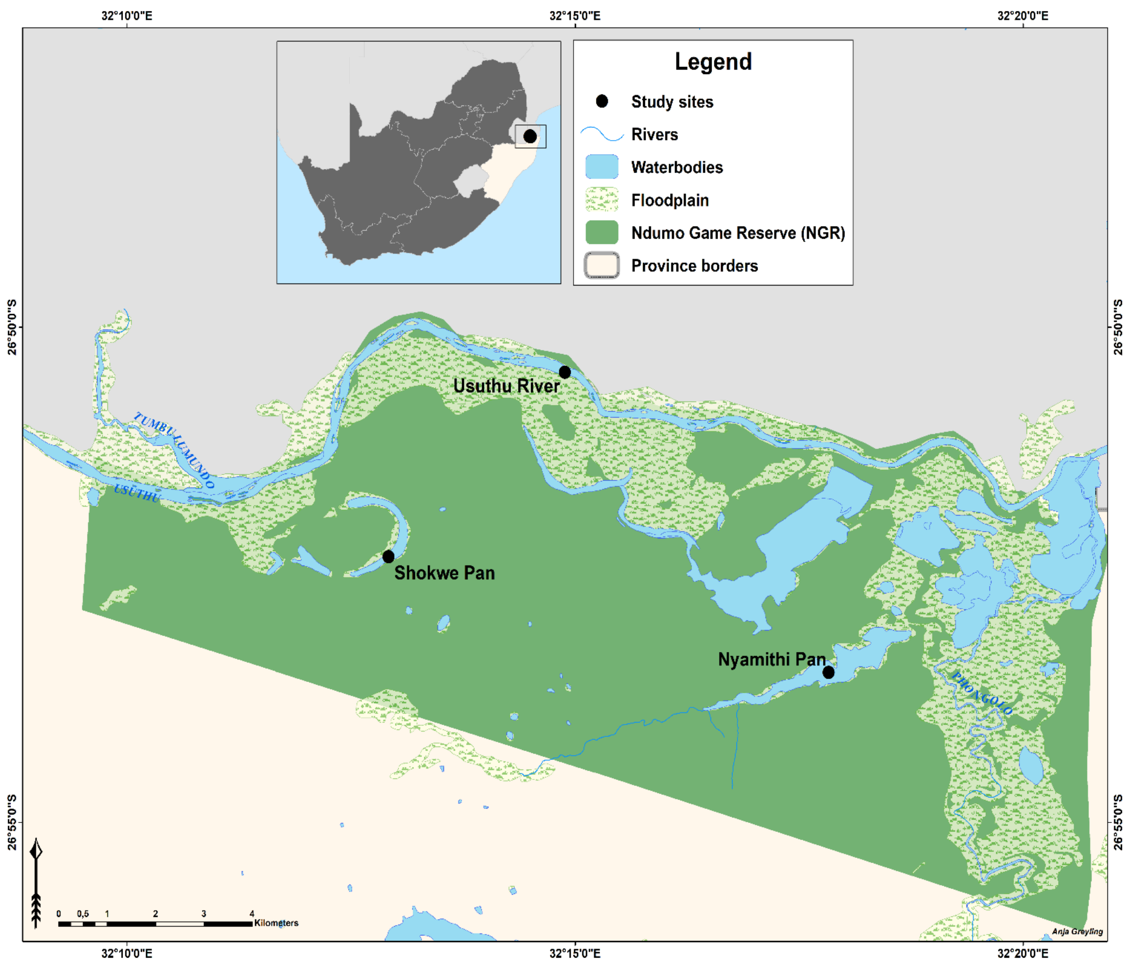

Figure 2-1: Map of the protected Ramsar site of the Ndumo Game Reserve with the three selected sites indicated on the map – the sites are the lotic system: Usuthu River, the freshwater pan: Shokwe Pan and the saline pan: Nyamithi Pan.

2.2.3. Site description

2.2.3.1. Site 1: Usuthu River

The site consists of a 400 m stretch of deep fast-flowing water (Fig. 2.2A & B). On the riverbed there are many reeds and overhanging vegetation. The site was accessible through a channel in the reeds made by hippopotami. The site has a sandy substrate, with some submerged rocks.

2.2.3.2. Site 2: Shokwe Pan

Shokwe Pan is a temporary freshwater pan that receives its water from the Usuthu River when the river is in flood (Fig. 2.2C & D). The pan is situated in the western region of NGR. Shokwe Pan is classified as a lake with no outlet. The pan receives its water through rainfall and seasonal flooding from the Usuthu River (Whittington *et al.*, 2013). The pan is surrounded by *Ficus sycomorus* (fig trees) on the west side and *Vachellia xanthophloea* (fever trees) on the east side with reed beds. Access to the water is created by animals going in and out of the water. The pan has a clay substrate with *Nymphaeaceae* (water lilies) all over the site.

2.2.3.3. Site 3: Nyamithi Pan

Nyamithi Pan is classified as a non-tidal, semi-permanent pan that gets water predominantly from flooding in the Phongolo River system but can also receive water from the Usuthu River when in flood (Whittington *et al.*, 2013) (Fig. 2.2E & F). Since 2014 Nyamithi Pan had solely received water from the Usuthu River because of no flood release from the Pongolapoort Dam. The lake is 4.4 km long from west to east and about 700 m at the widest part (Ezat *et al.*, 2018). According to Pooley (1982) the depth of Nyamithi Pan varies from 5 m during the rainy season to less than 1 m during the dry season. Nyamithi Pan has the highest density of crocodiles in NGR (Calverley & Downs, 2015). The shallow nature of the lake results in higher temperatures than in the Phongolo River channels (Calverley & Downs, 2014). Nyamithi Pan has permanent water channels that are formed by the movement of hippopotami. The pan is surrounded by *Vachellia xanthophloea* all around the banks with no reed beds present. Nyamithi Pan has no aquatic vegetation with a very coarse substrate.

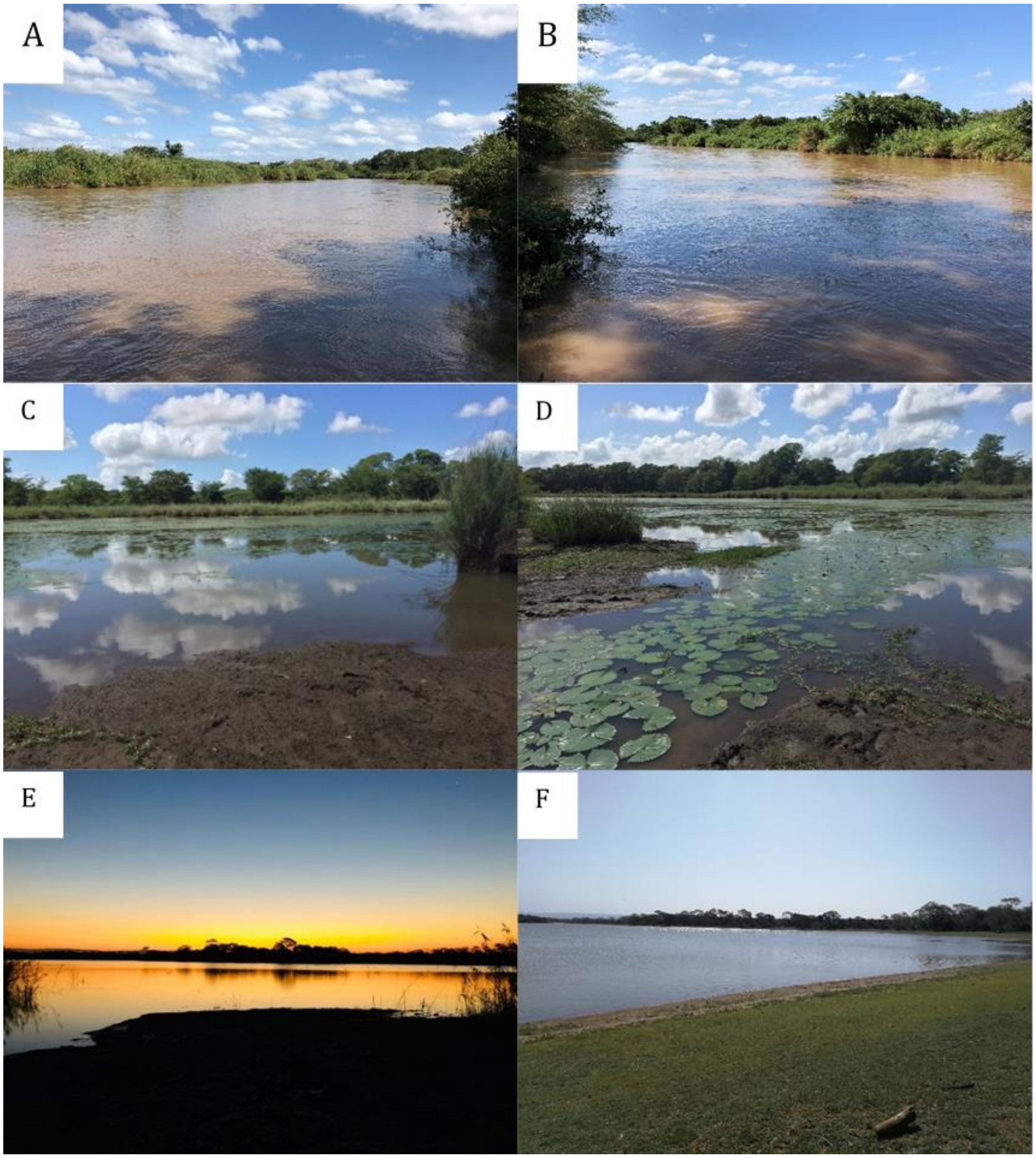

Figure 2-2: Photographs of the three selected sites and the typical marginal and aquatic vegetation, showing firstly, the lotic system: Usuthu River (A & B), the lentic freshwater system: Shokwe Pan (C & D) and the lentic saline system: Nyamithi Pan (E & F).

2.2.4. Sampling

Samples were collected during the rainy season of November 2018. Samples of water and sediment were collected for metal analyses in triplicate for each site. Water samples were collected in acid-washed polypropylene bottles and sediment samples were taken in acid-washed glass bottles. All sediment and water samples were frozen and transported back to the laboratory for further analysis.

2.2.5. Laboratory methods

2.2.5.1. Metal concentration in water samples

Fifty mL of water was filtered through cellulose nitrate filter paper (0.45 µm pore size). After filtration the water was acidified to 1% nitric acid by adding 50 µL 65% nitric acid. The acidified samples were then decanted into 50 mL amber glass bottles, labelled and sent for Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Agilent technologies, 7500CE) analysis to determine the dissolved metal concentrations for the following metals (Al, Fe, Mn, Co, Ni, Cu, Zn, As, Se, Ag, Cd and Pb). The metal concentrations were expressed in µg/L. Equation derived from Dahms *et al.,* (2016).

$$\text{Metal concentration } (\mu g/L) = \text{ICP-MS reading} \times 1000$$

2.2.5.2. Metal concentration in sediment samples

A known weight of each sediment sample from the survey was freeze-dried using a Labconco FreeZone® Plus 6 Liter Cascade Console Freeze Dry System (7753030). Afterwards 0.2 g of the dried sample was placed in Teflon tubes, and 2.5 mL nitric acid (HNO_3) and 7.5 mL 32% hydrochloric acid (HCl) were added to each sample. The samples were digested using an Ethos Easy Maxi-44 Microwave Digestion system at 1800 W in 200˚C for 45 minutes. The digested sediment was then filtered and diluted to 50 mL with 1% HNO_3. The samples were then decanted into amber glass bottles that were labelled and sent for analysis on the ICP-MS. All concentrations of metals were converted into mg/kg. Equation derived from Dahms-Verster *et al.,* (2016).

$$\text{Metal concentration (mg/kg)} = \frac{ICP-MS\ reading}{Sample\ dry\ mass\ (g)} \times \text{dilution volume (50 mL)}$$

To ensure the quality of the digestion and ICP-MS analysis, certified reference material ([NCS DC 73310] Stream Sediment from the China National Analysis Centre for Iron & Steel) was treated and digested in the same manner as the samples. The measured concentrations and the recovery rates are indicated in Table 2.1. Although the recovery rates were not within the 20% range it was close enough for most of the elements.

Table 2-1: Measured concentrations of the different metals of the certified reference material ([NCS DC 73310] Stream Sediment from the China National Analysis Centre for Iron & Steel) with the percentage recovery rates (all concentrations are expressed as mg/kg).

Sediment			
	Reference	Measured	% Recovery
Mn	1400 ± 73	881.5	66
Co	8.8 ± 1.1	5.9	77
Ni	12.8 ± 1.9	8.4	77
Cu	1230 ± 51	841.9	71
Zn	498 ± 27	317.5	67
As	115 ± 9	73.3	69
Cd	4.0 ± 0.4	3.2	87
Pb	285 ± 16	219.7	82

2.2.6. Statistical analysis

Univariate statistical analyses were conducted using GraphPad Prism®7 software. All data was tested for homogeneity using the Shapiro-Wilk test. If found to be normally distributed, a one-way analysis of variance (ANOVA) was used with a Tukey post-hoc analysis; when the data was found to not conform, a Kruskal-Wallis test was performed followed by a Dunn’s multiple comparison test. Post-hoc analyses were performed to indicate significant differences ($p < 0.05$) between the three sites, if any. A Discriminant Function Analysis (DFA) was performed using SPSS version 24 (PASW Statistic, IBM, USA) to determine the drivers that discriminate the sites from each other by looking at the metal concentration in the water and sediment of the different ecosystems, and the accuracy of reclassifying individual samples into their respective sites.

2.3. Results

The results of the environmental metal concentrations are divided into essential (Fig. 2.3) and non-essential (Figs. 2.4 & 2.5).

2.3.1 Essential metals in water and sediment

The concentration of essential metals in sediments showed the trend that the lentic freshwater system, Shokwe Pan, had significantly higher concentrations followed by the lentic saline system, Nyamithi Pan, while the lotic system had the lowest concentration of essential metals in the sediment.

The water concentrations for essential metals followed the trend that the lentic saline system had significantly higher concentrations for all the essential metals except for Zn. Shokwe Pan had higher levels of iron (Fe) in the sediment than both the Usuthu River ($p = 0.0001$) and Nyamithi Pan ($p = 0.0002$) (Fig. 2.3A). Although Shokwe Pan had the highest sediment concentration of 50027 mg/kg, the water concentration was the lowest with 30.49 µg/L. Nyamithi Pan had significantly higher ($p = 0.0011$) Fe concentrations in the water (71.45 µg/L) than both the other sites (Fig. 2.3B). Nyamithi Pan had significantly higher water concentrations of Co ($p = 0.006$), with Shokwe Pan having significantly higher sediment concentrations of Co ($p < 0.0001$) (Fig. 2.3C & D). Shokwe Pan had the highest sediment concentration at 20.23 mg/kg and Nyamithi Pan had the highest water concentration at 0.4856 µg/L.

Shokwe Pan had a significantly higher sediment concentration (85.92 mg/kg) of Ni than both Usuthu River (15.05 mg/kg, $p < 0.0001$) and Nyamithi Pan (21.35 mg/kg, $p = 0.0003$) (Fig. 2.3E). A significant difference occurs between Usuthu River and both Shokwe Pan ($p = 0.0017$) and Nyamithi Pan ($p = 0.0004$) with the Usuthu River having significantly lower water concentrations of Ni than the other two sites (Fig. 2.3F). The zinc (Zn) concentration in the sediment (Fig. 2.3G) across the sites was significantly different ($p < 0.0001$) with Shokwe Pan having the highest concentration of Zn (50.24 mg/kg). There were no significant differences in the water concentration of Zn across the three sites (Fig. 3.2H). A significant difference is present in both the sediment and water concentrations of Cu across the three sites (Fig. 2.3I & J). The sediment concentration of Cu was significantly higher (Fig. 2.3I) at Shokwe Pan (55.88 mg/kg) ($p < 0.0001$) than at both the other sites. Nyamithi Pan had significantly higher water concentration of Cu (34.23 µg/L) ($p = 0.0023$) when compared to the other sites sampled.

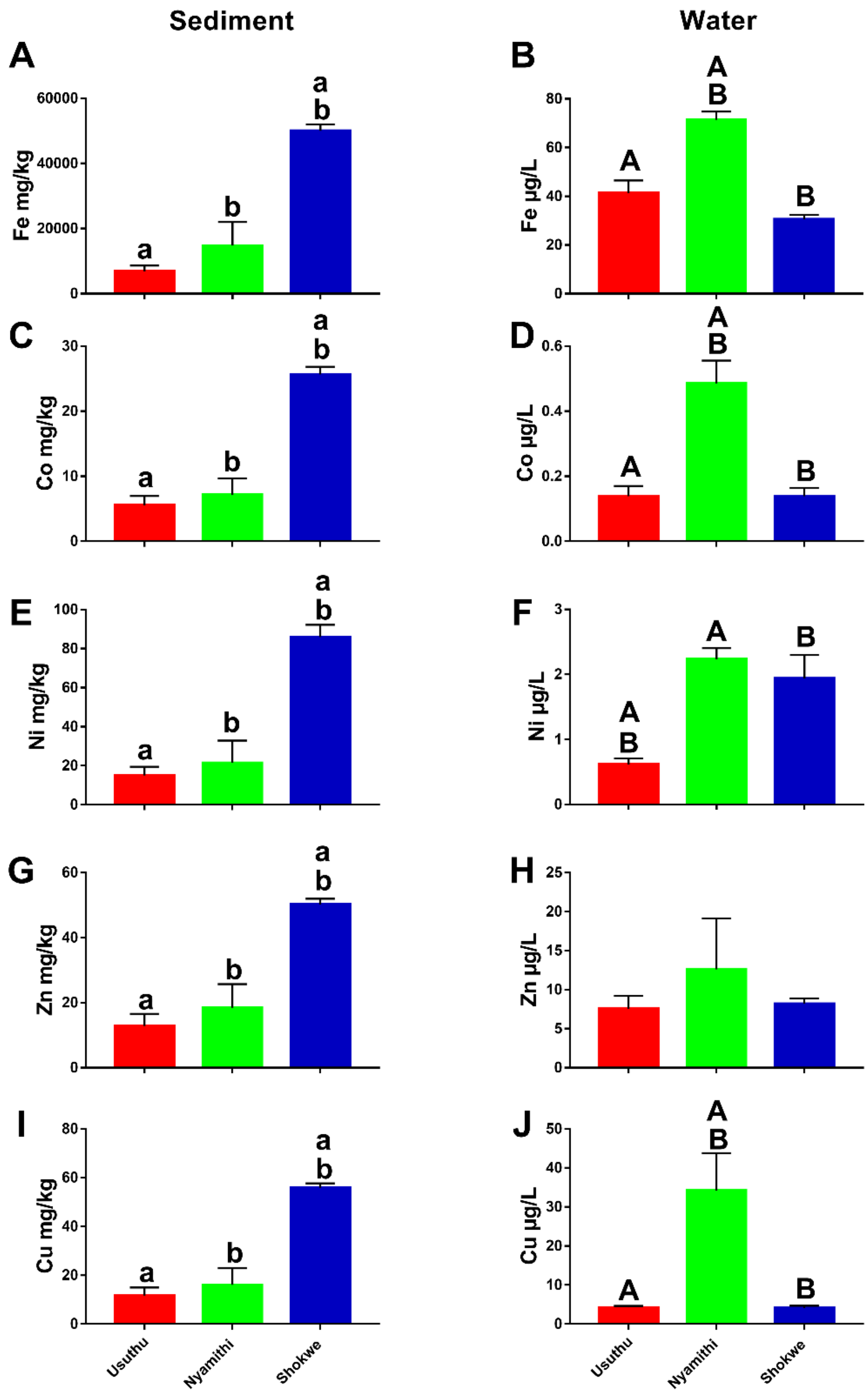

Figure 2-3: Bar graphs showing the concentration (mean and SEM) of different essential metals (Fe, Co, Ni, Zn, Cu) in both the sediment and water across the three sites located in Ndumo Game Reserve (common superscripts are used to indicate significant differences between sites).

2.3.2 Non-essential metals in water and sediment

The non-essential metal concentrations in the sediment followed the same trend as that of the essential metals with the freshwater lentic system having the highest concentrations followed by the saline lentic system and the freshwater lotic system having the lowest concentrations (Figs. 2.4 & 2.5). There was no trend in the concentrations of non-essential metals in the water. (Figs. 2.4 & 2.5).

Shokwe Pan had higher sediment concentrations of Al than the Usuthu River ($p = 0.0386$) (Fig. 2.4A). The water concentration of Al was slightly higher in the Usuthu River (26.59 µg/L) than in Shokwe Pan and the concentration was below the detection limit in the Nyamithi Pan (Fig. 2.4B). The water concentration of manganese (Mn) showed no significant differences (Fig. 2.4 D) with a large standard deviation in the level of Mn at both the Shokwe Pan and Usuthu River sites. There was a significant difference between the three sites when looking at the Mn concentration in the sediment (Fig. 2.4E), with Shokwe Pan having a significantly higher concentration at 418.2 mg/kg than Usuthu River at 139 mg/kg ($p = 0.0121$) but not significantly higher than Nyamithi Pan at 252.6 mg/kg. The sediment concentration of As (Fig. 2.4E) is significantly higher in the Shokwe Pan at 3.951 mg/kg than in the Usuthu River (1.031 mg/kg) ($p = 0.0085$). Arsenic (As) in the water across the three sites showed significant differences (Fig 2.4F). Nyamithi Pan had a significantly higher water concentration ($p < 0.0001$) at 1.424 µg/mL than both Shokwe Pan (0.5466 µg/mL) and Usuthu River (0.4309 µg/mL) (Fig. 2.4 F).

The cadmium (Cd) concentration showed no significance differences in both the sediment and water across the three sites (Fig. 2.5A & B). Although Shokwe Pan had the highest concentrations of Cd in both the sediment (0.124 mg/kg) (Fig. 2.5A) and water (0.1023 µg/mL) (Fig. 2.5B), large standard deviations occurred, therefore no significance could be evidenced. Shokwe Pan showed significantly higher ($p < 0.0001$) concentrations of lead (Pb) (16.36 mg/kg) present in the sediment (Fig. 2.5C) than both the Usuthu River (4.157 mg/kg) and Nyamithi Pan (5.74 mg/kg). Nyamithi Pan had higher concentrations of Pb (0.8583 µg/mL) in the water (Fig. 2.5D) but because of a large standard deviation there was no significant difference between the three sites. There was no significant difference in the concentration of Se (Figs. 2.5E & F) and Ag (Figs. 2.5G & H) in the sediment across the three sites with Shokwe Pan having the highest concentrations. Nyamithi Pan had significantly higher concentrations of Se in the water than the Usuthu River ($p = 0.0219$) (Fig. 2.5F).

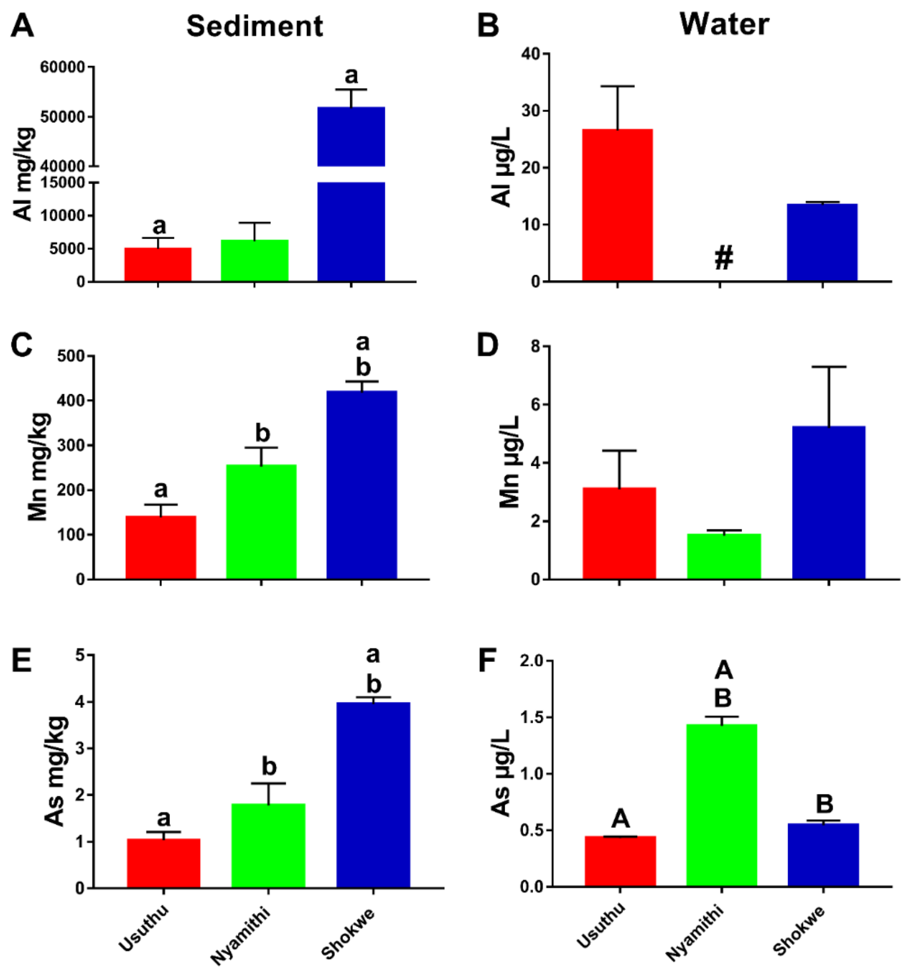

Figure 2-4: Bar graphs showing the concentration (mean and SEM) of different non-essential (Al, Mn, As) metals in both the sediment and water across the three sites (common superscripts are used to indicate significant differences between sites; # indicates a concentration below detection limit).

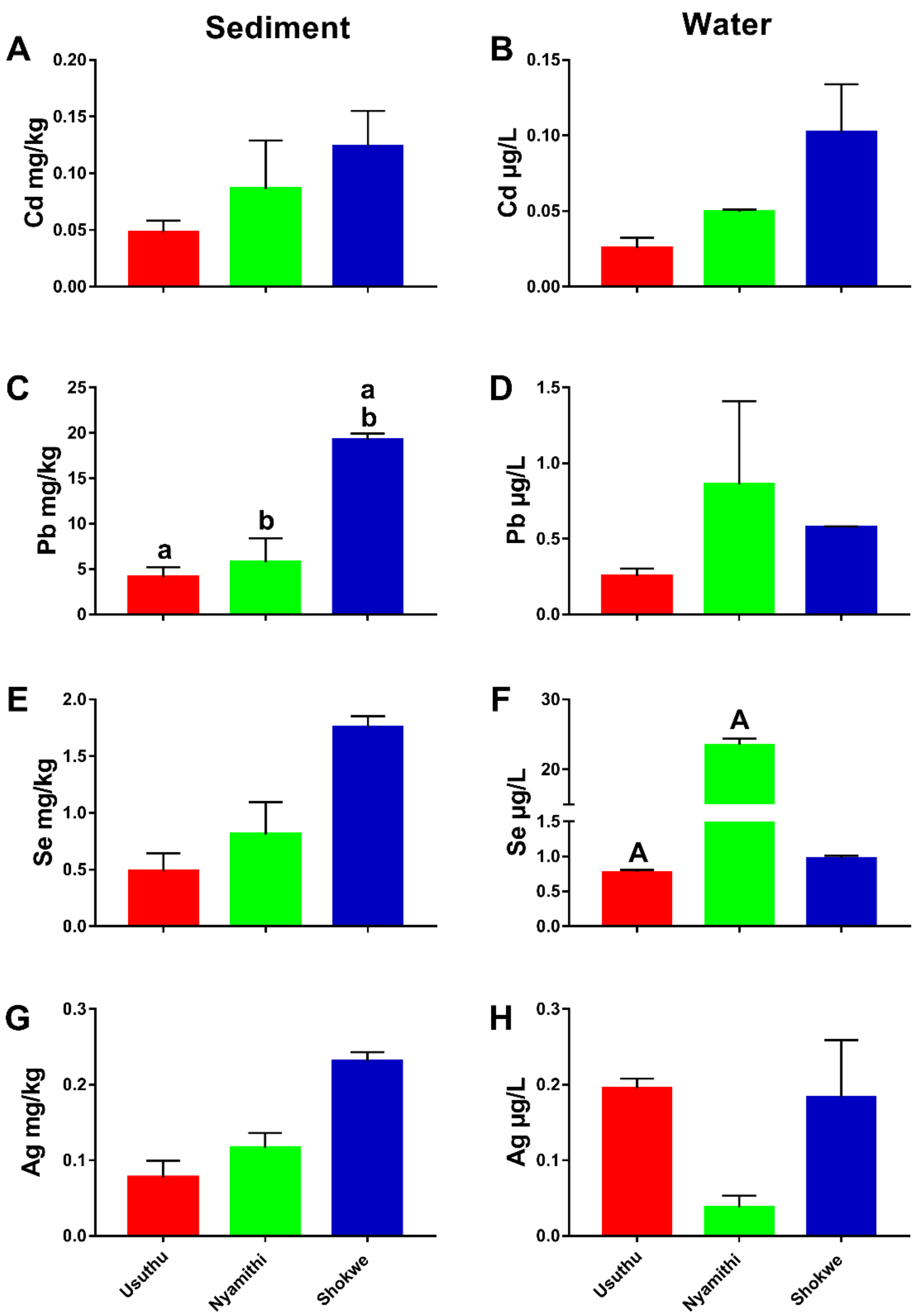

Figure 2-5: Bar graphs showing the concentration (mean and SEM) of different non-essential metals (Cd, Pb, Se, Ag) in both the sediment and water across the three sites located in Ndumo Game Reserve (common superscripts are used to indicate significant differences between sites).

A biplot of the canonical variates derived from the results of a Discriminant Function Analysis (DFA) in Figure 2.7 shows the variation in the metal concentrations present in sediment samples of the three sites in Ndumo Game Reserve. The structure matrix of the two functions can be seen in Table 2.2. Functions 1 and 2 describe 100% of the variance in metal concentration at the different sites. The first function (X axis) describes 77.8% of the variance, where the three sites are separated by the concentration of metals in the sediment which was driven by a decrease in Cd concentration and an increase in Al concentrations. The second function (y axis) describes 22.2% of the variance and was mainly driven by Co, Zn, Cu and As. There was a 100% accuracy for the reclassification of the variant data to their predefined groups (Table 2.3).

Table 2-2: The structure matrix of the first two functions of a Discriminant Function Analysis (DFA) using various metal concentrations present in the water from three different sites in Ndumo Game Reserve (the largest absolute correlation between each variable and any discriminant function is indicated in bold).

Structure Matrix												
Metal		% explained	Cd	Al	Co	Zn	Cu	As	Fe	Mn	Ni	Pb
Function	1	77.8	**-0.439**	**0.057**	0.006	-0.102	0.005	-0.056	0.056	0.116	-0.004	-0.119
	2	22.2	-0.281	0.036	**0.411**	**0.372**	**0.354**	**0.332**	**0.319**	**0.294**	**0.292**	**0.252**

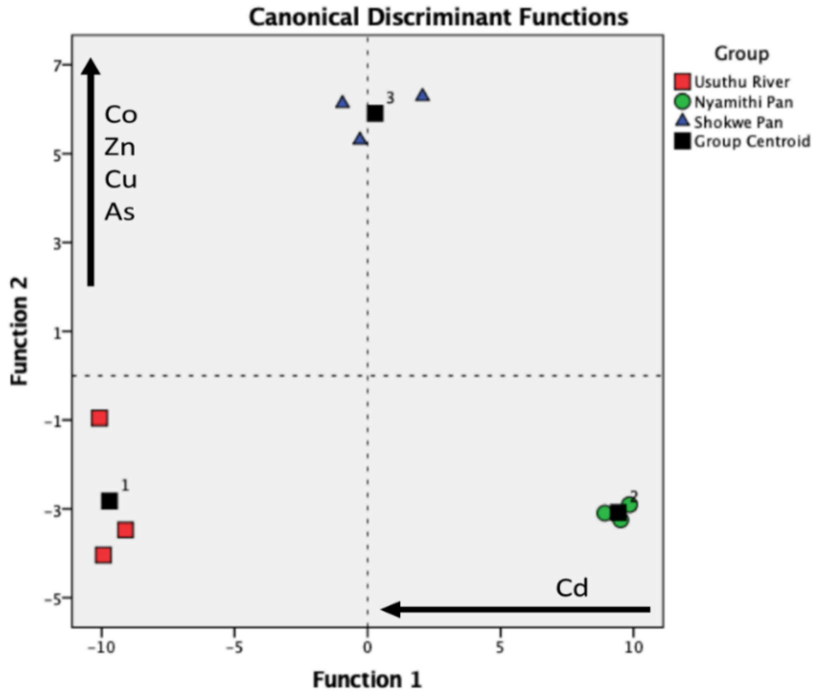

Figure 2-6: Biplot of the canonical variates obtained from a Discriminant Function Analysis (DFA) comparing the metal concentration of the different metals in the sediment of three sites located in Ndumo Game Reserve.

Table 2-3: Classification summary of the different metal concentrations from a Discriminant Function Analysis (DFA) of metal concentrations in the sediment of the three different sites at Ndumo Game Reserve.

Classification Results[a]				
Group	**% Predicted Group Membership**			**Total**
	Usuthu River	**Nyamithi Pan**	**Shokwe Pan**	
Usuthu River	100	0	0	100
Nyamithi Pan	0	100	0	100
Shokwe Pan	0	0	100	100

[a] 100% of the original grouped cases correctly classified

The results of a Discriminant Function Analysis of the different metal concentrations present in the water across the three sites is used to form a biplot of canonical variates (Fig. 2.6). The structure matrix of the first two axes can be seen in Table 2.4. The Discriminant Function Analysis of functions 1 and 2 describe 100% of the variation in the mean metal concentrations in the water of the three sites. The first function (X axis) explains 99.5% of the variance where the three sites were separated according to the metal concentrations in the water and was mainly driven by the gradient of As across the sites. Nyamithi Pan had the highest concentration of As followed by Usuthu River and Shokwe Pan with the lowest concentration of metals. The second function (y axis) describes the remaining 0.5% with Zn and Al as the main drivers. There was a 100% accuracy for the reclassification of the variant data into their predefined groups (Table 2.5).

Table 2-4: The structure matrix of the first two functions of a Discriminant Function Analysis (DFA) using various metal concentrations present in the water from three different sites in Ndumo Game Reserve (the largest absolute correlation between each variable and any discriminant function is indicated in bold).

Structure Matrix												
Metal		% explained	As	Zn	Al	Ni	Pb	Cd	Cu	Co	Fe	Mn
Function	1	99.5	**-0.190**	-0.151	0.074	-0.012	-0.092	0.141	-0.047	-0.036	-0.064	0.015
	2	0.5	-0.151	**0.765**	**-0.581**	**0.436**	**0.341**	**-0.253**	**0.226**	**0.176**	**-0.163**	**0.082**

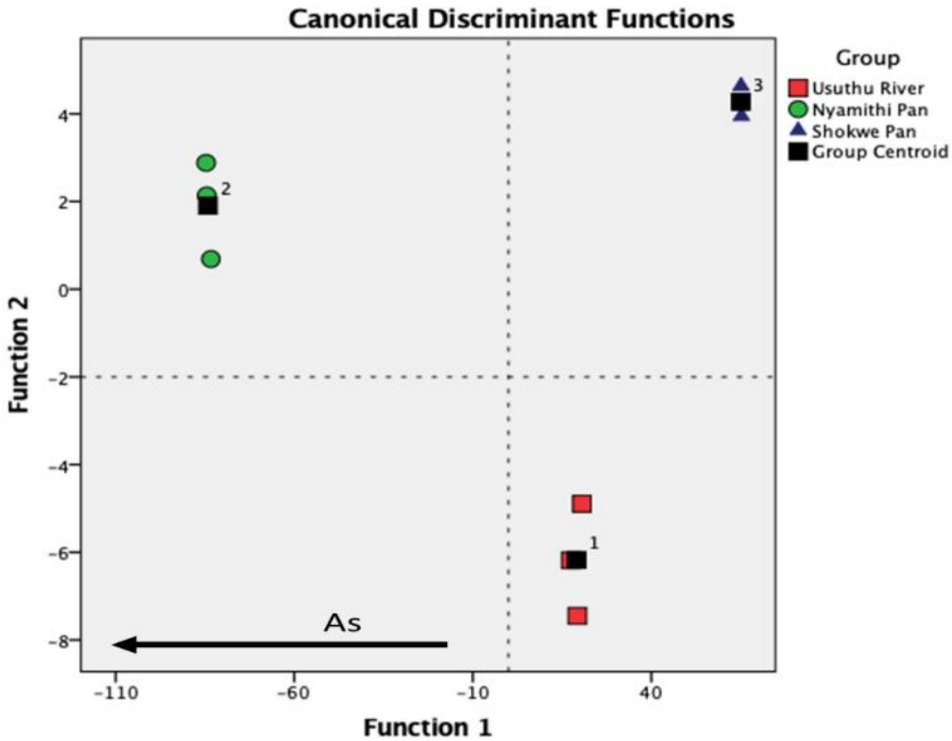

Figure 2-7: Biplot of the canonical variates obtained from a Discriminant Function Analysis (DFA) comparing the concentration of 12 different metals in water samples across three sites located in Ndumo Game Reserve South Africa.

Table 2-5: Classification summary of the different metal concentrations from a Discriminant Function Analysis (DFA) of metal concentrations in the sediment of the three different sites at Ndumo Game Reserve.

Classification Results[a]				
Group	**% Predicted Group Membership**			**Total**
	Usuthu River	**Nyamithi Pan**	**Shokwe Pan**	
Usuthu River	100	0	0	100
Nyamithi Pan	0	100	0	100
Shokwe Pan	0	0	100	100

[a] 100% of the original grouped cases correctly classified

2.4. Discussion

In order to establish the condition of the aquatic ecosystem in NGR the sediment and water metal concentrations were compared to guidelines. As there are currently no sediment guidelines for South Africa (Gordon & Muller, 2010), the sediment in this study was compared with the Canadian sediment guidelines (CCME, 2014). The water concentrations were compared to the South African guideline for water quality set up by DWAF (1996), as well as the Australian, New Zealand (Anzecc, 2000) and European quality guidelines (Cole *et al.*, 1999).

The three aquatic sites were also compared with different aquatic ecosystems in South Africa as well as two international floodplain pans namely the River Lahn and River Severn floodplains. The lotic system was compared to three rivers in the Kruger National Park (KNP) because it is the biggest conservation area in South Africa and the lentic systems were compared to the Nyl River floodplain of South Africa and two international floodplains. The Nyl River floodplain is one of South Africa's largest floodplains (McCarthy *et al.*, 2011). The concentrations of Zn, Cd, As and Pb were lower at all three sites than the SQG of the CCME (2014).

When comparing the metal concentrations in the sediment of the lotic system in the study areas to the two lentic systems as well as the three different rivers in the KNP, the lotic system had the lowest concentrations of metal in the sediment compared to the lentic systems, because of the flowing water flushing the sediment downstream (Extence *et al.*, 2013, Meena *et al.*, 2018). The lotic system has a sandy substrate while the two lentic systems have a clay substrate; metals bind to greater effect to a clay substrate than to a sandy substrate (Pekey, 2006).

Both the essential and non-essential metal concentrations of the sediment of the Usuthu River lotic system are compared with the sediment levels of three different rivers (Olifants, Letaba and Luvuvhu) located in the KNP in Table 2.4. The KNP is one of the most studied protected areas in South Africa and there are results available of the metal concentrations of the main rivers (Gerber *et al.*, 2016). The three biggest east-flowing rivers were chosen to compare with the east-flowing river of this study. The KNP is 332 km north-north-west of NGR. The Olifants River is known to be one of the most mining-polluted rivers in South Africa (McCarthy, 2011).

Usuthu River had lower concentrations of both the essential and non-essential metals than the Olifants and Luvuvhu Rivers. This is because the Usuthu River has no major mining activities in its catchment area and is mainly used for small-scale agriculture. When comparing the Usuthu

River with the Letaba River, Usuthu River had higher concentrations of Ni and Pb, but lower concentrations of the other metals. In comparing with the CCME (2014) it was found that all metal concentrations in the sediment of the Usuthu River were below their guidelines and thus proves that the Usuthu River is in a moderately good condition.

When comparing the metal concentrations of the two lentic systems of NGR to the lotic system it was found that both the lentic systems had higher concentrations of all the metals than the lotic system. This is because of the fact that metals that flow into a pan or lake adsorb to the sediment and the sediment acts as a sink (Tendaupenyu & Magadza, 2019). The two lentic systems also have a clay substrate where metals have more surface area to bind to and thus they bind more than the lotic system's sandy substrate (Pekey, 2006).

The two lentic systems were compared with each other in order to determine the effect of saline on the bioaccumulation of metals. The freshwater lentic system had the highest concentrations of all the metals in the sediment. The saline pan has high concentrations of salts that form complexes with the metals, which make adsorption to the sediment more difficult (Wright & Zamuda, 1987, Kumar *et al.*, 2015).

The comparison of the metal concentrations in the sediment of the two lentic systems in NGR to the floodplain of the Nyl River as well as to two international floodplains can be seen in Table 2.5. Shokwe Pan had the higher concentrations of all the essential metals compared to the Nyl River and the international floodplains. The elevated levels of concentration at Shokwe Pan could be due to the geological qualities of the system. The concentrations of essential metals in the sediment of Nyamithi Pan were lower than the Nyl River and the two international floodplains. The salts in Nyamithi Pan play a big role in preventing the adsorption of metals to the sediment.

The concentrations of the non-essential metal manganese in the sediment of the two lentic pans were well below the concentration in the Nyl River sediment. The Cd and Pb concentrations in the two lentic systems were well below the concentrations in both the international floodplain sites. The non-essential metals were also well below the concentrations for the CCME target sediment guidelines (CCME, 2014).

Table 2-6: Comparison of the metal concentrations (mg/kg) in the sediment of the Usuthu River lotic system, the Shokwe Pan freshwater lentic system and the Nyamithi Pan saline lentic system with the metal concentrations found in the sediment of three rivers in the Kruger National Park (Olifants, Letaba, Luvuvhu) and in three floodplains (Nyl River, River Lahn, River Severn).

	Metals	**Essential**					**Non-essential**						
		Fe	**Co**	**Ni**	**Zn**	**Cu**	**Al**	**Mn**	**As**	**Cd**	**Pb**	**Se**	**Ag**
River sediment (mg/kg)	**Usuthu River**	**6864**	**5.551**	**15.05**	**12.86**	**11.73**	**4950**	**139**	**1.031**	**0.048**	**4.16**	**0.49**	**0.08**
	Olifants River (Gerber *et al.*, 2016)	17501	11.52	31.34	59.29	45.07	12333	382.32	7.14	0.14	4.78	1.07	
	Letaba River (Gerber *et al.*, 2016)	11504	61.98	11.11	25.87	19.3	14858	156.55	1.22	0.08	1.74	1.58	
	Luvuvhu River (Gerber *et al.*, 2016)	21917	12.22	54.05	106.82	52.15	16678	451.48	1.73	0.21	7.8	1.87	
Floodplain sediment (mg/kg)	**Shokwe Pan**	**50027**	**25.67**	**85.92**	**502.4**	**55.88**	**51217**	**418.2**	**3.95**	**0.12**	**19.24**	**1.76**	**0.23**
	Nyamithi Pan	**14795**	**7.188**	**21.35**	**18.46**	**16.02**	**6130**	**252.6**	**1.78**	**0.09**	**5.74**	**0.81**	**0.12**
	Nyl River Floodplain (Dahms *et al.*, 2017)	3600.8		58.23	149.4	6.117		4324.1					
	River Lahn Floodplain (Martin, 2000)		11.8		121	27.8				0.6	41.9		
	River Severn (Taylor & Lewin, 1996)			80.47	338.1	55.48				1.91	69.26		

When comparing the metal concentrations in the water across the three sites in NGR it can be seen that for most of the metals the lotic system had the lowest concentrations in the water. The lotic system has a continuous flow of water that carries metal concentrations downstream.

The study found that the saline lentic system had the highest concentration of all metals in the water except for Al that was below detection level in the water of Nyamithi Pan. The high salt concentrations in the water form complexes with the metals that make adsorption harder than in fresh water and thus the metals stay in the water (Wright & Zamuda, 1987, Kumar *et al.*, 2015). Adsorption is possible in the freshwater lentic system and thus the lentic system has lower water concentrations of metals than the saline lentic system. The concentration of all essential metals except for Zn in the water of the three sites in NGR was below the TWQR of South Africa with Zn being higher than the South African guidelines but lower than the Australian and European guidelines.

The concentration of Fe at the three sites was very similar to the concentrations found in the three rivers of the KNP (Table 2.6). Iron is found naturally in all ecosystems and is considered to be the fourth most abundant metal in natural ecosystems and comprises 3.5% of the earth's crust (Mucha *et al.*, 2003). Iron can also enter aquatic ecosystems through anthropogenic activities such as mining (Mucha *et al.*, 2003). There are no major mining activities in the catchment areas of the Usuthu River. Currently there is no TWQR for Co in South Africa (DWAF, 1996), but the concentration in the three study sites was lower than in the four rivers namely the Olifants, Letaba, Luvuvhu and Nyl rivers (Table 2.6). Cobalt can be introduced into aquatic ecosystems naturally through forest fires, seawater spray, volcanoes or through anthropogenic activities such as burning fossil fuels or phosphate fertilisers (Zadnipryany *et al.*, 2017). In the Ndumo area as well as the Usuthu River catchment area there are small-scale subsistence farmers who use small amounts of fertilisers as well as engage in small-scale burning of fossil fuels as heat or cooking source.

Although there is no TWQR for Ni in South Africa the concentration of Ni across the three sites was lower than the TWQR for Australia, America and Europe. The concentration of Ni in both Shokwe and Nyamithi pans was higher than the concentration in the three KNP rivers (Table 2.6). Nickel is released into aquatic ecosystems naturally from weathering of sandstone, slate and clay. It can also be introduced into the environment through mining activities (Cempel & Nikel, 2006). The Olifants River catchment area is full of mining activities that release mining effluence through runoff into the environment (Gerber *et al.*, 2016). There are no mining activities close to the NGR or in the Usuthu River catchment area and thus the lower concentrations of Ni in the water.

The concentration of Cu was higher in the three sites than in the four rivers (Table 2.6). Nyamithi Pan had the highest concentration of Cu present. Copper is an important trace element and is used for enzyme functions (de Oliveira-Filho *et al.*, 2004), and can be found at approximately 60 mg/kg in the earth's surface (Flemming & Trevors, 1989). Anthropogenic activities such as mining and burning of fossil fuels release Cu into the environment (de Oliveira-Filho *et al.*, 2004).

The zinc concentrations in the water of all three sites were above the TWQR of South Africa which is 2 µg/L for aquatic ecosystems. The concentrations fall within the range of the Australian TWQR which is between 2.4 µg/L for 100% of species and 32 µg/L for 80% of species. The Zn concentrations of the three sites were higher than in the three KNP rivers but lower than the concentrations found in the Nyl River (Table 2.6). Zinc enters aquatic ecosystems naturally through the weathering of rocks and sulphides (DWAF, 1996). Anthropogenic influences such as exhaust fumes, fertilisers and insecticides can also contribute to zinc levels in aquatic ecosystems through runoff (DWAF, 1996). There are no mining activities close to the NGR and very little use of fertilisers close to NGR thus it is believed that the Zn concentrations were high due to geological characteristics of the NGR.

The concentrations of the non-essential metals in the water of the three different sites in NGR were compared to those of the three KNP rivers as well as the Nyl River (Table 2.5). The water concentration of Al in the three sites was higher than the Target Water Quality Range (TWQR) set by DWAF (DWAF, 1996) of 0.1 to 1.5 µg/L. Although the concentration was higher than the TWQR it was still lower than the Olifants, Letaba, Luvuvhu and Nyl rivers of South Africa. Aluminium is naturally abundant in ecosystems and comprises 8.1% of the earth's crust (Tria *et al.*, 2007). Therefore, higher levels of aluminium can be the result of natural corrosion of the earth's crust. Anthropogenic activities such as the addition of Al salts to purify water and coal combustion are also sources of Al in surface water (Tria *et al.*, 2007). Ndumo Game Reserve is not located close to any big coal combustion sites, but there is small-scale burning of coal for cooking. According to WHO (2017) the concentration of Al in the water of the three sites, although exceeding the TWQR, is still considered as tolerable for surface waters.

The TWQR for Mn in South Africa is a concentration of 180 µg/L. Therefore, all three sites were well below the concentration set for TWQR in South Africa. The concentration of Mn in the three sites was higher than the concentration of Mn in both the Olifants and Luvuvhu rivers but lower than in the Letaba River and within the range of that of the Nyl River. Although Mn is released into the environment through mining industries there is no evidence of that at the sites. The low concentration of Mn in the water does not suggest Mn pollution at the selected sites.

The concentration of As in the water of all three sites exceeded the TWQR of South Africa. Arsenic is released into the aquatic ecosystem through the natural weathering of As containing rocks or ores (DWAF, 1996). Arsenic is a by-product of copper smelting (Ng *et al.*, 2003), and is also a by-product of insecticides or herbicides (Mohan *et al.*, 1982, Alain *et al.*, 1993). The increased levels of As could be due to the herbicides and insecticides the locals use for their sustainable farming. The concentration of Cd in the water of all three sites was below the TWQR of South Africa (0.15-0.4 µg/mL) but a bit higher than the TWQR of both Australia (0.8 µg/mL) and Europe's (<0.08-0.25 µg/mL). The concentration of cadmium in all three sites was lower than the concentrations in the KNP rivers (Table 2.7). Cadmium is normally found in association with other elements such as Cu, Zn and sulphides, and enter aquatic ecosystems through natural weathering (DWAF, 1996). Mining and industrial activities release Cd into aquatic ecosystems through runoff (DWAF, 1996).

The concentrations of Pb in the water at the three sites were below the TWQR of South Africa (1.2 µg/mL). The lead concentrations were also lower than in the four rivers (Table 2.5). Most lead present in aquatic ecosystems is introduced through natural weathering of sulphide ores (DWAF, 1996). Anthropogenic activities such as sewage treatment and fuel combustions can also introduce Pb into the environment (Gioia *et al.*, 2010). The toxicity of lead is dependent on the pH of the water body: the more alkaline the water the more the toxicity increases (DWAF, 1996). Only the concentration of Se in Nyamithi Pan was higher than the concentrations found in the three rivers of the KNP (Table 2.7). According to Lemly (2002), selenium is a natural element, but Se pollution occurs worldwide through anthropogenic activities such as mining, agriculture and industry.

Table 2-7: Comparison of the metal concentrations (µg/L) in the water of the lotic site (Usuthu River) and the two lentic sites (freshwater Shokwe Pan and saline Nyamithi Pan) of Ndumo Game Reserve to three rivers of the Kruger National Park (Olifants River, Letaba River, Luvuvhu River) as well as the Nyl River.

	Metals	**Essential**					**Non-essential**						
		Fe	**Co**	**Ni**	**Zn**	**Cu**	**Al**	**Mn**	**As**	**Cd**	**Pb**	**Se**	**Ag**
Water (µg/L)	**Usuthu River**	**41.35**	**0.139**	**0.613**	**7.561**	**4.28**	**26.59**	**3.099**	**0.431**	**0.026**	**0.255**	**0.78**	**0.20**
	Shokwe Pan	**30.49**	**0.139**	**1.937**	**8.206**	**4.079**	**12.58**	**5.198**	**0.547**	**0.102**	**0.575**	**0.98**	**0.18**
	Nyamithi Pan	**71.45**	**0.486**	**2.236**	**12.62**	**34.23**	**ND**	**1.506**	**1.424**	**0.0494**	**0.858**	**23.54**	**0.04**
	Olifants River (Gerber *et al.*, 2016)	26.48	0.31	1.18	4.71	1.52	51.19	1.29	0.95	0.14	2.96	2.42	
	Letaba River (Gerber *et al.*, 2016)	41.77	4.38	1.76	5.42	1.19	99.27	5.4	0.6	5.77	5.68	2.09	
	Luvuvhu River (Gerber *et al.*, 2016)	65.25	0.18	1	3.85	1.68	50.85	1.23	0.34	0.13	1.15	1.18	
	Nyl River (Dahms-Verster *et al.*, 2018)	34.88 – 915.65	0 – 0.71	0.48 – 10.5	14.62 – 89.67	2.10 – 11.01	40.74 – 115.59	0.10 – 16.7		0- 0.76	0.5 – 11.27		

2.5. Conclusion

The hypothesis for this chapter was that because lentic systems function as natural sinks for metals, they would have higher concentrations of metals than the lotic system, and that concentrations of metals present in all sites of the study would be below the international guidelines for target water quality as well as international sediment quality guidelines. This study is the first to report on the environmental concentrations of metals in NGR. In the study it was found that the freshwater lentic system had the highest concentrations of metals in the sediment across the three sites followed by the saline lentic system whereas the lotic system had the lowest concentrations of metals in the sediment. The metal concentrations in the water was the highest in the saline lentic system. Although the lotic system did not have the lowest concentrations of all the metals in the water it had the lowest overall concentrations of metals in the water.

All the metals were below the Canadian sediment quality range and most of the metal concentrations in the water were found to be lower than the TWQR of South Africa. The essential metal, Zn, was higher than the TWQR of South Africa but lower than the TWQR of Australia and Europe. The non-essential metals, Al and As, had higher concentrations in the water at the three sites than the TQWR of South Africa, and can thus be a source for concern in the aquatic ecosystems. Overall the hypothesis can be accepted with the lentic systems having higher environmental concentrations of both the essential and non-essential metals. Although the Al and As concentrations in the water exceeded that of the TWQR of South Africa the hypothesis that states that the metal concentrations in the study areas would be below the guidelines can still be accepted.

Now that the environmental concentration of metals in the study sites is known it can be used to determine the exposure of the environmental concentrations on the aquatic organisms in the ecosystem, and their role as bio-indicators.

3. Bioaccumulation of essential and non-essential metals in the tissues of the bio-indicator – *Clarias gariepinus* – from three sites with differing abiotic metal profiles.

3.1. Introduction

As previously indicated metals can enter aquatic ecosystems through many different pathways, ranging from geological weathering and atmospheric deposition to anthropogenic impacts such as agricultural, industrial and residential waste products (Dhanakumar *et al.*, 2015), where these metals would be detrimental to organisms at high concentrations (Eneji *et al.*, 2011). Scientists use biomonitoring to investigate the presence of increased metals in aquatic ecosystems and monitor these levels over time. Biomonitoring is the use of feral or transplanted organisms as an early warning system for pollution of the environment, i.e. aquatic organisms in aquatic ecosystems (Adams & Greeley, 2000). Fish are considered to be one of the best bio-indictors used for biomonitoring of metal pollution in aquatic ecosystems (Authman *et al.*, 2015).

Fish have long lifespans and can therefore integrate fluctuations of metals over an extended period, where it is anticipated that the most abundant of the metals present will have higher concentrations in older organisms (Lamas *et al.*, 2007). Fish spend their entire lives in water and can therefore be used for continuous monitoring of metals. Bioaccumulation in fish also improves the accuracy of analysing trace metals near the detection limits and lowers the costs of the analysis (Rainbow, 1998). Ayandiran *et al.* (2009) state that in a polluted environment, fish tend to accumulate metals in their organs, which is supported by many previous studies which have found that metals accumulate in organs such as the gonads, heart, kidney, bone, liver and brain, but many studies focus on the edible muscle tissue (Avenant-Oldewage & Marx, 2000, Eneji *et al.*, 2011, Murtala *et al.*, 2012, El-Moselhy *et al.*, 2014).

Biomagnification is the increase in concentration of pollution in aquatic organisms across the trophic levels of the environment up to the top predator (Thomann, 1989, Ribeiro *et al.*, 2005, Juncos *et al.*, 2019). The accumulation patterns of metals for fish are dependent on both the elimination and uptake rates (Mansour & Sidky, 2002). According to Brezonik *et al.* (1991) and Burger *et al.* (2002), there are two main uptake routes for metals in fish. Firstly, the transport of metals via ionic exchange through the gills of the fish; secondly, through ingestion, sediment and food particles containing the metals passing through the digestive tract where the metals along with nutrients are absorbed in the intestines of the fish. Previous studies have found that the latter route can be considered as the most important (Fleischer *et al.*, 1974, Bervoets *et al.*, 2001, Bervoets & Blust, 2003, Bury *et al.*, 2003, Sauliutė & Svecevičius, 2015).

Clarias gariepinus (Burchell 1822) is a commercially important fish species that serves as a major food source in third world countries (Farombi *et al.*, 2007). According to Olufemi *et al.* (1991), the catfish is widely distributed in the rivers, lakes and dams across the entire African continent. It is considered that *C. gariepinus* is the most important tropical catfish species for aquaculture in West Africa (Huisman & Richter, 1987, Gabriel *et al.*, 2007, Oké & Goosen, 2019). *Clarias gariepinus* can be considered to be a good bio-indicator of metal pollution, because, it is a bottom-dwelling fish that comes into contact with the sediment regularly (Gamal *et al.*, 2012). As the catfish disturbs the sediment, metals are released back into the water column and the metals become available for uptake (Simpson *et al.*, 2002).

The sharptooth catfish is known to have a long lifespan with Booth *et al.* (2010) reporting catfish of more than 15 years old. They are omnivores that feed on anything from plants to other predators in the water body (Skelton, 2012). The catfish can endure harsh conditions and therefore is able to survive and thrive even in highly polluted systems (Skelton, 2012). *Clarias gariepinus* is a fish species without scales, which may contribute to higher metal accumulation in the skin and muscle tissues of the fish (Coetzee *et al.*, 2002). Furthermore, numerous previous studies in several freshwater aquatic ecosystems across Africa have shown that *C. gariepinus* is an important fish species which can be used for biomonitoring as it is able to accumulate and thus reflect metal contamination in its direct environment (Coetzee *et al.*, 2002, Asagba *et al.*, 2008, Crafford & Avenant-Oldewage, 2010, Eneji *et al.*, 2011, Jooste *et al.*, 2015).

This chapter serves to investigate the metal accumulation profiles in two tissues of *C. gariepinus* from the three study sites to ascertain whether these catfish can reflect their unimpacted environments (as shown in Chapter 2). The hypothesis for this chapter states that the bioaccumulation of metals in *C. gariepinus* at all three sites will reflect the environmental conditions of the sites, with both the lentic systems (fresh and saline) having higher bioaccumulation factors than the lotic system. *Clarias gariepinus* will also bioaccumulate the non-essential metals to higher levels than the essential metals. The aim of this chapter is to determine the concentration of essential and non-essential metals in both the muscle and liver tissues of the host *C. gariepinus* and to establish if these concentrations differ across the various types of aquatic ecosystems.

3.2. Materials and methods

3.2.1. Study area and sampling

Sampling took place during November 2018 at sites within Shokwe and Nyamithi pans and along the Usuthu River all located within the NGR (For detailed background and site descriptions refer to Chapter 2 Sections 2.2.1 and 2.2.2, as well as Figs. 2.1 and 2.2).

3.2.2. Field collections

For the study 25 *C. gariepinus* individuals were collected at both Nyamithi and Shokwe pans and 24 at the Usuthu River. Different sampling techniques were used, including rod and reel and fyke nets. Rod and reel sampling worked best at the lentic sites (Shokwe and Nyamithi pans) whilst the fyke nets worked best at the lotic site (Usuthu River). As the catfish were collected, they were placed in temporary holding/transport tanks and transported to the field station where they were euthanised by severing the spinal cord (SOP NWU-00267-17-S5, see appendix 10). All sampling procedures were approved by the NWU-AnimCare Committee (Ethics Committee on Animal Care, Health and Safety in Research) (AREC-130913-015). The necessary muscle from the left side of the catfish and liver tissues were sampled and placed in polypropylene tubes before freezing (-20˚C) for later laboratory analysis.

3.2.3. Laboratory analysis of tissues and quality control

Samples were removed from the freezer and allowed to defrost prior to drying. A known weight (~10 g) of both tissue types were freeze-dried using a Labconco FreeZone® Plus 6 Liter Cascade Console Freeze Dry System (7753030). The dried tissue samples were reweighed (in order to calculate the moisture content for wet weight comparisons if needed), whereafter 0.2 g of the dried samples were placed into Teflon tubes and 2.5 mL nitric acid (HNO_3) as well as 7.5 mL 32% hydrochloric acid (HCl) were added to each tube. The samples and blanks (2.5 mL HNO_3, 7.5 mL 32% HCl) were then digested using the Ethos Easy Maxi-44 Microwave Digestion system at 1800

W at 200˚C for 45 minutes. The digested samples were diluted to 50 mL with 1% HNO_3 and decanted into amber glass bottles, labelled and sent for ICP-MS analysis. All metal concentrations were converted into mg/kg using the following equation derived from Dahms-Verster *et al.,* (2016).

$$\text{Metal concentration (mg/kg)} = \frac{ICP-MS\ reading\,(mg/L)}{Sample\ dry\ mass\ (g)} \text{ x dilution volume.}$$

To ensure quality control for the digestion and ICP-MS analysis, eight reference samples (DORM-4: Fish protein certified reference material for trace metals) were treated in the same way as the muscle and liver tissue of the catfish and analysed. The results found for the concentration of metals in the certified reference material (CRM) and the percentage recovery are shown in Table 3.1. The recovery ranged from 96% for Ni to 149% for Pb. All the elements except for Cd and Pb showed recoveries within the 20% of the certified range (Erasmus *et al.,* 2020).

Table 3-1: The mean measured concentrations of several metals of the certified reference material (DORM-4: Fish protein certified reference material for trace metals) with the percentage recovery rates (all concentrations were converted into mg/kg).

	Tissue		
	Reference (mg/kg)	**Measured (mg/kg)**	**% Recovery**
Mn	3.17 ± 0.26	3.25	102
Ni	1.34 ± 0.14	1.42	96
Cu	15.7 ± 0.46	19.19	119
Zn	51.6 ± 2.8	49.81	97
As	6.87 ± 0.44	8.38	115
Cd	0.299 ± 0.018	0.38	127
Pb	0.404 ± 0.062	0.70	149

3.2.4. Statistical analysis

Univariate statistics were analysed using GraphPad Prism®7 software. Homogeneity was tested by using the Shapiro-Wilk test and if the data was found to be distributed normally, a one-way analysis of variance (ANOVA) with Tukey post-hoc analysis were used. If the data was not distributed normally, a Kruskal-Wallis followed by the Dunn’s multiple comparison test was performed. A Discriminant Function Analysis (DFA) was performed using SPSS version 18

(PAWS Statistics, IBM, USA) to determine if metal concentrations in host tissues can be used to reclassify individual catfish into their respective sites, as well as to determine which of the metals were the most successful in discriminating between sites. Tests were done in order to establish if any significant differences ($p < 0.05$) between the three sites of the study occurred. The bioaccumulation factor (BAF) was calculated using the following equation derived from Arnot and Gobas (2006):

$$BAF = \frac{Concentration\ in\ host\ tissue}{Concentration\ in\ water}$$

3.3. Results

3.3.1. Biometric data of *Clarias gariepinus* across the three sites

From the 74 *C. gariepinus* samples selected during the study 40 individuals were females, 33 individuals were males and only one had not yet reached sexual maturity (Table 3.2). The catfish of lotic and freshwater lentic systems were of similar size with the catfish at Nyamithi Pan significantly larger than those at the other two sites. The catfish across the three sites ranged in weight from 70 g to 4280 g.

Table 3-2: Biometric data of the *Clarias gariepinus* sampled at the three different sites located in the Ndumo Game Reserve (bold numbers indicate significantly different values).

Site	Standard length (cm)	Total length (cm)	Weight (g)	# of males	# of females	# of juveniles
Usuthu River	398.75 ± 87.11 (178 – 578)	450.67 ± 99.54 (209 – 654)	733.79 ± 413.44 (70 – 1700)	13	10	1
Nyamithi Pan	**505.08 ± 115.76 (259 – 710)**	**563.8 ± 121.95 (391 – 790)**	**1648.16 ± 1193.05 (170 – 4280)**	15	10	0
Shokwe Pan	406.08 ± 116.62 (255 – 705)	456.2 ± 130.91 (290 – 775)	830.8 ± 841.78 (100 – 3840)	5	20	

3.3.2. Metal concentrations in hosts

The concentrations of the essential metals (Fe, Co, Ni, Zn and Cu) (Fig. 3.1) and non-essential metals (Al, Mn, As, Cd, Se, Ag and Pb) (Fig. 3.2 & Fig. 3.3) were analysed and compared across the three different aquatic ecosystems. The mean and standard deviation of the 12 metals at the three different sites are compared in bar graphs and the concentrations are expressed in mg/kg.

3.3.2.1. Essential metals

The concentrations of the essential metals in both the muscle and liver tissues of *C. gariepinus* across the three sites are shown in Fig. 3.1. There were no significant differences in the Fe concentrations in both the muscle and liver tissues of the hosts across the three sites. The catfish in the lotic system had higher concentrations of most of the essential metals in both the muscle and liver tissues.

Muscle tissue:

There were no significant differences in the Fe (Fig. 3.1A) and Co (Fig. 3.1C) concentrations in the muscle tissue across the three sites. The catfish from the Usuthu River had the highest concentration of Fe (Fig. 3.1A) in muscle (34.06 mg/kg) followed by catfish from Shokwe Pan (33.6 mg/kg) and Nyamithi Pan (31.7 mg/kg). Usuthu River had the highest concentration of Co (Fig. 3.1C) with a concentration of 0.065 mg/kg, followed by the catfish at Shokwe Pan (0.059 mg/kg) and the catfish at Nyamithi Pan (0.054 mg/kg).

The *C. gariepinus* of the Usuthu River had significantly lower concentrations of Ni (Fig. 3.1E) ($p = 0.031$) and Zn (Fig. 3.1G) ($p = < 0.0001$ for both) in the host muscle tissue than *C. gariepinus* at Nyamithi and Shokwe pans as well as significantly higher levels of Cu (Fig. 3.1I) than *C. gariepinus* at Nyamithi and Shokwe pans. The catfish at Shokwe Pan had higher levels of all the essential metals than in the catfish of the second lentic system (Nyamithi Pan), but only significantly higher levels of Ni (Fig. 3.1E) and Zn (Fig. 3.1G). Shokwe Pan's catfish had the highest concentration of Ni in the muscle tissue at 0.177 mg/kg, followed by the catfish at Nyamithi Pan (0.150 mg/kg) and the catfish in the Usuthu River (0.083 mg/kg).

The Zn in the muscle tissue of *C. gariepinus* at Shokwe Pan was the highest with a concentration of 18.4 mg/kg, closely followed by the *C. gariepinus* at Usuthu River (13.69 mg/kg). Nyamithi Pan's *C. gariepinus* had the lowest concentration at 13.29 mg/kg. The concentration of Cu (4.086 mg/kg) in the muscle tissue of catfish at the Usuthu River was significantly higher than for both the catfish at Shokwe Pan (3.038 mg/kg) ($p = 0.002$) and Nyamithi Pan (2.657 mg/kg) ($p = 0.028$).

Liver tissue:

The liver of the catfish had higher concentrations than the muscle tissue for all the essential metals (Fig. 3.1). All the essential metals except Ni (Fig. 3.1F) were significantly higher in the liver than in the muscle tissue. The concentrations of essential metals in the liver tissue of *C. gariepinus* showed the highest concentrations of Fe (Fig. 3.1B), Co (Fig. 3.1D) and Cu (Fig. 3.1J) in the lotic system (Usuthu River). The catfish of Usuthu River had Fe (Fig. 3.1B) concentrations of 1951 mg/kg followed by the catfish at both Nyamithi Pan (1800 mg/kg) and Shokwe Pan (1420 mg/kg).

The Co (Fig. 3.1D) concentrations followed the same trend with *C. gariepinus* of the Usuthu River (0.913 mg/kg) having the significantly highest concentration followed by the *C. gariepinus* of Nyamithi Pan (0.514 mg/kg) ($p < 0.0001$) and Shokwe Pan (0.408 mg/kg) ($P < 0.0001$). The concentrations of Ni (Fig. 3.1F) and Zn (Fig. 3.1H) in Usuthu River's catfish were lower than in the Shokwe Pan's catfish but still higher than the Nyamithi Pan's catfish. The catfish of Nyamithi Pan had the lowest concentrations of all the essential metals except Fe in the liver in comparison to the catfish at the other two sites. The concentrations of Ni in the liver of *C. gariepinus* was higher in Shokwe Pan than the *C. gariepinus* of both Usuthu River (0.156 mg/kg) and Nyamithi Pan (0.249 mg/kg), but only significantly higher than the *C. gariepinus* at Nyamithi Pan ($p = 0.0283$).

Clarias gariepinus of Shokwe Pan had significantly higher Zn (89.58 mg/kg) concentrations followed by *C. gariepinus* of both Usuthu River (70.52 mg/kg) ($p < 0.0001$) and Nyamithi Pan (65.98 mg/kg) ($p < 0.0001$). Again, Usuthu River's catfish had significantly higher concentrations of Cu (58.45 mg/kg) compared to Shokwe Pan's catfish ($p = 0.028$) and Nyamithi Pan's catfish ($p = 0.0018$). The fish at Shokwe Pan had the second highest concentration at 33.28 mg/kg and Nyamithi Pan's catfish had the lowest concentration at 8.049 mg/kg. The catfish which had significantly greater muscle concentrations of the essential metals also had significantly higher levels in the liver at the respective sites.

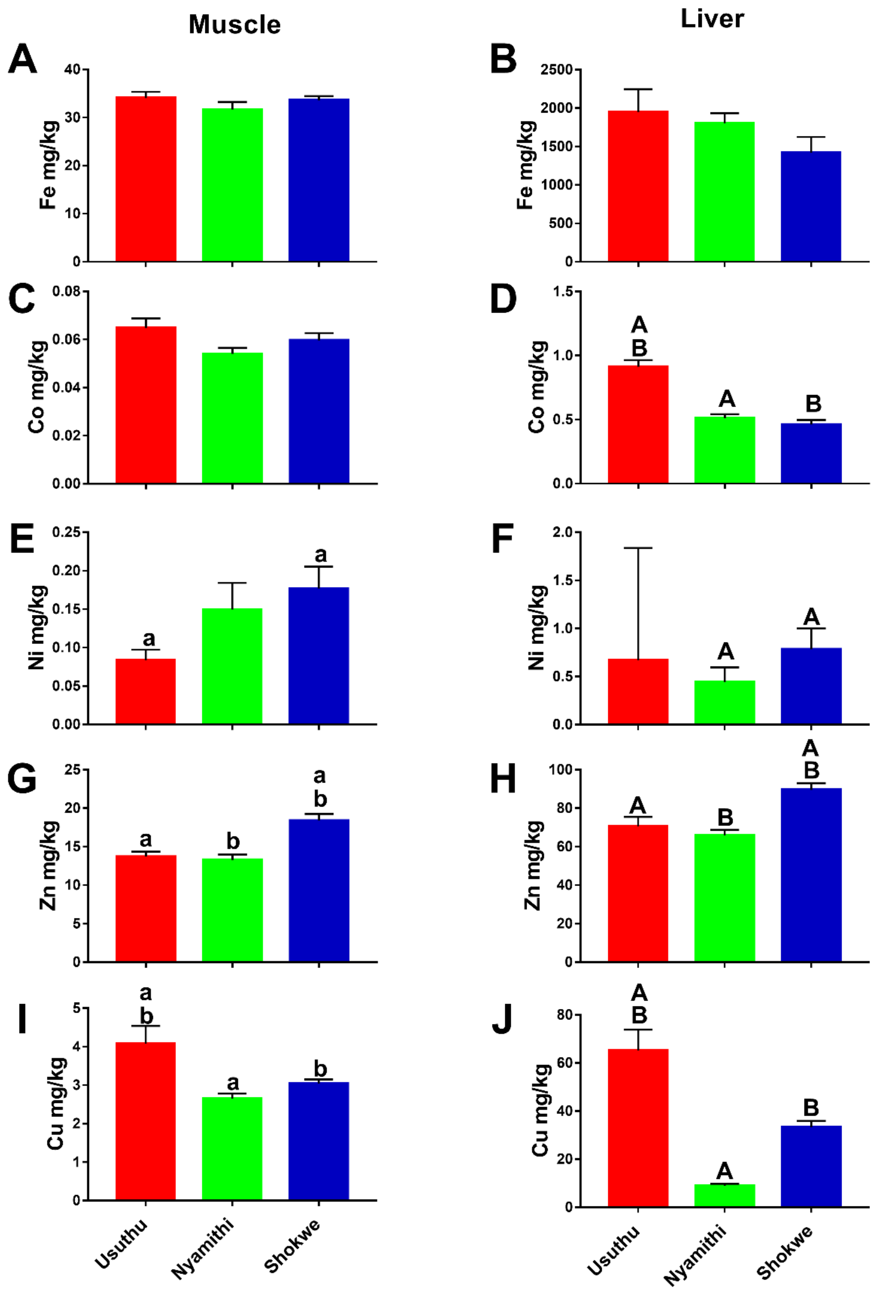

Figure 3-1: Bar graphs showing the mean concentration (± 1 SEM) of the different essential metals (Fe, Co, Ni, Zn, Cu) in both the muscle and liver tissues (mg/kg) of the catfish *Clarias gariepinus* from the Usuthu River and Nyamithi and Shokwe pans inside the Ndumo Game Reserve (common superscripts for each of the tissues indicate significant differences between the sites).

3.3.2.2. Non-essential metals

When comparing the concentration of non-essential metals in the muscle and liver tissues of *C. gariepinus* across the three sites (Fig. 3.2 & Fig. 3.3) the liver had significantly higher concentrations of Al, Mn, Se and Ag than in the muscle tissue across all sites. The concentrations of As, Cd and Pb were similar in the muscle tissue and the liver tissue at all sites. The catfish at Shokwe had significantly higher concentrations of all the non-essential metals except for As, Se and Ag in the muscle tissue. The catfish of Nyamithi had the lowest concentrations of all the non-essential metals except for As and Ag in the muscle tissue.

Muscle tissue:

Aluminium (Fig. 3.2A) was the metal with the significantly highest ($p < 0.0001$) concentration in the muscle tissue of *C. gariepinus* at Shokwe Pan (20.82 mg/kg) followed by that of *C. gariepinus* at both Nyamithi Pan (14.35 mg/kg) and Usuthu River (14.19 mg/kg). Shokwe Pan's catfish had a Mn concentration (Fig. 3.2C) of 1.625 mg/kg in the muscle tissue which was significantly higher than Usuthu River's catfish (1.233 mg/kg) ($p < 0.0001$) and Nyamithi Pan's catfish (1.188 mg/kg) ($p < 0.0001$). The catfish at Nyamithi Pan had the highest concentration of As (Fig. 3.2E) in the muscle tissue (0.197 mg/kg) which was significantly higher ($p = 0.003$) than the concentration in the catfish at Shokwe Pan (0.139 mg/kg) but similar to the muscle tissue concentrations in catfish from the Usuthu River (0.181 mg/kg).

Clarias gariepinus at Shokwe had significantly higher concentrations of Cd (Fig. 3.3 A) at 0.391 mg/kg than *C. gariepinus* from both the Nyamithi Pan (0.072 mg/kg) ($p < 0.0001$) and Usuthu River (0.085 mg/kg) ($p < 0.0001$). The concentration of Pb (Fig. 3.3C) in the catfish was significantly higher at Shokwe Pan (3.726 mg/kg) ($p < 0.0001$) than in the catfish at Usuthu River (0.941 mg/kg) and in the catfish at Nyamithi Pan (0.798 mg/kg). Usuthu River's catfish had the highest concentrations of both Se (Fig. 3.3E) (0.229 mg/kg) and Ag (Fig. 3.3G) (0.031 mg/kg), but not significantly present at both.

Liver tissue:

Clarias gariepinus of the lotic system had the highest concentrations of the non-essential metals, Al (Fig. 3.2B), As (Fig. 3.2F) and Cd (Fig. 3.3B), in their liver tissue (Figs. 3.2 & 3.3). Usuthu River's catfish had a significantly higher Al (Fig. 3.2A) concentration in their liver at 24.72 mg/kg followed by Shokwe Pan's catfish (20.64 mg/kg) ($p = 0.007$) and Nyamithi Pan's catfish (18.59 mg/kg) ($p = 0.011$). The catfish of Usuthu River had significantly lower concentrations of Mn at a level of 5.184 mg/kg than both the catfish of Shokwe Pan (7.467 mg/kg) ($p = 0.0007$), and Nyamithi Pan (6.627 mg/kg) ($p = 0.0447$).

Clarias gariepinus of Nyamithi Pan had significantly lower concentrations of As (0.147 mg/kg) ($p < 0.0001$) than both the *C. gariepinus* of Usuthu River (0.293 mg/kg) and Shokwe Pan (0.2588 mg/kg). The concentration of Cd in the liver tissue was significantly different across all three sites ($p < 0.0001$) with Usuthu River's catfish at 0.655 mg/kg and the catfish at Nyamithi Pan at 0.160 mg/kg and at Shokwe Pan at 0.363 mg/kg. Nyamithi Pan's catfish had a Pb concentration of 1.165 mg/kg followed by the catfish of both Shokwe Pan (0.972 mg/kg) and Usuthu River Pan (0.69 mg/kg). *Clarias gariepinus* of the lotic system had significantly lower concentrations of both Se ($p < 0.0001$) and Ag ($p < 0.0001$) in the liver tissue. For the most part the non-essential metals did not follow the same accumulation pattern in the catfish as the essential metals – catfish from sites which showed the highest accumulation in the muscle did not necessarily show the highest accumulation in the liver tissue.

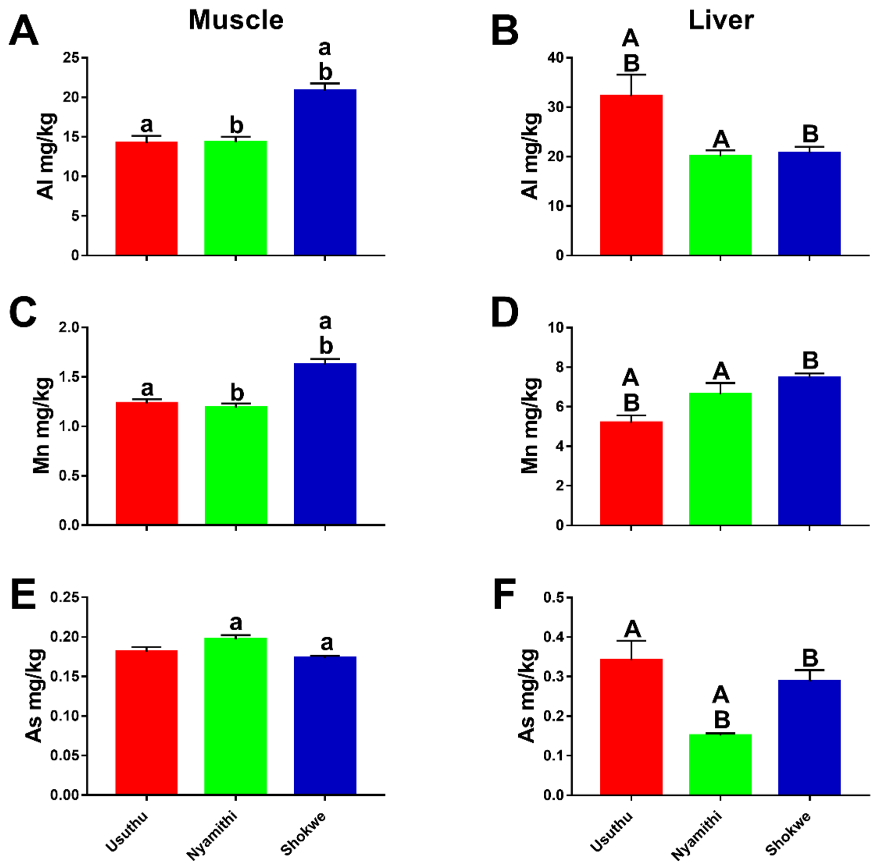

Figure 3-2: Bar graphs showing the mean concentration (± 1 SEM) of the different non-essential metals in both the muscle and liver tissues (mg/kg) of the catfish *Clarias gariepinus* from the Usuthu River and Nyamithi and Shokwe pans inside the Ndumo Game Reserve (common superscripts indicate significant differences between the sites).

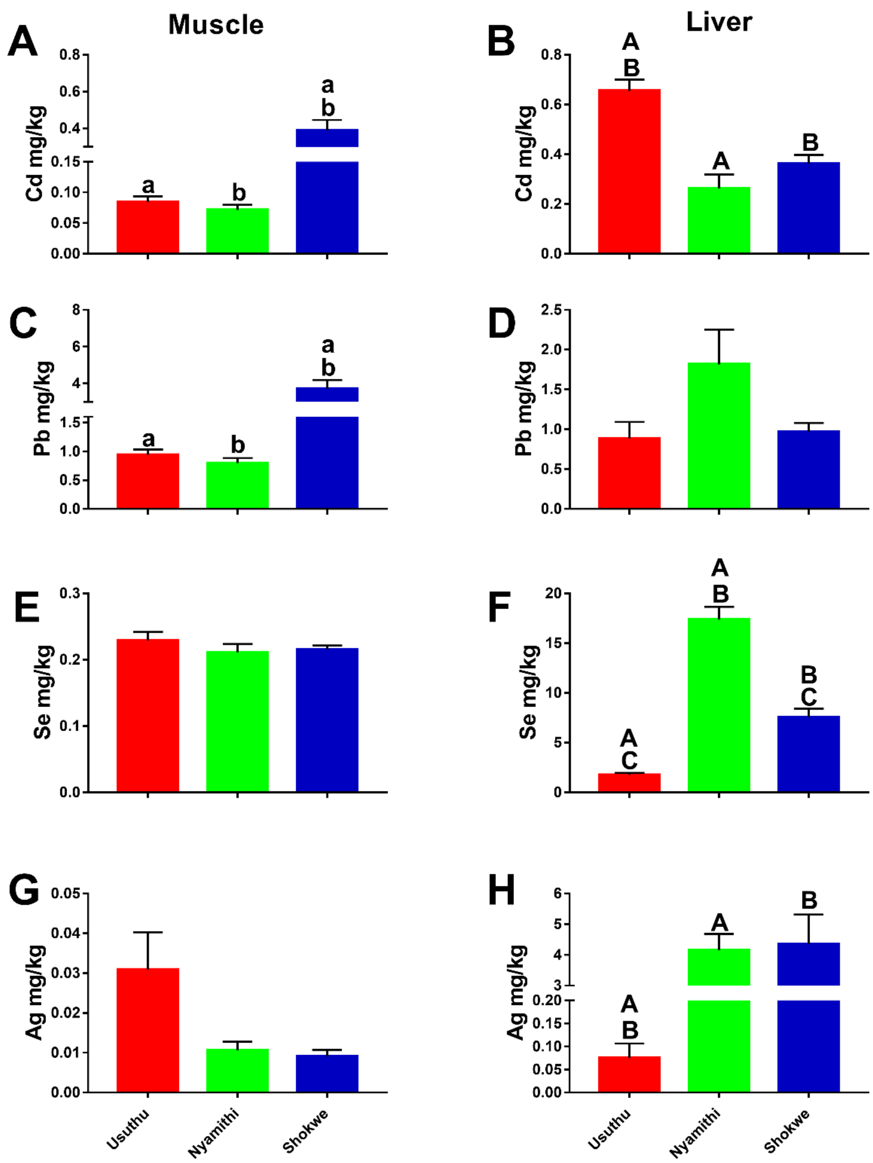

Figure 3-3: Bar graphs showing the mean concentration (± 1 SEM) of the different non-essential metals in both the muscle and liver tissues (mg/kg) of the catfish *Clarias gariepinus* from the Usuthu River and Nyamithi and Shokwe pans inside the Ndumo Game Reserve (common superscripts indicate significant differences between the sites).

The results derived from a Discriminant Function Analysis are shown on a biplot (Fig 3.4) of the canonical variates calculated during the analysis; functions 1 and 2 described 100% of the variation in the metal concentrations in the muscle tissue of *C. gariepinus*. The first function (X axis) explained 65.9% of the variance, which was mainly driven by an increase in the metals, Ag (0.0778), Co (0.097) and Se (0.086). These drivers separated the lotic system from the rest of the groups indicating significantly higher concentrations of the metals. The second function (Y axis) explained 34.1% of the variance through the metals, Pb (0.390), Mn (0.371) and Cd (0.336). These drivers separated Shokwe Pan from Usuthu River and Nyamithi Pan, with Shokwe Pan having higher concentrations of Pb, Mn, As and Zn followed by Usuthu River and then Nyamithi Pan. There was a 96.1% accuracy for the reclassification success for individual catfish into their predefined groups.

Table 3-3: The structure matrix of the first two functions of a Discriminant Function Analysis (DFA), using the various metal concentrations present in the muscle tissue of *Clarias gariepinus* from the three different sites in Ndumo Game Reserve (the largest absolute correlation between each variable and any discriminant function is indicated in bold).

Structure Matrix														
Metal		% explained	Ag	Co	Se	Pb	Mn	Cd	As	Zn	Al	Fe	Ni	Cu
Function	1	65.9	**.0778**	**.097**	**.086**	-.254	-.174	-.234	.018	-0.148	-.162	.04	-.097	.077
	2	34.1	.255	.064	-.011	**.390**	**.371**	**.336**	**-.205**	**.185**	**.181**	**.138**	**-.107**	**.104**

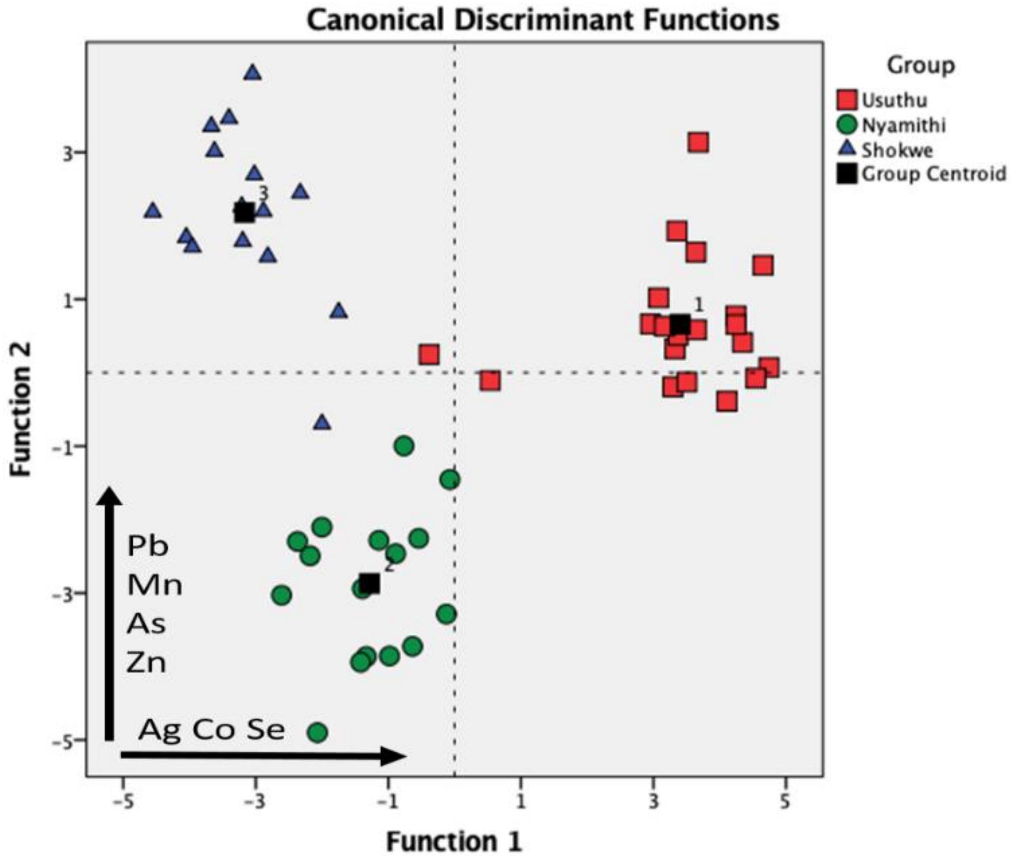

Figure 3-4: Biplot of the canonical variates obtained from a Discriminant Function Analysis (DFA) comparing the 12 different metal concentrations in the muscle tissue of *Clarias gariepinus* across three sites located in Ndumo Game Reserve, South Africa.

Table 3-4: Classification summary of the different metal concentrations from a Discriminant Function Analysis (DFA) of metal concentrations in the muscle tissue of *Clarias gariepinus* from three sites in Ndumo Game Reserve.

Classification Results[a]				
Group	**% Predicted Group Membership**			**Total**
	Usuthu River	**Nyamithi Pan**	**Shokwe Pan**	
Usuthu River	95	5	0	100
Nyamithi Pan	0	100	0	100
Shokwe Pan	0	6.7	93.3	100

[a] 96.1% of the original grouped cases correctly classified

Figure 3.5 is a biplot of the results of the canonical variates calculated from a Discriminant Function Analysis, where the sites are separated by the metal concentrations in the liver tissue of *C. gariepinus*. Functions 1 and 2 describe 100% of the variation in the mean metal concentration present in the liver tissue of *C. gariepinus*. The first function (X axis) describes 90.8% of the variance, which was mainly driven by the Ag (0.406), Co (-0.376), Se (0.182), Pb (0.175) and Mn (0.07) concentrations. These drivers separate the catfish from the three sites from each other, respectively, with the catfish from the lotic system having the highest concentrations of Cu, Cd, Ni and Al and the lowest concentration of Se, followed by individuals from Shokwe and Nyamithi pans, respectively. The second function (y axis) describes 9.2% of the variance through Cd (-0.488), As (0.433), Zn (0.313) and Al (0.264). The drivers separate the freshwater pan form the other two systems with Shokwe Pan having higher concentrations of Zn, Mn and Ag and the lowest concentration of Co. There was a 95.8% reclassification of the catfish into their predefined groupings.

Table 3-5: The structure matrix of the first two functions of a Discriminant Function Analysis (DFA), using the various metal concentrations present in the liver tissue of *Clarias gariepinus* from the three different sites in Ndumo Game Reserve (the largest absolute correlation between each variable and any discriminant function is indicated in bold).

Structure Matrix														
Metal		% explained	Ag	Co	Se	Pb	Mn	Cd	As	Zn	Al	Fe	Ni	Cu
Function	1	90.8	**.406**	**-.376**	**.182**	**.175**	**.070**	.216	.003	-.085	-.252	.138	-.026	-.066
	2	9.2	.370	-.025	-.099	-.127	-.030	**-.488**	**.433**	**.313**	**.264**	**.163**	**-.145**	**-.066**

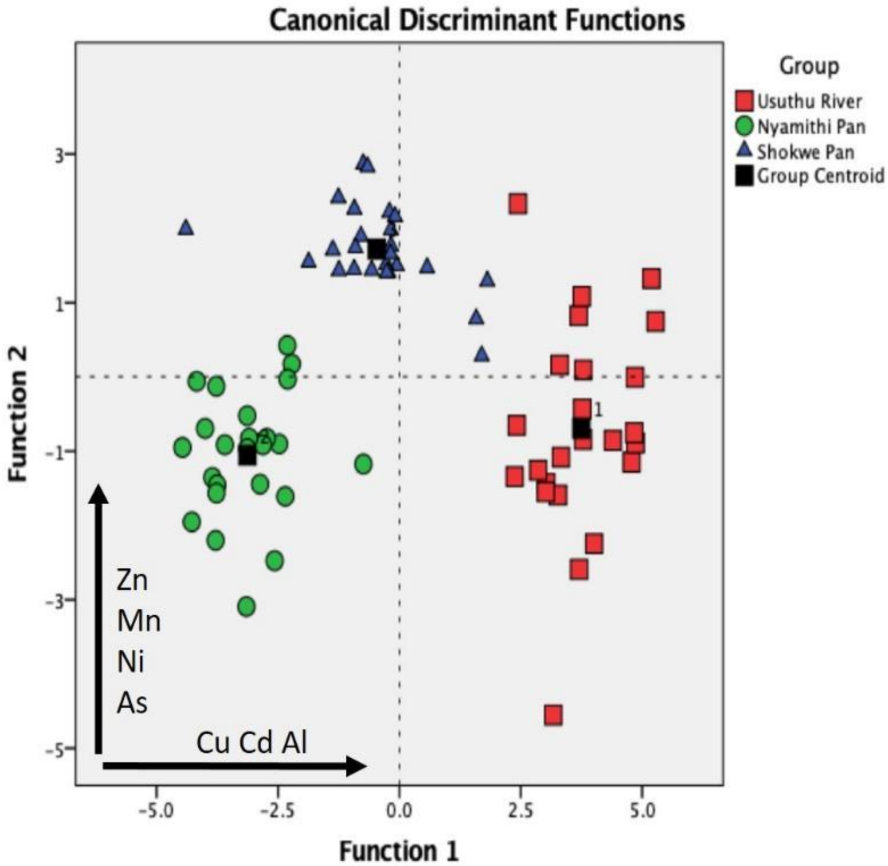

Figure 3-5: Canonical variates obtained from a Discriminant Function Analysis (DFA) comparing the metal concentration of 12 different metals in the liver tissue of *Clarias gariepinus* across three sites located in Ndumo Game Reserve, South Africa.

Table 3-6: Classification summary of the different metal concentrations from a Discriminant Function Analysis (DFA) of metal concentrations in the liver tissue of *Clarias gariepinus* from three sites in Ndumo Game Reserve.

Classification Results[a]				
Group	**% Predicted Group Membership**			**Total**
	Usuthu River	**Nyamithi Pan**	**Shokwe Pan**	
Usuthu River	95.8	0	4.2	100
Nyamithi Pan	0	100	0	100
Shokwe Pan	4	4	92	100

[a] 95.8% of the original grouped cases correctly classified

3.3.3. Bioaccumulation factors of essential and non-essential metals (BAF)

The BAF of both essential and non-essential metals in muscle and liver tissues are shown in Table 3.7. In order to assist with interpretation, the classification system according to Arnot and Gobas (2006) was used where a substance is bioaccumulative when it has a BAF ≥ 2000, and very bioaccumulative with a BAF ≥ 5000. It is evident that the BAF of the liver is higher than the BAF of the muscle tissue.

In the muscle samples there was a BAF range of 83 to 6511 for all the metals across the three sites. Nickel had the lowest BAF with an average of 211 across the three sites. Cadmium had the highest BAF in the muscle tissue with an average of 5523 across the three sites. For the BAF in the liver tissue of *C. gariepinus* (Table 3.7) the samples had a range from 60 to 47177. The BAF of Ni, Al, As and Pb was similar between the muscle and liver tissue. For all the other metals the liver had a higher BAF than the muscle tissue. The muscle tissues of the *C. gariepinus* across the three sites were bioaccumulative for Zn, Al, Cd and Pb. The liver tissue was bioaccumulative for Co, Al, Mn, Cd, Pb and Se and very bioaccumulative for Fe, Zn, Cu and Ag.

Table 3-7: Bioaccumulation factors (BAF) of metals calculated from the water to the muscle and liver tissues of *Clarias gariepinus* in three sites located in the Ndumo Game Reserve (# indicates levels under the detection limits were present in the water of Nyamithi Pan and therefore no calculations could be done).

Bioaccumulation Factors						
	Usuthu River		Nyamithi Pan		Shokwe Pan	
Metals	**Muscle**	**Liver**	**Muscle**	**Liver**	**Muscle**	**Liver**
Essential						
Fe	899 ± 394 (594 – 2621)	47177 ± 34925 (3714 – 116152)	465 ± 148 (313 – 985)	25199 ± 9386 (9638 – 41245)	1238 ± 364 (828 – 2517)	46577 ± 33587 (6324 – 149991)
Co	466 ± 145 (273-719)	6580 ± 1791 (2442 – 10878)	127 ± 65 (82 – 397)	1058 ± 292 (578 – 1579)	429 ± 108 (275 – 661)	2932 ± 655 (2015 – 4650)
Ni	249 ± 363 (23 – 1406)	254 ± 135 (62 – 442)	226 ± 576 (0.001 – 6)	111 ± 73 (49 – 299)	160 ± 290 (0.7 – 1479)	278 ± 213 (43 – 817)
Zn	1810 ± 426 (1270 – 3180)	9327 ± 3240 (3933 – 19291)	1159 ± 450 (753 – 2453)	5036 ± 1073 (3858 – 8092)	2447 ± 906 (1524 – 6075)	10924 ± 2120 (7255 – 17493)
Cu	955 ± 523 (460 – 2082)	13656 ± 6596 (4128 – 26578)	83 ± 33 (44 – 2016)	235 ± 45 (165 – 356)	784 ± 236 (519 – 1717)	8159 ± 3202 (3260 – 14986)
Non-essential						
Al	625 ± 475 (267 – 2718)	1210 ± 812 (588 – 3499)	#	#	2091 ± 1930 (879 – 9455)	1535 ± 492 (687 – 2456)
Mn	420 ± 124 (300 – 924)	1673 ± 603 (767 – 3392)	789 ± 140 (562 – 1150)	4400 ± 1887 (1893 – 9272)	324 ± 77 (224 – 599)	1437 ± 215 (905 – 1942)
As	421 ± 65 (244 – 534)	681 ± 161 (339 – 899)	138 ± 18 (113 – 178)	103 ± 12 (80 – 126)	315 ± 47 (164 – 431)	474 ± 176 (275 – 954)
Cd	3585 ± 2115 (1371 – 9755)	25699 ± 8696 (9574 – 40866)	1603 ± 1128 (569 – 5367)	3231 ± 2255 (928 – 9883)	3817 ± 2730 (484- 10399)	3546 ± 1668 (539 – 7541)
Pb	3698 ± 1711 (1707 – 8125)	2710 ± 1268 (827 – 6834)	930 ± 501 (422 – 2216)	1357 ± 1331 (232 – 4947)	6479 ± 4060 (1198 – 15684)	1690 ± 943 (469 – 4021)
Se	295 ± 80 (129 – 514)	2265 ± 1278 (827 – 6834)	9 ± 3 (1 – 13)	738 ± 274 (205 – 1322)	221 ± 27 (172 – 284)	7734 ± 4386 (854 – 15250)
Ag	159 ± 234 (82 – 513)	384 ± 797 (32 -4005)	279 ± 286 (13 – 1374)	109425 ± 68043 (26687 - 326560	49 ± 47 (15 – 194)	23663 ± 26116 (25 – 101917)

3.4. Discussion

Metal concentration in the environment plays an essential role in metabolism and growth of organisms but high concentrations of metals can become detrimental for organisms. The increasing amounts of pollutants that flow into the aquatic ecosystems pose a great threat to the biodiversity of aquatic ecosystems. Biomonitoring is an important aspect of aquatic ecosystem management as it is a tool that can be used to safeguard the health and sustainability of our limited aquatic resources. Previous studies have used *C. gariepinus* successfully as bio-indicators of pollution in polluted systems. The aim of the study was to investigate the use of *C. gariepinus* as a bio-indicator of metals in three different aquatic systems, two lentic and one lotic; reflecting natural conditions as closely as possible; and to ascertain the bioaccumulative properties of muscle and liver tissue for both essential and non-essential metals.

Barwick and Maher (2003) state that although muscle tissues do not accumulate metals to the highest levels in comparison with other organs of an organism, it is important to investigate their accumulation due to the high rate of human consumption. In this study it was evident that even in natural (non-polluted) systems fish will bioaccumulate essential and non-essential metals in their muscle and liver tissue, with the liver accumulating most of the metals at higher levels. Fish have the ability to regulate metal concentrations in their tissues until a certain level whereafter the metals will accumulate in the different organs of the organism (Heath, 2018). The concentration of metals in *C. gariepinus* was found to be higher in the Usuthu River catfish than in the two lentic systems with the catfish at Nyamithi Pan having the lowest concentrations of metals.

Clarias gariepinus in the study had the highest concentrations of Fe, Zn and Al across the three sites which can be due to the fact that Fe and Al are two of the most abundant elements in nature along with Zn that is very common (Jones & Das, 1960, Faust, 2018). Although Fe, Al and Zn can be released into the environment through anthropogenic activities, in this study it is believed to be due to the natural weathering of rocks in the area of the sites. Previous studies found the same trends of Fe, Zn and Al having the highest concentrations in the fish studied (Grobler *et al.*, 1994, Gerber *et al.*, 2016).

When looking at the bioaccumulation of the different metals in the muscle and liver tissues of the host it was found that Ni, Al, As and Pb had similar BAFs in both the liver and muscle tissue. As the bioaccumulation of metals is dependent on the availability of metals in the environment (Arnot & Gobas, 2006), the low environmental concentration in this study can be the reason for the low bioaccumulation in catfish. According to a European Commission Directive (EC, 2013) Zn, Cd, Pb and Al are bioaccumulative in the muscle tissue of the hosts. Muscle tissue reflects exposure to metals over a longer period of time. Cadmium and lead are two of the most dangerous metals that can cause fatalities of organisms when accumulating to a high level. Major causes of Cd and Pb pollution in ecosystems are mining activities, sewage treatment and fertiliser applications (Satarug *et al.*, 2003).

In the liver tissue of *C. gariepinus* Co, Al, Mn, Cd, Pb and Se were classified as bioaccumulative according to a European Commission Directive (EC, 2013) with Fe, Zn, Cu and Ag classified as very bioaccumulative. The liver tissue reflects more recent exposures of metals from the environment and can thus be used as an early warning indicator of metal pollution. The liver functions as detoxification mechanism and binds to non-essential metals (Bremner, 1987, Seixas *et al.*, 2007), thus accumulating metals to higher levels than the muscle tissue. Chen and Chen (1999) found that toxic metals (Cd and Pb) accumulate at higher levels in the liver because of the liver's role in regulating metals.

There is currently no information on the concentrations of metals in the environment of NGR. To evaluate the metal concentrations found in the study they were compared to historical data on metal concentrations in *C. gariepinus* of aquatic ecosystems at other areas throughout South Africa (Tables 3.8 and 3.9). The Vaal River system and the Olifants River system are known to be located in mining districts (Coetzee *et al.*, 2002, Crafford & Avenant-Oldewage, 2010, 2011, Jooste *et al.*, 2015). The Nylsvlei floodplain is classified as a Ramsar site just like NGR and can thus be a good comparison site for this study (Greenfield *et al.*, 2012, Musa *et al.*, 2017). The Fe, Co, Ni and Zn concentrations in the muscle tissue of the host at the study sites were well below the concentrations at the other sites indicated in Tables 3.8 and 3.9 (Coetzee *et al.*, 2002, Crafford & Avenant-Oldewage, 2010, 2011, Jooste *et al.*, 2015, Musa *et al.*, 2017).

The copper concentration in the host muscle tissue was similar to the concentrations found in the Vaal river system (Crafford & Avenant-Oldewage, 2010, 2011) and at the Phalaborwa Barrage and Flag Boshielo Dam (Jooste *et al.*, 2015) but lower than at the rest of the sites. The Mn concentration in the muscle and liver tissue at NGR was similar to the concentrations in the muscle tissue of the Vaal River Barrage (Crafford & Avenant-Oldewage, 2011). The rest of the non-essential metals at the study sites were at lower concentration in the muscle and liver tissues in comparison to the other sites in Tables 3.8.

Table 3-8: Comparison of metal concentrations in the muscle tissue of *Clarias gariepinus* at the three sites in the Ndumo Game Reserve with the concentrations of metals in the muscle tissue at other aquatic ecosystems in other areas of South Africa (the concentrations of metals are indicated in mg/kg).

	Metals	Essential					Non-essential						
		Fe	Co	Ni	Zn	Cu	Al	Mn	As	Cd	Pb	Se	Ag
Muscle Tissue	**Usuthu River**	**34.06**	**0.07**	**0.083**	**13.69**	**4.086**	**14.19**	**1.23**	**0.182**	**0.085**	**0.941**	**0.229**	**0.031**
	Shokwe Pan	**33.6**	**0.06**	**0.177**	**18.4**	**3.038**	**20.82**	**1.63**	**0.173**	**0.391**	**3.726**	**0.216**	**0.009**
	Nyamithi Pan	**31.7**	**0.05**	**0.150**	**13.29**	**2.657**	**14.35**	**1.19**	**0.197**	**0.072**	**0.798**	**0.211**	**0.011**
	Vaal Dam Crafford and Avenant-Oldewage, 2010 & 2011)	60.63		1.91	40.40	2.87	32.73	1.39			3.05		
	Vaal River Barrage (Crafford and Avenant-Oldewage, 2010 & 2011)	56.90		2.48	26.43	2.80	30.78	1.35			4.20		
	Phalaborwa Barrage (Jooste *et al.*, 2015)	90.1	0.3	1.1	42.6	3.9	42.6	3.4	1.0	0.1	1.6		
	Flag Boshielo Dam (Jooste *et al.*, 2015)	948	10.2	2.2	26.5	5.8	57	6.1	1.2	0.0	7.0		
	Donkerpoort Dam (Musa *et al.*, 2017)	591.68				47.69	19.45	4.09	15.83				
	Nylsvlei (Musa *et al.*, 2017)	212.05				71.53	44.06	10.8	6.12				
	Klein Olifants River (Coetzee *et al.*, 2002)	121 - 246		14 - 21	31 - 53	3 - 14	26 - 109	3 - 4			5 - 11		
	Olifants River (Coetzee *et al.*, 2002)	72 - 272		9 - 31	31 - 73	2 - 35	11 - 54	2 - 8			3 - 8		

Table 3-9: Comparison of metal concentrations in the liver tissue of *Clarias gariepinus* at the three sites in the Ndumo Game Reserve with the concentrations of metals in the liver tissue at other aquatic ecosystems in other areas of South Africa (the concentrations of metals are indicated in mg/kg wet weight).

	Metals	Essential					Non-essential						
		Fe	Ni	Zn	Cu	Co	Al	Mn	As	Cd	Pb	Se	Ag
Liver Tissue	**Usuthu River**	**1951**	**0.156**	**70.52**	**58.45**	**0.913**	**24.72**	**5.18**	**0.293**	**0.655**	**0.69**	**1.762**	**0.075**
	Shokwe Pan	**1420**	**0.538**	**89.58**	**33.28**	**0.408**	**20.64**	**7.47**	**0.259**	**0.363**	**0.972**	**7.564**	**4.354**
	Nyamithi Pan	**1800**	**0.249**	**65.98**	**8.049**	**0.514**	**18.59**	**6.63**	**0.147**	**0.160**	**1.165**	**17.361**	**4.158**
	Vaal Dam (Crafford and Avenant-Oldewage, 2010 & 2011)	2041.8	1.55	76.61	40.22		49.53	5.29			4.44		
	Vaal River Barrage (Crafford and Avenant-Oldewage, 2010 & 2011)	1386.7	1.69	74.59	30.28		36.08	5.84			3.44		
	Donkerpoort Dam (Musa *et al.*, 2017)	35974.5		200.01	64.36		54.68	19.53					
	Nylsvlei (Musa *et al.*, 2017)	15607.3		292.11	109.53		89.05	24.44					
	Klein Olifants River (Coetzee *et al.*, 2002)	646 - 1997	11 - 17	98 - 122	20 - 42		35 - 109	4 - 10			5 – 9		
	Olifants River (Coetzee *et al.*, 2002)	72 - 272	8 - 31	31 - 73	2 - 35		11 - 54	2 - 8			3 – 8		

There were clear differences in the metal concentrations of the catfish in the lotic system – Usuthu River had the higher Fe, Co and Cu concentrations in the host in both the muscle and liver tissue. All three of these essential elements are naturally present in the environment and can enter the aquatic ecosystems due to natural weathering of rocks (Chen *et al.*, 2000, Shrivastava, 2009).

For the non-essential metals, the catfish in the lotic system had lower concentrations of Al, Mn, As, Cd and Pb than at least one lentic system in both the muscle and liver tissues of the host. The catfish in the lotic system had the highest concentration of Se and Ag in the muscle tissue of the host, but the lowest concentration in the liver of the host. Lotic systems continuously flush the sediment downstream and thus limit the metal binding capacity at a specific site in the system (Orr *et al.*, 2006). Lentic systems act as a sink for non-essential metals and can thus be readily available for bioaccumulation (Adams *et al.*, 1980, Pinedo-Hernández *et al.*, 2015, Meena *et al.*, 2018). Fish in lentic systems are also confined to a smaller waterbody than fish in lotic systems and thus accumulate metals at higher levels.

In this study two different lentic ecosystems were investigated: the first being a freshwater pan (Shokwe) and the second a saline pan (Nyamithi). The freshwater pan had the highest concentrations of all metals (essential and non-essential) in the muscle and liver tissues of the hosts. In saline water the chloride forms complexes with metals, which make the metals less bioavailable for uptake by organisms (Wright & Zamuda, 1987, Kumar *et al.*, 2015). Metals, whether essential or non-essential, have many possible sources and effects on organisms when accumulating to high levels. Iron is one of the most abundant and important essential metals in the geosphere (Faust, 2018). The highest concentrations of Fe were present in the liver of the fish which coincide with various previous studies (Crafford & Avenant-Oldewage, 2010, Osman & Kloas, 2010, Sunjog *et al.*, 2019). Ibrahim and Omar (2013) state that Fe accumulates in the liver as hemosiderin and ferritin (proteins to which Fe binds). The high levels of Fe in the host can be as a result of Fe being abundant in the environment. Cobalt is a by-product of industrial waste (Barałkiewicz & Siepak, 1999). According to the Agency for Toxic Substances and Disease Registry ATSDR (2013b) an excess Co causes cardiovascular, respiratory, haematological as well as developmental issues in humans.

Just like other essential metals, Ni becomes detrimental for organisms at high concentrations (Pickering, 1974). Nickel and Zn are by-products of many industrial activities and mining activities (Mudd, 2010), which are not very common close to Ndumo Game Reserve. According to Javed and Usmani (2013) metals will accumulate in the liver as a result of the presence of metallothioneins (MTs) that bind to Zn. As the World Health Organization (2017) set the limit of Zn for human consumption at 100 mg/kg, the concentrations at the three sites were well below the limit. Exceeding the limit can alter the health of organisms and can cause anaemia or pancreas damage (ATSDR, 2013c).

The presence of copper in water is due to natural weathering of rocks, or activities such as agriculture and the use of pesticides (Shrivastava, 2009). Although the concentration of Cu in the muscle tissues was high across the three sites, it was still lower than the limit of 30 mg/kg set for consumption by the World Health Organization WHO (2017). Although Cu is essential for good health, exceeding the limit can cause damage in gills, liver and kidneys (Javed & Usmani, 2013). Aluminium is one of the most used metals on Earth because of its light weight-to-strength ratio as well as its ability to resist corrosion (Jones & Das, 1960). Smelting of Al is the main source of aluminium pollution and causes acid rains (Arnesen *et al.*, 1995). An excess of Al accumulation in an organism causes respiratory disturbances (Alwan *et al.*, 2009).

A major source of Mn pollution is municipal wastewater discharge as well as sewage water. According to the Ndumo Game Reserve's Integrated Management Plan (2009) the sewage treatment in the Ndumo Game Reserve is through septic tanks and French drain systems that are known to cause pollution of ground waters. They are not up to standard and can be the cause of the elevated levels of Mn in the ecosystem. According to Javed and Usmani (2013), exposure to Mn will cause lung, liver and vascular disturbances. The concentrations present in the muscle tissue of the three sites exceeded the limit for consumption set by WHO (2017) which is 1 mg/kg.

Arsenic is detected in low concentrations in all environmental matrices (ATSDR, 2013a). An excess of As can have serious health effects and can affect virtually all organ systems (Tchounwou *et al.*, 2012). High levels of Cd are known to target the blood vessels of organisms and can lead to death (Tchounwou *et al.*, 2012). Lead is present in small quantities in the earth's crust (Tchounwou *et al.*, 2012). The limit for consumption set by the WHO (2017) for Pb is 0.5 mg/kg which means all three our sites exceeded the limits. High concentrations of Pb can have serious health implications for organisms. The nervous system is targeted by Pb poisoning and can lead to death at extremely high concentrations (Tchounwou *et al.*, 2012). Selenium is a natural trace element that is released into the environment through anthropogenic activities such as mining, agricultural, industrial and petrochemical operations (Lemly, 2004).

3.5. Conclusion

This is the first study reporting the metal concentrations of fish from the floodplains of the NGR as well as in the lower Usuthu River system. The study found that *C. gariepinus* accumulated metals even when present in aquatic ecosystems close to a natural condition. There were clear spatial differences in the accumulation of metals with the catfish in the lotic system accumulating the essential metals to higher levels than in the two lentic systems. The catfish in the lotic system had the lowest accumulation of the non-essential metals. It was also clear that salinity played a role in the accumulation of metals with the catfish in the freshwater lentic system accumulating all metals to higher concentrations than the catfish in the saline lentic system, however metal concentrations in this saline water were predominantly higher than the freshwater systems.

The hypotheses of this chapter stated that the bioaccumulation of metals in *C. gariepinus* would reflect the environmental conditions of the sites, with both the lentic systems (fresh and saline) having higher bioaccumulation factors than the lotic systems and secondly that *C. gariepinus* would bioaccumulate the non-essential metals to higher levels than the essential metals The first hypothesis of this chapter is rejected because the catfish in the lotic system had higher BAFs than the catfish in the saline lentic system and higher BAFs for some metals than the catfish in the freshwater lentic system.

The second hypothesis is also partially rejected as only Cd and Pb were bioaccumulated to higher levels than the essential metals.

Clarias gariepinus has proven to be a good bio-indicator of metal concentrations even in a non-polluted ecosystem. Now that the bioaccumulation of *C. gariepinus* at the three aquatic ecosystems is known it can be used to see if endohelminth parasites will accumulate metals to a greater degree than their hosts and thus potentially be better bio-indicators of metal pollution.

4. Bioaccumulation of metals in endohelminth parasites of *Clarias gariepinus*: correlation with host accumulation, ecosystem type and infection site location-specific accumulation patterns.

4.1. Introduction

Parasites are organisms which are dependent on host individuals for survival, whether as a food source, a safe environment to survive in or a place to complete their life cycle (Lafferty & Kuris, 1999, Pietrock & Marcogliese, 2003, Sures *et al.*, 2017). Parasites can be found in all ecosystems of the world and are present in abundance (Sures, 2001, Vázquez *et al.*, 2005). Parasites are functionally important organisms, particularly in aquatic communities with regard to their trophic level in the food web making them useful bio-indicators for anthropogenic pollution (Sures, 2004). Mackenzie (1999) states that there are three reasons to use parasites as bio-indicators: firstly, there are many more parasitic than free-living species (biological diversity); secondly, some parasites have complex life cycles (occupying multiple hosts) which can increase the number of potential indicators; and lastly, some parasites are sensitive to changes in their environment. For these reasons parasites are considered to be good bio-indicators of the health of an ecosystem (Sures, 2003, Sures, 2004, Marcogliese, 2005, Marcogliese & Pietrock, 2011, Sures *et al.*, 2017)

Sures *et al.* (2017) state, that from all the studies conducted on parasites as biological indicators, it has been found that parasites can be utilised in three different ways; the first of which as parasites and biomarkers, where the effect of parasites on the physiological homeostasis of the host is investigated (Sures, 2004, Sures *et al.*, 2017). Organisms have a physiological response to the uptake and accumulation of environmental pollutants (Sures *et al.*, 2017). Sures *et al.* (2017) also mention that the interaction of parasites and pollutant-induced biomarker responses of their host has been receiving increased awareness and has been investigated as early as 1977 by Boyce and Yamada (1977), where the studies found that parasite infections had an effect on the resistance of their hosts towards environmental chemicals.

The second way in which parasites is being used, are as effect indicators by focusing on the populations and community structure of parasites as well as individual parasitic organisms (Sures, 2004). Single organisms are used by looking at the effect of direct toxicity on the free-living larval stages (Sures, 2004). Numerous studies have found that pollutants such as metals bind to receptors causing the deactivation of enzymes causing fatalities of both the host and its parasites (Pietrock & Marcogliese, 2003, Sibley, 2004, Avilán *et al.*, 2011, Osman *et al.*, 2012). Many studies have been conducted on the effect of environmental pollution on the changes in population and communities of parasites within their hosts (Kennedy, 1997, Lafferty & Kuris, 1999, Mackenzie, 1999, Vidal-Martinez *et al.*, 2010).

Despite the many studies that have been conducted, only limited parasite populations indicated predictable changes due to environmental pollution; however, the general consensus is that pollution does have an effect on parasite populations and communities both at host level and directly (Sures, 2004).

Lastly, parasites are also used as accumulation indicators of pollution in aquatic ecosystems (Sures, 2004). Pollution can be fatal to the organisms present in polluted systems thus resulting in certain organisms being absent in some systems. Certain organisms have a higher tolerance for pollution and have the ability to accumulate pollutants (Sures *et al.*, 1999, Sures, 2003). Most studies up until now have focused on metal pollution, with a few cases focusing on organic pollutants (Sures, 2004, Sures *et al.*, 2017). Studies have found that parasites with a low percentage of fat are unable to bio-concentrate lipophilic substances such as organic pollutants (Heinonen *et al.*, 1999, Heinonen *et al.*, 2000, Ruus *et al.*, 2001). Parasites can also alter the uptake of metals of their hosts (Boyce & Yamada, 1977, Bergey *et al.*, 2002, Sures, 2003, Sures, 2004, Sures *et al.*, 2017). Evans *et al.* (2001) found that Digenea infection in hosts causes destruction of the hepatopancreas cells, which reduces the capacity of hosts to store metals.

To date more than 50 different species of parasites have been studied for their bioaccumulation properties; these parasites belong predominantly to four endohelminth taxa (Cestoda, Nematoda, Acanthocephala and Digenea) (Sures *et al.*, 2017). Studies found that freshwater acanthocephalans and cestodes had the greatest capacity to accumulate various elements to high levels (Sures *et al.*, 2017). However, many of these studies have been done in Europe where Acanthocephala are particularly abundant, which is not the case for Africa, with a distinct lack of spiny-headed worms in freshwater ecosystems (Boomker, 1994). Previous studies conducted within the Ndumo Game Reserve (NGR) have found four different intestinal helminth parasites and one body cavity helminth parasite in *Clarias gariepinus,* which includes two Cestodes

(*Proteocephalus* sp. Weinland, 1858 and *Tetracampos ciliotheca* Wedl, 1861) and three Nematodes (*Contracaecum* sp. Railliet and Henry, 1912, *Procamallanus pseudolaeviconchus* Moravec et Van As, 2015 and *Paracamallanus cyathopgharynx* Baylis, 1923) (Smit *et al.*, 2016, Svitin *et al.*, 2019).

The two cestode species (*T. ciliotheca* and *Proteocephalus* sp.) are found in the intestine of their intermediate hosts. Cestode species feed by absorbing nutrients and pollutants from their host through their teguments and membranes (Dalton *et al.*, 2004). Two of the nematode species are also to be found in the digestive tract of *C. gariepinus*: the first species, *P. pseudolaeviconchus*, is found in the stomach (Svitin *et al.*, 2019) and the second, *Pa. cyathopgharynx*, is found in the rectum (Svitin *et al.*, 2019). The third, a *Contracaecum* sp., is found in the body cavity of the host (Barson, 2004). Both species found in the intestinal tract absorb nutrients and pollutants through their tegument and membranes (Hassan *et al.*, 2016), whereas the body cavity species feed on the blood and tissue of the host (Barson, 2004).

It was hypothesised that the endohelminth parasites of *C. gariepinus* would prove to be better bio-indicators of both essential and non-essential metals than their hosts due to the bioconcentration and biomagnification of metals in the endohelminth parasites. It was also hypothesised that the endohelminth parasites in the intestines would have higher accumulation rates for metals than the *Contracaecum* sp. in the body cavity due to higher concentrations of metals in the intestinal tract. Lastly, it was hypothesised that there would be a gradient of metal accumulation in the nematode parasites with the highest being in the stomach due to the low pH making metals there more bioavailable than in the rectum of the hosts.

To test these hypotheses, the aim of this chapter is to determine the concentrations of metals in the parasites and to evaluate the use of different helminth parasites as bio-indicators of both essential and non-essential metals to establish if these parasites can be used for bioaccumulation studies. In this study we compared the accumulation capacity of all the endohelminth parasites found in *C. gariepinus* to see if the position of the parasites in the host played a role in the accumulation capacity of the parasites. This part of the study also examined the effect of parasite infection and accumulation on host accumulation and *vice versa*.

4.2. Materials and methods

4.2.1. Field collection

Following euthanisation of the hosts (described in Chapter 3 Section 3.2.2), their body cavities were inspected for the presence of *Contracaecum* sp. (Fig. 4.1A, B). When found, these parasites were then collected, and all the organs removed to gain access to the gastrointestinal tract of the host. The digestive system was removed from the esophagus to the anus and thoroughly checked under a dissection microscope for the presence of helminth parasites. The parasites collected, included *P. pseudolaeviconchus* in the stomach (Fig. 4.1C), two different cestode species: *T. ciliotheca* and *Proteocephalus* sp. in the intestine (Fig. 4.1D, E, F) and *Pa. cyathopgharynx* in the rectum (Fig. 4.1G). Collected parasites were counted and frozen for further analyses.

4.2.2. Laboratory analysis of the body cavity nematode (*Contracaecum* sp.) and quality control

In order to get sufficient weight (0.05 g – 0.2 g) for metal analysis, individual parasites were pooled. Thereafter the metal concentration was analysed in the same manner as that of the host tissue (described in Chapter 3 Section 3.2.3). The quality control for the digestion and ICP-MS analysis of the *Contracaecum* sp. was assessed in the same way as that of the host tissue. The Certified Reference Material (CRM) (DORM-4: Fish protein certified reference material for trace metals); results and recoveries are shown in Table 3.1 (refer to Chapter 3 Section 3.2.3). Recoveries ranged from 96% for Ni to 149% for Pb.

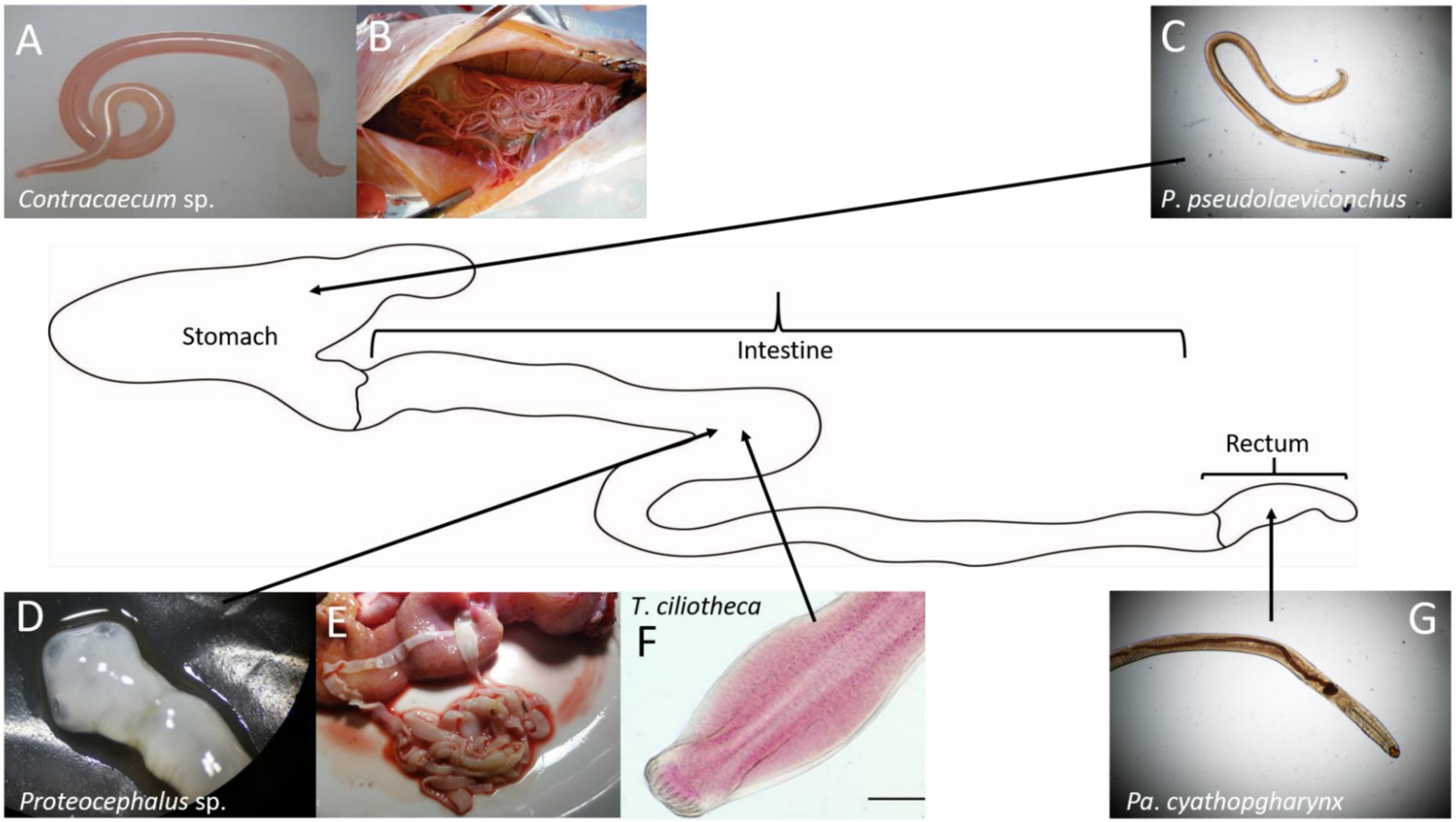

Figure 4-1: The digestive tract of *Clarias gariepinus* with photographs of the five different endohelminth parasites at the location where they were found in their hosts: *Contracaecum* sp. in the body cavity (A,B), *Procamallanus pseudolaeviconchus* in the stomach (C), *Proteocephalus* sp. (D,E) and *Tetracampos ciliotheca* (F) in the intestine and *Paracamallanus cyathopgharynx* in the rectum of the host (G).

4.2.3. Laboratory analysis of collected gastro-intestinal helminth parasites

Since the sizes of the different gastro-intestinal species of parasites are so small, it was decided to pool samples of *P. pseudolaeviconchus*, *T. ciliotheca* and *Pa. cyathopgharynx* to provide enough weight for digestions (0.0004 g – 0.01 g) (Table 4.1). *Proteocephalus* sp. had enough weight not to be pooled but was only present at Nyamithi Pan. Pooled samples were weighed and placed in different wells of a Teflon heat block, and 1 mL 65% HNO_3 was added. The heat block was then placed in an oven at 70˚C for 24 hours. Following cooling, the sample in each well was removed with a clean sterile pipette and placed in a 15 mL polypropylene tube, and then diluted with 1% HNO_3 to a volume of 10 mL. The digested samples were labelled and sent for ICP-MS analysis. All metal concentrations were converted to mg/kg. To ensure quality control for the digestion and ICP-MS analysis CRM (DORM-4: Fish protein certified reference material for trace metals) was treated in the same way as for the different parasite species, as set out above. The results for the concentration of metals in the CRM and the percentage recovery are shown in Table 4.2. The recoveries ranged from 91% for As to 161% for Pb.

Table 4-1: Pooled sample numbers with the weight of the *Procamallanus pseudolaeviconchus*, *Tetracampos ciliotheca* and *Paracamallanus cyathopgharynx* across the three sites of Ndumo Game Reserve (each number in brackets represents a specific sample number for *Clarias gariepinus* sample at a specific site).

P. pseudolaeviconchus	**#**	**Weight (g)**	***T. ciliotheca***	**#**	**Weight (g)**	***Pa. cyathopgharynx***	**#**	**Weight (g)**
Usuthu	11	0.0004	Usuthu (1, 3, 4, 12)	6	0.0016	Usuthu (1, 2, 4)	15	0.004
Nyamithi (1, 4, 5, 8)	17	0.0013	Usuthu (14, 16, 18, 19, 22)	7	0.0005	Usuthu (5, 6, 7, 8, 10)	27	0.0011
Nyamithi (9, 10, 11, 13)	11	0.0024	Usuthu (23, 24)	7	0.0008	Usuthu (11, 12, 13, 14)	37	0.002
Nyamithi (14, 17, 18)	24	0.0015	Nyamithi (1, 2)	7	0.004	Usuthu (16, 17, 19, 21-24)	43	0.0025
Nyamithi (20, 22, 25)	7	0.0008	Nyamithi (7, 10)	8	0.0074	Nyamithi (2, 7, 9, 10, 11)	10	0.0008
Shokwe	33	0.001	Nyamithi (13, 14, 16)	14	0.003	Nyamithi (12, 13, 17, 18, 19, 25)	7	0.001
			Nyamithi (17, 21, 23, 24)	9	0.002	Shokwe (1, 2)	31	0.0065
			Shokwe (1, 2, 5, 6)	23	0.0107	Shokwe (4 - 17)	99	0.0133
			Shokwe (8, 9, 12, 13, 14, 17)	15	0.0055	Shokwe (18 - 25)	42	0.0025
			Shokwe (19, 22, 23, 24)	11	0.0015			

Table 4-2: The mean (1 ± SEM) measured concentrations for different metals of the certified reference material (CRM) (DORM-4: Fish protein certified reference material for trace metals) with percentage recovery rate (all concentrations were converted into mg/kg).

Tissue			
	Reference	**Measured**	**% Recovery**
Mn	3.17 ± 0.26	3.710	117
Ni	1.34 ± 0.14	1.243	93
Cu	15.7 ± 0.46	15.758	100
Zn	51.6 ± 2.8	58.966	114
As	6.87 ± 0.44	6.250	91
Cd	0.299 ± 0.018	0.414	139
Pb	0.404 ± 0.062	0.750	161

4.2.4. Statistical analysis

Univariate statistics were done using GraphPad Prism®7 software. Homogeneity was tested by using the Shapiro-Wilk test and if the data was found to be homogeneous a one-way ANOVA with Tukey post-hoc was performed. If the data was not homogenous a Kruskal-Wallis test was performed with a Dunn's multiple comparison test. Significance was set at $p < 0.05$. The different parameters for parasite infections were calculated according to Lafferty *et al.* (1997). Canoco version 5 (ter Braak & Smilauer, 2012) was used to perform a redundancy analysis (RDA) of the effect of parasites on the metal concentrations of their host, both in terms of parasite numbers as well as metal concentrations in the parasites. Further RDAs were performed to relate metal concentrations in the parasites to concentrations in their environment, namely host muscle and liver tissues. A redundancy analysis (RDA) works on a linear response model that relates two sets of variables to the sampling sites – in this case individual catfish or parasite samples. Placement of the variables indicates similarities between variables, with variables at ≤ 90˚ positively correlating and variables at > 90 negatively correlating. The bioaccumulation factor (BAF) was calculated using the following equation derived from Arnot and Gobas (2006):

$$BAF = \frac{Concentration\ in\ parasite}{Concentration\ in\ host\ muscle\ tissue}$$

4.3. Results:

4.3.1. Parasites collected:

A total of 74 *C. gariepinus* were examined for helminth parasites (Table 4.3). *Contracaecum* sp. had the highest prevalence (73.3%) across the three sites, where Nyamithi Pan had a 100% infection rate, followed by Shokwe Pan (72%) and the Usuthu River catfish (50%). *Contracaecum* sp. also had the highest mean abundance with an abundance of 92.8 individuals per catfish. *Procamallanus pseudolaeviconchus* had the lowest prevalence for *Clarias gariepinus* across the three sites with only 47% of the catfish infected. The *P. pseudolaeviconchus* also had the lowest mean abundance of 1.39 individuals per catfish of the helminth parasites. The catfish in the Usuthu River had the lowest prevalence for *P. pseudolaeviconchus* (25%) with Shokwe Pan the highest where 60% of the catfish were infected. *Proteocephalus* sp. was only present in catfish in the Nyamithi Pan and had a prevalence of 24% of the catfish infected, the overall abundance of

Proteocephalus sp. was 0.8 individuals per catfish. The prevalence of *T. ciliotheca* in *C. gariepinus* was 54.1% with a mean abundance of 7.13 individuals per catfish across the three sites. The *T. ciliotheca* at Nyamithi Pan had the highest prevalence (60%) and abundance (3.96) followed by Shokwe Pan (56%, 0.64) and Usuthu River (45.8%, 0.83). *Paracamallanus cyathopgharynx* had the second highest prevalence (71.6%) across the three sites. Nyamithi Pan had the lowest prevalence of *Pa. cyathopgharynx* (44%) followed by Usuthu River (79.2%) and Shokwe Pan (92%).

Table 4-3: The prevalence, mean abundance and intensity of the endohelminth parasites *Procamallanus pseudolaeviconchus*, *Proteocephalus* sp., *Tetracampos ciliotheca*, *Paracamallanus cyathopgharynx* and *Contracaecum* sp. found in 74 *Clarias gariepinus* across three sites located in the Ndumo Game Reserve.

	P. pseudolaeviconchus			***Proteocephalus* sp.**			***T. ciliotheca***			***Pa. cyathopgharynx***			***Contracaecum* sp.**		
Site plus number sampled	**Prevalence**	**Abundance**	**Intensity**	**Prevalence**	**Abundance**	**Intensity**	**Prevalence**	**Abundance**	**Intensity**	**Prevalence**	**Abundance**	**Intensity**	**Prevalence**	**Abundance**	**Intensity**
Usuthu (24)	6 (25%)	0.46	1.83	0	0	0	11 (45.8%)	0.83	1.82	19 (79.2%)	4.96	6.26	12 (50%)	2.13	4.25
Nyamithi (25)	14 (56%)	2.36	4.21	6 (24%)	0.80	3.33	15 (60%)	3.96	6.19	11 (44%)	0.68	1.55	25 (100%)	264.36	264.36
Shokwe (25)	15 (60%)	1.44	7.20	0	0	0	14 (56%)	0.64	4.00	23 (92%)	0.72	6.00	18 (72%)	5.84	6.08
Total/mean	35 (47.3%)	1.88	5.56	6 (8%)	0.27	3.33	40 (54.1%)	2.53	6.03	53 (71.6%)	4.41	9.88	55 (73.3%)	94.74	114.93

4.3.2. Essential metal concentrations in parasites:

The concentration of iron (Fe) in all the different parasites across the three sites was higher than in the host muscle tissue but lower than in the liver (Fig. 4.2A, B, C). *Contracaecum* sp. had significantly lower concentrations of Fe than the other parasites from both the Usuthu River and Shokwe Pan sites. *T. ciliotheca*, had the highest concentration of Fe at both the Usuthu River and Shokwe Pan sites. Whereas *Pa. cyathopgharynx* had the highest concentrations of Fe at Nyamithi Pan (Fig. 4.2B), the *Proteocephalus* sp. had significantly lower concentrations of Fe than the other parasites at Nyamithi Pan Fig. 4.2B).

Cobalt concentrations in the different parasites were higher than the concentrations of Co found in the host muscle and liver tissue (Fig. 4.2D, E, F). There was no significant difference in Co concentrations in the different parasites at any of the sites. Once again *T. ciliotheca* had the highest concentrations of Co at the three sites, whereas *Proteocephalus* sp. had the lowest concentration of Co at the Nyamithi Pan site (Fig. 4.2E).

The concentration of nickel (Fig. 4.2G, H, I) in the different parasites was higher than the concentrations in the host muscle tissue across the three sites. At the Nyamithi Pan site *Proteocephalus* sp. was the only parasite with a lower concentration than in the host liver tissue (Fig. 4.2H). There was no significant difference in the Ni concentrations in the different parasites at both Usuthu River and Shokwe Pan sites with *Contracaecum* sp. parasites having the lowest concentration of Ni. At both the Usuthu River and Nyamithi Pan sites the *Pa. cyathopgharynx* had the highest concentration of Ni. The *Proteocephalus* sp. had significantly lower concentrations than all the other parasites and even lower concentrations than in the host muscle tissue.

Concentrations of Zn in all parasites across all sites were higher than the host muscle tissue concentrations and only the *Proteocephalus* sp. and *Contracaecum* sp. at both Nyamithi Pan and Shokwe Pan had lower concentrations than the host liver tissue (Fig. 4.3A, B, C). The *Contracaecum* sp. had significantly lower concentrations of Zn at both the Usuthu River (Fig. 4.3A) and Shokwe Pan (Fig. 4.3C) sites with only the *Proteocephalus* sp. having lower concentrations than the *Contracaecum* sp. at Nyamithi Pan (Fig 4.3B). Both the *P. pseudolaeviconchus* and *Pa. cyathopgharynx* parasites had higher concentrations of Zn at all three sites with the *T. ciliotheca* having higher concentrations of Zn at the Usuthu River site.

The copper (Cu) concentrations in the parasites across the three sites were higher than in the host muscle tissue except for *Proteocephalus* sp., at Nyamithi (Fig. 4.3D, E, F). At both the Usuthu River and Shokwe Pan sites the parasites had lower concentrations of Cu than present in the host's liver tissue. There was no significant difference in the concentration of Cu between the different parasites at the Shokwe Pan site; only *Contracaecum* sp. had lower concentrations at the Usuthu River site. The two intestinal nematodes (*P. pseudolaeviconchus* and *Pa. cyathopgharynx*) and a single cestode species (*T. ciliotheca*) had very similar concentrations of Cu at the three sites.

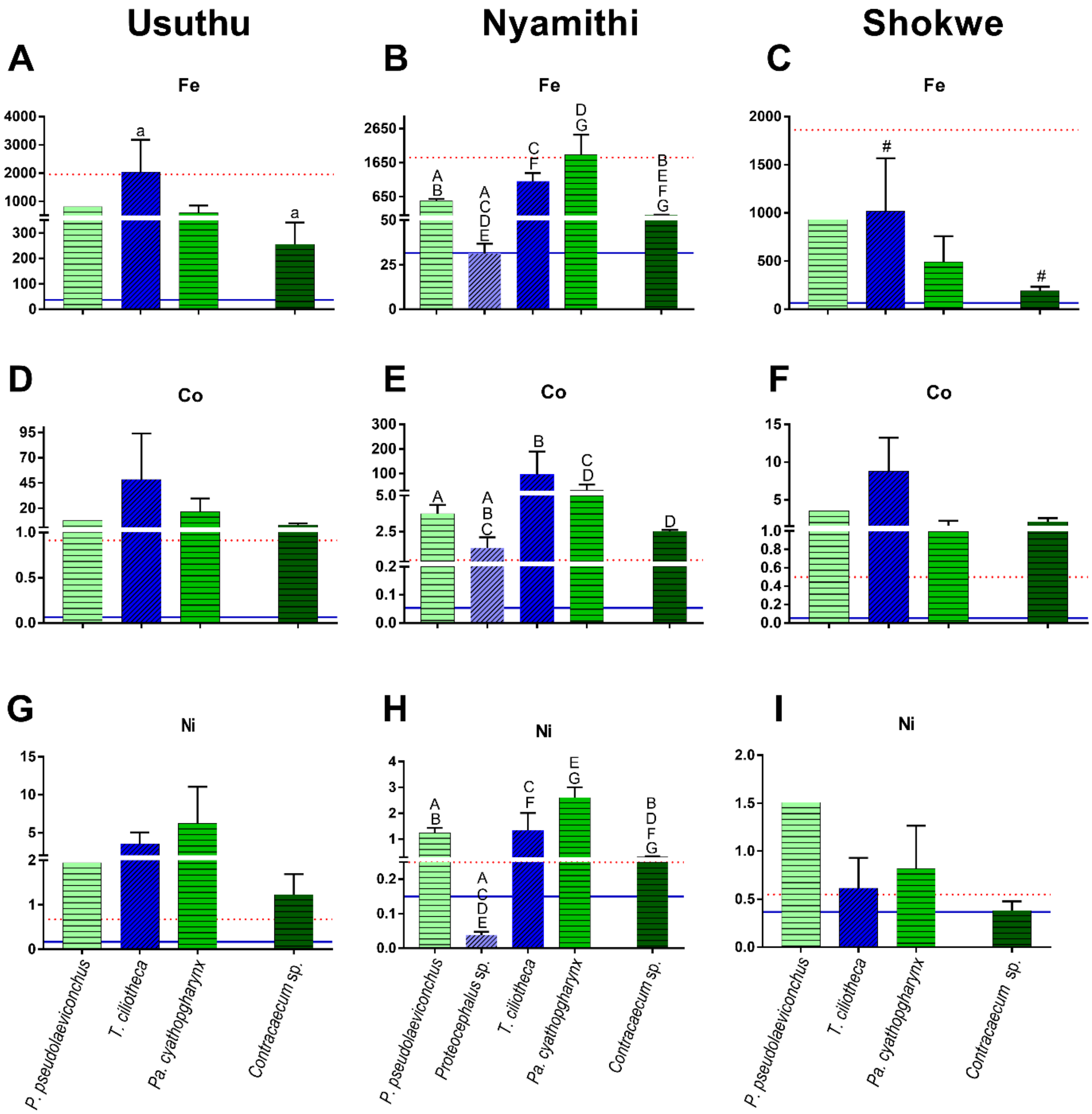

Figure 4-2: Bar graphs showing the concentration (mg/kg) (mean ± SE) of the essential metals: Fe (A, B, C), Co (D, E, F) and Ni (G, H, I), in five different endohelminth parasites of *Clarias gariepinus* across three sites in Ndumo Game Reserve (the blue solid line and red dotted lines indicate the mean concentration of metals in the host muscle and liver tissues, respectively).

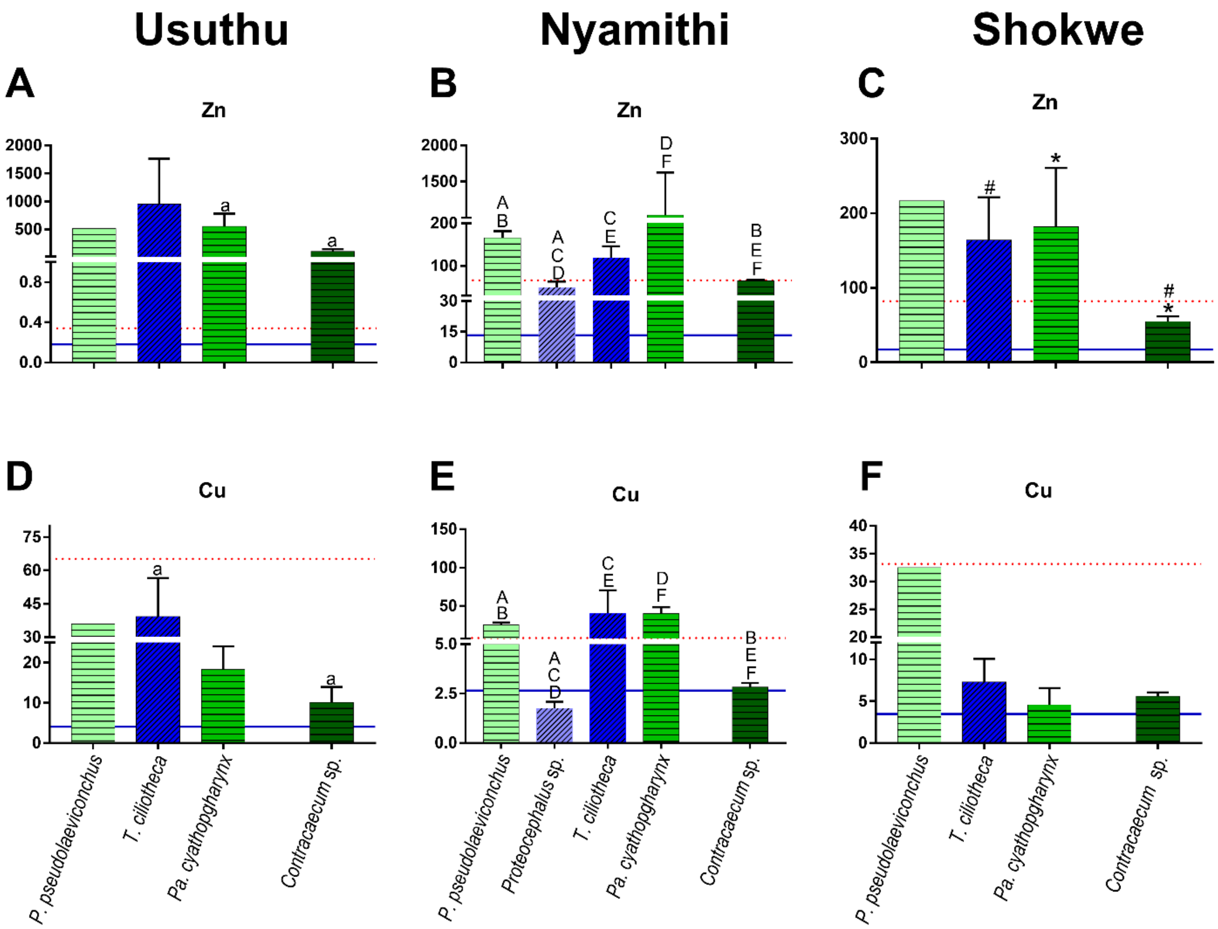

Figure 4-3: Bar graphs showing the concentration (mg/kg) (mean ± SE) of the essential metals: Zn (A, B, C) and Cu (D, E, F), in five different endohelminth parasites of *Clarias gariepinus* across three sites in Ndumo Game Reserve (the blue solid line and red dotted lines indicate the mean concentration of metals in the host muscle and liver tissues, respectively).

4.3.3. Non-essential metal concentrations in parasites:

The concentration of Al was higher in all the parasites than in the muscle and liver tissue of the hosts across the three sites (Fig. 4.4A, B, C). The *Pa. cyathopgharynx* had the highest concentration of Al at both Usuthu River and Nyamithi Pan sites, with the *Contracaecum* sp. parasites having significantly lower Al concentrations than all the other parasites across all three sites. The *P. pseudolaeviconchus* had the second highest concentrations of Al at the three sites.

The concentration of Mn in all the parasites exceeds the concentration of Mn in the host muscle tissue at the three sites (Fig. 4.4D, E, F). Only the *Proteocephalus* sp. and *Contracaecum* sp. parasites had lower concentration of Mn than in the host liver tissue across the three sites (Fig. 4.4D, E, F). The *Contracaecum* sp. parasites had significantly lower concentrations of Mn than the other nematode parasites and very similar concentrations to the *Proteocephalus* sp. present at Nyamithi Pan. At the Usuthu River site the cestode species *T. ciliotheca* had higher concentrations of Mn than the nematodes: *P. pseudolaeviconchus* and *Pa. cyathopgharynx*. At Nyamithi Pan and Shokwe Pan both *P. pseudolaeviconchus* and *Pa. cyathopgharynx* had higher concentrations of Mn than *T. ciliotheca*.

Procamallanus pseudolaeviconchus had the highest concentrations of As at both the Usuthu River and Shokwe Pan sites (Fig. 4.4G, H, I). The *Contracaecum* sp. parasites had the lowest concentration of As at both Usuthu River and Shokwe Pan, with the Usuthu River site being significantly lower than the *T. ciliotheca*. At the Nyamithi Pan site both the *Proteocephalus* sp. and the *Contracaecum* sp. had lower concentrations of As than in the hosts' muscle and liver tissue (Fig. 4.4H). The *Pa. cyathopgharynx* had the highest concentration of As at Nyamithi Pan site followed by the *T. ciliotheca*.

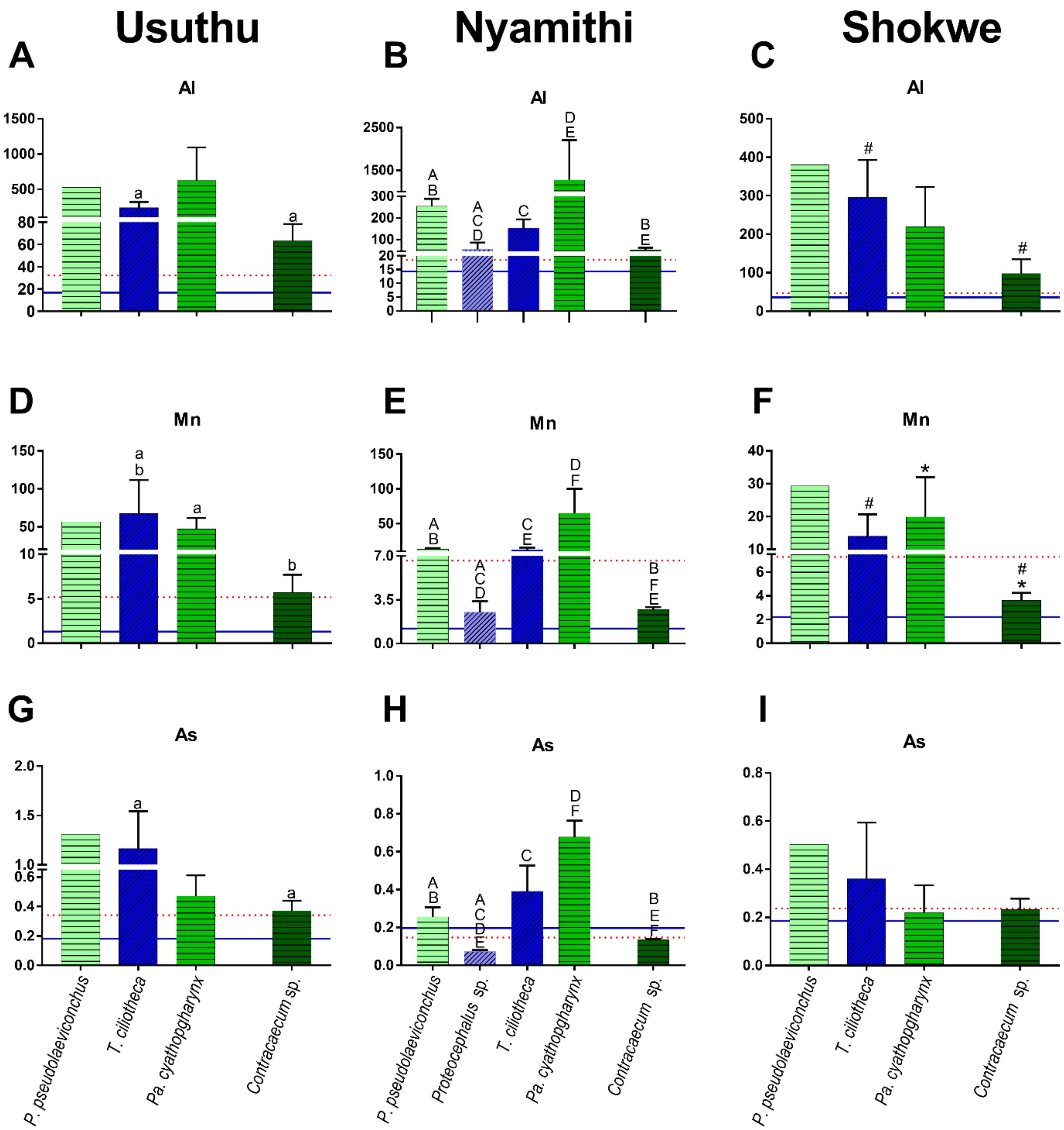

Figure 4-4: Bar graphs showing the concentration (mg/kg) (mean ± SE) of the non-essential metals: Al (A, B, C), Mn (D, E, F) and As (G, H, I), in five different endohelminth parasites of *Clarias gariepinus* across three sites in Ndumo Game Reserve (the blue solid line and red dotted lines indicate the mean concentration of metals in the host muscle and liver tissues, respectively).

The concentration of cadmium (Cd) in the different parasites was higher than the concentrations in the host muscle tissue, except for *Proteocephalus* sp. and *Contracaecum* sp. parasites at Nyamithi Pan and Shokwe Pan (Fig. 4.5A, B, C). *Contracaecum* sp. parasites had the lowest concentration of Cd at both the Usuthu River and Shokwe Pan sites, and the second lowest concentration at Nyamithi Pan with only *Proteocephalus* sp. having lower concentrations. *Procamallanus pseudolaeviconchus* had the highest concentration of Cd at both the Usuthu River and Shokwe Pan sites, with *Pa. cyathopgharynx* having the highest concentration at Nyamithi Pan followed by *P. pseudolaeviconchus*.

The concentrations of lead (Pb) in the different parasites had higher concentrations than in the host muscle tissue with the exceptions of *Proteocephalus* sp. and *Contracaecum* sp. parasites at Nyamithi Pan and Shokwe Pan (Fig. 4.5D, E, F). *Contracaecum* sp. had the lowest concentrations of Pb at both the Usuthu River and Shokwe Pan sites. *Procamallanus pseudolaeviconchus* had the highest concentrations of Pb at both the Usuthu River and Shokwe Pan sites, with *Pa. cyathopgharynx* having the highest concentration of Pb at Nyamithi Pan.

Selenium (Se) was present in lower concentrations in all parasites, except *Contracaecum* sp. at Shokwe Pan, than in both the host muscle and liver tissue (Fig. 4.5G, H, I). *Contracaecum* sp. parasites had the highest concentration of Se at both the Usuthu River and Shokwe Pan sites with the second highest concentration present in *T. ciliotheca* at all three sites.

The concentration of silver (Ag) in the parasites was higher than the concentrations in both the muscle and liver tissues of the hosts (Fig. 4.5J, K, L). *Contracaecum* sp. parasites had the lowest concentration of Ag at the three sites. At both the Usuthu River and Nyamithi Pan sites *Pa. cyathopgharynx* had the highest concentration of Ag, while *Procamallanus pseudolaeviconchus* had the second highest concentration. *Tetracampos ciliotheca* had the highest concentration of Ag at the Shokwe Pan site which was significantly higher than the concentration in *Contracaecum* sp. parasites.

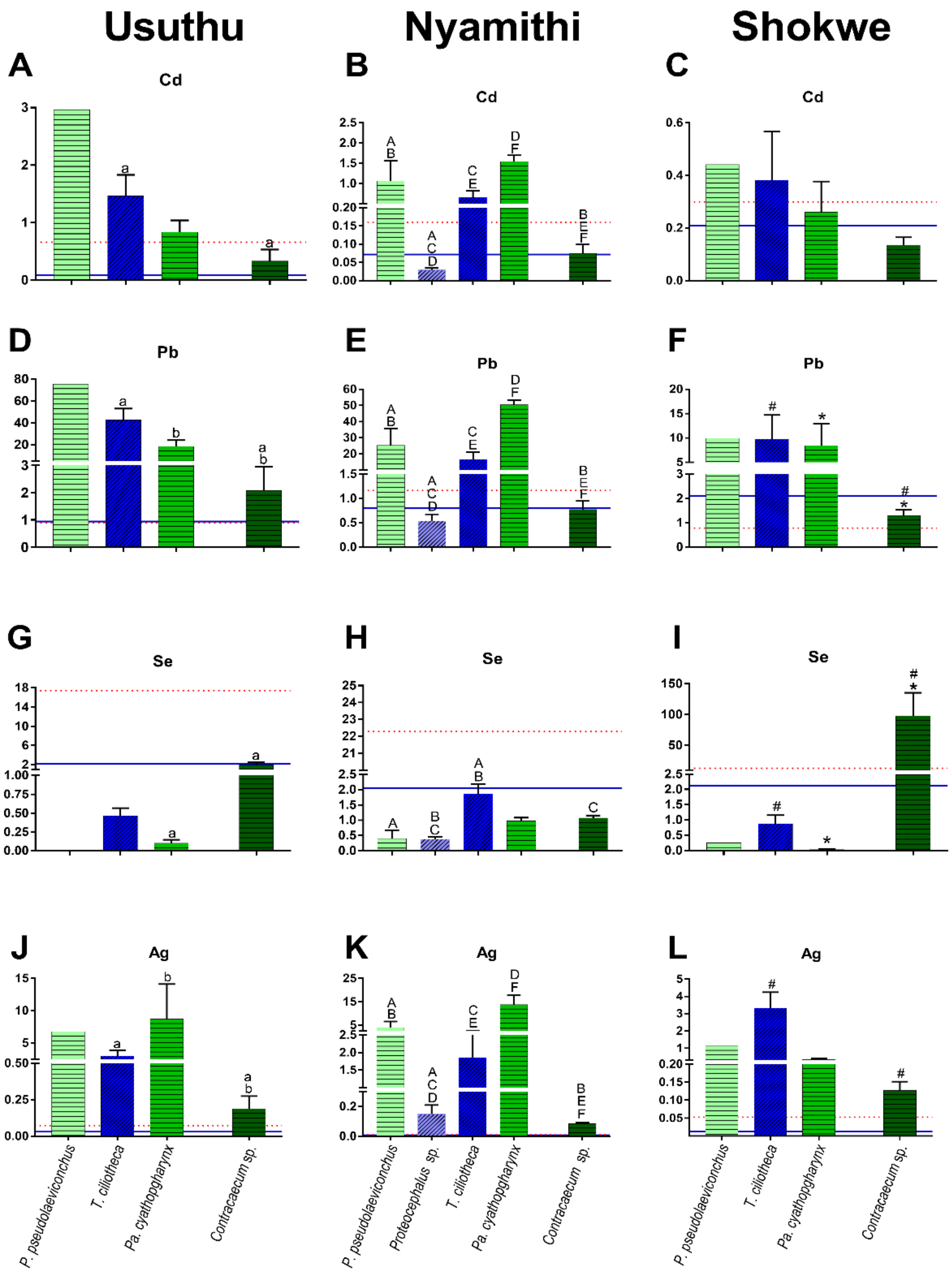

Figure 4-5: Bar graphs showing the concentration (mg/kg) (mean ± SE) of the non-essential metals: Cd (A, B, C), Pb (D, E, F), Se (G, H, I), and Ad (J, K, L), in five different endohelminth parasites of *Clarias gariepinus* across three sites in Ndumo Game Reserve (the blue solid line and red dotted lines indicate the mean concentration of metals in the host muscle and liver tissues, respectively).

4.3.4. Bioaccumulation factors for essential and non-essential metals (BAF)

The bioaccumulation factors for metals in all the different parasites found in *C. gariepinus* provided interesting insights into bioaccumulation in these parasites (Table 4.4). The BAF for *P. pseudolaeviconchus* ranged from 0 for Se to 414 for Ag. The *P. pseudolaeviconchus* had the highest BAF for Ag across the three sites with an average of 241.7 and the lowest for Se with an average of 0. The BAF of *T. ciliotheca* ranged from 0 for Se to 1548 for Co. *Tetracampos ciliotheca* had a BAF average of 821.3 for Co and a 0 average for Se across the three sites. *Pa. cyathopgharynx* had a BAF range of 0 for Se to 1445 for Ag. The BAF for Ag averaged 586.3 across the three sites in the *Pa. cyathopgharynx*. *Proteocephalus* sp. had a BAF that ranged from 0 for Se, As and Cd to 22 for Co, and therefore had the lowest bioaccumulation ability of all the different parasite species. The *Contracaecum* sp. parasites had a BAF ranging from 1 for six different metals to 53 for Co. *Tetracampos ciliotheca* was found to be the best bioaccumulator for Al, Fe, Mn, Co, Zn and Cu (Table 4.4), while *P. pseudolaeviconchus* had the highest ability to bioaccumulate As, Cd and Pb. *Paracamallanus cyathopgharynx* had the highest BAF for both Ni and Ag and the *Contracaecum* sp. parasites had the highest BAF for Se.

Table 4-4: Mean ± SE and range (in parentheses) of bioaccumulation factors (BAF) for metals, calculated from the *Clarias gariepinus* muscle tissue for each of the different endohelminth parasites from three sites located in the Ndumo Game Reserve (ND indicates where the concentrations were below detection in the parasites).

	Usuthu River				**Nyamithi Pan**					**Shokwe Pan**			
Metals	*P. pseudolaeviconchus*	*T. ciliotheca*	*Pa. cyathopgharynx*	*Contracaecum* sp.	*P. pseudolaeviconchus*	*Proteocephalus* sp.	*T. ciliotheca*	*Pa. cyathopgharynx*	*Contracaecum* sp.	*P. pseudolaeviconchus*	*T. ciliotheca*	*Pa. cyathopgharynx*	*Contracaecum* sp.
						Essential metals							
Fe	22	55 ± 53 (14 – 115)	16 ± 14 (3 – 36)	7 ± 3 (2 – 9)	16 ± 4 (12 – 20)	1 ± 0 (0 – 2)	33 ± 15 (14 – 48)	56 ± 25 (38 – 74)	4 ± 2 (2 – 11)	14	15 ± 14 (4 – 31)	7 ± 7 (2 – 15)	3 ± 3 (1 – 11)
Co	119	749 ± 1215 (48 – 2155)	259 ± 401 (16 – 859)	32 ± 36 (8 – 102)	60 ± 20 (31 – 77)	22 ± 34 (1 – 78)	1548 ± 3003 (16 – 6052)	493 ±558 (98 - 887)	41 ± 8 (20 – 56)	67	167 ± 148 (27 – 323)	29 ± 23 (10 – 54)	40 ± 36 (10 – 103)
Ni	12	21 ± 16 (7 – 38)	37 ± 58 (3 – 124)	6 ± 7 (0 – 20)	2 ± 1 (1 – 3)	ND	2 ± 2 (1 – 5)	4 ± 1 (3 – 5)	1 ± 0 (0 – 2)	4	2 ± 1 (1 – 3)	2 ± 2 (0 – 5)	1 ± 1 (0 – 3)
Zn	37	70 ± 103 (8 – 188)	40 ± 33 (8 – 87)	8 ± 8 (3 – 23)	11 ± 2 (10 – 15)	3 ± 3	115 ± 213 (5 – 434)	71 ± 57 (31 – 111)	4 ± 1 (2 – 6)	12	9 ± 6 (3 – 15)	10 ± 8 (5 – 19)	3 ± 2 (1 – 7)
Cu	9	10 ± 7 (5 – 18)	4 ± 3 (2 – 8)	2 ± 2 (1 – 8)	9 ± 2 (7 – 12)	1 ± 0 (0 – 1)	14 ± 21 (3 – 46)	14 ± 4 (11 – 17)	1 ± 0 (0 – 2)	9	2 ± 1 (1 – 4)	1 ± 1 (1 – 2)	2 ± 0 (1 – 3)
						Non-essential metals							
Al	32	112 ± 169 (9 – 306)	37 ± 57 (4 – 122)	4 ± 2 (1 – 9)	15 ± 4 (9 – 18)	3 ± 5 (0 – 12)	239 ± 459 (5 – 927)	78 ± 80 (21 – 134)	3 ± 4 (1 – 19)	11	8 ± 5 (4 – 13)	6 ± 5 (1 – 11)	3 ± 4 (0 – 16)
As	7	6 ± 4 (4 – 11)	3 ± 2 (1 – 4)	2 ± 1 (1 – 3)	1 ± 1 (1 – 2)	0	2 ± 1 (1 – 4)	3 ± 1 (3 – 4)	1 ± 0 (0 – 1)	3	2 ± 2 (1 – 4)	1 ± 1 (1 – 2)	1 ± 1 (0 – 4)
Cd	32	16 ± 7 (9 – 23)	9 ±5 (3 – 14)	4 ± 6 (0 – 16)	13 ± 13 (2 – 32)	0 ± 0 (0 – 1)	8 ± 5 (4 – 14)	19 ± 3 (17 – 21)	1 ± 1 (0 – 6)	2	2 ± 2 (1 – 4)	1 ± 1 (1 – 2)	1 ± 1 (0 – 2)
Mn	43	52 ± 59 (10 – 119)	36 ± 22 (8 – 63)	4 ± 4 (2 – 13)	11 ± 3 (9 – 15)	2 ± 2 (0 – 6)	97 ± 173 (6 356)	55 ± 42 (25 – 84)	2 ± 1 (1 – 4)	13	6 ± 5 (2 – 12)	9 ± 9 (2 – 20)	2 ± 1 (1 – 5)
Pb	80	45 ± 19 (24 – 62)	20 ± 12 (2 – 27)	2 ± 2 (0 – 5)	32 ± 25 (4 – 66)	1 ± 0 (0 – 1)	21 ± 11 (11 – 34)	63 ± 3 (59 – 63)	1 ± 1 (0 – 4)	5	5 ± 4 (2 – 9)	4 ± 4 (1 – 8)	1 ± 0 (0 – 2)
Se	ND	ND	ND	1 ± 1 (0 – 2)	ND	ND	1 ± 0 (1 – 1)	ND	1 ± 0 (0 – 1)	ND	ND	ND	46 ± 68 (8 – 267)
Ag	225	991 ± 533 (512 – 1564)	2896 ± 3620 (126 – 7855)	62 ± 78 (3 – 194)	414 ± 557 (68 – 1244)	16 ± 18 (4 -58)	194 ± 151 (59- 328)	1445 ± 593 (1026 – 1864)	13 ± 17 (3 – 86)	86	253 ± 122 (125 – 368)	25 ± 10 (14 – 34)	10 ± 7 (1 – 24)

4.3.5. Correlations between essential and non-essential metal concentrations in parasite species and the environmental and host muscle and liver concentrations

The Spearman's correlation coefficient for all metals in all parasite species in relation to their environment the host muscle and liver tissue concentrations as well as to their hosts environment the water and sediment can be found in Table 4.5 and Table 4.6. The data suggest that for essential metals, *P. pseudolaeviconchus* and *Contracaecum* sp. have a negative relationship with corresponding water concentrations, whereas the correlations for Zn and Cu and Fe were significant in these two species, respectively. The opposite is true for *T. ciliotheca* and *Pa. cyathopgharynx* where parasite essential metal concentrations were positively correlated with levels in the water and for Co significantly so in both species. For the non-essential metals, *P. pseudolaeviconchus* and *Contracaecum* sp. had a negative relationship with corresponding water concentrations, whereas the correlations for Cd and Mn in *P. pseudolaeviconchus* and Mn and Se in *Contracaecum* sp was significantly positive. *Tetracampos ciliotheca* had positive correlations for Cd and Ag, while *Pa. cyathopgharynx* had positive correlations for As, Pb and Se but a negative correlation for Ag.

The essential metals in *P. pseudolaeviconchus*, *T. ciliotheca*, *Pa. cyathopgharynx* and *Contracaecum* sp. had a positive relationship corresponding with the sediment. *Procamallanus pseudolaeviconchus* had correlations with Co; *T. ciliotheca* had positive correlations for both Fe; and Ni; *Pa. cyathopgharynx* had a positive correlation for Ni; and *Contracaecum* sp. had positive correlations for both Co and Zn. The non-essential metals *P. pseudolaeviconchus*, *T. ciliotheca*, *Pa. cyathopgharynx* and *Contracaecum* sp. had positive relationships with the corresponding sediment, and the negative correlations were between *T. ciliotheca* and the sediment for Ag. *P. pseudolaeviconchus* had positive correlations for Cd and Pb; *T. ciliotheca* had positive correlations with As, Cd and Pb; *Pa. cyathopgharynx* had positive correlations for both Se and Ag and *Contracaecum* sp. only had a positive correlation for Se. The correlation of the concentration of essential and non-essential metals in the four different parasites and their concentrations in both the host muscle and liver tissue showed some significance (Table 4.6).

The essential metals only had significantly negative correlations between the parasites and the host muscle tissue. *Tetracampos ciliotheca* had a negative correlation for Ni, and *Pa. cyathopgharynx* had negative correlations for both Fe and Zn. *Procamallanus* *pseudolaeviconchus* and *Contracaecum* sp. had no significant correlations between their concentration of essential metals and the concentration in the host muscle tissue. The essential metals in *P. pseudolaeviconchus* and *Contracaecum* sp. had no significant relationships with their corresponding muscle tissue, while *T. ciliotheca* and *Camallanus* sp. had a negative relationship with the corresponding muscle tissue. *Tetracampos ciliotheca* had a negative correlation for Ni, and *Pa. cyathopgharynx* for Fe and Zn.

The non-essential metals in *P. pseudolaeviconchus* and *Contracaecum* sp. had positive relationships with their corresponding muscle tissue. Sellenium in *P. pseudolaeviconchus* and As, Cd and Pb in *Contracaecum* sp., *Paracamallanus cyathopgharynx* and *Contracaecum* sp. had negative relationship with corresponding muscle tissue. Cadmium, Mn and Pb in *Pa. cyathopgharynx* and Mn in *Contracaecum* sp. had negative correlations with the muscle tissue of the hosts. The essential metals, Ni and Co respectively, in *T. ciliotheca* and *Contracaecum* sp. had negative relationships with the concentration in their corresponding host liver tissues. The non-essential metals, Cd and Pb respectively, in *P. pseudolaeviconchus* and *Contracaecum* sp. had positive relationships with that in the corresponding liver tissue.

Table 4-5: Spearman correlation coefficients (r) between the concentration of both essential and non-essential metals in four different helminth parasites and the concentration of the metals in both the water and sediment at sites within the Ndumo Game Reserve (bold number indicates significant differences; * is < 0.005** is < 0.001).

	P. pseudolaeviconchus	*T. ciliotheca*	*Pa. cyathopgharynx*	*Contracaecum*		*P. pseudolaeviconchus*	*T. ciliotheca*	*Pa. cyathopgharynx*	*Contracaecum*
Water					**Sediment**				
Essential metals									
Fe	-0.500	-0.500	0.500	-1.000**	**Fe**	-0.500	1.000**	0.500	0.500
Co	0.500	1.000**	1.000**	0.500	**Co**	1.000**	0.500	0.500	1.000**
Ni	-0.500	0.500	0.500	-0.500	**Ni**	0.500	1.000**	1.000**	0.500
Zn	-1.000**	0.500	0.500	-0.500	**Zn**	0.500	0.500	0.500	1.000**
Cu	-1.000**	0.500	0.500	-1.000**	**Cu**	0.500	0.500	0.500	0.500
Non-essential metals									
Al	-1.000	-1.000	-1.000	1.000	**Al**	0.500	0.500	0.500	-0.500
As	-0.500	0.500	1.000**	-0.500	**As**	0.500	1.000**	0.500	0.500
Cd	1.000**	1.000**	0.500	0.500	**Cd**	1.000**	1.000**	0.500	0.500
Mn	1.000**	-0.500	-0.500	1.000**	**Mn**	0.500	0.500	0.500	0.500
Pb	0.500	0.500	1.000**	-0.500	**Pb**	1.000**	1.000**	0.500	0.500
Se	1.000	0.500	1.000**	1.000**	**Se**	1.000	0.500	1.000**	1.000**
Ag	-0.500	1.000**	-1.000**	0.500	**Ag**	0.500	-1.000**	1.000**	-0.500

Table 4-6: Spearman correlation coefficients (r) between the concentration of both essential and non-essential metals in four different helminth parasites and the concentration of the metals in the muscle and liver tissue of *Clarias gariepinus* at sites within the Ndumo Game Reserve (bold number indicates significant differences; * is < 0.005** is < 0.001).

	P. pseudolaeviconchus	***T. ciliotheca***	***Pa. cyathopgharynx***	***Contracaecum***		***P. pseudolaeviconchus***	***T. ciliotheca***	***Pa. cyathopgharynx***	***Contracaecum***
	Muscle					**Liver**			
	Essential metals								
Fe	0.771	-0.430	**-.767***	0.038	**Fe**	-0.371	-0.139	-0.483	-0.108
Co	0.714	-0.455	0.400	-0.201	**Co**	0.600	-0.200	0.367	**-.392****
Ni	-0.429	**-.648***	-0.333	0.154	**Ni**	0.029	**-.667***	-0.117	-0.195
Zn	0.371	0.273	**-.867****	0.091	**Zn**	0.314	0.139	-0.167	-0.063
Cu	0.371	0.042	0.033	-0.164	**Cu**	0.314	-0.297	-0.267	0.031
	Non-essential metals								
Al	0.486	-0.248	-0.433	0.081	**Al**	0.543	-0.188	-0.317	0.031
As	0.200	-0.248	0.500	**.296***	**As**	0.429	0.455	0.000	0.101
Cd	-0.657	-0.491	**-.933****	**.409****	**Cd**	**.829***	0.309	-0.100	-0.026
Mn	0.771	-0.539	**-.933****	**-.289***	**Mn**	0.486	-0.442	0.117	-0.056
Pb	-0.200	-0.552	**-.867****	**.962****	**Pb**	0.657	-0.600	0.083	**.315***
Se	**.943****	-0.248	-0.050	0.159	**Se**	-0.314	0.079	0.467	0.050
Ag	0.500	-0.467	0.143	0.199	**Ag**	-0.500	-0.400	-0.429	0.080

4.3.6. Correlation between the metal concentrations of parasites and their environment in both long and short-term accumulation

In order to relate the correlation of the metal concentrations in the endohelminth parasites with the long-term accumulation of metals in the host *C. gariepinus* muscle tissue, an RDA triplot was constructed (Fig. 4.6). The host metal concentration was overlaid with the metal concentration of the endohelminth parasites to provide an indication of the correlation between the parasite metals and their host muscle tissue. The first and second axes combine to explain 8.43% of the variance in the data where the three sites separate from each other. The first axis explains 5.48% of the variance in data where the different endohelminth parasites across the three sites separated.

Some separation based on sampling location can be seen: the figure shows that metal concentrations in parasites collected from *C. gariepinus* in the Usuthu River predominantly cluster with increased metal concentrations in the host muscle tissue. There was a positive correlation between the concentration of Ag and Fe in the endohelminth parasites and their host, and a negative correlation between Al, As and Co in the endohelminth parasites and their hosts.

In order to relate the correlation of metal concentration in the endohelminth parasites with the short-term accumulation of metals in the host *C. gariepinus* liver tissue, an RDA triplot was constructed (Fig. 4.7). The first and second axes combine to explain 18.01% of the variance in the data where the endohelminth parasites are separated in terms of their metal concentrations. The first axis explains 15.77% of the variance in the data and the second axis explains 2.24%. The metal concentrations in the parasites of Nyamithi Pan as well as the *Contracaecum* sp. of Shokwe Pan separated away from the other parasites. The metal concentrations in the helminth parasites had correlations with the metal concentrations in their host liver tissue. There was a positive correlation between the As, Cd and Ag concentration in the parasites and the host liver tissue. There was a negative correlation in the Mn and Zn in the parasites and their concentrations in the *C. gariepinus* liver tissue.

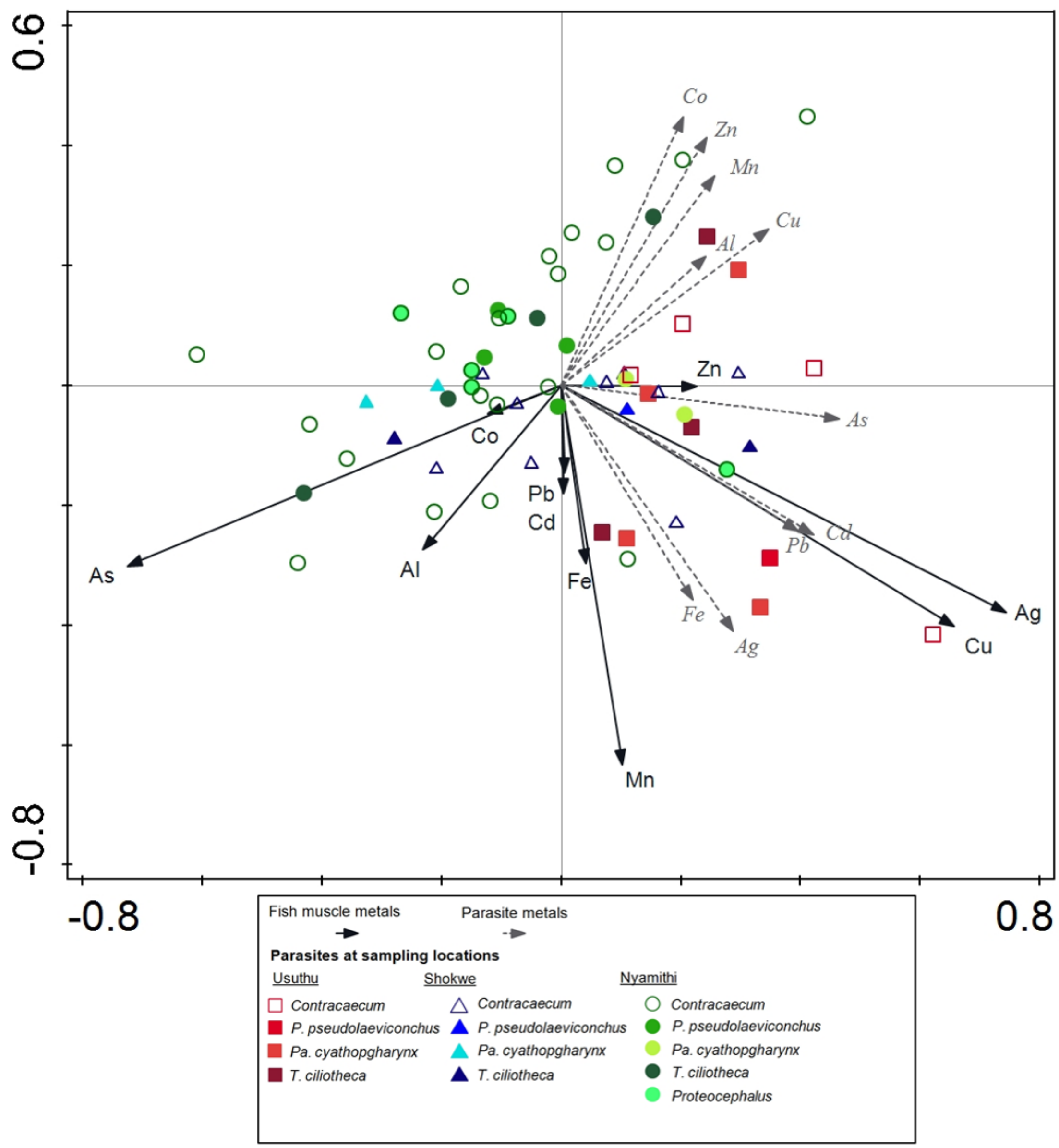

Figure 4-6: Constrained Redundancy Analysis triplot of the concentration of metals in the different endohelminth parasites across three different sites in Ndumo Game Reserve (Squares – Usuthu River, Circles – Nyamithi Pan, Triangles – Shokwe Pan; with the metal concentration in the muscle tissue of *Clarias gariepinus* overlaid; the first [horizontal] axis explains 5.48% and the second [vertical] axis a further 2.98%, for a cumulative 8.43%).

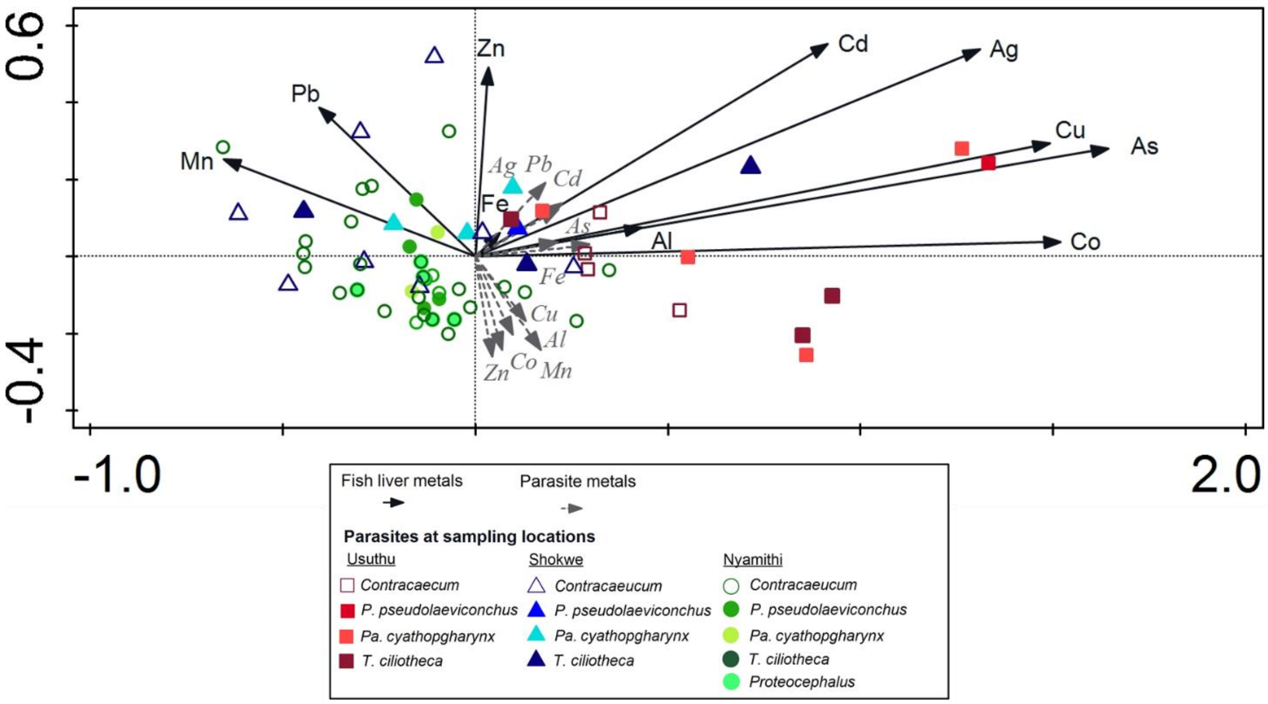

Figure 4-7: Constrained Redundancy Analysis triplot of the concentration of metals in the different endohelminth parasites across three different sites in Ndumo Game Reserve (Squares – Usuthu River, Circles – Nyamithi Pan, Triangles – Shokwe Pan; with the metal concentration in the liver tissue of *Clarias gariepinus* overlaid; the first [horizontal] axis explains 15.77% and the second [vertical] axis a further 2.24%, for a cumulative 18.01%).

4.4. Discussion

This chapter focused on the accumulation of metals in different endohelminth parasites located in different parts of their host *C. gariepinus*. The metal concentration found in the different endohelminth parasites was compared firstly with the metal concentrations in the environment of the *C. gariepinus* (chapter 3 section 3.3) and secondly the concentrations in their ecosystem (Chapter 2 section 2.3).

The *Clarias gariepinus* in the Ndumo Game Reserve area were infected with five different helminth parasites that consisted of three species of nematodes (*Contracaecum* sp., *P. pseudolaeviconchus* and *Pa. cyathopgharynx*) and two species of cestodes (*Proteocephalus* sp. and *T. ciliotheca*). The high prevalence and abundance of parasites in Nyamithi Pan are most likely due to the fact that Nyamithi Pan is a permanent pan with high numbers of intermediate hosts (copepods) (Smit *et al.*, 2016). A permanent pan has water throughout the wet and dry seasons and thus the intermediate hosts can multiply to higher levels than in temporary pans (Williams & Bunkley-Williams, 2019). The low number of parasites in the lotic system of Usuthu River is possibly because catfish is not confined to a small space and can move around, making infections difficult. In order for the endohelminth parasites in the study to complete their life cycles water birds act as definitive hosts; thus if there is no water the lifecycle cannot be completed in temporary pans (Garbin *et al.*, 2019).

Procamallanus pseudolaeviconchus across the three sites had a similar prevalence than in previous studies (Mashego, 1981, Boomker, 1982, Boomker, 1994). Moravec (1975) stated that *P. pseudolaeviconchus* was a widespread parasite in African freshwater fishes and was found in the stomach of their hosts. Previous studies found a lower prevalence of *Pa. cyathopgharynx* in *C. gariepinus* than present in this study (Boomker, 1982, Mashego, 1989, Boomker, 1994, Gulelat *et al.*, 2013). The prevalence of *Contracaecum* sp. was similar to prevalence found by Mashego (1989) who found a prevalence ranging from 10 to 100% across nine sites, with five sites having a prevalence between 65 and 78%. The *Contracaecum* sp. was located in the body cavity of their hosts. All the nematodes have copepods as intermediate hosts and their abundance is directly related to the abundance of copepods present in the ecosystem.

The prevalence of the cestodes (*Proteocephalus* sp. and *T. ciliotheca*) was very similar to the prevalence found by Madanire-Moyo and Avenant-Oldewage (2013) in *C. gariepinus* of the Vaal Dam. The study found that out of the 12 metals analysed all, except As and Se, were found at higher levels than in their hosts. However, there was a difference in the range of accumulation between the different parasites. The first nematode species was *Contracaecum* sp. located in the body cavity of its hosts. The study found similar concentrations of metal than Otachi *et al.* (2014) found in *Contracaecum* sp. from *Oreochromis leucostictus* from Lake Naivasha in Kenya. The *Contracaecum* sp. were found to accumulate metals at higher levels than their host muscle tissue, but lower than the other helminth parasites. This is believed to be because of the location of the *Contracaecum* sp. in the body cavity of their hosts and the feeding mechanisms being used (Otachi *et al.*, 2014). *Contracaecum* sp. feed on the blood and tissue of their hosts and do not absorb nutrients through their skin (Otachi *et al.*, 2014).

The second nematode *P. pseudolaeviconchus* is located in the stomach of its host. The only previous study conducted on *P. pseudolaeviconchus* as a bioaccumulator of metal was that of

Akinsanya and Kuton (2016). They found that the *P. pseudolaeviconchus* of *Synodontis clarias* in the Lekki Lagoon in Nigeria accumulated Fe (BAF = 1.37) and Pb (BAF = 23.167) at high levels. In this study it is evident that along with those two metals *P. pseudolaeviconchus* accumulated most metals at higher levels than their hosts. The last nematode *Pa. cyathopgharynx* is located in the rectum of its hosts. No previous studies have been conducted on *Pa. cyathopgharynx* as bioaccumulator of metals. In this study we found that *Pa. cyathopgharynx* had similar accumulation rates than *P. pseudolaeviconchus.* As the *Pa. cyathopgharynx* is located in the rectum of its hosts, it is located on the opposite side of the intestinal tract than *P. pseudolaeviconchus.* This shows that the position in the intestinal tract does not play a major role in helminth parasites' ability to accumulate metals.

Two different species of cestodes (*Proteocephalus* sp., *T. ciliotheca*) were analysed in this study and it was found that *T. ciliotheca* had the best bioaccumulation rate. Cestodes absorb nutrients and pollutants through their tegument and membranes (Izvekova *et al.*, 1997). The cestodes in this study showed bioaccumulation of both Ag and Co at levels not yet recorded in any previous studies. In this study we found that the cestode *T. ciliotheca* had the highest bioaccumulation ability that is consistent with previous studies (Sures *et al.*, 2017). It can however be seen that the cestode, *Proteocephalus* sp., had the lowest bioaccumulation ability. This consequently illustrates that different species of cestodes have different abilities to accumulate metals and that not all cestodes will be good bio-indicators of metals.

It was also found that the nematode species *P. pseudolaeviconchus* and *Pa. cyathopgharynx* proved to be adequate species to be bio-indicator species of metal pollution. The study found significant correlations between the concentrations of essential and non-essential metals in the different parasites and in the environment. The essential metals in the water had a predominantly negative correlation, with only Co having positive correlations with *T. ciliotheca* and *Pa. cyathopgharynx*. It is possible that when the concentrations of essential metals are low in the environment the parasite will store the essential metals in their tissue. The non-essential metals had positive correlations between the environmental concentrations and the parasite concentrations. This is because parasites cannot use non-essential metals, which in turn accumulate in the parasites. The essential metal concentrations in the sediment had positive correlations with the concentrations in the different parasites.

The different helminth parasites showed negative correlations for the essential metals in both the host muscle and liver tissues. This is because of the competition between the parasite and their hosts for the essential metals (Sures, 2002). These results are supported by the results found by many previous studies (Szefer *et al.*, 1998, Sures, 2002, Oyoo-Okoth *et al.*, 2010). For the non-essential metals *Contracaecum* sp. had significantly positive correlations with As, Cd and Pb in the muscle tissue which is in line with the results of Leite *et al.* (2017) where *Contracaecum* sp. parasitised *Acestrorhynchus lacustris. Procamallanus pseudolaeviconchus* had a positive correlation for Se and the host muscle tissue that was in line with the findings of Morris *et al.* (2016).

There was a significant negative correlation between the concentration of Cd, Mn and Pb in *Pa. cyathopgharynx* and their concentration in the host muscle tissue. *Procamallanus pseudolaeviconchus* had a positive correlation for Cd compared to the liver concentrations. This is supported by a study done by Thielen *et al.* (2004) on *Pomphorhynchus laevis* in barbel, who found that *P. laevis* bioaccumulate Cd at higher levels than in their host liver tissue. *Contracaecum* sp. had a positive correlation for Pb in the liver tissue of their hosts. Correlation analyses in this study showed some associations between the metal concentrations in the host tissue and the abundance of helminth parasites. For essential metals it was found that no significant correlations were found for Fe and Ni, which can be due to the abundance of Fe and Ni found in the environment (Chapter 2 section 2.3) being enough to support both host and parasites. The same results were found by Oyoo-Okoth *et al.* (2010) through the metal partitioning in cestode *Ligula intestinalis* in *Rastrineobola argentea*. Most significant correlations were found to be between the liver tissue of the host and the abundance of parasites.

There was a negative correlation between the catfish liver and number of parasites for Co for both *P. pseudolaeviconchus* and *Contracaecum* sp. The same negative correlation for Co was found by Sures (2002) between *Acanthocephalus lucii* and their host *Perca fluviatilis* and Thielen *et al.* (2004) between *Pomphorhynchus laevis* and their host barbel as well as Oyoo-Okoth *et al.* (2010) between *Ligula intestinalis* and their host *Rastrineobola argentea.* This negative correlation is believed to be due to competition between host and parasites for the essential element Co (Sures, 2002, Thielen *et al.*, 2004, Oyoo-Okoth *et al.*, 2010). The positive correlation between the Zn concentration of *T. ciliotheca* in the muscle and *Pa. cyathopgharynx* in the liver is supported by the findings of Oyoo-Okoth *et al.* (2010) and Oyoo-Okoth *et al.* (2012) who found the same correlations of Zn between *Ligula intestinalis* and their host *Rastrineobola argentea*. Parasites absorb binding proteins from their hosts, which in turn mess with the detoxification of some metals in the host, that can lead to higher levels of Zn in the host (Oyoo-Okoth *et al.*, 2012).

Copper had significantly negative correlations for both *T. ciliotheca* and *Contracaecum* sp. between the concentration in the liver and number of parasites. This result is supported by findings from Alonso *et al.* (2004) and Jankovská *et al.* (2012) who found a negative correlation between *Moniezia expansa* and their host sheep. *Paracamallanus cyathopgharynx* had a positive correlation for Cu between the liver and number of parasites. All the non-essential metals in this study showed significant correlations for at least one parasite. Aluminium showed a positive correlation between concentration in the muscle tissue of the host and the number of *P. pseudolaeviconchus,* and a negative correlation between concentration in the liver and the number of *Contracaecum* sp. *Paracamallanus cyathopgharynx* had a positive correlation between the concentration of Mn in the host muscle tissue and the number of parasites. These findings are supported by Thielen *et al.* (2004) who found a positive correlation between *Pomphorhynchus laevis* and their host barbel in that both Mg and Mn positively correlate with the concentration in the muscle tissue.

4.5. Conclusion

This study is one of the first studies to focus on the metal accumulation in different parasite species at different aquatic ecosystems. This study found that the different aquatic systems of NGR had different parasite loads, and that the type of aquatic system played a role in the bioaccumulation of metals by the parasites. The study also found that that there was no gradient present for metal accumulation through the intestinal tract. The first hypothesis stated that the parasites in the intestines would have higher accumulation rates for metals than the *Contracaecum* sp. in the body cavity due to the higher concentration of metals in the intestinal tract. This hypothesis was accepted because *Contracaecum* sp. had the lowest BAF of all the endohelminth parasites.

The second hypothesis stated that there would be a gradient of metal accumulation in the endohelminth parasites with the highest being in the parasites in the stomach due to the low pH making metals more bio-available than in the rectum of the host. This hypothesis was rejected as the study found that the position in the intestinal tract did not play a significant role in the bioaccumulation ability of the parasites, with the *Pa. cyathopgharynx* having similar BAF than the *P. pseudolaeviconchus*. Now that the accumulation of metals from the environment into *C. gariepinus* and then into their endohelminth parasites have been established, the next chapter of this dissertation focuses on the biological responses of *C. gariepinus* to metal exposure as well as determine if endohelminth parasites have an effect on the biological responses of their hosts.

5. Host-parasite systems: interactions of parasite load, biomarkers of exposure and biomarkers of effect

5.1. Introduction

The increase of anthropogenic stress on aquatic ecosystems can have adverse effects (acute or chronic) on the well-being of organisms in aquatic ecosystems (Adams & Greeley, 2000, Van der Oost *et al.*, 2003, Palma *et al.*, 2015, Gerber *et al.*, 2018). The presence of pollutants such as metals and organochlorine pesticides in aquatic ecosystems does not in itself indicate detrimental effects on organisms (Van der Oost *et al.*, 2003). According to Sevcikova *et al.* (2011) metals are an important contaminant because they accumulate in aquatic organisms. It is thus of utmost importance to make a connection between levels of exposure and internal effects (Van der Oost *et al.*, 2003). Van der Oost *et al.* (2003) state that the detrimental effect of pollution of organisms is difficult to detect, because the effects only manifest after long periods of time. Bucheli and Fent (1995) state that these early warning indicators were established and called biomarkers.

The term biomarker is derived from "biological markers" (Strimbu & Tavel, 2010). Biomarkers have many definitions; the definition used in this chapter is the definition of Shugart *et al.* (1992) who state that a biomarker is a cellular or biochemical response to a chemical or anthropogenic stress in an organism that can be measured. Biomarkers can therefore be used to determine the biological responses of organisms to metals that are accumulated in the organisms from the environment (Wepener *et al.*, 2011, Sures *et al.*, 2015, Gerber *et al.*, 2018).

Previous studies have successfully measured the response of *Clarias gariepinus* to pollutant exposures (Farombi *et al.*, 2007, Du Preez & Wepener, 2016). Since the early 2000s numerous studies have focused on the effect of parasites on the biological response of their hosts to pollution (Morley *et al.*, 2006, Sures, 2006, 2008, Morley, 2010). For example, in a study of Sanchez *et al.* (2016) it was demonstrated that parasites cause an increase in resistance to arsenic in their hosts. Freshwater clams with digenean trematodes were less affected by pentachlorophenol and had lower mortalities than uninfected clams (Heinonen *et al.*, 2001). Chen *et al.* (2015) found that gammarids infected with acanthocephalans had higher levels of glycogen.

Biomarkers are often divided into biomarkers of exposure and biomarkers of effect (Shugart *et al.*, 1992, Van der Oost *et al.*, 2003). A biomarker of exposure is defined as the detection and quantification of an external substance, its metabolite or the product of an interaction between organism cells and a xenobiotic that can be quantified (Van der Oost *et al.*, 2003). The biomarker of exposure chosen for this study is the metal concentrations in *C. gariepinus* and the endohelminth parasites. In this study we found that *C. gariepinus* have bioaccumulated metals to much higher levels than the metals in the environment (Chapter 3). The study also found that the different endohelminth parasites bioaccumulate metals to even higher levels than their hosts (Chapter 4).

Biomarkers of effect are defined as the potential health impairment caused by toxicants that can be measured as physiological, biochemical or other changes in an organism (Van der Oost *et al.*, 2003). The biomarkers of effect chosen for this study are the effect of the endohelminth parasites infection on their hosts (infected vs. uninfected).

According to Marcogliese and Pietrock (2011), parasites are natural stressors for their hosts and have negative impacts on their hosts. Sindermann (1987) states that parasites can have lethal and sub-lethal effects on their hosts. The lethal effects of parasites only occur at enormously high infection rates. Sub-lethal effects of parasites include but are not limited to disruption of organ functions, growth impairment, skeletal deformation, deficiencies in nutrients, reproductive failure and cardiac problems (Sindermann, 1987).

Although it is a fact that parasites have negative effects on their hosts, there are some contradictory cases where parasites are reported to have positive effects on their hosts (Sures *et al.*, 2017). Sures *et al.* (2017) state that theoretically you would expect a lesser amount of effects of pollution on an organism if concentrations of pollutants in an organism are reduced by parasites. Endohelminth parasites accumulate metals to higher concentrations than their hosts (Sures *et al.*, 2017).

Previous studies found that parasites had an effect on biomarkers of effect responses such as on the synthesis of metallothioneins (MTs) in the host organism (Baudrimont *et al.*, 2006, Baudrimont & De Montaudouin, 2007). Metallothioneins (MTs), the non-enzymatic reduced glutathione (GSH) and cellular energy allocation (CEA) of the host can be impacted by parasite infections. According to Mijošek *et al.* (2019) the main role of the protein, MT, is to regulate essential metals and detoxify non-essential metals. Metallothioneins bind to multiple metals and thus, in a polluted system (with a mixture of metals), it is impossible to connect an increase in MTs to a specific element (Mijošek *et al.*, 2019). Although you cannot connect MTs to a specific element, the MTs will still be induced by an increase in metals in the environment; thus MTs can be used as an early indicator of metal pollution.

The function of GSH is to synthesise proteins and DNA, help with amino acid transport as well as defend against oxidising and potentially harmful metals (Atli & Canli, 2007). Reduced glutathione's key role is to assist in the detoxification of xenobiotics in an organism and it can thus be an effective biomarker (Van der Oost *et al.*, 2003). Many previous studies have used GSH to determine the oxidative stress of fish to metal concentrations (Farombi *et al.*, 2007, Doherty *et al.*, 2010, Otitoloju & Olagoke, 2011, Saliu & Bawa-Allah, 2012). These studies found that the GSH levels in organisms increase with an increase in metal accumulation.

The CEA is an indicator of the energy utilisation of cells in stress-induced conditions (Smolders *et al.*, 2004, Gerber *et al.*, 2018). Previous studies found that the CEA of an organism decreases significantly under high environmental stress (De Coen & Janssen, 2003, Moolman *et al.*, 2007, Wang *et al.*, 2012), and also found that an increase in metal pollution causes a decrease in cellular energy budget of fish (Smolders *et al.*, 2003, Smolders *et al.*, 2004, Moolman *et al.*, 2007, Gerber *et al.*, 2018, Louis *et al.*, 2019)

The aim of this chapter is to determine the biological responses (CEA, MTs and GSH) to metal concentrations in both *C. gariepinus* and their associated endohelminth parasites, and to evaluate the possible positive effect of an increase in endohelminth parasite numbers on the biological responses to metal accumulation of their hosts. It was hypothesised that an increase in endohelminth parasite numbers would alleviate the body burdens of metals in the hosts thereby resulting in less stress experienced by the hosts to detoxify and remove metals from their organs.

5.2. Materials and methods

5.2.1. Sampling

Approximately 1 g of the muscle and liver tissues from the catfish used in chapter 3 was dissected and placed in polypropylene tubes and frozen, the frozen samples were transported back to the laboratory and stored at -80˚C in a freezer. The endohelminth parasite infection rates in this study were not high enough to have enough sample mass to determine both the metal accumulation (Chapter 4) as well as the biological responses. Only the *Contracaecum* sp. at Nyamithi Pan had enough sampled mass per individual host to determine the three biological responses of the study and Shokwe Pan had enough *Contracaecum* sp. to determine their CEA.

5.2.2. Biomarker of exposure

The metal concentrations in *C. gariepinus* was determined in Chapter 3 sections 3.2.2 and 3.2.3 and the metal concentrations in the endohelminth parasites were determined in Chapter 4 sections 4.2.1 to 4.2.3 by using standard ICP-MS analysis.

5.2.3. Tissue preparation for biomarkers of effect

Samples were taken out of the -80˚C freezer and allowed to defrost on ice; approximately 0.2 g of either catfish liver or whole *Contracaecum* sp. samples were placed in Eppendorf tubes labelled A and B, for GSH and MT analysis, respectively; a further 0.2 g of the *Contracaecum* sp. and catfish muscle tissue were placed in Eppendorf tubes labelled C and D, respectively. All samples were homogenised on ice using the Velp Scientifica™ OV5 homogeniser and centrifuged using the Lasec HERMLE Z32 HK centrifuge.

Eppendorf A was homogenised in 2000 µL of General Homogenising Buffer (GHB) (made up of 0.1 M potassium phosphate buffer (pH 7.4), KCl, 0.001 M ethylene diamine tetraacetate (EDTA), 0.0001 M phenylmethanesulfonyl fluoride solution and 20% glycerol) and centrifuged at 5590 G for 10 minutes at 4˚C and 50 µL of the supernatant taken for GSH analysis.

Eppendorf B was homogenised in 1000 µL Homogenising Buffer (made up of Tris buffer with 0.006 mM leupeptine, 0.0005 M phenylmethylsulphynolflouride and 0.01% b-mercaptoethanol), and then centrifuged at 30 000 G and the supernatant used for MT analysis.

Eppendorf C was homogenised in 1000 µL deionised water and Eppendorf D in 1000 µL ETS Homogenising Buffer (made up of 0.1 M Tris-HCl (pH 8.5), 0.2% (v/v) Triton X-100, 15% (w/v) polyvinylpyrrolidone and 153 µM magnesium sulfate ($MgSO_4$); both were centrifuged at 2938 G for 10 minutes and the supernatant used for the different analyses. Supernatant for Eppendorf C was divided into protein, lipid and glycogen analysis, and the supernatant of Eppendorf D was used for the determination of the electron transport activity.

5.2.4. Biomarker of effect

To determine GSH, the method of Cohn and Lyle (1966) was followed, where 75 µL phosphoric acid is added to 500 µL of sample in Eppendorf A and left on ice for 10 minutes to let proteins precipitate. After incubation the samples were centrifuged at 500 G at 4˚C for 10 minutes. One hundred µL of the supernatant was added to 1 mL of deionised water in a new Eppendorf tube and mixed. Six µL of each sample was loaded onto a 96 well polyethylene microtiter plate in triplicate and 232 µL sodium phosphate buffer and 12 µL o-phthalaldehyde was added and left to incubate in the dark for 15 minutes at room temperature. The fluorescence was measured at 420 nm resulting from excitation at 350 nm on an analytical and diagnostic microplate reader. To determine the protein concentrations of the supernatant the method described by Bradford (1976) was used in the calculations.

The methodology of Viarengo *et al.* (1997) and Viarengo *et al.* (1999) was adapted using the modifications by Atli and Canli (2008) and Fernandes *et al.* (2008) and was used to assess the metallothionein (MT) content. Five hundred µL of supernatant was taken from Eppendorf B and placed into a new sterile Eppendorf tube (2.5 mL). Five hundred µL of cold (4˚C) absolute ethanol and 40 µL chloroform were added, vortexed and centrifuged at 2750 G at 4˚C for 10 minutes. Afterwards, three volumes of cold absolute ethanol were added, vortexed and then stored at -20˚C until a pellet formed. After pellet formation, the supernatant was decanted and the pellet washed twice with 1 mL of washing buffer (87% ethanol, 1% chloroform, 12% homogenising buffer) and then vortexed and centrifuged at 500 G at 4˚C for 20 minutes. The washed pellet was dried using compressed air, and 300 µL of Tris-ethylene diamine tetraacetate (EDTA) were added and vortexed. Fifteen µL of each of the samples were added to 96 well polyethylene microtiter plates in triplicate along with 210 µL Ellman's reagent. The microplate was incubated at room temperature for 15 minutes. The absorbance was measured using a Berthold Tristar LB 941 Germany analytical and diagnostic microplate reader at 412 nm. Reduced glutathione (GSH) stock solution was used to prepare a calibration curve. The standard curve was used to calculate MT concentration in $nM.mg^{-1}$ protein. To determine the protein concentrations of the supernatant the method described by Bradford (1976) was used in the calculations.

5.2.5. Cellular energy allocation in muscle tissue

Eppendorf tubes labelled C and D were used to determine the CEA. The method used to calculate the CEA was adapted from De Coen and Janssen (1997) and De Coen and Janssen (2003).

5.2.5.1. Energy available (Ea)

To determine the energy allocation available for organisms, one must determine the glycogen, lipids and proteins present in the muscle tissue (Eppendorf C).

The glycogen was determined with a phenol-sulfuric microassay adapted from Rasouli *et al.* (2014). Samples were prepared according to the method of Rasouli *et al.* (2015). Fifty milligrams of muscle sample were placed in 2 mL Eppendorf tubes and 200 µL of 30% KOH added. The Eppendorf tubes were then incubated in a water bath for 10 minutes at 95˚C. Samples were left until room temperature was achieved and 275 µL ethanol was added. The samples were vortexed and centrifuged for 10 minutes at 1700 G at room temperature. The supernatant was discarded, and the pellet re-suspended in 2 mL deionised water. Fifty µL sample was added to a 2 mL Eppendorf tube and 50 µL deionised water, 100 µL 6.5% phenol and 500 µL H_2SO_4 added. The samples were vortexed and left for 30 minutes. The samples were then loaded in triplicate onto 96 well polyethylene microtiter plates and the absorbance measured at 492 nm, with glycogen used as standard.

The total protein content was determined according to the method of Bradford (1976), where 5 µL of the homogenate was added to 245 µL Bradford reagent. It was left for 5 minutes and then the absorbance measured at 595 nm using the microplate reader Tristar LB 941 from Berthold technologies.

The total lipids were extracted using the method of Bligh and Dyer (1959). Two hundred and fifty µL of homogenate was added to 500 µL of chloroform and vortexed. After the first vortex, 500 µL methanol and 250 µL deionised water was added and vortexed again. The samples were then centrifuged for 5 minutes at 3000 G (4˚C) using the Lasec HERMLE Z32 HK centrifuge. After centrifugation 100 µL organic phase (bottom layer) was placed into glass tubes – one tube for every sample and one extra blank tube that contained 100 µL chloroform. Five hundred µL H_2SO_4 was added to all the glass tubes and covered with foil. The tubes were then incubated at 200˚C for 15 minutes in a MINILAB oven. After incubation, 1 mL deionised water was added and allowed to cool. When the samples reached room temperature, they were placed in triplicate onto 96 well polyethylene microtiter plates. The absorbance was measured by using an analytical and diagnostic microplate reader at 360 nm.

5.2.5.2. Energy consumption (Ec)

The samples in Eppendorf D were used to determine the cellular respiration rate, also known as energy consumption, by measuring the ETS activity. Twenty-five µL of the ETS homogenising buffer was placed in triplicate at the beginning of the microplate as blanks. After centrifugations, 25 µL of the supernatant were taken and placed in triplicate onto a 96 well polyethylene microtiter plate and 75 µL BSS (0.3% [v/v] Triton X-100) was added. Twenty-five µL of the NAD(P)H solution was then added to the mixture, and the reaction started by adding 50 µL INT (p-iodonitrotetrazolium violet/chloride). The mixture was then placed in the microplate reader and the absorbance measured at 490 nm (20˚C) at one-minute intervals for a five-minute period. The data were then used to construct a curve for each sample.

5.2.5.3. CEA calculations

The different Ea fractions for the individual organisms were transformed into energetic equivalents by using the enthalpy of combustion (De Coen & Janssen, 1997). These values were as follows: glycogen (17500 $mJ.mg^{-1}$), proteins (24000 $mJ.mg^{-1}$) and lipids (500 $mJ.mg^{-1}$). The Ec was determined by using the ETS data which used the theoretical stoichiometric relationship. The relationship states that for each 2 µmol of formazan formed, 1 µmol of oxygen is consumed. The quantity of oxygen consumed was transformed into an energy value by using the oxyenthalpic equivalents that equals 484 kJ/mol O_2. The total net energy budget was then determined using the following equation derived from De Coen & Janssen, (1997):

$CEA = Ea – Ec$

Where: $Ea = E_{glycogen} + E_{proteins} + E_{lipids}$

$Ec = E_{ETS}$

5.2.6. Statistical analysis

All data that was recorded was then analysed to determine the effect of metals on the biological responses of *C. gariepinus* and its associated endohelminth parasites, and then to determine the interaction between an increase in parasite numbers on the biological response of and metal accumulation in their hosts. GraphPad Prism® 7 software was used to perform statistical analysis on the biomarker responses and reported as mean ± 1 SEM (standard error of the mean).

Homogeneity was tested by using the Shapiro-Wilk test and if the data was found to be distributed normally, a one-way ANOVA with Tukey post-hoc analysis was used. If the data was not distributed normally, a Kruskal-Wallis followed by the Dunn's multiple comparison test was performed. Student T-test was performed where needed to calculate significant differences. Significance was set at $p < 0.05$. Canoco version 5 (ter Braak & Smilauer, 2012) was used to form a Redundancy Analysis (RDA) of the effect of metals' concentrations on the biomarker response of *C. gariepinus* and their associated endohelminth parasites and to determine the interaction of an increase in parasites numbers on the biological response of their hosts. A Redundancy Analysis (RDA) works on a linear response model that relates two variables to the sampling sites. Placement of the variables indicates similarities between variables, with variables at ≤ 90˚ positively correlating and variables at > 90 negatively correlating.

5.3. Results

5.3.1. Biomarkers of effect

Endohelminth parasite infection had significant effect on some of the metal concentrations in both the muscle and liver tissues of the host located in the lotic system of the Usuthu River (Table 5.1). Where significant differences occurred the infected catfish always had higher metal concentrations than the uninfected catfish. There were no significant differences in the essential metal concentrations in the muscle tissue, and only *T. ciliotheca* had a significant effect on the Co concentrations in the liver tissue with the infected catfish having higher concentrations. Only *P. pseudolaeviconchus* showed significant differences in the concentration of metals in the muscle tissue of the host with Al ($p = 0.0183$) and Ag ($p = 0.033$) being significantly higher in the infected catfish (Table 5.1). In the liver tissue *P. pseudolaeviconchus* had a significant effect on the Al ($p = 0.0472$), *T. ciliotheca* on Co ($p = 0.0218$) and Se ($p = 0.0352$), *Pa. cyathopgharynx* on Mn ($p = 0.0024$) and Cd ($p = 0.012$) and *Contracaecum* sp. on Ag ($p = 0.0284$) (Table 5.1).

The endohelminth parasites had significant effect on some of the host metal concentrations in both the muscle and liver tissues at Shokwe Pan (Table 5.2). In terms of the essential metals only *T. ciliotheca* had a significant effect on the Co ($p = 0.0211$) in the muscle tissue of the host, and *Contracaecum* sp. on the Fe ($p = 0.0474$) and Zn ($p = 0.0171$). For the non-essential metals there were no significant differences in the muscle tissue of the host with only the *Contracaecum* sp. having a significant effect for Cd ($p = 0.0474$) and Pb ($p = 0.0474$) on the liver concentrations of

C. gariepinus (Table 5.2). As all *C. gariepinus* at Nyamithi Pan were infected by *Contracaecum* sp. no correlation between the metal concentrations in infected and uninfected catfish could be made (Table 5.3). In terms of the concentrations of metals in the muscle tissue of the host there were no significant differences in the concentration of essential metals between the infected and uninfected catfish for all the endohelminth parasites.

The non-essential metals in the muscle showed the same trend as was observed at Usuthu River and Shokwe Pan, with the infected *C. gariepinus* having higher concentrations of the metals where significant differences occurred. *Procamallanus pseudolaeviconchus* had a significant effect on the Co ($p = 0.0333$) concentrations in the liver tissue of *C. gariepinus*. *Clarias gariepinus* infected with *T. ciliotheca* had significantly higher concentrations of Zn ($p = 0.0369$) in their liver tissues than in the uninfected hosts. *Paracamallanus cyathopgharynx* had a significant effect on the Ni ($p = 0.0211$) concentrations in the host at Nyamithi Pan.

Table 5-1: Spearman's comparison of the concentrations of the different metals in both the muscle and liver tissues of the host *Clarias gariepinus* infected or uninfected by four different endohelminth parasites in the lotic system of the Usuthu River in Ndumo Game Reserve (bold numbers indicate significant differences with * is $p < 0.05$ and ** $p < 0.001$).

		P. pseudolaeviconchus		***T. ciliotheca***		***Pa. cyathopgharynx***		***Contracaecum sp.***	
	Muscle								
	Essential metals								
		Infected	**Uninfected**	**Infected**	**Uninfected**	**Infected**	**Uninfected**	**Infected**	**Uninfected**
Usuthu River	Fe	47.03	33.92	37.22	37.11	33.61	31.81	32.02	35.02
	Co	0.07	0.06	0.07	0.06	0.08	0.05	0.06	0.07
	Ni	0.21	0.21	0.12	0.22	0.14	0.19	0.21	0.10
	Zn	13.22	13.83	14.63	13.01	12.68	13.47	13.23	12.57
	Cu	4.81	3.84	4.21	4.02	3.14	3.38	3.88	2.94
	Non-essential metals								
	Al	**27.52***	**13.03***	14.64	18.28	15.74	11.63	15.44	11.92
	Mn	1.61	1.24	1.32	1.34	1.21	1.19	1.22	1.23
	As	0.21	0.20	0.20	0.20	0.20	0.20	0.20	0.20
	Cd	0.07	0.09	0.09	0.08	0.08	0.09	0.09	0.08
	Pb	0.84	1.03	1.01	0.94	0.84	1.01	0.92	0.91
	Se	2.11	2.12	2.09	2.11	2.08	1.98	2.01	2.10
	Ag	**0.04***	**0.03***	0.02	0.04	0.02	0.04	0.04	0.02
	Liver Tissue								
	Essential metals								
	Fe	1332.64	2156.81	2010.61	1900.22	1835.04	2390.91	1947.01	1955.05
	Co	0.82	0.92	**1.10***	**0.80***	0.91	0.83	0.89	0.91
	Ni	0.11	0.99	0.24	1.24	0.70	0.64	0.44	0.97
	Zn	74.68	69.12	72.71	68.70	74.54	55.64	66.68	74.38
	Cu	74.75	61.94	75.74	56.22	70.41	45.42	59.98	70.29
	Non-essential metals								
	Al	**42.24***	**28.81***	25.52	37.84	35.10	20.89	33.25	31.24
	Mn	6.21	4.94	5.04	5.33	**5.60***	**3.60***	4.82	5.63
	As	0.49	0.33	0.35	0.41	0.45	0.34	0.33	0.39
	Cd	0.78	0.64	0.62	0.72	**0.70***	**0.40***	0.62	0.67
	Pb	1.41	0.71	0.59	1.15	0.94	0.72	0.61	1.18
	Se	17.98	17.25	**22.30***	**13.30***	17.31	17.79	19.05	15.84
	Ag	0.23	0.04	0.05	0.09	0.09	0.02	**0.03***	**0.10***

Table 5-2: Spearman's comparison of the concentrations of the different metals in both the muscle and liver tissues of the host *Clarias gariepinus* infected or uninfected by four different endohelminth parasites in the lentic system of Shokwe Pan in Ndumo Game Reserve (bold numbers indicate significant differences with * is $p < 0.05$ and ** $p < 0.001$).

		P. pseudolaeviconchus		***T. ciliotheca***		***Pa. cyathopgharynx***		***Contracaecum sp.***	
		Muscle							
		Essential metals							
		Infected	**Uninfected**	**Infected**	**Uninfected**	**Infected**	**Uninfected**	**Infected**	**Uninfected**
Shokwe	Fe	33.98	35.03	40.84	33.81	34.03	36.70	34.72	34.91
	Co	0.06	0.05	**0.07***	**0.05***	0.06	0.05	0.06	0.06
	Ni	0.24	0.22	0.23	0.46	0.21	0.22	0.21	0.40
	Zn	17.85	16.74	21.74	17.04	17.85	15.41	17.63	17.65
	Cu	2.91	3.30	3.15	3.32	3.13	2.73	3.12	2.94
		Non-essential metals							
	Al	21.64	21.43	34.21	20.31	21.75	20.71	22.33	20.45
	Mn	1.61	1.65	1.69	1.54	1.61	1.53	1.59	1.67
	As	0.22	0.21	0.22	0.22	0.23	0.24	0.21	0.23
	Cd	0.33	0.41	0.41	0.31	0.41	0.13	0.41	0.36
	Pb	3.84	3.44	4.29	3.10	3.87	1.64	3.80	3.35
	Se	1.79	2.01	2.01	2.03	1.86	1.81	1.95	1.91
	Ag	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01
		Liver Tissue							
		Essential metals							
	Fe	1104.03	1309.01	1120.04	1257.99	1257.96	1508.04	**1409.01***	**869.12***
	Co	0.43	0.44	0.41	0.4	0.39	0.38	0.41	0.39
	Ni	0.65	0.32	0.55	0.5	0.55	2.81	0.64	0.34
	Zn	88.21	87.69	88.12	87.9	88.23	79.70	**91.91***	**83.42***
	Cu	29.51	31.87	28.39	34.2	29.51	31.94	34.81	27.52
		Non-essential metals							
	Al	23.54	16.54	19.91	19.52	20.69	14.40	17.91	25.14
	Mn	7.51	7.40	7.13	7.61	7.48	8.11	7.42	7.60
	As	0.19	0.21	0.22	0.24	0.21	0.22	0.21	0.21
	Cd	0.41	0.35	0.40	0.37	0.39	0.24	**0.41***	**0.33***
	Pb	0.92	0.84	0.61	0.91	0.92	0.54	**0.93***	**0.61***
	Se	11.24	10.99	11.92	10.14	10.61	14.22	11.45	11.22
	Ag	0.06	0.06	0.05	0.07	0.06	0.04	0.06	0.05

Table 5-3: Spearman's comparison of the concentrations of the different metals in both the muscle and liver tissues of the host *Clarias gariepinus* infected or uninfected by four different endohelminth parasites in the lentic system of Nyamithi Pan in Ndumo Game Reserve (# indicates where there were no uninfected hosts; bold numbers indicate significant differences with * is p < 0.05 and ** p < 0.001).

		P. pseudolaeviconchus		***T. ciliotheca***		***Pa. cyathopgharynx***		***Contracaecum sp.***	
	Muscle								
	Essential metals								
		Infected	**Uninfected**	**Infected**	**Uninfected**	**Infected**	**Uninfected**	**Infected**	**Uninfected**
Nyamithi	Fe	33.87	30.62	34.31	28.73	33.87	30.30	33.29	#
	Co	0.05	0.05	0.05	0.05	0.06	0.05	0.06	#
	Ni	0.11	0.19	0.24	0.23	0.21	0.20	0.74	#
	Zn	13.84	11.23	12.72	11.21	12.74	11.80	14.63	#
	Cu	2.43	2.94	2.73	2.63	2.65	2.60	2.79	#
	Non-essential metals								
	Al	14.91	13.80	14.22	14.34	**15.64***	**13.80***	16.38	#
	Mn	1.19	1.14	1.21	1.25	1.19	1.14	1.25	#
	As	0.21	0.23	0.22	0.23	0.23	0.21	0.21	#
	Cd	0.07	0.05	0.06	0.06	0.06	0.06	0.19	#
	Pb	0.74	0.65	0.74	0.61	0.71	0.63	0.78	#
	Se	1.91	2.23	**2.20***	**1.70***	2.03	2.14	2.14	#
	Ag	0.01	0.00	0.01	0.00	0.01	0.00	0.02	#
	Liver Tissue								
	Essential metals								
	Fe	1479.04	1778.98	1449.03	2104.00	1961.00	1476.03	1637.01	#
	Co	**0.41***	**0.64***	0.54	0.60	0.60	0.59	0.55	#
	Ni	0.23	0.22	0.23	0.20	**0.10***	**0.30***	0.49	#
	Zn	58.99	67.14	**61.04***	**80.00***	61.50	62.84	61.54	#
	Cu	7.48	8.21	8.09	8.6	8.20	8.21	8.53	#
	Non-essential metals								
	Al	18.62	18.11	18.39	18.33	17.21	18.83	19.14	#
	Mn	4.83	8.64	5.64	6.87	6.63	5.64	6.15	#
	As	0.21	0.15	0.13	0.19	0.18	0.14	0.21	#
	Cd	0.20	0.22	0.14	0.23	0.13	0.21	0.29	#
	Pb	0.85	1.43	0.81	2.01	0.64	1.49	1.20	#
	Se	20.79	19.81	20.14	21.34	19.74	21.21	21.95	#
	Ag	0.01	0.01	0.01	0.01	0.01	0.01	0.021	#

5.3.2. Correlations between the number of parasites and metal concentration in host muscle tissue

The correlation between the number of parasites and the concentrations of the essential and non-essential metals in both the muscle and liver tissues of *Clarias gariepinus* is shown in Table 5.4. Iron and cobalt had positive correlations for *P. pseudolaeviconchus*, *T. ciliotheca* and *Pa. cyathopgharynx* but a negative correlation for *Contracaecum* sp. *Paracamallanus cyathopgharynx* had a negative correlation for Ni with the other three parasites having positive correlations. There was a significant positive correlation for the number of *T. ciliotheca* and the concentration of Zn in the host and the rest of the parasites had almost no correlations. *Paracamallanus cyathopgharynx* and *T. ciliotheca* had a positive correlation with Cu where *P. pseudolaeviconchus* and *Contracaecum* sp. had negative correlations.

The correlation between the parasite infection rate and the concentration of non-essential metals is indicated in Table 5.4. Al and Mn were positive in *P. pseudolaeviconchus*, *T. ciliotheca* and *Pa. cyathopgharynx* with *P. pseudolaeviconchus* being significant for Al and *Pa. cyathopgharynx* being significant for Mn. Both the correlations for Al and Mn were negative in *Contracaecum* sp. Arsenic had a significant positive correlation with *Contracaecum* sp. *Procamallanus pseudolaeviconchus* had no significant correlation for the different non-essential metals, but positive correlations for Cd, Pb and Ag and a negative correlation for Se.

Tetracampos ciliotheca had no significant correlations but had positive correlations for Cd, Pb and Se and a negative correlation for Ag. *Paracamallanus cyathopgharynx* had a significant positive correlation for Pb and positive correlations for Cd and Ag and a negative correlation for Se. *Contracaecum* sp. had significantly negative correlations for both Cd and Pb as well as a slightly negative correlation for Ag and a slightly positive correlation for Se.

5.3.3. Correlations between the number of parasites and metal concentrations in host liver tissue

The Spearman's correlation between the number of parasites and the concentrations of the essential and non-essential metals in both the muscle and liver tissues of *Clarias gariepinus* from all the sites together are shown in Table 5.4. *Procamallanus pseudolaeviconchus* had negative correlations for all the essential metals compared to the concentrations of the metals in the host liver tissue, the only significant correlation was for Co. *Tetracampos ciliotheca* had negative correlations for all the essential metals but only the correlation of Cu was significant. *Paracamallanus cyathopgharynx* had significant positive correlations for both Zn and Cu, and a slightly positive correlation for Co and negative correlations for Fe and Ni. *Contracaecum* sp. had significantly negative correlations for Co, Zn and Cu, a slightly negative correlation for Ni and slightly positive correlation for Fe.

Procamallanus pseudolaeviconchus had no significant correlations with the non-essential metals in Table 5.4, but had negative correlations with Mn and As and a positive correlation with Al. *Tetracampos ciliotheca* had negative correlations for Al, Mn and As with, As being significant. *Paracamallanus cyathopgharynx* had positive correlations for Al, Mn and As with As being significant. *Contracaecum* sp. had significantly negative correlations for both Al and As and a slightly positive correlation for Mn. *Procamallanus pseudolaeviconchus* had no significant correlations, with Cd and Ag having slightly negative correlations and Pb and Se having slightly positive correlations. *Tetracampos ciliotheca* had significantly positive correlations for Se and a significantly negative correlation for Cd, and slightly negative correlations for both Pb and Ag. *Paracamallanus cyathopgharynx* had significantly positive correlations for Cd, and negative correlations for Se and Ag, and slightly negative correlations for Pb. *Contracaecum* sp. also had significant correlations for Cd and Ag (both negative) and Se (positive) with Pb having a slightly negative correlation.

Table 5-4: Spearman's correlations between the number of parasites (*Procamallanus pseudolaeviconchus*, *Tetracampos ciliotheca*, *Paracamallanus cyathopgharynx* and *Contracaecum* sp.) and the metal concentrations in *Clarias gariepinus* muscle and liver tissues in three different aquatic ecosystems in Ndumo Game Reserve (bold numbers indicate significant differences with * is < 0.05 and ** < 0.001).

Metal	***P. pseudolaeviconchus***		***T. ciliotheca***		***Pa. cyathopgharynx***		***Contracaecum* sp.**	
	Muscle	**Liver**	**Muscle**	**Liver**	**Muscle**	**Liver**	**Muscle**	**Liver**
Essential metals								
Fe	0.076	-0.078	0.228	-0.066	0.069	-0.164	0.117	0.164
Co	0.09	**-0.301****	0.208	-0.066	0.1936	0.193	-0.092	**-0.274***
Ni	0.033	-0.008	0.047	-0.068	0.035	-0.106	0.193	-0.22
Zn	0.128	-0.037	**0.244***	-0.168	0.065	0.281	-0.118	**-0.248***
Cu	-0.156	-0.202	0.046	**-0.212***	0.165	**0.414****	-0.176	**-0.736****
Non-essential								
Al	**0.303****	0.145	0.121	-0.132	0.202	0.196	-0.058	**-0.270***
Mn	0.172	-0.056	0.13	-0.143	**0.237***	0.057	-0.222	0.019
As	-0.006	-0.166	0.227	**-0.276***	-0.271	**0.441****	**0.299****	**-0.695****
Cd	0.031	-0.099	0.113	**-0.310****	0.217	**0.380****	**-0.321****	**-0.484****
Pb	0.06	0.062	0.127	-0.194	0.241*	-0.191	**-0.326****	-0.028
Se	-0.1767	0.090	0.156	**0.244***	-0.035	**-0.297***	-0.024	**0.465****
Ag	0.044	-0.017	-0.157	-0.085	0.164	**0.311****	-0.192	**-0.567****

5.3.4. Effect of endohelminth infection rate on the long-term metal exposure

In order to determine the effect of different parasite numbers on the long-term metal concentration in the muscle tissue of *C. gariepinus* across the three sites in NGR, an RDA triplot was constructed (Fig. 5.1). Axes 1 and 2 combine to explain 9.34% of the variance in the data, where catfish from each of the three sites are plotted according to the concentration of metals in the muscle tissue of the host. The first axis explains 7.36% of the total variance, where catfish from Nyamithi Pan separate from both the Shokwe Pan and Usuthu River sites. The separation is largely due to lower concentrations of metals associated with an increase in *Contracaecum* sp. numbers in the host catfish from Nyamithi Pan. From the figure it can also be seen that the catfish from the Usuthu River and Shokwe Pan had higher numbers of the other parasites and that this did not correlate with a decrease in long-term bioaccumulation.

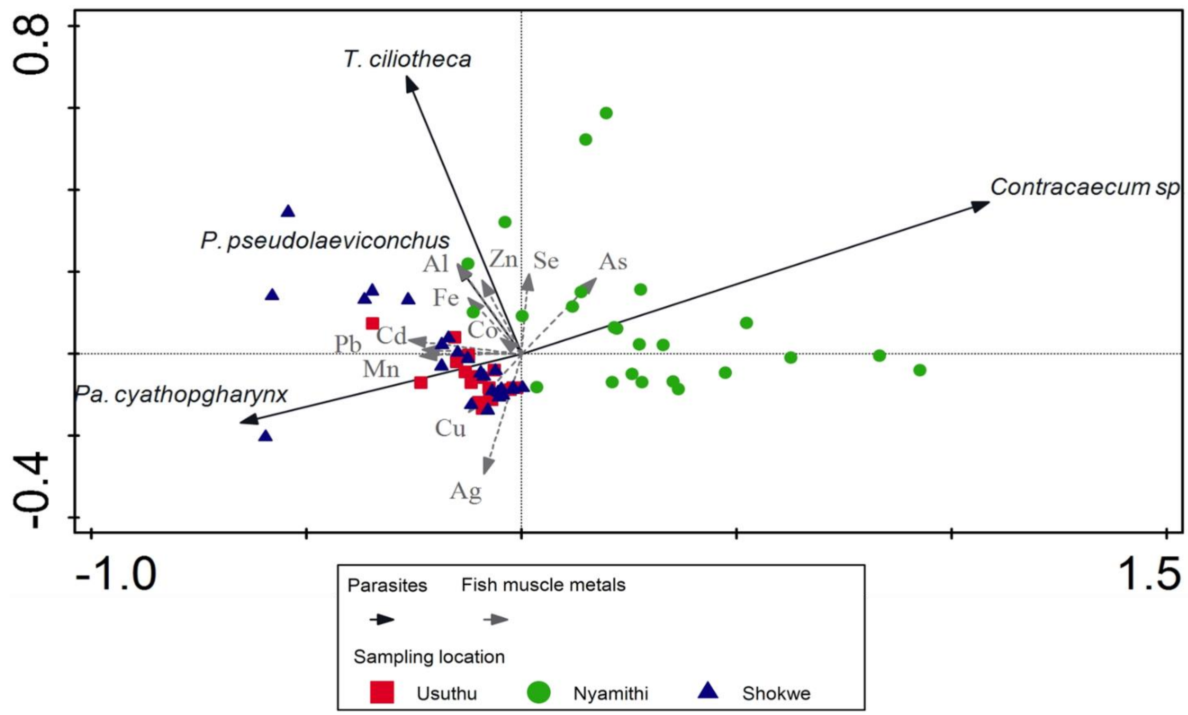

Figure 5-1: Constrained Redundancy Analysis triplot of the concentration of metals in the muscle tissue of *Clarias gariepinus* across three different sites in Ndumo Game Reserve (Squares – Usuthu River, Circles – Nyamithi Pan, Triangles – Shokwe Pan; with the parasite numbers of the four different endohelminth parasites overlaid; the first [horizontal] axis explains 7.36% and the second [vertical] axis a further 1.99%, for a cumulative 9.34%).

5.3.5. Effect of endohelminthic infection rate on the long-term metal exposure

In order to determine the effect, the number of parasites have on the short-term accumulation of metals using the liver concentrations of *C. gariepinus* as proxy a Constrained Redundancy Analysis triplot was prepared (Fig. 5.2). Axes 1 and 2 combine to explain 14.03% of the variance in the data where catfish from the three sites are plotted according to their metal concentrations in the liver tissue of the host. The first axis explains 11.76% of the total variance where catfish from Nyamithi are separated from the other two sites according to their metal concentrations. The separation is largely due to a decrease in metal concentrations in the catfish of Nyamithi Pan with an increase in three of the four endohelminth parasites. The second axis explains 2.27% of the variance in the data. It can also be seen that the catfish of Usuthu River and Shokwe Pan had higher numbers of *Pa. cyathopgharynx* parasites than the catfish of Nyamithi Pan.

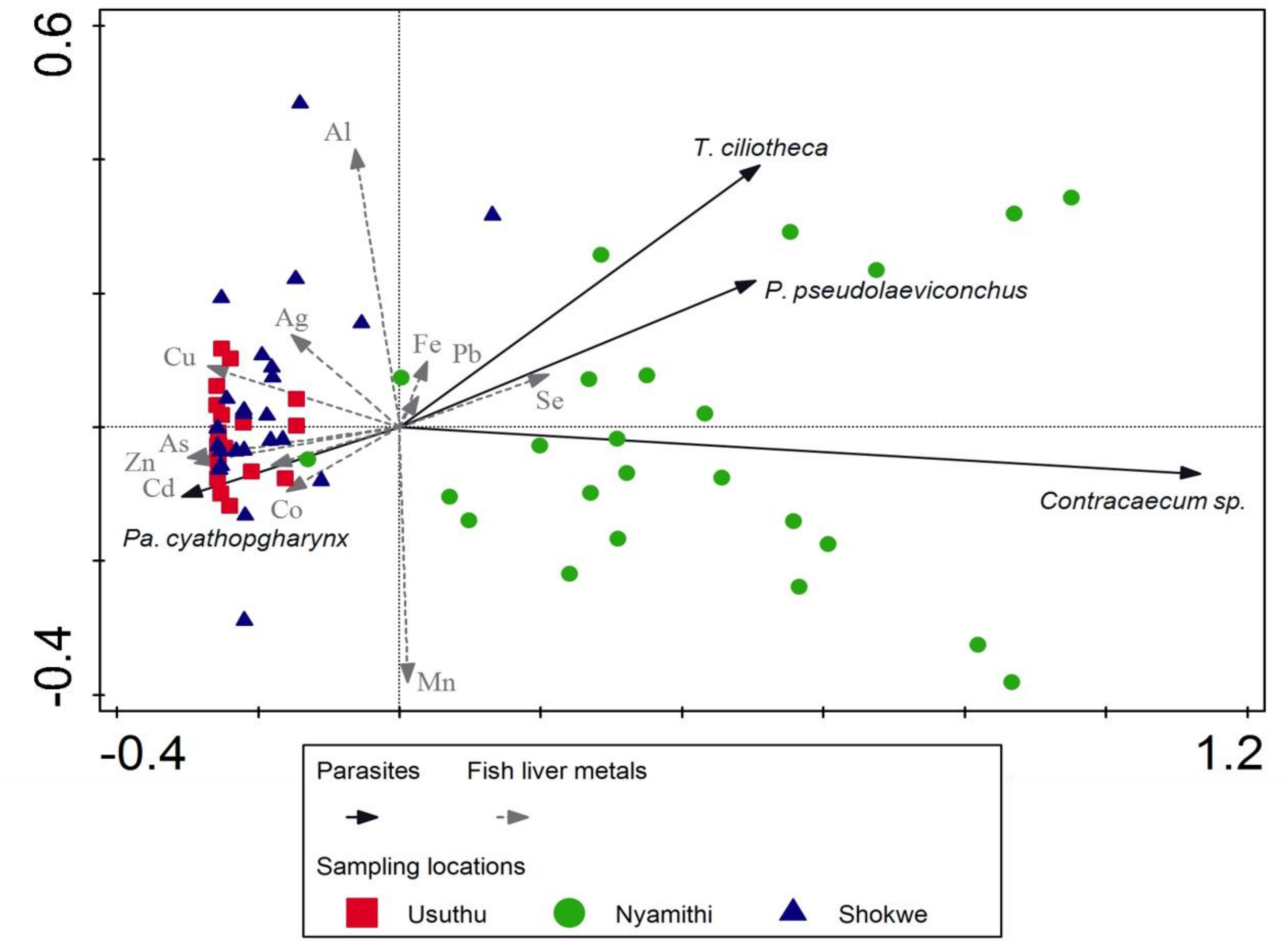

Figure 5-2: Constrained Redundancy Analysis triplot of the concentration of metals in the liver tissue of *Clarias gariepinus* across three different sites in Ndumo Game Reserve (Squares – Usuthu River, Circles – Nyamithi Pan, Triangles – Shokwe Pan; with the parasite numbers of the four different endohelminth parasites overlaid; the first [horizontal] axis explains 11.76% and the second [vertical] axis a further 2.27%, for a cumulative 14.03%).

5.3.6. Biomarker of effect

The biomarkers of effect measured in *C. gariepinus* and their *Contracaecum* sp. from NGR are presented in Fig. 5.3A & B. Metallothioneins were significantly higher in the Usuthu River (lotic system) than in the two lentic systems. Although the MTs was lower in the *Contracaecum* sp. than in their hosts it was not significantly lower. The reduced glutathione showed no significant differences between the three sites. *Contracaecum* sp. had significantly higher GSH than their associated *C. gariepinus* from Nyamithi Pan with a p-value of < 0.0001.

The CEA and its components of *C. gariepinus* from NGR are shown in Figure 5.4A - F. When considering the Ea, *Contracaecum* sp. had significantly higher levels of glycogen and lipids than all the hosts. The catfish from Shokwe Pan had significantly higher proteins than the catfish of Nyamithi Pan and their *Contracaecum* sp. with p-values of 0.0387 and < 0.0001 respectively. Although *Contracaecum* sp. had significantly higher levels of glycogen and lipids there was no significant difference in the E_a across the sites and species. The energy consumption (Ec) showed a significant difference (p < 0.0001) between the *Contracaecum* sp. and its host *C. gariepinus* but no differences between the sites.

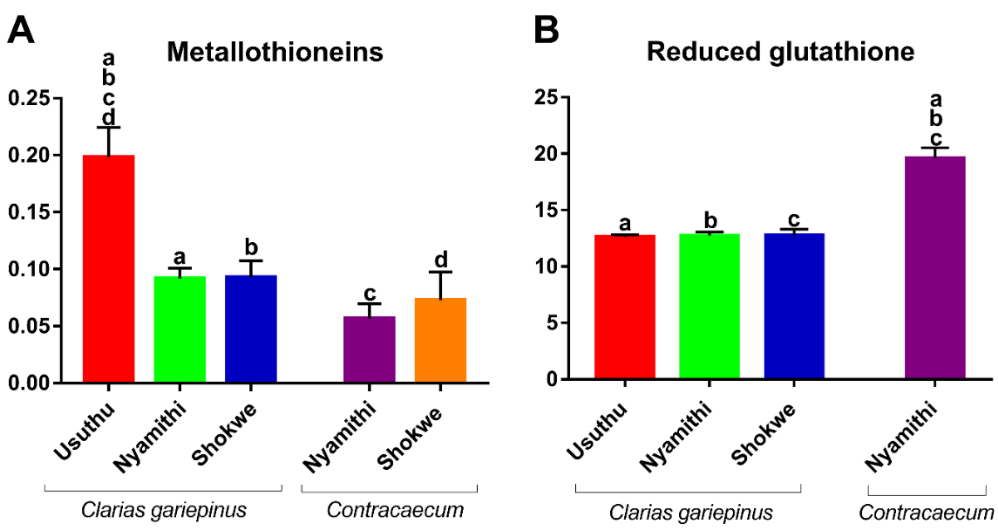

Figure 5-3: Concentrations of metallothioneins (A) (nmol MT/mg) and reduced glutathione (B) (µg/g) in the host *Clarias gariepinus* and the *Contracaecum* sp. across the three sites in Ndumo Game Reserve ([mean ± SEM]; bars with common superscripts are significantly different [p < 0.05]).

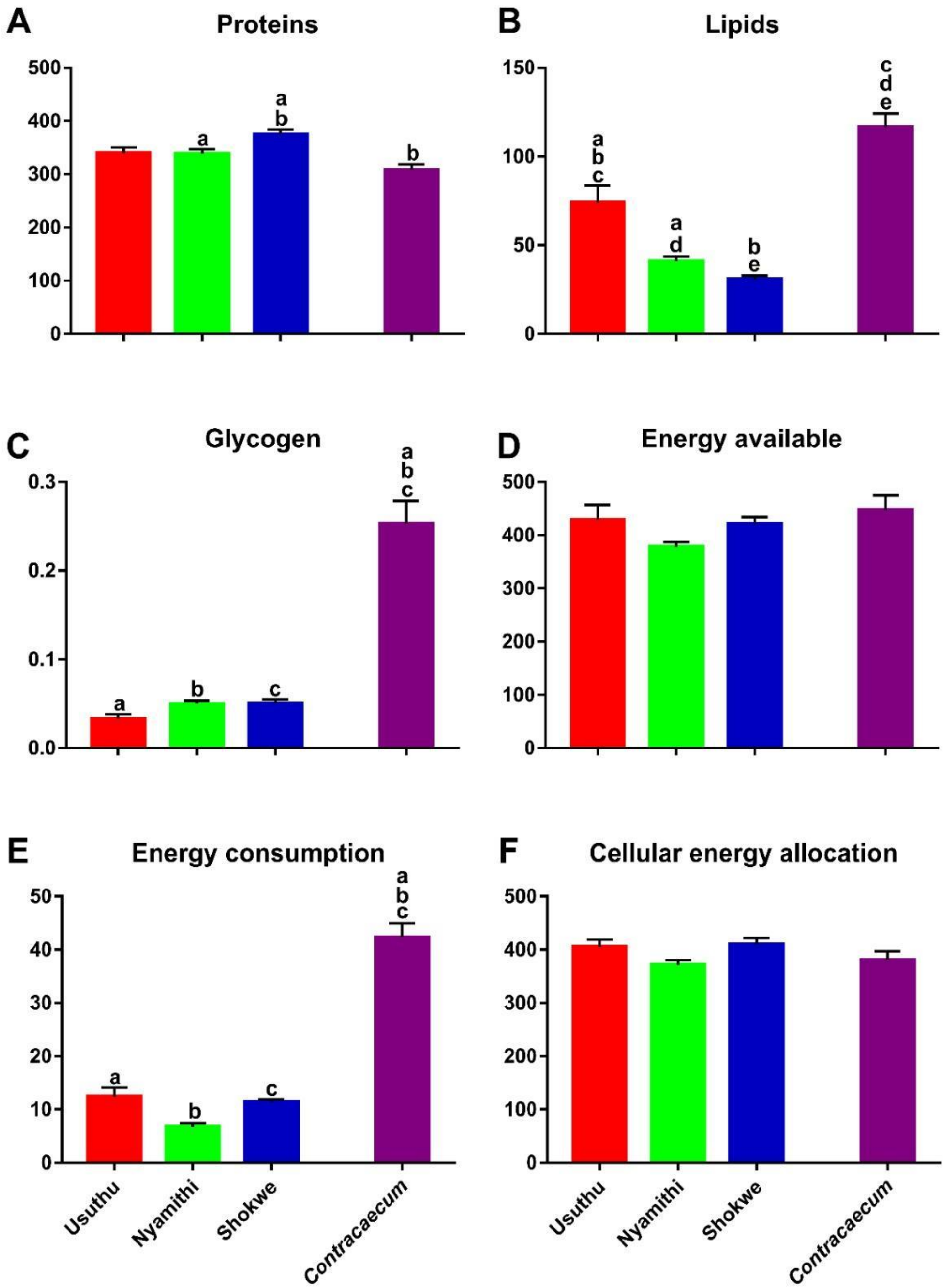

Figure 5-4: Concentrations of all components of cellular energy allocations (CEA) of *Clarias gariepinus* at Ndumo Game Reserve – available energy (D) consists of proteins (A), lipids (B) and glycogen (C) and energy consumption (E) and the final cellular energy allocation (F) (bars with common superscripts are significantly different [$p < 0.05$]).

5.3.7. Biological response of *Clarias gariepinus* in terms of endohelminth parasite infection

There was no significance present in the biological response in the host *Clarias gariepinus* between the catfish infected with endohelminth parasites and uninfected catfish when all the sites' data are combined (Table 5.5). The CEA showed a trend of being higher in the uninfected catfish; the uninfected catfish had slightly higher available energy and higher energy consumption. The metallothioneins' activity in the uninfected hosts was also slightly higher than in the infected hosts with the GSH being slightly higher in the infected catfish.

The *C. gariepinus* infected with *P. pseudolaeviconchus* at Usuthu River had a significantly higher concentration of glycogen ($p = 0.0344$) and those infected with *Contracaecum* sp. had significantly higher lipids ($p < 0.0001$) than the uninfected catfish. The *C. gariepinus* infected with *P. pseudolaeviconchus* at Shokwe Pan had significantly higher MT concentrations ($p = 0.0315$) than the uninfected hosts. There were no significant differences in the biological responses of the infected and the uninfected *C. gariepinus* at Nyamithi Pan.

Table 5-5: Spearman's comparison of different biological responses taking place in the host *Clarias gariepinus*, infected or uninfected, by four different endohelminth parasites in Ndumo Game Reserve (# indicates where there were no uninfected hosts; bold numbers indicate significant differences with * is p < 0.05 and ** p < 0.001).

		P. pseudolaeviconchus		***T. ciliotheca***		***Pa. cyathopgharynx***		***Contracaecum sp.***	
		Infected	**Uninfected**	**Infected**	**Uninfected**	**Infected**	**Uninfected**	**Infected**	**Uninfected**
All sites	Cellular energy allocation (J / g)	384.44	411.79	389.50	410.48	395.74	406.73	398.05	401.18
	Energy consumption (J / g)	9.97	10.30	10.24	10.02	10.75	8.63	9.51	11.99
	Available energy (J / g)	394.41	412.11	399.74	420.50	406.48	415.36	407.56	413.17
	Proteins (J / g)	347.25	353.40	345.20	357.07	355.14	338.78	348.64	355.88
	Lipids (J / g)	48.28	68.64	54.49	65.14	52.14	76.54	58.88	60.03
	Glycogen (J / g)	0.05	0.04	0.05	0.04	0.05	0.04	0.05	0.04
	Metallothionein (nM / mg protein)	0.13	0.15	0.13	0.16	0.14	0.15	0.13	0.18
	Reduced glutathione (nM / mg protein)	12.70	12.69	12.82	12.53	12.63	12.85	12.88	12.14
Usuthu River	Cellular energy allocation (J / g)	435.29	358.88	392.07	436.60	387.05	526.93	447.21	385.17
	Energy consumption (J / g)	11.81	14.23	13.57	11.44	12.79	10.98	13.32	11.51
	Available energy (J / g)	447.11	373.10	405.64	448.04	399.84	537.91	460.53	396.68
	Proteins (J / g)	341.43	299.99	322.68	338.18	330.00	335.14	317.75	344.39
	Lipids (J / g)	105.63	73.09	82.93	109.83	69.80	202.75	**142.75****	**52.25****
	Glycogen (J / g)	**0.04***	**0.02***	0.03	0.03	0.04	0.03	0.03	0.04
	Metallothionein (nM / mg protein)	0.23	0.22	0.21	0.25	0.21	0.30	0.24	0.22
	Reduced glutathione (nM / mg protein)	12.54	12.86	12.58	12.63	12.64	12.48	12.94	12.25
Nyamithi Pan	Cellular energy allocation (J / g)	372.02	370.31	370.02	373.49	385.53	360.06	371.27	#
	Energy consumption (J / g)	7.16	6.07	7.00	6.11	5.53	7.59	6.68	#
	Available energy (J / g)	379.18	376.37	377.02	379.60	391.05	367.65	377.95	#
	Proteins (J / g)	341.47	334.88	334.31	346.14	349.32	330.12	338.57	#
	Lipids (J / g)	37.67	41.44	42.66	33.41	41.68	37.48	39.33	#
	Glycogen (J / g)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	#
	Metallothionein (nM / mg protein)	0.10	0.08	0.10	0.08	0.08	0.10	0.09	#
	Reduced glutathione (nM / mg protein)	12.65	12.79	12.62	12.89	12.50	12.88	12.71	#
Shokwe Pan	Cellular energy allocation (J / g)	406.25	415.12	409.74	409.87	407.79	432.86	402.48	428.63
	Energy consumption (J / g)	10.89	12.23	11.33	11.55	11.55	10.03	10.89	12.81
	Available energy (J / g)	417.14	427.36	421.07	421.42	419.34	442.90	413.36	441.44
	Proteins (J / g)	371.56	395.33	375.35	388.34	378.68	408.46	383.21	375.56
	Lipids (J / g)	48.26	31.98	45.67	35.60	42.12	34.41	30.11	75.58
	Glycogen (J / g)	0.05	0.05	0.05	0.05	0.05	0.03	0.05	0.05
	Metallothionein (nM / mg protein)	**0.13***	**0.07***	0.10	0.11	0.11	0.04	0.11	0.10
	Reduced glutathione (nM / mg protein)	12.70	12.85	13.23	12.09	12.68	13.51	13.08	11.96

5.3.8. Biomarker response of *C. gariepinus* in relation to accumulated metal concentrations

In order to determine the effect that metals have over the long term in *C. gariepinus* across the three sites in NGR, an RDA was done and the triplot is shown in Fig. 5.5. Axes 1 and 2 combine to explain 17.89% of the variance in the data, where catfish from each of the three sites are plotted according to the concentrations of metals in their muscle tissue. Axis 1 explains 11.28% of the total variance, where catfish from Nyamithi Pan separate to the left, according to their energy reserves with the catfish in Nyamithi Pan having less energy than those at the other two systems. Axis 2 explains 6.61% of the variance in the total data with Usuthu River separating down with an increase in Cu, Ag and As concentrations and higher MTs, and Shokwe Pan separating to the top with an increase in most of the metals as well as an increase in CEA and GSH.

In order to determine the effect that metals have over a short term in *C. gariepinus* across three sites in NGR, an RDA triplot was done and the triplot is shown in Fig. 5.6. Axes 1 and 2 combine to explain 14.12% of the total variance in the data, where catfish from each of the three sites are plotted according to the concentration of metals in the liver tissue of the host. The first axis explains 8.43% of the total variance, where catfish from the lotic system (Usuthu River) separates from both the lentic systems (Nyamithi and Shokwe pans). The separation is largely due to an increase in Cu, Co and Cd metal concentrations in the liver associated with an increase in MTs. The second axis explains 5.69% of the total variance in the data. The separation on the second axis is due to an increase in most of the metal concentrations which is associated with a decrease in CEA and GSH, but an increase in MTs and increased energy consumption.

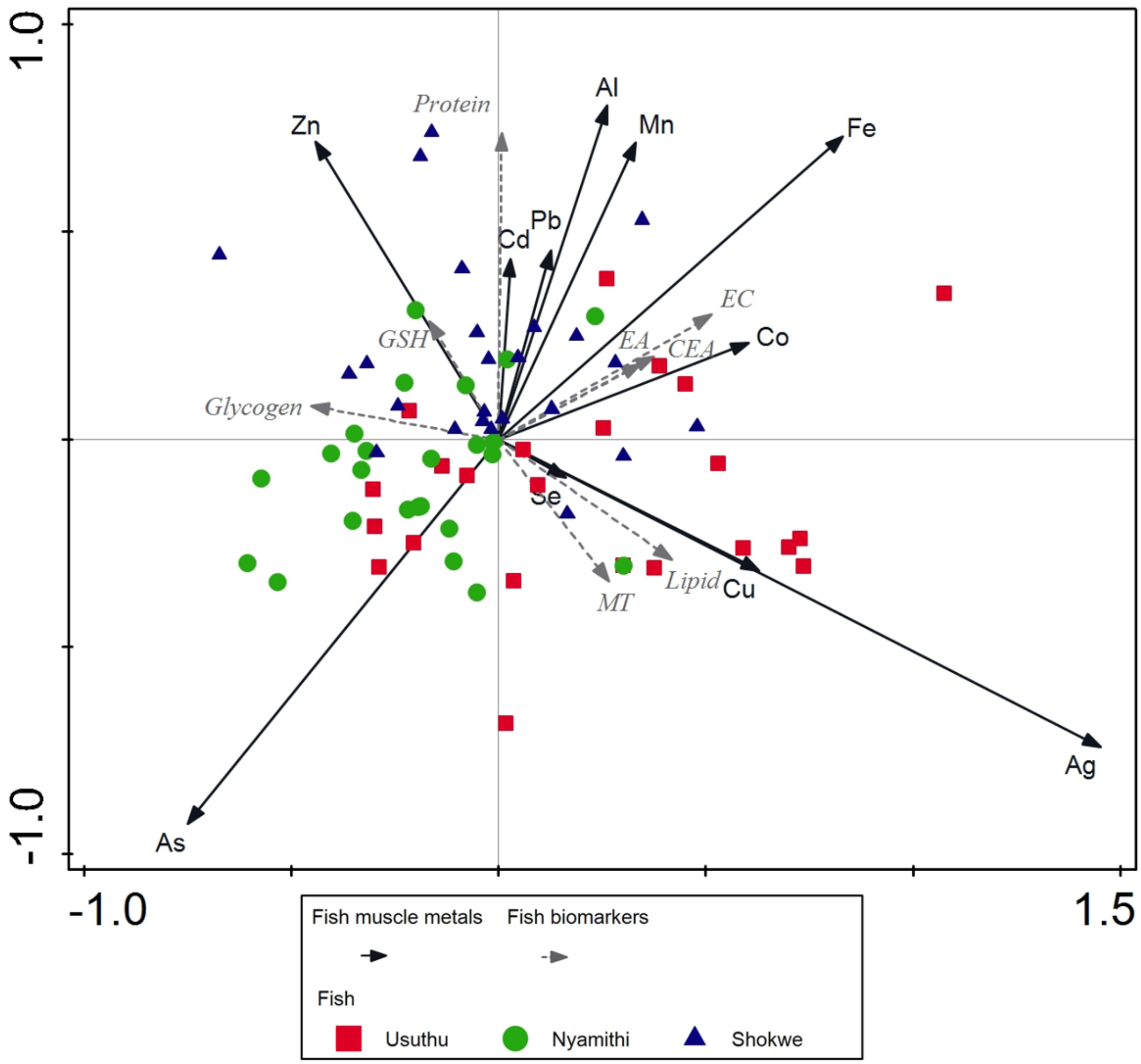

Figure 5-5: Constrained Redundancy Analysis triplot of the concentration of metals in the muscle tissue of *Clarias gariepinus* across three different sites in Ndumo Game Reserve (Squares – Usuthu River, Circles – Nyamithi Pan, Triangles – Shokwe Pan; with their corresponding biomarker responses: cellular energy allocation [CEA], metallothioneins [MT] and reduced glutathione [GSH] overlaid; the first [horizontal] axis explains 11.28% and the second [vertical] axis a further 6.61%, for a cumulative 17.89%).

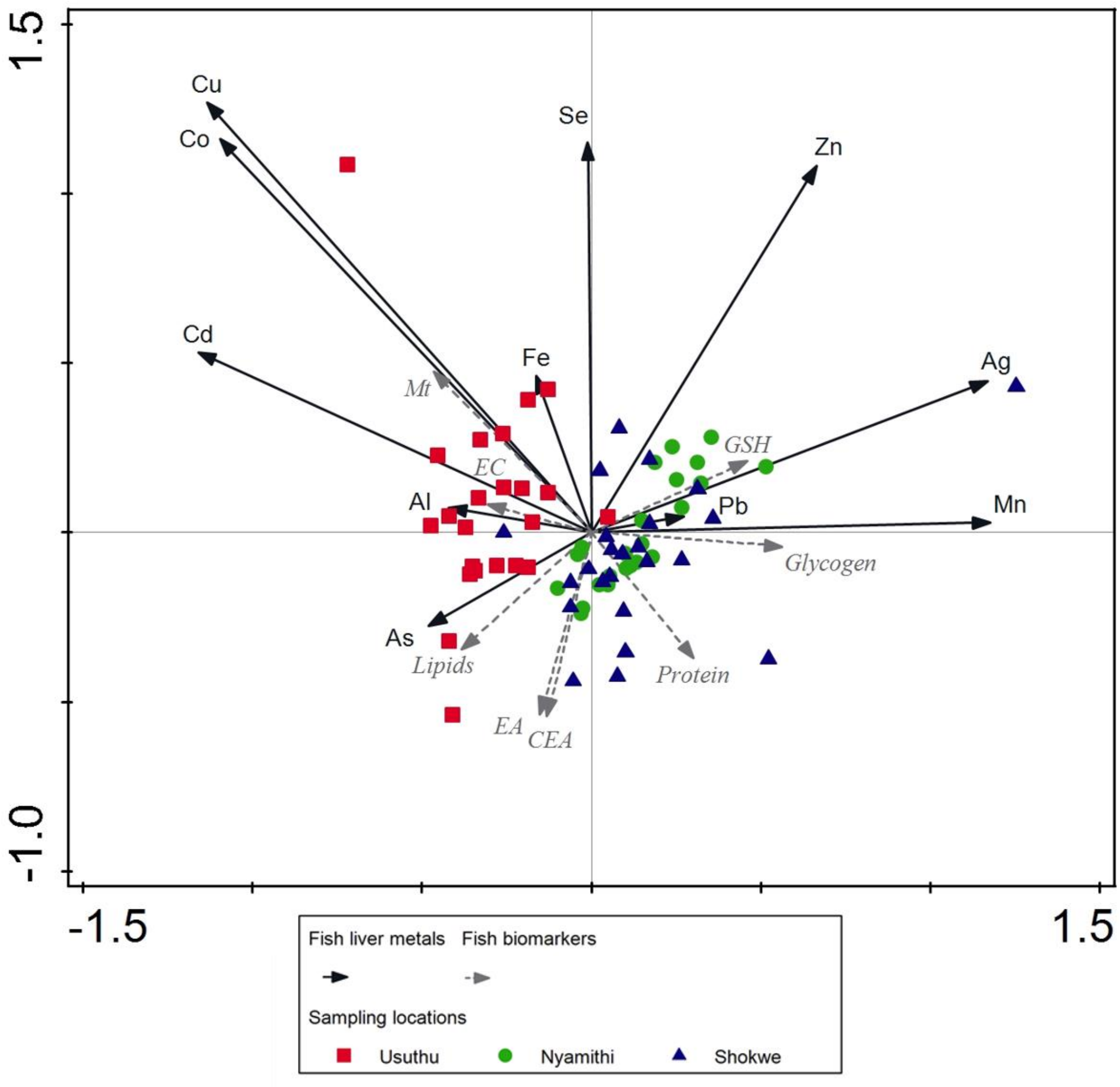

Figure 5-6: Constrained Redundancy Analysis triplot of the concentration of metals in the liver tissue of *Clarias gariepinus* across three different sites in Ndumo Game Reserve (Squares – Usuthu River, Circles – Nyamithi Pan, Triangles – Shokwe Pan; with their corresponding biomarker responses: cellular energy allocation [CEA], metallothioneins [MT] and reduced glutathione [GSH] overlaid; the first [horizontal] axis explains 8.43% and the second [vertical] axis a further 5.69%, for a cumulative 14.12%).

5.3.9. Biomarker response of *C. gariepinus* in relation to different endohelminth parasite numbers

To investigate the effect of the number/load of the four different endohelminth parasites on the biological responses from their hosts *C. gariepinus* an RDA was done and the triplot is shown in Fig. 5.7. Axes 1 and 2 combine to explain 5.75% of the total variance in the data, where catfish from each of the three sites are plotted according to their number of endohelminth parasites. The first axis explains 4.27% of the variance in the total data where Nyamithi Pan separates away from Usuthu River and Shokwe Pan due to higher infection rates of the endohelminth parasites, which was accompanied by lower MT and energy reserves. The second axis explains 1.51% of the variance in the data and shows a slight separation between Shokwe Pan (down) and Usuthu River (up); this separation is due to an increase in MTs at Shokwe Pan and an increase in CEA and *Camallanus* sp. at Usuthu River.

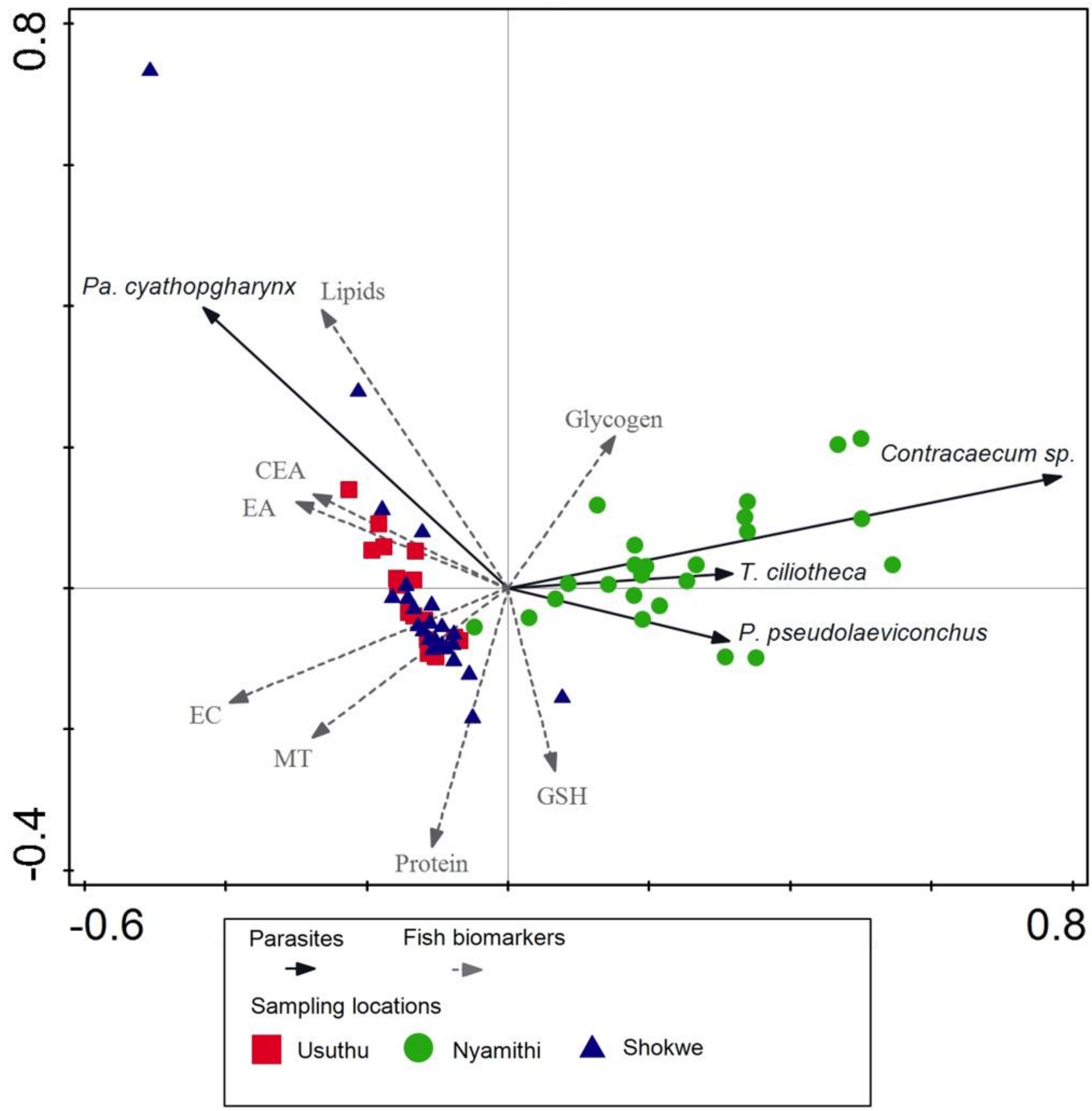

Figure 5-7: Constrained Redundancy Analysis triplot of the biomarker responses: cellular energy allocation (CEA), metallothioneins (MT) and reduced glutathione (GSH) of the host *Clarias gariepinus* across three different sites in Ndumo Game Reserve with the parasite counts of the four endohelminth parasites overlaid (The first [horizontal] axis explains 4.27% and the second [vertical] axis a further 1.51%, for a cumulative 5.78%).

5.3.10. Biological response of *Contracaecum* sp. in relation to their accumulation and their environment (host accumulation)

To determine the effect of metal concentrations in the *Contracaecum* sp. at Nyamithi Pan, on their biomarkers (CEA, MTs and GSH) an RDA was done and the triplot is shown in Fig. 5.8, where the catfish is plotted according to their metal concentrations. The first and second axes combine to explain 36.08% of the variance in the total data, where the parasites are separated by their metal concentrations, as those individuals with a greater MT response accumulated less metals but also had less energy reserves. The first axis explains 19.24% of the total variance in data, where the parasites are separated according to their biological responses. The parasites with low concentrations of the different metals showed an increase in the total CEA as well as an increase in available energy. The *C. gariepinus* and the parasites with an increase in Pb and Cd metal concentrations had an increase in GSH response and higher energy consumption. The parasites with an increase in MTs showed a decrease in most of the metal concentrations.

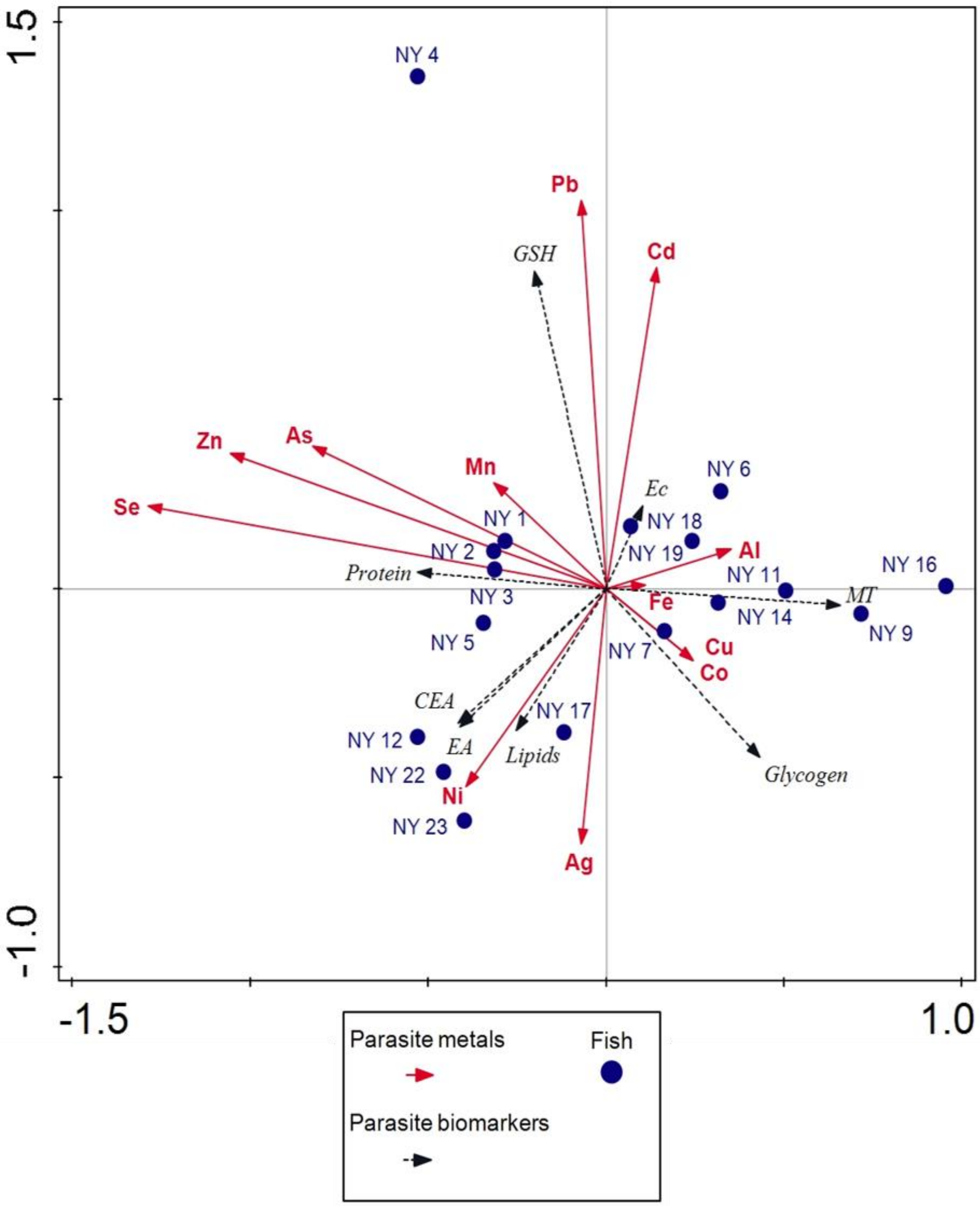

Figure 5-8: Unconstrained Redundancy Analysis triplot of the concentration of metals in *Contracaecum* sp. across two different sites in Ndumo Game Reserve with their biomarker responses: cellular energy allocation (CEA), metallothioneins (MT) and reduced glutathione (GSH) overlaid (The first [horizontal] axis explains 19.49% and the second [vertical] axis a further 17.11%, for a cumulative 36.08%).

The RDA triplot constructed in Figure 5.9 indicates the long-term effect of concentration of metals accumulating in the environment of *Contracaecum* sp. (in the muscle tissue of *C. gariepinus*) on the biomarker responses of the *Contracaecum* sp. Axes 1 and 2 combine to explain 37.90% of the total variance in the data. Axis 1 explains 24.77% of the variance in data where the parasites are separated by their biomarker response. The second axis explains 13.13% of the total variance where the parasites are separated due to the concentration of metals in their environment. *Contracaecum* sp. present in *C. gariepinus* with higher concentrations of Pb and Cd had higher GSH response and energy consumption. The parasites with the highest CEA, especially available energy as well as MT response, were present in the hosts with lower concentrations of most of the metals.

In order to investigate the biological responses (CEA, MT and GSH) of *Contracaecum* sp. to the short-term metal concentrations in their environment an RDA was done and the triplot is shown in Fig. 5.10 where *Contracaecum* sp. is plotted according to the metal concentrations in their host liver tissue. The first and second axes combine to explain 42.47% of the total variance in the data. The first axis explains 23.14% of the variance with the parasites being separated by their biological responses. The *Contracaecum* sp. parasites separate equally with nine individuals separating left, because of an increase in available energy with an increase in Se, Zn and As in their host liver tissue. The second axis explains 19.13% of the variance in the data where the parasites are separated by the concentration of metals present in their environment. The parasites present in hosts with higher concentrations of metals had higher MT responses. The parasites with higher CEA, especially available energy, were present in catfish with lower concentrations of most of the metals.

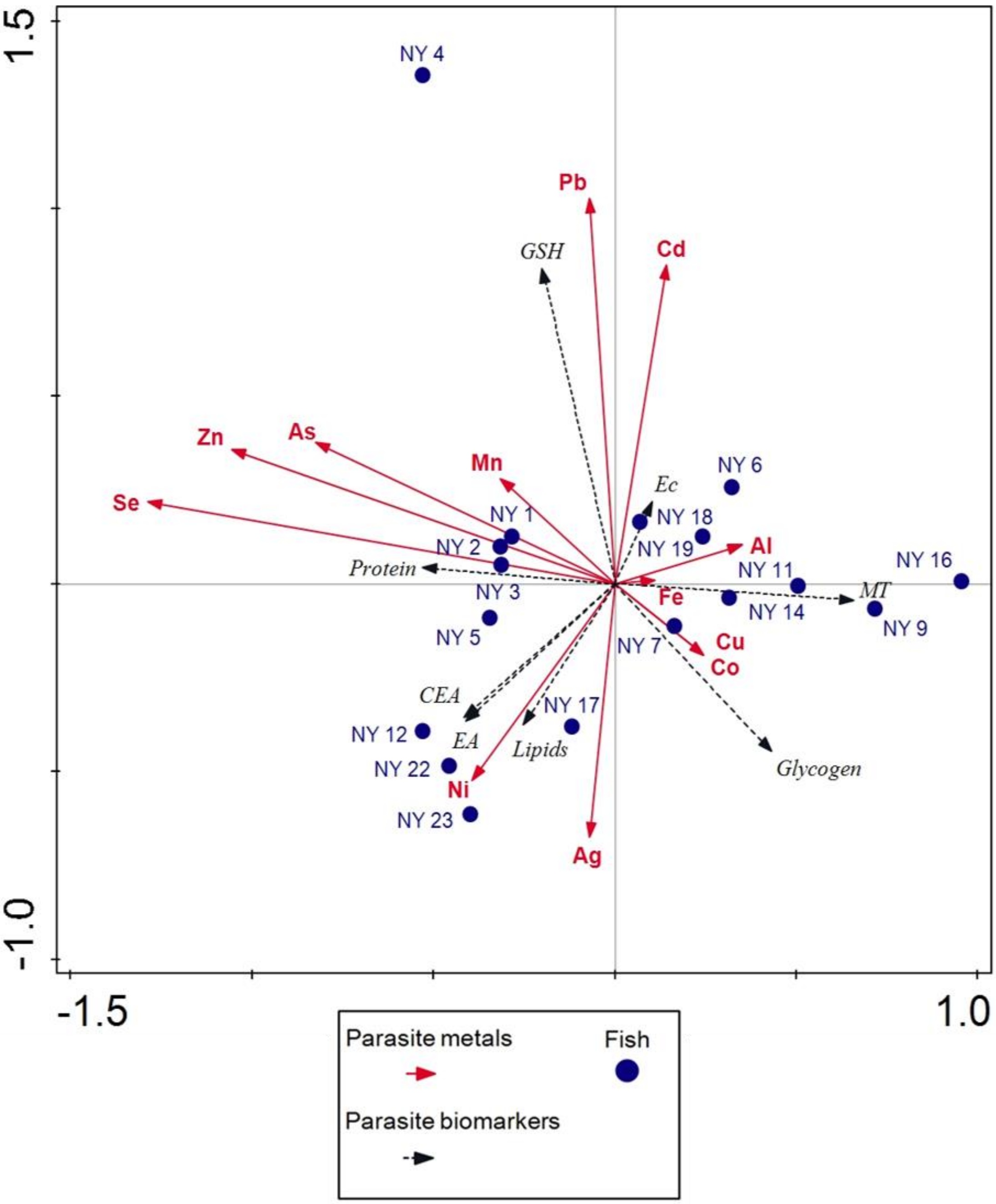

Figure 5-9: Unconstrained Redundancy Analysis triplot of the biological responses: cellular energy allocation (CEA), metallothioneins (MT) and reduced glutathione (GSH) of the *Contracaecum* sp. across three different sites in Ndumo Game Reserve with the metal concentration in the muscle tissue of the host *Clarias gariepinus* overlaid (The first [horizontal] axis explains 24.77% and the second [vertical] axis a further 13.13%, for a cumulative 37.90%).

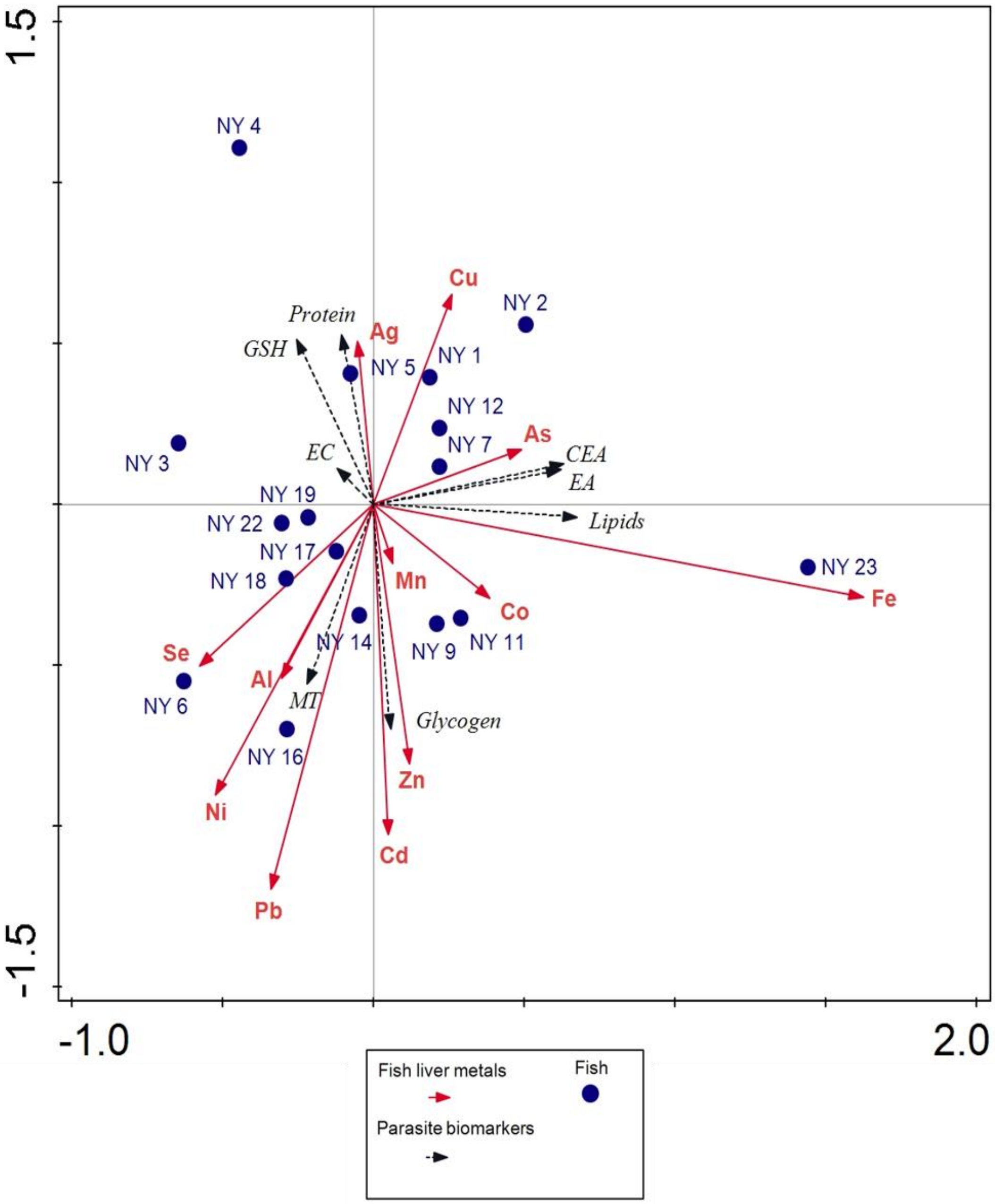

Figure 5-10: Unconstrained Redundancy Analysis triplot of the biological responses: cellular energy allocation (CEA), metallothioneins (MT) and reduced glutathione (GSH) of the *Contracaecum* sp. of the *Clarias gariepinus* in Nyamithi Pan in Ndumo Game Reserve with the metal concentrations in the liver tissue of the host Clarias gariepinus overlaid (The first [horizontal] axis explains 23.14% and the second [vertical] axis a further 19.13%, for a cumulative 42.47%).

5.4. Discussion

Pollutants such as metals are known to cause stress in organisms that can be quantified by measuring biological responses at cellular levels (Gerber *et al.*, 2018). Along with pollution parasites will also cause stress to their hosts by taking up nutrients from their hosts. Many previous studies have found that parasites will have negative effects on the biological response of their hosts. In recent times studies have started to focus on the possible positive effect of parasites on their hosts in terms of biological responses to pollutants. This study focussed on the biological responses of both *C. gariepinus* and *Contracaecum* sp. to the metal concentrations in their environment, both in the short and long term, to evaluate the possible positive effect of endohelminth parasites on the biological responses of their hosts.

5.4.1. Effect of endohelminth parasites on their host biomarker responses

Parasites are dependent on energy from their hosts for their development, growth and reproduction (Barber, 2005). Parasite infection can alter their hosts' use of energy, having a negative effect on the host (Robar *et al.*, 2011). Parasitisation can therefore interfere with host growth rate, swimming behaviour and maturation time (Walkey & Meakins, 1970). Organisms infected with parasites in a polluted system will thus have to use almost all of their energy to survive but will have no energy for growth and reproduction (Marcogliese & Pietrock, 2011). Since the early 2000s researchers have started to investigate the effects of parasites on the biomarkers of their hosts in polluted systems (Morley *et al.*, 2006, Sures, 2006, 2008, Morley, 2010).

Although many studies have proven the negative effect of parasites and pollution on their hosts, some studies have found positive effects of parasites on their host in terms of pollutants (Sures *et al.*, 2017). It is believed that at a certain infection rate, parasites can reduce pollutant stress on their hosts sufficiently to have a beneficial effect on the host. Parasites remove metals and other pollutants from their hosts lowering the energy required by the hosts to detoxify and remove the pollutants themselves. Parasites such as some acanthocephalans and cestodes species are known to lower metal concentrations in their hosts through biomagnification (Sures *et al.*, 2017). The decrease in metals in the host organisms will cause less stress on the hosts through pollution, but the hosts still undergo stress caused by the parasite infection. When the stress caused by the parasite infection is lower than the stress the metals would have had on the hosts the parasite infection can be seen to have a positive effect on the host.

5.4.2. Biomarker of exposure

In this study positive correlations were found between the number of *T. ciliotheca, Pa. cyathopgharynx* and *Contracaecum* sp. and the short-term metal accumulation in their hosts, as well as between *Contracaecum* sp. and the long-term metal exposure in their hosts.

In this study a negative correlation was shown between the number of *T. ciliotheca* and *Contracaecum* sp. and the concentration of Cd in the liver, and between *Contracaecum* sp. and the concentration in the muscle tissue. This supports the findings of Sures (1996) and Sures *et al.* (1997) who found that acanthocephalans (parasites) reduce the uptake of Cd by their hosts. However, this study also found a positive correlation between the number of *Pa. cyathopgharynx* and the concentration of Cd in the liver of the host.

The study found that in the short term parasites can have a positive effect on the host metals with an increase in the number of *T. ciliotheca*, *P. pseudolaeviconchus* and *Contracaecum* sp. leading to a decrease in metal concentration in the liver tissue of the host. In the short term parasites and hosts compete for the essential metals needed by both the host and parasite species. Endohelminth parasites have adapted to absorb nutrients at higher levels than their hosts to ensure survival (Dalton *et al.*, 2004). Endohelminth parasites developed an acellular epithelial syncytium, to help absorb nutrients (and metals) from their host's digestive tract (Dalton *et al.*, 2004). Helminth parasites are also not able to produce their own cholesterol or fatty acids, and thus evolved to be efficient in absorbing them from their hosts (Sures & Siddall, 1999).

The study also found that an increase in *Contracaecum* sp. parasites in a host species will have a positive effect on the metal concentrations inside the host muscle tissue over a long term. The higher the number of *Contracaecum* sp. parasites in a host the lower the concentrations of the metals, both essential and non-essential, present in the host. According to Sures and Siddall (1999) the accumulation of metals in helminth parasites will affect the hosts' ability to metabolise pollutants. Parasites will act as a sink for metals in the long term by accumulating the metals (Sures *et al.*, 2017). Gabrashanska and Nedeva (1996) state that the uptake of metals by parasites is considered as an efflux of metals from their hosts and therefore reduces the concentrations inside the hosts. These results are supported by many previous studies that also found a decrease in the metal concentration in the host liver with an increase in different cestode species numbers (Oyoo-Okoth *et al.*, 2010, Brázová *et al.*, 2012, Khalil *et al.*, 2014) and (Evans *et al.*, 2001, Hursky & Pietrock, 2015) for different intestinal nematode species.

5.4.3. Biomarker of effect

Induction of metallothionein stress proteins are caused by an increase in intracellular and intercellular metal (essential and non-essential) concentrations (Thiele, 1992). According to Hamer (1986), the importance of MTs as a biomarker is increasing because most living organisms possess the necessary protein to synthesise MT and thus MTs can be measured in almost all organisms. Metallothioneins in organisms increase when concentrations of metals increase (Hamer, 1986, Viarengo *et al.*, 1999, Van der Oost *et al.*, 2003, Dahms-Verster *et al.*, 2019, Mijošek *et al.*, 2019). In this study it was found that the MT concentrations did not show the same trend, as the catfish in the Usuthu River that had the lowest environmental metal concentrations had the highest MT concentrations. This shows that MT concentrations can be affected by more than just the metal concentrations in the environment.

The reason for the increased levels of MTs is thought to be because of lower endohelminth parasite numbers in the lotic system. This is supported by Baudrimont and De Montaudouin (2007) who found that uninfected fish had approximately fourfold higher concentrations of MTs than infected fish. The MT levels correlate with the endohelminth numbers at the two lentic systems with the saline system having the highest number of parasites and the lowest MT concentrations. Metallothioneins' concentrations increase with the initial exposure to metals in their environment to help detoxify and remove the metals from the hosts (Bremner, 1987, Van der Oost *et al.*, 2003, Baudrimont & De Montaudouin, 2007). As arsenic accumulation increases over time the MT concentrations in the organism tend to stabilise and stay at a constant level (Baudrimont *et al.*, 2003, Cho *et al.*, 2008). Metallothioneins also compete with GSH to bind with pollutants, thus an increase in GSH leads to a decrease in MTs.

In this study it was found that *Contracaecum* sp. had higher concentrations of GSH than their hosts. Reduced glutathione is a non-protein tripeptide that consists of a g-glutamine, a cysteine and a glycine, that can be conjugated in the beginning of the formation of mercapturic acid (George, 1994). Its two main functions are detoxification and acting as an antioxidant (Stegeman *et al.*, 1992). Reduced glutathione is a major detoxification mechanism of helminth parasites (Brophy & Barrett, 1990).

The GSH concentrations in the study correlate negatively with the MT concentrations found, i.e. when MTs increase the GSHs decrease and vice versa. With an initial increase in metal concentrations the GSH concentrations decrease as the MT concentrations increase due to the competition for resources (Van der Oost *et al.*, 2003, Gerber *et al.*, 2018). *Contracaecum* sp. had elevated GSH levels due to the low MT concentrations. As MT's concentrations reach a stable level over the time of the exposure the GSH will increase in the organism (Van der Oost *et al.*, 2003). At higher concentrations of metals GSH have an affinity for Cd and Pb so that if the concentration of these metals increase the GSH concentration also increases (Dudley *et al.*, 1985).

Reduced glutathione is also part of the anti-oxidant system in organisms and helps combat oxidative stress with reactive oxygen species (ROS) forming due to pollutants (Gerber *et al.*, 2018). Organisms use their cellular energy allocation for important functions such as growth and reproduction. With the accumulation of pollutants such as metals, organisms must use their available energy to detoxify and remove pollutants from their tissues. As metals increase both in the short and long term, the CEA of organisms tends to decrease, as the organisms spend energy in the detoxification and excretion of metals due to the "chronic" exposure to metals (Smolders *et al.*, 2004, Ibrahim, 2006, Moolman *et al.*, 2007, Bednarska *et al.*, 2013).

As the accumulation of metals increase over time the glycogen decreases, but protein levels increase, this is because the readily available energy (glycogen) is used to remove metals from the organisms. This is supported by the findings of previous studies (Schramm *et al.*, 1998, Smolders *et al.*, 2004, Heath, 2018). The proteins are believed to be at elevated levels because organisms use the readily available energy reserves before using proteins (Smolders *et al.*, 2004). Smolders *et al.* (2004) state that low levels of pollution cause an increase in protein synthesis (for detoxification). Lipids have a negative correlation with increased metal exposure – a decrease in lipids due to metal exposure has been documented numerous times (Sancho *et al.*, 1998, Handy *et al.*, 1999, Smolders *et al.*, 2004). Handy *et al.* (1999) found that the decrease in lipids was due to the organism having to use reserve energy for bodily functions, while the available energy was being used to detoxify metals.

5.5. Conclusion

This study was the first study to look at the biomarker responses in *Contracaecum* sp. on metal accumulations as well as to determine the effect of *Contracaecum* sp. on their host biomarker response. In this study it was found that as the concentrations of metals in an organism (both) host and *Contracaecum sp*. increased the MT response. It was seen that with an increase in parasite infection there was a decrease in MT concentration in the host organism which supports the hypothesis that an increase in parasite load will decrease the effect of metals on the hosts. But overall it was found that the organisms with the highest parasite infections also had the lowest available energy and overall CEA levels. It is thus a “catch-22” where an increase in parasites helps deal with the effect of metals on their host, but at a cost to the energy budget of the host.

6. Conclusion and recommendations

6.1. Conclusion

Most freshwater ecosystems on Earth are threatened by anthropogenic activities such as mining and agriculture that release metals into the environment. Over the last decade, research on the effects of human impact on aquatic ecosystems, both lentic and lotic, has increased dramatically. Bio-indicators are currently being used to assess the health of aquatic ecosystems, with parasites gaining increasing attention due to the possibility of using them as early warning bio-indicators.

The aims of the study were firstly to determine the metal concentrations in three different aquatic ecosystems, secondly to determine if the different ecosystem types have an impact on the bioaccumulation of metals in *Clarias gariepinus*, thirdly to determine if different helminth parasites can act as early warning bio-indicators for metal pollution, and lastly to see if endohelminth parasites affect the biological responses of their hosts to metal accumulation.

6.1.1. Lentic aquatic ecosystems

6.1.1.1. Freshwater lentic ecosystem (Shokwe Pan)

Water and sediment samples were collected in triplicate at the Shokwe Pan in November 2018. The study found that Shokwe Pan had the highest concentrations of all the different metals in the sediment in comparison with the saline lentic system and the lotic system. Lentic systems are known to act as a sink for metals. Although the concentrations of metals in the water of Shokwe Pan were the highest of the three sites in the study, it was still below the target water quality guidelines. Shokwe Pan had higher concentrations of most metals (Se, Pb, Cd, As and Mn) than the lotic system, but lower concentrations of Al and similar concentrations of Ag. The freshwater lentic system had lower water concentrations of all metals except for Al than the saline lentic system. Although the water at Shokwe Pan had higher concentrations of Al, Zn and As than the TWQR, the environmental concentrations of metals of Shokwe Pan were found to be in a natural condition with little to no anthropogenic impact.

Clarias gariepinus in Shokwe Pan had the highest concentrations of Zn, Ni, Pb, Cd, Mn and Al in their muscle tissue which reflects the environmental concentrations. Muscle tissue indicates accumulation over a longer period and indicates a positive relationship with the sediment concentrations. The bioaccumulation factors for Fe, Co, Zn, Cu, As, Cd, Pb and Se of the catfish in Shokwe Pan were higher than those of the saline lentic pan, the salt in the saline pan binds to the metals forming complexes and complicate metal accumulation. Shokwe Pan also had higher BAFs for Fe, Zn, Al, Cd and Pb than the lotic system, the lentic systems had the highest environmental concentrations of metals, thus metals was readily available for bioaccumulation. The metal concentrations in the liver tissue of *C. gariepinus* was higher for Ni, Zn, Mn, Pb, Se and Ag than in the lotic system and higher for Ni, Zn, Cu, Mn, As and Cd than in the saline lentic system. Therefore, the bioaccumulation of metals over the long and short term was the highest in the freshwater lentic system.

The parasite infections in the freshwater lentic system, Shokwe Pan had the highest prevalence for both the *Procamallanus* sp. and *Camallanus* sp. Shokwe Pan had a higher number of parasites than the lotic system but less than the saline lentic pan. Shokwe Pan is a temporary pan that dries up during the dry season. This kills the intermediate hosts of the parasites negatively influencing the parasite numbers in the pan. *Tetracampos* sp. (cestode) had the highest concentrations of Fe, Co, and Ag, with *Procamallanus* sp. having the highest concentrations of Ni, Zn, Cu, Al, Mn, As, Cd and Pb, and *Contracaecum* sp. for Se. The BAFs of the four different endohelminth parasites show that *Tetracampos* sp. had the highest BAF for Fe, Co, Cd, Pb and Ag with *Procamallanus* sp. having the highest for Ni, Zn, Cu, Al, As, Cd, Mn and Pb. *Procamallanus* sp. was therefore the helminth parasite with the best bioaccumulation of metals in the freshwater lentic system.

6.1.1.2. Saline lentic ecosystem (Nyamithi Pan)

Water and sediment samples were collected in triplicate from Nyamithi Pan in November 2018. The present study found that Nyamithi Pan had higher concentrations of all the metals in the sediment than the lotic system. The freshwater lentic system of Shokwe Pan, had the highest levels of all the metals in the sediment. Nyamithi Pan is a permanent saline pan where the pan acts as a sink for the metals, but the salt in the water form complexes with the metals and therefore makes sediment adsorptions more difficult than in a freshwater pan. Nyamithi Pan had the highest water concentrations of all the essential metals across the three sites as well as the highest water concentrations of the non-essential metals: As, Pb and Se. Nyamithi Pan also had the lowest concentrations of Al, Mg and Ag with Al being below detection limits.

The salt in the water binds to metals and keeps the metals from being adsorbed by the sediment and therefore keeping the concentrations higher in the water. Although the concentrations of metals in the water were the highest in Nyamithi Pan, it was not above the TQWR. Thus, the environmental concentrations at Nyamithi Pan showed no anthropogenic disturbances. The *C. gariepinus* at Nyamithi Pan had the lowest concentrations of most of the metals in the muscle tissue. This is due to a combination of the lower concentrations of metals in the sediment of the pan and the salt in the water of the pan forming complexes with the metals and making it harder for uptake by the organisms.

The BAF for Nyamithi Pan was the lowest in terms of the concentrations of Fe, Co, Zn, Cu, As, Cd, Pb and Se in the muscle and liver tissues of *C. gariepinus*. *Clarias gariepinus* at Nyamithi Pan had the highest BAF for Mn and Ag compared with the other sites. The bioaccumulation factors show that in a saline pan, bioaccumulations are more difficult than in fresh water. The concentrations of Ni, Zn, Cu, As and Cd were also lower in the liver tissue than in the liver tissue of catfish from the other two sites. It is therefore evident that saline lentic systems accumulate metals less than its freshwater counterpart and even less than the lotic system.

The parasite infections in the saline water lentic system, Nyamithi Pan had the highest prevalence for both the *Tetracampos* sp. and *Contracaecum* sp. Nyamithi Pan had the highest numbers of parasites in comparison with the other two sites. Nyamithi Pan is a permanent saline pan, which means that the intermediate hosts of the parasites can multiply throughout the year, positively influencing the parasite numbers in the pan.

Tetracampos sp. had the highest concentrations of Co and Se, with *Camallanus* sp. having the highest concentrations of Fe, Ni, Zn, Al, Mn, As, Cd, Pb and Ag. The BAFs of the five endohelminth parasites located at Nyamithi Pan showed that *Tetracampos* sp. had the highest BAFs for Co, Zn, Cu, Al, Mn and Se, with *Camallanus* sp. having the highest for BAFs for Fe, Ni, Cu, As, Cd, Pb and Ag. Therefore, in the saline lentic system, the *Camallanus* sp. had the highest bioaccumulation.

6.1.2. Lotic aquatic ecosystem (Usuthu River)

Water and sediment samples were collected in triplicate from the Usuthu River in November 2018. The study found that the Usuthu River had the lowest concentrations of all the metals in the sediment. Lotic systems flush the sediment downstream as a result of the running water, preventing adsorption of metals. The lotic system had the lowest concentrations of Ni, As, Cd, Pb and Se in the water when compared to the other two sites. The Usuthu River had the highest concentrations of Al and Ag in the water. The water transports pollutants over great distances and therefore diluting the metals to lower concentrations. Both the sediment and water concentrations were well below the target quality guidelines and it is therefore safe to say that the lower Usuthu River is in natural conditions without any major anthropogenic impact. The *C. gariepinus* in the Usuthu River had the highest concentrations of Fe, Co, Cu, Se and Ag in their muscle tissue. For the rest of the metals, the freshwater lentic system had the highest concentrations while the freshwater lotic system and saline lentic system had very similar concentrations of the metals.

The *C. gariepinus* in the freshwater lotic system had the highest concentrations of Fe, Co, Cu, Al, As and Cd in their liver tissue in comparison to *C. gariepinus* in the other systems. The BAFs of *C. gariepinus* in the freshwater lotic system were the highest for Co, Ni, As and Se proving to have higher BAFs than the *C. gariepinus* located in the saline lentic pan. The lotic system had the lowest parasite numbers when compared to the other two sites. This can be as a result of the lotic system not being confined and having larger areas for the catfish to swim and more places where the intermediate hosts can live; therefore a smaller chance of infection.

The *Tetracampos* sp. in the *C. gariepinus* of the lotic system had the highest concentrations of Fe, Co, Zn, Cu and Mn; with the *Procamallanus* sp. having the highest concentrations of As, Cd and Pb; and the *Camallanus* sp. the highest concentrations for Ni, Al and Ag. The BAFs of the different endohelminth parasites show that the *Tetracampos* sp. had the highest BAFs for Fe, Co, Zn, Al and Mn, with the *Camallanus* sp. having the highest BAFs for Ni and Ag and the *Procamallanus* sp. the highest for As, Cd and Pb proving that the *Tetracampos* sp. had the highest bioaccumulation properties in the lotic system.

6.1.3. Parasites and biomarkers

The present study found that parasites plays an important role in the biomarker response of their hosts, with the first impact being the stress caused by the parasites themselves. Infected fish are more stressed due to high infection rates, which in turn cause an increase in energy usage by the fish. The parasites also feed on nutrients required by the fish for normal bodily functions, this cause even more stress on the fish. High metal concentrations can affect the biomarker response of both the hosts as well as the parasites in different ways. The study found that *C. gariepinus* produces higher levels of MTs when exposed to higher levels of metals while the *Contracaecum* sp. produces higher levels of GSH at higher metal concentrations. Parasites are known to bioaccumulate metals from their hosts by removing metals from their hosts. Parasites can therefore have a positive effect on the biological response of their hosts to metal concentrations. The study found that hosts with the highest number of parasites had the lowest MT production, this indicates that the parasites had an effect on the biological response of their hosts to metal concentrations. Although the host with the highest number of parasites had the lowest concentrations of MTs they also had the lowest available energy as well as the lowest overall CEA levels. The stress caused by the parasite infection thus had the greater effect on their hosts than higher metal concentrations.

6.1.4. General conclusions

- This is the first study to determine the metal concentrations in different aquatic ecosystems of the Ndumo Game Reserve. The study found that all three of the sites were in natural conditions without any major anthropogenic impact.
- The freshwater lentic system had the highest concentrations of metals in the sediment followed by the saline lentic system and the freshwater lotic system which had the lowest concentrations of metals in the sediment.
- The saline lentic system had the highest concentrations of essential metals in the water with the freshwater lentic system having the highest concentrations of most of the non-essential metals and the freshwater lotic system having the lowest concentrations of most of the essential metals in the water.
- The freshwater lentic system had the highest concentrations of metals present in the muscle tissue of *C. gariepinus,* followed by the freshwater lotic system and lastly the saline lentic system having the lowest concentrations of metals in the muscle tissue of *C. gariepinus*.
- The freshwater lotic system had the highest concentrations of metals in the liver tissue of *C. gariepinus* followed by the by the freshwater lentic system and the saline lentic system having the lowest concentrations in the liver of *C. gariepinus.*

- The saline lentic system had the highest number of parasites across the three different sites and even having a cestode species (*Proteocephalus* sp.) which was not present at any of the other sites.
- It was important to analyse all the different endohelminth parasites as bio-indicators for metals because different parasites accumulate different metals better.
- A high number of *Contracaecum* sp. in a host will remove metals from the host and lower the bioaccumulations of the metals in the host muscle tissue.
- *Procamallanus* sp. had the highest bioaccumulation rates in the freshwater lentic system.
- *Camallanus* sp. had the highest bioaccumulation rates in the saline lentic system.
- *Tetracampos* sp. had the highest bioaccumulation rates in the freshwater lotic system.
- An increase in parasite load will cause a decrease in the biological response to metals by lowering the MT response of the host due to the removal of metals.
- Hosts with the highest number of parasites have the lowest amount of CEA and therefore they have less metals to deal with, but the parasites drain their energy.

6.2. Hypotheses

Hypothesis 1: Because lentic systems function as natural sinks for metals, they will have higher concentrations of metals than lotic systems. The concentrations of metals present in all sites of the study will be below the international guidelines for target water quality as well as international sediment quality guidelines.

After the analysis of the water and sediment samples of the three sites, it was found that the freshwater lentic system had the highest concentrations of all the metals in the sediment and most metals in the water. It was only found that the saline lentic system had the highest concentrations of most of the metals in the water when compared to the other two sites. It was also found that both the water and sediment concentrations were below the TWQR and therefore the three sites were in natural conditions with no major metal pollution present. Therefore, the two lentic systems had higher metal concentrations in the environment and as a result the hypothesis is <u>accepted.</u>

Hypothesis 2: The bioaccumulation of metals in *C. gariepinus* at all three sites will reflect the environmental conditions of the sites, with both the lentic systems (fresh and saline) having higher bioaccumulation factors than the lotic system. *Clarias gariepinus* will also bioaccumulate the non-essential metals to higher levels than the essential metals.

After analysing the metal concentrations in both the muscle and liver tissue of the *C. gariepinus* from the three different sites and comparing them with the water concentrations, it was found that the freshwater pan had the highest bioaccumulation rate in the organisms as was expected. It was found that the freshwater lotic system had higher bioaccumulation rates than the saline lentic system because of the salt forming complexes with the metals. Therefore, the hypothesis is rejected as only one lentic system had higher BAFs.

Hypothesis 3: The parasites will prove to be better bio-indicators of both essential and non-essential metals than their hosts due to the bioconcentration and biomagnification of the metals in the parasites.

The metal concentrations in the five different endohelminth parasites were determined and compared to the concentrations in both the host muscle and liver tissues. It was found that all endohelminth parasites had higher concentrations of the different metals than their host muscle tissue and higher than the liver tissue for most of the metals. Therefore, the hypothesis is accepted.

Hypothesis 4: The parasites in the intestines will have higher accumulation rates of metals than the *Contracaecum* sp. in the body cavity due to the higher concentration of metals in the intestinal tract.

After the metal concentrations in the endoparasites were determined, they were compared with each other to determine if a difference in accumulation had occurred. It was found that the nematode *Contracaecum* sp. located in the body cavity had the lowest concentrations of all metals except for Se. Therefore, the hypothesis is accepted as there was a clear difference in accumulation of the parasites in the intestines than the body cavity parasites, with the parasites in the intestines having higher concentrations of metals than the *Contracaecum* sp. in the body cavity.

Hypothesis 5: There will be a gradient of metal accumulation in the nematode parasites with the highest being in the parasites in the stomach due to the low pH making metals there more bioavailable than in the rectum of the host.

When comparing the metal accumulation of the endohelminth parasites in the intestines of the hosts we found that the different parasites accumulated different metals to higher concentrations with no specific parasite accumulating metals to higher levels. Therefore, the hypothesis is rejected as the location of parasites in their hosts did not have an effect on their accumulation rates,

Hypothesis 6: An increase in endohelminth parasite numbers will alleviate the body burdens of metals in the hosts thereby resulting in less stress experienced by the hosts to detoxify and remove metals from their organs.

To analyse the effect of parasites on the biomarker responses of their hosts, the biomarker responses of the hosts were compared between hosts with low parasite numbers and ones with high parasite numbers. The study found that with an increase in parasite numbers, there was a decrease in the metallothionein production in hosts as well as a decrease in available energy and total cellular energy allocation. Therefore, the hypothesis is accepted as an increase in parasite numbers lowers the MT's production. It is also important to note that this advantage to the hosts comes with a decrease in energy and may not necessarily be beneficial for the hosts.

6.3. Recommendations

For future studies, the author recommends that the following aspects be considered for inclusion:

- Conducting the study over different seasons as the parasite infections may be different.
- Increasing the number of hosts to 30 per site to ensure the collection of enough of the endohelminth parasites to have more replicates for metal and biomarker analysis.
- Use different host species as different species have different parasite infection rates.
- Conduct the same type of study with different types of parasites to compare different parasites effects on their hosts.
- Including more biomarkers in the study to search for more effects of parasites on their hosts' biomarkers and to be able to make a better case for the positive effect of parasites on their hosts in the presence of metal pollution.

References

Adams, S. & Greeley, M. 2000. Ecotoxicological indicators of water quality: using multi-response indicators to assess the health of aquatic ecosystems. *Water, air, and soil pollution*, 123(1-4):103-115.

Adams, T.G., Atchison, G.J. & Vetter, R.J. 1980. The impact of an industrially contaminated lake on heavy metal levels in its effluent stream. *Hydrobiologia*, 69(1-2):187-193.

Agah, H., Leermakers, M., Elskens, M., Fatemi, S.M.R. & Baeyens, W. 2009. Accumulation of trace metals in the muscle and liver tissues of five fish species from the Persian Gulf. *Environmental monitoring and assessment*, 157(1-4):499-514.

Ahmad, M., Islam, S., Rahman, M., Haque, M. & Islam, M. 2010. Heavy metals in water, sediment and some fishes of Buriganga River, Bangladesh. *International journal of environmental research*, 4(2):321-332.

Akcil, A. & Koldas, S. 2006. Acid Mine Drainage (AMD): causes, treatment and case studies. *Journal of cleaner production*, 14:1139-1145.

Akinsanya, B. & Kuton, M.P. 2016. Bioaccumulation of heavy metals and parasitic fauna in *Synodontis clarias* (Linnaeus, 1758) and *Chrysichthys nigrodigitatus* (Lacepede, 1803) from Lekki Lagoon, Lagos, Nigeria. *Asian Pacific journal of tropical disease*, 6(8):615-621.

Akinsanya, B. & Otubanjo, O.A. 2006. Helminth parasites of *Clarias gariepinus* (Clariidae) in Lekki Lagoon, Lagos, Nigeria. *Revista de biología tropical*, 54(1):93-99.

Alain, G., Tousignant, J. & Rozenfarb, E. 1993. Chronic arsenic toxicity. *International journal of dermatology*, 32(12):899-901.

Alonso, M.L., Montaña, F.P., Miranda, M., Castillo, C., Hernández, J. & Benedito, J.L. 2004. Interactions between toxic (As, Cd, Hg and Pb) and nutritional essential (Ca, Co, Cr, Cu, Fe, Mn, Mo, Ni, Se, Zn) elements in the tissues of cattle from NW Spain. *Biometals*, 17(4):389-397.

Alwan, S., Hadi, A. & Shokr, A. 2009. Alterations in hematological parameters of fresh water fish, Tilapia zillii, exposed to aluminum. *Journal of science and its applications*, 3(1):12-19.

Amiard, J.-C., Amiard-Triquet, C., Barka, S., Pellerin, J. & Rainbow, P. 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. *Aquatic toxicology*, 76(2):160-202.

Anderson, R. 2009. Fluvial dynamics of the Usuthu River and Banzi Pan, Ndumo Game Reserve. Pretoria: University of Pretoria. (Dissertation – BSc Hons).

ANZECC (Australian and New Zealand Environment and Conservation Council) & ARMCANZ (Agriculture and Resource Management Council of Australia and New Zealand). 2000. Australian and New Zealand guidelines for fresh and marine water quality. Canberra. https://www.waterquality.gov.au/sites/default/files/documents/anzecc-armcanz-2000-guidelines-vol1.pdf Date of access: 10 Jun. 2019.

Arnesen, A., Abrahamsen, G., Sandvik, G. & Krogstad, T. 1995. Aluminium-smelters and fluoride pollution of soil and soil solution in Norway. *Science of the total environment*, 163(1-3):39-53.

Arnot, J.A. & Gobas, F.A. 2006. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. *Environmental reviews*, 14(4):257-297.

Asagba, S.O., Eriyamremu, G.E. & Igberaese, M.E. 2008. Bioaccumulation of cadmium and its biochemical effect on selected tissues of the catfish (*Clarias gariepinus*). *Fish physiology and biochemistry*, 34(1):61-69.

Atli, G. & Canli, M. 2007. Enzymatic responses to metal exposures in a freshwater fish *Oreochromis niloticus*. *Comparative biochemistry and physiology part C: toxicology & pharmacology*, 145(2):282-287.

Atli, G. & Canli, M. 2008. Responses of metallothionein and reduced glutathione in a freshwater fish *Oreochromis niloticus* following metal exposures. *Environmental toxicology and pharmacology*, 25(1):33-38.

ATSDR (Agency for Toxic Substances and Disease Registry). 2013a. Toxicological profile for arsenic. U.S. Department of Health & Human Services, Atlanta, Georgia.

ATSDR (Agency for Toxic Substances and Disease Registry). 2013b. Toxicological profile for cobalt. U.S. Department of Health & Human Services, Atlanta, Georgia.

ATSDR (Agency for Toxic Substances and Disease Registry). 2013c. Toxicological profile for zinc. U.S. Department of Health & Human Services, Atlanta, Georgia.

Authman, M.M., Zaki, M.S., Khallaf, E.A. & Abbas, H.H. 2015. Use of fish as bio-indicator of the effects of heavy metals pollution. *Journal of aquaculture research & development*, 6(4):1-13.

Avenant-Oldewage, A. & Marx, H. 2000. Bioaccumulation of chromium, copper and iron in the organs and tissues of *Clarias gariepinus* in the Olifants River, Kruger National Park. *Water SA*, 26(4):569-582.

Avilán, L., Gualdrón-López, M., Quiñones, W., González-González, L., Hannaert, V., Michels, P.A. & Concepción, J.-L. 2011. Enolase: a key player in the metabolism and a probable virulence factor of trypanosomatid parasites: perspectives for its use as a therapeutic target. *Enzyme research*, doi:10.4061/2011/932549.

Ayandiran, T., Fawole, O., Adewoye, S. & Ogundiran, M. 2009. Bioconcentration of metals in the body muscle and gut of *Clarias gariepinus* exposed to sublethal concentrations of soap and detergent effluent. *Journal of cell and animal biology*, 3(8):113-118.

Babault, J., Bonnet, S., Crave, A. & Van den Driessche, J. 2005. Influence of piedmont sedimentation on erosion dynamics of an uplifting landscape: an experimental approach. *Geology*, 33(4):301-304.

Bady, P., Dolédec, S., Fesl, C., Gayraud, S., Bacchi, M. & Schöll, F. 2005. Use of invertebrate traits for the biomonitoring of European large rivers: the effects of sampling effort on genus richness and functional diversity. *Freshwater biology*, 50:159-173.

Bailey, H.H. & Fairbairn, D. 1968. Lipid metabolism in helminth parasites: V. Absorption of fatty acids and monoglycerides from micellar solution by *Hymenolepis diminuta* (Cestoda). *Comparative biochemistry and physiology*, 26:819-836.

Barałkiewicz, D. & Siepak, J. 1999. Chromium, nickel and cobalt in environmental samples and existing legal norms. *Polish journal of environmental studies*, 8(4):201-208.

Barber, I. 2005. Parasites grow larger in faster growing fish hosts. *International journal for parasitology*, 35(2):137-143.

Barbour, M. & Yoder, C. 2000. The multimetric approach to bioassessment, as used in the United States of America: assessing the biological quality of fresh waters: RIVPACS and other techniques. (*In* Proceedings of an International Workshop held in Oxford, UK, on 16-18 September 1997. Freshwater Biological Association (FBA). p. 281-292).

Barson, M. 2004. The occurrence of *Contracaecum sp. larvae* (Nematoda: Anisakidae) in the catfish *Clarias gariepinus* (Burchell) from Lake Chivero, Zimbabwe. *Onderstepoort journal of veterinary research*, 71(1):35-39.

Bartram, J. & Ballance, R., *eds.* 1996. Water quality monitoring: a practical guide to the design and implementation of freshwater quality studies and monitoring programmes. UNEP/WHO. https://www.who.int/water_sanitation_health/resourcesquality/waterqualmonitor.pdf Date of access: 29 Nov. 2019.

Barwick, M. & Maher, W. 2003. Biotransference and biomagnification of selenium copper, cadmium, zinc, arsenic and lead in a temperate seagrass ecosystem from Lake Macquarie Estuary, NSW, Australia. *Marine environmental research*, 56:471-502.

Baudrimont, M., Andres, S., Durrieu, G. & Boudou, A. 2003. The key role of metallothioneins in the bivalve *Corbicula fluminea* during the depuration phase, after in situ exposure to Cd and Zn. *Aquatic toxicology*, 63:89-102.

Baudrimont, M. & De Montaudouin, X. 2007. Evidence of an altered protective effect of metallothioneins after cadmium exposure in the digenean parasite-infected cockle (*Cerastoderma edule*). *Parasitology*, 134(2):237-245.

Baudrimont, M., De Montaudouin, X. & Palvadeau, A. 2006. Impact of digenean parasite infection on metallothionein synthesis by the cockle (*Cerastoderma edule*): a multivariate field monitoring. *Marine pollution bulletin*, 52(5):494-502.

Bednarska, A.J., Stachowicz, I. & Kuriańska, L. 2013. Energy reserves and accumulation of metals in the ground beetle *Pterostichus oblongopunctatus* from two metal-polluted gradients. *Environmental science and pollution research*, 20(1):390-398.

Belfiore, N.M. & Anderson, S.L. 2001. Effects of contaminants on genetic patterns in aquatic organisms: a review. *Mutation research-reviews in mutation research*, 489(2-3):97-122.

Bergey, L., Weis, J.S. & Weis, P. 2002. Mercury uptake by the estuarine species *Palaemonetes pugio* and *Fundulus heteroclitus* compared with their parasites, *Probopyrus pandalicola* and *Eustrongylides sp*. *Marine pollution bulletin*, 44(10):1046-1050.

Bervoets, L. & Blust, R. 2003. Metal concentrations in water, sediment and gudgeon (*Gobio gobio*) from a pollution gradient: relationship with fish condition factor. Enviro*nmental pollution*, 126(1):9-19.

Bervoets, L., Blust, R. & Verheyen, R. 2001. Accumulation of metals in the tissues of three spined stickleback (*Gasterosteus aculeatus*) from natural fresh waters. *Ecotoxicology and environmental safety*, 48(2):117-127.

Birungi, Z., Masola, B., Zaranyika, M., Naigaga, I. & Marshall, B. 2007. Active biomonitoring of trace heavy metals using fish (*Oreochromis niloticus*) as bioindicator species. The case of Nakivubo wetland along Lake Victoria. *Physics and chemistry of the earth, parts A/B/C*, 32:1350-1358.

Bligh, E.G. & Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology*, 37(8):911-917.

Boomker, J.D.F. 1982. Parasites of South African freshwater fish: I. Some nematodes of the catfish [*Clarias gariepinus* (Burchell, 1822)] from the Hartbeespoort Dam. The *Onderstepoort journal of veterinary research*, 49(1):41-51.

Boomker, J.D.F. 1994. Parasites of South African freshwater fish: VI. Nematode parasites of some fish species in the Kruger National Park. The *Onderstepoort journal of veterinary research,* 61(1):35-43.

Booth, A.J., Traas, G.R.L. & Weyl, O.L.F. 2010. Adult African sharptooth catfish, Clarias gariepinus, population dynamics in a small invaded warm-temperate impoundment. *African zoology*, 45(2):299-308.

Boyce, N.P. & Yamada, S.B. 1977. Effects of a parasite, *Eubothrium salvelini* (Cestoda: Pseudophyllidea), on the resistance of juvenile sockeye salmon, Oncorhynchus nerka, to zinc. *Journal of the Fisheries Board of Canada*, 34(5):706-709.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72(1-2):248-254.

Brázová, T., Torres, J., Eira, C., Hanzelová, V., Miklisová, D. & Šalamún, P. 2012. Perch and its parasites as heavy metal biomonitors in a freshwater environment: the case study of the Ružín water reservoir, Slovakia. *Sensors*, 12(3):3068-3081.

Bremner, I. 1987. Interactions between metallothionein and trace elements. *Progress in food & nutrition science*, 11(1):1-37.

Brezonik, P.L., King, S.O. & Mach, C.E. 1991. The influence of water chemistry on trace metal bioavailability and toxicity to aquatic organisms. (*In* Newman, M.C. & McIntosh, A.W. *eds.* Metal ecotoxicology: concepts and applications. Chelsea, Michigan: Lewis Publishers. p. 1-31). (Advances in trace substances research).

Brophy, P. & Barrett, J. 1990. Glutathione transferase in helminths. *Parasitology*, 100(2):345-349.

Brown, C., Joubert, A., Tlou, T., Birkhead, A., Marneweck, G., Paxton, B. & Singh, A. 2018. The Pongola Floodplain, South Africa–part 2: holistic environmental flows assessment. *Water SA*, 44(4):746-759.

Bucheli, T.D. & Fent, K. 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical reviews in environmental science and technology*, 25(3):201-268.

Burger, J., Gaines, K.F., Boring, C.S., Stephens, W.L., Snodgrass, J., Dixon, C., McMahon, M., Shukla, S., Shukla, T. & Gochfeld, M. 2002. Metal levels in fish from the Savannah River: potential hazards to fish and other receptors. *Environmental research*, 89(1):85-97.

Bury, N.R., Walker, P.A. & Glover, C.N. 2003. Nutritive metal uptake in teleost fish. *Journal of experimental biology*, 206(Pt 1):11-23.

Calmano, W., Ahlf, W. & Förstner, U. 1990. Exchange of heavy metals between sediment components and water. (*In* Broekaert, J.A.C., Güçer, Ş. & Adams, F., *eds.* Metal speciation in the environment. Berlin: Springer. p. 523-543).

Calverley, P.M. & Downs, C.T. 2014. Habitat use by Nile crocodiles in Ndumo Game Reserve, South Africa: a naturally patchy environment. *Herpetologica*, 70(4):426-438.

Calverley, P.M. & Downs, C.T. 2015. Movement and home range of Nile Crocodiles in Ndumo Game Reserve, South Africa. *Koedoe*, 57(1):1-13.

CCME (Canadian Council of Ministers of the Environment). 2014. Canadian Environmental Quality Guidelines. http://ceqg-rcqe.ccme.ca/en/index.html Date of access: 1 Jul. 2019.

Cempel, M. & Nikel, G. 2006. Nickel: a review of its sources and environmental toxicology. *Polish journal of environmental studies*, 15(3):375-382.

Chen, H.-W., Liu, W.-C., Davis, A. & Jordán, F. 2008. Network position of hosts in food webs and their parasite diversity. *Oikos*, 117(12):1847-1855.

Chen, H.-Y., Grabner, D. S., Nachev, M., Shih, H.-H. & Sures, B. 2015. Effects of the acanthocephalan *Polymorphus minutus* and the microsporidian *Dictyocoela duebenum* on energy reserves and stress response of cadmium exposed *Gammarus fossarum*. *PeerJ*, 3(10):e1353. doi:10.7717/peerj.1353.

Chen, J., Blume, H.-P. & Beyer, L. 2000. Weathering of rocks induced by lichen colonization: a review. *Catena*, 39(2):121-146.

Chen, M.-H. & Chen, C.-Y. 1999. Bioaccumulation of sediment-bound heavy metals in grey mullet, *Liza macrolepis*. *Marine pollution bulletin*, 39:239-244.

Cheng, S., Grosse, W., Karrenbrock, F. & Thoennessen, M. 2002. Efficiency of constructed wetlands in decontamination of water polluted by heavy metals. *Ecological engineering*, 18(3):317-325.

Chenoweth, J., Hadjikakou, M. & Zoumides, C. 2014. Quantifying the human impact on water resources: a critical review of the water footprint concept. *Hydrology and earth system sciences*, 18(6):2325-2342.

Cho, Y. S., Lee, S. Y., Kim, K.-Y., Bang, I.C., Kim, D.S. & Nam, Y.K. 2008. Gene structure and expression of metallothionein during metal exposures in *Hemibarbus mylodon*. *Ecotoxicology and environmental safety*, 71(1):125-137.

Clarke, R. 2013. Water: the international crisis. https://doi.org/10.4324/9781315070261 Date of access: 24 Jul. 2019.

Coetzee, L., Du Preez, H. & Van Vuren, J. 2002. Metal concentrations in *Clarias gariepinus* and *Labeo umbratus* from the Olifants and Klein Olifants River, Mpumalanga, South Africa: zinc, copper, manganese, lead, chromium, nickel, aluminium and iron. *Water SA*, 28(4):433-448.

Cohn, V.H. & Lyle, J. 1966. A fluorometric assay for glutathione. *Analytical biochemistry*, 14(3):434-440.

Cole, S., Codling, I., Zabel, T., Zabel, T., Nature, E. & Heritage, S.N. 1999. Guidelines for managing water quality impacts within UK European marine sites. *Report prepared by WRc for UK Marine SACs project.*

Combrink, A.S. 2004. Population survey of *Crocodylus niloticus* (Nile crocodile) at Lake Sibaya, Republic of South Africa. Pietermaritzburg: University of KwaZulu-Natal. (Dissertation – Master of Environment and Development).

Crafford, D. & Avenant-Oldewage, A. 2010. Bioaccumulation of non-essential trace metals in tissues and organs of *Clarias gariepinus* (sharptooth catfish) from the Vaal River system: strontium, aluminium, lead and nickel. *Water SA*, 36(5):621-640.

Crafford, D. & Avenant-Oldewage, A. 2011. Uptake of selected metals in tissues and organs of *Clarias gariepinus* (sharptooth catfish) from the Vaal River System: chromium, copper, iron, manganese and zinc. *Water SA*, 37(2):181-200.

Dahms, S., Baker, N. & Greenfield, R. 2017. Ecological risk assessment of trace elements in sediment: a case study from Limpopo, South Africa. *Ecotoxicology and environmental safety*, 135:106-114.

Dahms-Verster, S., Baker, N. & Greenfield, R. 2018. A multivariate examination of 'artificial mussels' in conjunction with spot water tests in freshwater ecosystems. *Environmental monitoring and assessment*, 190(7):427. doi:10.1007/s10661-018-6764-6.

Dahms-Verster, S., Nel, A., Van Vuren, J. & Greenfield, R. 2019. Biochemical responses revealed in an amphibian species after exposure to a forgotten contaminant: an integrated biomarker assessment. *Environmental toxicology and pharmacology*, 73:103272. doi:10.1016/j.etap.2019.103272

Dalton, J.P., Skelly, P. & Halton, D.W. 2004. Role of the tegument and gut in nutrient uptake by parasitic platyhelminths. *Canadian journal of zoology*, 82(2):211-232.

Damm, B. & Hagedorn, J. 2010. Holocene floodplain formation in the southern Cape region, South Africa. *Geomorphology*, 122(3-4):213-222.

DEAT (Department of Environmental Affairs and Tourism). 1996. Ndumo Game Reserve, South Africa: information sheet for the site designated to the List of Wetlands of International Importance in terms of the Convention on Wetlands of International Importance especially as Waterfowl Habitat. South African Wetlands Conservation Programme. Document No: 24/21/3/3/3/15. Pretoria.

De Buron, I., James, E., Riggs-Gelasco, P.J., Ringwood, A.H., Rolando, E. & Richardson, D. 2009. Overview of the status of heavy metal accumulation by helminths with a note on the use of in vitro culture of adult acanthocephalans to study the mechanisms of bioaccumulation. *Neotropical helminthology*, 3:101-110.

De Coen, W.M. & Janssen, C. 1997. The use of biomarkers in *Daphnia magna* toxicity testing: IV. Cellular energy allocation: a new methodology to assess the energy budget of toxicant-stressed *Daphnia* populations. *Journal of aquatic ecosystem stress and recovery*, 6(1):43-55.

De Coen, W.M. & Janssen, C.R. 2003. The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. *Environmental toxicology and chemistry: an international journal*, 22:1632-1641.

De Oliveira-Filho, E.C., Lopes, R.M. & Paumgartten, F.J.R. 2004. Comparative study on the susceptibility of freshwater species to copper-based pesticides. *Chemosphere*, 56:369-374.

Demirak, A., Yilmaz, F., Tuna, A.L. & Ozdemir, N. 2006. Heavy metals in water, sediment and tissues of *Leuciscus cephalus* from a stream in southwestern Turkey. *Chemosphere*, 63:1451-1458.

Dhanakumar, S., Solaraj, G. & Mohanraj, R. 2015. Heavy metal partitioning in sediments and bioaccumulation in commercial fish species of three major reservoirs of river Cauvery delta region, India. *Ecotoxicology and environmental safety*, 113:145-151.

Doherty, V., Ogunkuade, O. & Kanife, U. 2010. Biomarkers of oxidative stress and heavy metal levels as indicators of environmental pollution in some selected fishes in Lagos, Nigeria. *American-Eurasian journal of agricultural & environmental sciences*, 7(3):359-365.

Downs, C.A., Dillon, R.T., Jr., Fauth, J.E. & Woodley, C.M. 2001. A molecular biomarker system for assessing the health of gastropods (*Ilyanassa obsoleta*) exposed to natural and anthropogenic stressors. *Journal of experimental marine biology and ecology*, 259:189-214.

Du Preez, G. & Wepener, V. 2016. Influence of mining pollution on metal bioaccumulation and biomarker responses in cave dwelling fish, *Clarias gariepinus*. *Bulletin of environmental contamination and toxicology*, 97:18-23.

Dube, T., Wepener, V., Van Vuren, J., Smit, N. & Brendonck, L. 2015. The case for environmental flow determination for the Phongolo River, South Africa. *African journal of aquatic science*, 40(3):269-276.

Dudgeon, D., Arthington, A.H., Gessner, M.O., Kawabata, Z.-I., Knowler, D.J., Lévêque, C., Naiman, R.J., Prieur-Richard, A.-H., Soto, D. & Stiassny, M.L. 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. *Biological reviews*, 81(2):163-182.

Dudley, R.E., Gammal, L.M. & Klaassen, C.D. 1985. Cadmium-induced hepatic and renal injury in chronically exposed rats: likely role of hepatic cadmium-metallothionein in nephrotoxicity. *Toxicology and applied pharmacology*, 77(3):414-426.

DWAF (Department of Water Affairs and Forestry) 1996. South African water quality guidelines, vol. 7: Aquatic Ecosystems. Pretoria: The Government Printer.

Dykaar, B.B. & Wigington, P.J., Jr. 2000. Floodplain formation and cottonwood colonization patterns on the Willamette River, Oregon, USA. *Environmental management*, 25(1):87-104.

EC (European Commission). 2013. Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:226:0001:0017:EN:PDF Date of access: 29 Nov. 2019.

El-Moselhy, K.M., Othman, A., Abd El-Azem, H. & El-Metwally, M. 2014. Bioaccumulation of heavy metals in some tissues of fish in the Red Sea, Egypt. *Egyptian journal of basic and applied sciences*, 1(2):97-105.

Eneji, I.S., Sha'Ato, R. & Annune, P. 2011. Bioaccumulation of heavy metals in fish (*Tilapia zilli* and *Clarias gariepinus*) organs from River Benue, North-Central Nigeria. *Pakistan journal of analytical & environmental chemistry*, 12:25-31.

Erwin, K.L. 2009. Wetlands and global climate change: the role of wetland restoration in a changing world. *Wetlands ecology and management*, 17:71-84.

Evans, D., Irwin, S. & Fitzpatrick, S. 2001. The effect of digenean (Platyhelminthes) infections on heavy metal concentrations in *Littorina littorea*. *Journal of the Marine Biological Association of the United Kingdom*, 81:349-350.

Extence, C.A., Chadd, R.P, England, J., Dunbar, M.J., Wood, P.J. & Taylor, E.D. 2013. The assessment of fine sediment accumulation in rivers using macro-invertebrate community response. *River research and applications*, 29(1):17-55.

Ezat, M.A., Fritsch, C.J. & Downs, C.T. 2018. Use of an unmanned aerial vehicle (drone) to survey Nile crocodile populations: a case study at Lake Nyamithi, Ndumo game reserve, South Africa. *Biological conservation*, 223:76-81.

Farombi, E., Adelowo, O. & Ajimoko, Y. 2007. Biomarkers of oxidative stress and heavy metal levels as indicators of environmental pollution in African cat fish (*Clarias gariepinus*) from Nigeria Ogun River. *International journal of environmental research and public health*, 4(2):158-165.

Faust, B.C. 2018. A review of the photochemical redox reactions of iron (III) species in atmospheric, oceanic, and surface waters: influences on geochemical cycles and oxidant formation. (*In* Helz, G.R., Zepp, R.G. & Crosby, D.G., *eds.* Aquatic and surface photochemistry. Boca Raton: CRC Press. p. 3-38).

Fernandes, D., Zanuy, S., Bebianno, M.J. & Porte, C. 2008. Chemical and biochemical tools to assess pollution exposure in cultured fish. *Environmental pollution*, 152(1):138-146.

Fleischer, M., Sarofim, A. F., Fassett, D.W., Hammond, P., Shacklette, H.T., Nisbet, I.C. & Epstein, S. 1974. Environmental impact of cadmium: a review by the Panel on Hazardous Trace Substances. *Environmental health perspectives*, 7:253-323.

Flemming, C. & Trevors, J. 1989. Copper toxicity and chemistry in the environment: a review. *Water, air, and soil pollution*, 44(1-2):143-158.

Förstner, U. & Wittmann, G.T. 2012. Metal pollution in the aquatic environment. Berlin: Springer.

Gabrashanska, M. & Nedeva, I. 1996. Content of heavy metals in the system fish-cestodes. *Parassitologia*, 38:58.

Gabriel, U., Akinrotimi, O., Bekibele, D., Onunkwo, D. & Anyanwu, P. 2007. Locally produced fish feed: potentials for aquaculture development in subsaharan Africa. *African journal of agricultural research*, 2(7):287-295.

Gamal, A., Elsheikh, E. & Nasr, E. 2012. Morphological adaptation of the buccal cavity in relation to feeding habits of the omnivorous fish *Clarias gariepinus*: a scanning electron microscopic study. *The journal of basic & applied zoology*, 65:191-198.

Garbin, L., Diaz, J., Morgenthaler, A., Millones, A., Kuba, L., Fuchs, D. & Navone, G. 2019. Cormorant pellets as a tool for the knowledge of parasite-intermediate host associations and nematode diversity in the environment. *Helminthologia*, 56(4):296-302.

George, S.G. 1994. Enzymology and molecular biology of phase II xenobiotic-conjugating enzymes in fish. (*In* Malins, D.C. & Ostrander, G.K. *eds.* Aquatic toxicology: molecular, biochemical and cellular perspectives. Boca Raton: CRC Press. p. 37-85).

Gerber, R., Smit, N., Van Vuren, J.H., Ikenaka, Y. & Wepener, V. 2018. Biomarkers in tigerfish (*Hydrocynus vittatus*) as indicators of metal and organic pollution in ecologically sensitive subtropical rivers. *Ecotoxicology and environmental safety*, 157:307-317.

Gerber, R., Smit, N.J., Van Vuren, J.H. & Wepener, V. 2016. Metal concentrations in *Hydrocynus vittatus* (Castelnau 1861) populations from a premier conservation area: relationships with environmental concentrations. *Ecotoxicology and environmental safety*, 129:91-102.

Gioia, S.M., Babinski, M., Weiss, D.J. & Kerr, A.A. 2010. Insights into the dynamics and sources of atmospheric lead and particulate matter in São Paulo, Brazil, from high temporal resolution sampling. *Atmospheric research*, 98:478-485.

Gordon, A. & Muller, W. 2010. Developing sediment quality guidelines for South Africa. PHASE 1: Identification of international best practice and applications for South Africa to develop a research and implementation framework. Report to the Water Research Commission. http://www.wrc.org.za/wp-content/uploads/mdocs/KV%20242-10%20Conservation%20of%20Water%20Ecosystems.pdf Date of access: 29 Nov. 2019.

Grams, P.E. & Schmidt, J.C. 2002. Streamflow regulation and multi-level flood plain formation: channel narrowing on the aggrading Green River in the eastern Uinta Mountains, Colorado and Utah. *Geomorphology*, 44(3):337-360.

Gray, C., Baird, D.J., Baumgartner, S., Jacob, U., Jenkins, G.B., O'Gorman, E.J., Lu, X., Ma, A., Pocock, M.J. & Schuwirth, N. 2014. Ecological networks: the missing links in biomonitoring science. *Journal of applied ecology*, 51(5):1444-1449.

Greenfield, R., Van Vuren, J. & Wepener, V. 2012. Heavy metal concentrations in the water of the Nyl River system, South Africa. *African journal of aquatic science*, 37(2):219-224.

Grobler, D., Kempster, P. & Van der Merwe, L. 1994. A note on the occurrence of metals in the Olifants River, Eastern Transvaal, South Africa. *Water SA*, 20:195-203.

Gulelat, Y., Yimer, E., Asmare, K. & Bekele, J. 2013. Study on parasitic helminths infecting three fish species from Koka reservoir, Ethiopia. *SINET: Ethiopian journal of science*, 36(2):73-80.

Gunn, A.M., Winnard, D.A. & Hunt, D.T. 1988. Trace metal speciation in sediment and soils. (*In* Kramer, J.R. & Allen, H.E., *eds.* Metal speciation: theory, analysis and application. Boca Raton: Lewis Publications. p. 261-294).

Gupta, A., Rai, D.K., Pandey, R.S. & Sharma, B. 2009. Analysis of some heavy metals in the riverine water, sediments and fish from river Ganges at Allahabad. *Environmental monitoring and assessment*, 157(1-4):449-458.

Hamer, D.H. 1986. Metallothionein. *Annual review of biochemistry*, 55:913-951.

Handy, R., Sims, D., Giles, A., Campbell, H. & Musonda, M. 1999. Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout (*Oncorhynchus mykiss*) during chronic dietary exposure to copper. *Aquatic toxicology*, 47:23-41.

Hassan, A.H., Al-Zanbagi, N.A. & Al-Nabati, E.A. 2016. Impact of nematode helminthes on metal concentrations in the muscles of Koshar fish, Epinephelus summana, in Jeddah, Saudi Arabia. *Journal of basic & applied zoology*, 74:56-61.

Haybach, A., Schöll, F., König, B. & Kohmann, F. 2004. Use of biological traits for interpreting functional relationships in large rivers. *Limnologica*, 34(4):451-459.

Heath, A.G. 2018. Water pollution and fish physiology. Boca Raton: CRC press. doi.10.1201/9780203718896

Heathwaite, A. 2010. Multiple stressors on water availability at global to catchment scales: understanding human impact on nutrient cycles to protect water quality and water availability in the long term. *Freshwater biology*, 55(1):241-257.

Heberer, T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicology letters*, 131(1-2):5-17.

Heeg, J. & Breen, C.M. 1982. Man and the Pongolo floodplain. https://researchspace.csir.co.za/dspace/bitstream/handle/10204/2417/SANSP%20056.pdf?sequence=1&isAllowed=y Date of access: 30 Nov. 2019.

Heinonen, J., Kukkonen, J. & Holopainen, I. 2000. Toxicokinetics of 2, 4, 5-trichlorophenol and benzo (a) pyrene in the clam *Pisidium amnicum*: effects of seasonal temperatures and trematode parasites. *Archives of environmental contamination and toxicology*, 39(3):352-359.

Heinonen, J., Kukkonen, J.V. & Holopainen, I.J. 1999. The effects of parasites and temperature on the accumulation of xenobiotics in a freshwater clam. *Ecological applications*, 9(2):475-481.

Heinonen, J., Kukkonen, J.V. & Holopainen, I.J. 2001. Temperature-and parasite-induced changes in toxicity and lethal body burdens of pentachlorophenol in the freshwater clam Pisidium amnicum. *Environmental toxicology and chemistry: an international journal*, 20(12):2778-2784.

Huisman, E. & Richter, C. 1987. Reproduction, growth, health control and aquacultural potential of the African catfish, *Clarias gariepinus* (Burchell 1822). *Aquaculture*, 63(1-4):1-14.

Hursky, O. & Pietrock, M. 2015. Intestinal nematodes affect selenium bioaccumulation, oxidative stress biomarkers, and health parameters in juvenile rainbow trout (*Oncorhynchus mykiss*). *Environmental science & technology*, 49(4):2469-2476.

Ibrahim, A.T.A. & Omar, H.M. 2013. Seasonal variation of heavy metals accumulation in muscles of the African Catfish *Clarias gariepinus* and in River Nile water and sediments at Assiut Governorate, Egypt. *Journal of biology and earth sciences*, 3(2):B236-B248.

Ibrahim, M.M. 2006. Energy allocation patterns in *Biomphalaria alexandrina* snails in response to cadmium exposure and *Schistosoma mansoni* infection. *Experimental parasitology*, 112(1):31-36.

Islam, M.S., Ahmed, M.K., Raknuzzaman, M., Habibullah-Al-Mamun, M. & Islam, M.K. 2015. Heavy metal pollution in surface water and sediment: a preliminary assessment of an urban river in a developing country. *Ecological indicators*, 48:282-291.

Izvekova, G., Kuperman, B. & Kuz'mina, V. 1997. Digestion and digestive-transport surfaces in cestodes and their fish hosts. *Comparative biochemistry and physiology part A: physiology*, 118(4):1165-1171.

Jain, V., Fryirs, K. & Brierley, G. 2008. Where do floodplains begin? The role of total stream power and longitudinal profile form on floodplain initiation processes. *Geological Society of America bulletin*, 120(1-2):127-141.

Jankovská, I., Száková, J., Lukešová, D., Langrová, I., Válek, P., Vadlejch, J., Čadková, Z. & Petrtýl, M. 2012. Effect of lead in water on the absorption of copper, iron, manganese and zinc by sheep (*Ovis aries*) infected with sheep tapeworm (*Moniezia expansa*). *Experimental parasitology*, 131(1):52-56.

Javed, M. & Usmani, N. 2013. Assessment of heavy metal (Cu, Ni, Fe, Co, Mn, Cr, Zn) pollution in effluent dominated rivulet water and their effect on glycogen metabolism and histology of Mastacembelus armatus. *SpringerPlus*, 2(1):390. doi:10.1186/2193-1801-2-390

Jezierska, B. & Witeska, M. 2006. The metal uptake and accumulation in fish living in polluted waters. (*In* Twardowska, I., Allen, H.E., Häggblom, M.M. & Stefaniak, S. *eds.* Soil and water pollution monitoring, protection and remediation. Springer. p. 107-114). (NATO science series, 69).

Jiang, Z., Xu, N., Liu, B., Zhou, L., Wang, J., Wang, C., Dai, B. & Xiong, W. 2018. Metal concentrations and risk assessment in water, sediment and economic fish species with various habitat preferences and trophic guilds from Lake Caizi, Southeast China. *Ecotoxicology and environmental safety*, 157:1-8.

Jones, W. & Das, P. 1960. The mechanical properties of aluminium-lithium alloys. *Journal of the Institute of Metals*, 88:435-443.

Jooste, A., Marr, S.M., Addo-Bediako, A. & Luus-Powell, W.J. 2015. Sharptooth catfish shows its metal: a case study of metal contamination at two impoundments in the Olifants River, Limpopo river system, South Africa. *Ecotoxicology and environmental safety*, 112:96-104.

Juncos, R., Arcagni, M., Squadrone, S., Rizzo, A., Arribére, M., Barriga, J., Battini, M., Campbell, L., Brizio, P. & Abete, M. 2019. Interspecific differences in the bioaccumulation of arsenic of three Patagonian top predator fish: organ distribution and arsenic speciation. *Ecotoxicology and environmental safety*, 168:431-442.

Kadlec, R.H. & Wallace, S. 2008. Treatment wetlands. 2nd ed. Boca Raton: CRC Press.

Karadede, H. & Ünlü, E. 2000. Concentrations of some heavy metals in water, sediment and fish species from the Atatürk Dam Lake (Euphrates), Turkey. *Chemosphere*, 41:1371-1376.

Karr, J.R. 1999. Defining and measuring river health. *Freshwater biology*, 41:221-234.

Kennedy, C. 1997. Freshwater fish parasites and environmental quality: an overview and caution. *Parassitologia*, 39(3):249-254.

Khalil, M., Furness, D.N., Zholobenko, V. & Hoole, D. 2014. Effect of tapeworm parasitisation on cadmium toxicity in the bioindicator copepod, *Cyclops strenuus*. *Ecological indicators*, 37:21-26.

Kim, S.-K. & Kannan, K. 2007. Perfluorinated acids in air, rain, snow, surface runoff, and lakes: relative importance of pathways to contamination of urban lakes. *Environmental science & technology*, 41:8328-8334.

King, S.L., Sharitz, R.R., Groninger, J.W. & Battaglia, L.L. 2009. The ecology, restoration, and management of southeastern floodplain ecosystems: a synthesis. *Wetlands*, 29:624-634.

Kivaisi, A. K. 2001. The potential for constructed wetlands for wastewater treatment and reuse in developing countries: a review. *Ecological engineering*, 16(4):545-560.

Kramer, A. 2003. Managing freshwater ecosystems of international water resources: the case of the Maputo River in Mozambique (Working Paper on Management in Environmental Planning).

http://www.jav.tu-berlin.de/fileadmin/a0731/uploads/publikationen/workingpapers/wp00703.pdf Date of access: 30 Nov. 2019.

Kuklina, I., Kouba, A. & Kozák, P. 2013. Real-time monitoring of water quality using fish and crayfish as bio-indicators: a review. *Environmental monitoring and assessment*, 185(6):5043-5053.

Kumar, V., Sinha, A.K., Rodrigues, P.P., Mubiana, V.K., Blust, R. & De Boeck, G. 2015. Linking environmental heavy metal concentrations and salinity gradients with metal accumulation and their effects: a case study in 3 mussel species of Vitória estuary and Espírito Santo bay, Southeast Brazil. *Science of the total environment*, 523:1-15.

Lafferty, K.D. Dobson, A.P. & Kuris, A.M. 2006. Parasites dominate food web links. *Proceedings of the National Academy of Sciences of the United States of America*, 103(30):11211-11216.

Lafferty, K.D. & Kuris, A.M. 1999. How environmental stress affects the impacts of parasites. *Limnology and oceanography*, 44(3):925-931.

Lafferty, K., Shostak, A., Bush, A. & Lotz, J. 1997. Parasitology meets ecology on its own terms: Margolis et al. revisited. *The journal of parasitology*, 83(4):575-583.

Lamas, S., Fernández, J., Aboal, J. & Carballeira, A. 2007. Testing the use of juvenile *Salmo trutta* L. as biomonitors of heavy metal pollution in freshwater. *Chemosphere*, 67(2):221-228.

Leite, L.A., Pedro, N.H., De Azevedo, R.K., Kinoshita, A., Gennari, R.F., Watanabe, S. & Abdallah, V.D. 2017. *Contracaecum sp*. parasitizing *Acestrorhynchus lacustris* as a bioindicator for metal pollution in the Batalha River, southeast Brazil. *Science of the total environment*, 575:836-840.

Lemly, A.D. 2002. Selenium assessment in aquatic ecosystems: a guide for hazard evaluation and water quality criteria. New York: Springer. (Springer series on environmental management).

Lemly, A.D. 2004. Aquatic selenium pollution is a global environmental safety issue. *Ecotoxicology and environmental safety*, 59(1):44-56.

Li, L., Zheng, B. & Liu, L. 2010. Biomonitoring and bioindicators used for river ecosystems: definitions, approaches and trends. *Procedia environmental sciences*, 2:1510-1524.

Livingstone, D. 2003. Oxidative stress in aquatic organisms in relation to pollution and aquaculture. *Revue de médecine vétérinaire*, 154(6):427-430.

Lord, W.B. 1994. Flood hazard delineation: the one percent standard. *Water resources update*, 95(Spring):36-39.

Loucks, D.P. & Van Beek, E. 2017. Water resource systems planning and management: an introduction to methods, models, and applications. New York: Springer.

Louis, F., Devin, S., Giambérini, L., Potet, M., David, E. & Pain-Devin, S. 2019. Energy allocation in two dreissenid species under metal stress. *Environmental pollution*, 245:889-897.

Lucke, T. & Nichols, P.W. 2015. The pollution removal and stormwater reduction performance of street-side bioretention basins after ten years in operation. *Science of the total environment*, 536:784-792.

Macías-Zamora, J., Villaescusa-Celaya, J., Muñoz-Barbosa, A. & Gold-Bouchot, G. 1999. Trace metals in sediment cores from the Campeche shelf, Gulf of Mexico. *Environmental pollution*, 104(1):69-77.

MacKenzie, K. 1999. Parasites as pollution indicators in marine ecosystems: a proposed early warning system. *Marine pollution bulletin*, 38(11):955-959.

Madanire-Moyo, G. & Avenant-Oldewage, A. 2013. Occurrence of *Tetracampos ciliotheca* and *Proteocephalus glanduligerus* in *Clarias gariepinus* (Burchell, 1822) collected from the Vaal Dam, South Africa. *Onderstepoort journal of veterinary research*, 80(1):1-5.

Mansour, S. & Sidky, M. 2002. Ecotoxicological studies: 3. Heavy metals contaminating water and fish from Fayoum Governorate, Egypt. *Food chemistry*, 78(1):15-22.

Marchini, A., Munari, C. & Mistri, M. 2008. Functions and ecological status of eight Italian lagoons examined using biological traits analysis (BTA). *Marine pollution bulletin*, 56(6):1076-1085.

Marcogliese, D.J. 2005. Parasites of the superorganism: are they indicators of ecosystem health? *International journal for parasitology*, 35(7):705-716.

Marcogliese, D.J. & Pietrock, M. 2011. Combined effects of parasites and contaminants on animal health: parasites do matter. *Trends in parasitology*, 27(3):123-130.

Markert, B. 2007. Definitions and principles for bioindication and biomonitoring of trace metals in the environment. *Journal of trace elements in medicine and biology*, 21(S1):77-82.

Markert, B.A., Breure, A.M. & Zechmeister, H.G., *eds.* 2003. Bioindicators and biomonitors. Philadelphia: Elsevier. (Trace metals and other contaminants in the environment, 6).

Martin, C.W. 2000. Heavy metal trends in floodplain sediments and valley fill, River Lahn, Germany. *Catena*, 39(1):53-68.

Mashego, J. 1981. Observations on the prevalence of nematode parasites of the catfish, *Clarias gariepinus* (Burchell 1811), in Lebowa, South Africa. *South African journal of wildlife research*, 11(2):46-48.

Mashego, S. 1989. Nematode parasites of *Barbus* species in Lebowa and Venda, South Africa. *South African journal of wildlife research*, 19(1):35-37.

McCarthy, T.S. 2011. The impact of acid mine drainage in South Africa. *South African journal of science*, 107(5-6):1-7.

McCarthy, T. S., Tooth, S., Jacobs, Z., Rowberry, M.D., Thompson, M., Brandt, D., Hancox, P.J., Marren, P.M., Woodborne, S. & Ellery, W.N. 2011. The origin and development of the Nyl River floodplain wetland, Limpopo Province, South Africa: trunk-tributary river interactions in a dryland setting. *South African geographical journal*, 93(2):172-190.

McGeoch, M.A. & Chown, S.L. 1998. Scaling up the value of bioindicators. *Trends in ecology & evolution*, 13(2):46-47.

Meena, R.A.A., Sathishkumar, P., Ameen, F., Yusoff, A.R.M. & Gu, F.L. 2018. Heavy metal pollution in immobile and mobile components of lentic ecosystems: a review. *Environmental science and pollution research*, 25(5):4134-4148.

Miikue-Yobe, T. & Ibara, U. 2019. Determination of Pb, Hg, Cd, Cu, Ni and V in Periwinkle (*Tympanotonus fuscatus*), Crab (*Callinectes pali*) and Fish (*Rhodeus amarus*) from Bodo River. *International journal of pure & applied bioscience*, 7:14-21.

Mijošek, T., Marijić, V.F., Dragun, Z., Krasnići, N., Ivanković, D. & Erk, M. 2019. Evaluation of multi-biomarker response in fish intestine as an initial indication of anthropogenic impact in the aquatic karst environment. *Science of the total environment*, 660:1079-1090.

Mohan, M.S., Zingaro, R.A., Micks, P. & Clark, P.J. 1982. Analysis and speciation of arsenic in herbicide-treated soils by DC helium emission spectrometry. *International journal of environmental analytical chemistry*, 11:175-187.

Moolman, L., Van Vuren, J. & Wepener, V. 2007. Comparative studies on the uptake and effects of cadmium and zinc on the cellular energy allocation of two freshwater gastropods. *Ecotoxicology and environmental safety*, 68(3):443-450.

Moravec, F. 1975. The development of *Procamallanus laeviconchus* (Wedl, 1862) (Nematoda: Camallanidae). *Acta Societatis Zoologicae Bohemoslovacae*, 39:23-38.

Morley, N. 2010. Interactive effects of infectious diseases and pollution in aquatic molluscs. *Aquatic toxicology*, 96(1):27-36.

Morley, N., Lewis, J. & Hoole, D. 2006. Pollutant-induced effects on immunological and physiological interactions in aquatic host-trematode systems: implications for parasite transmission. *Journal of helminthology*, 80(2):137-149.

Morris, T., Avenant-Oldewage, A., Lamberth, S. & Reed, C. 2016. Shark parasites as bio-indicators of metals in two South African embayments. *Marine pollution bulletin*, 104(1-2):221-228.

Mucha, A.P., Vasconcelos, M.T.S. & Bordalo, A.A. 2003. Macrobenthic community in the Douro estuary: relations with trace metals and natural sediment characteristics. *Environmental pollution*, 121(2):169-180.

Mudd, G.M. 2010. Global trends and environmental issues in nickel mining: sulfides versus laterites. *Ore geology reviews*, 38(1-2):9-26.

Murtala, B.A., Abdul, W.O. & Akinyemi, A.A. 2012. Bioaccumulation of heavy metals in fish (*Hydrocynus forskahlii, Hyperopisus bebe occidentalis* and *Clarias gariepinus*) organs in downstream Ogun coastal water, Nigeria. *Journal of agricultural science*, 4(11):51-59.

Musa, R., Gerber, R. & Greenfield, R. 2017. A multivariate analysis of metal concentrations in two fish species of the Nyl River System, Limpopo Province, South Africa. *Bulletin of environmental contamination and toxicology*, 98(6):817-823.

Ng, J.C., Wang, J. & Shraim, A. 2003. A global health problem caused by arsenic from natural sources. *Chemosphere*, 52(9):1353-1359.

NRC (National Research Council). 1987. Biological markers in environmental health research. *Environmental health perspectives*, 74:3-9. doi:10.1289/ehp.74-1474499 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1474499/pdf/envhper00433-0008.pdf Date of access: 29 Nov. 2019.

Nunes, A.L., Zengeya, T.A., Hoffman, A.C., Measey, G.J. & Weyl, O.L. 2017. Distribution and establishment of the alien Australian redclaw crayfish, *Cherax quadricarinatus*, in South Africa and Swaziland. *PeerJ*, 5:e3135. doi:10.7717/peerj.3135.

Oké, V. & Goosen, N.J. 2019. The effect of stocking density on profitability of African catfish (*Clarias gariepinus*) culture in extensive pond systems. *Aquaculture*, 507:385-392.

Olufemi, B., Akinlabi, D. & Agbede, S. 1991. Aerobic bacterial pathogens isolated from the African catfish *Clarias gariepinus*. *Tropical veterinarian*, 9:177-180.

Omacini, M., Chaneton, E.J., Ghersa, C.M. & Müller, C.B. 2001. Symbiotic fungal endophytes control insect host-parasite interaction webs. *Nature*, 409(6816):78-81.

Orr, P.L., Guiguer, K.R. & Russel, C.K. 2006. Food chain transfer of selenium in lentic and lotic habitats of a western Canadian watershed. *Ecotoxicology and environmental safety*, 63(2):175-188.

Osman, A., Wang, C.K., Winter, A., Loukas, A., Tribolet, L., Gasser, R.B. & Hofmann, A. 2012. Hookworm SCP/TAPS protein structure: a key to understanding host-parasite interactions and developing new interventions. *Biotechnology advances*, 30(3):652-657.

Osman, A.G. & Kloas, W. 2010. Water quality and heavy metal monitoring in water, sediments, and tissues of the African Catfish *Clarias gariepinus* (Burchell, 1822) from the River Nile, Egypt. *Journal of environmental protection*, 1(4):389-400.

Ostroumov, S. 1998. Biological filtering and ecological machinery for self-purification and bioremediation in aquatic ecosystems: towards a holistic view. *Rivista di biologia*, 91(2):221-232.

Otachi, E.O., Körner, W., Avenant-Oldewage, A., Fellner-Frank, C. & Jirsa, F. 2014. Trace elements in sediments, blue spotted tilapia *Oreochromis leucostictus* (Trewavas, 1933) and its parasite *Contracaecum multipapillatum* from Lake Naivasha, Kenya, including a comprehensive health risk analysis. *Environmental science and pollution research*, 21:7339-7349.

Otitoloju, A. & Olagoke, O. 2011. Lipid peroxidation and antioxidant defense enzymes in *Clarias gariepinus* as useful biomarkers for monitoring exposure to polycyclic aromatic hydrocarbons. *Environmental monitoring and assessment*, 182(1-4):205-213.

Oyoo-Okoth, E., Admiraal, W., Osano, O., Hoitinga, L. & Kraak, M.H. 2010. Metal specific partitioning in a parasite-host assemblage of the cestode *Ligula intestinalis* and the cyprinid fish *Rastrineobola argentea*. *Science of the total environment*, 408:1557-1562.

Oyoo-Okoth, E., Admiraal, W., Osano, O., Kraak, M.H., Gichuki, J. & Ogwai, C. 2012. Parasites modify sub-cellular partitioning of metals in the gut of fish. *Aquatic toxicology*, 106:76-84.

Oyoo-Okoth, E., Wim, A., Osano, O., Kraak, M.H., Ngure, V., Makwali, J. & Orina, P.S. 2010. Use of the fish endoparasite *Ligula intestinalis* (L., 1758) in an intermediate cyprinid host (*Rastreneobola argentea*) for biomonitoring heavy metal contamination in Lake Victoria, Kenya. *Lakes & reservoirs: research & management*, 15(1):63-73.

Palma, P., Ledo, L. & Alvarenga, P. 2015. Assessment of trace element pollution and its environmental risk to freshwater sediments influenced by anthropogenic contributions: the case study of Alqueva reservoir (Guadiana Basin). *Catena*, 128:174-184. doi.10.1016/j.catena.2015.02.002

Pekey, H. 2006. Heavy metal pollution assessment in sediments of the Izmit Bay, Turkey. *Environmental monitoring and assessment*, 123(1-3):219-231.

Pickering, Q.H. 1974. Chronic toxicity of nickel to the fathead minnow. *Journal (Water Pollution Control Federation)*, 46:760-765.

Pietrock, M. & Marcogliese, D.J. 2003. Free-living endohelminth stages: at the mercy of environmental conditions. *Trends in parasitology*, 19(7):293-299.

Pinedo-Hernández, J., Marrugo-Negrete, J. & Díez, S. 2015. Speciation and bioavailability of mercury in sediments impacted by gold mining in Colombia. *Chemosphere*, 119:1289-1295.

Pooley, A. 1982. The status of African crocodiles in 1980. (*In* Crocodiles. Proceedings of the 5th Working Meeting of the IUCN/SSC Crocodile Specialist Group, Gainesville, Florida. Gland, Switzerland: IUCN Publications. p. 174-228).

Power, M.E., Parker, G., Dietrich, W.E. & Sun, A. 1995. How does floodplain width affect floodplain river ecology? A preliminary exploration using simulations. *Geomorphology*, 13:301-317.

Pracheil, B.M., Adams, S.M., Bevelhimer, M.S., Fortner, A.M., Greeley, M.S., Murphy, C.A., Mathews, T.J. & Peterson, M.J. 2016. Relating fish health and reproductive metrics to contaminant bioaccumulation at the Tennessee Valley Authority Kingston coal ash spill site. *Ecotoxicology*, 25:1136-1149.

Prawiranegara, M. 2014. Spatial multi-criteria analysis (SMCA) for basin-wide flood risk assessment as a tool in improving spatial planning and urban resilience policy making: a case study of Marikina river basin, metro Manila-Philippines. *Procedia-Social and behavioral sciences*, 135:18-24.

Rai, P.K. 2008. Heavy metal pollution in aquatic ecosystems and its phytoremediation using wetland plants: an ecosustainable approach. *International journal of phytoremediation*, 10(2):133-160.

Rainbow, P.S. 1998. Phylogeny of trace metal accumulation in crustaceans. (*In* Langston, W.J. & Bebianno, M.J., *eds.* Metal metabolism in aquatic environments. Berlin: Springer. p. 285-319). (Ecotoxicology series, 7).

Rasouli, M., Ostovar-Ravari, A. & Shokri-Afra, H. 2014. Characterization and improvement of phenol-sulfuric acid microassay for glucose-based glycogen. *European review for medical and pharmacological sciences*, 18(14):2020-2024.

Rasouli, M., Shokri-Afra, H. & Ostovar-Ravari, A. 2015. A new protocol for separation of acid soluble and insoluble fractions from total glycogen and simultaneous measurements. *European review for medical and pharmacological sciences*, 19(10):1785-1789.

Ribeiro, C.O., Vollaire, Y., Sanchez-Chardi, A. & Roche, H. 2005. Bioaccumulation and the effects of organochlorine pesticides, PAH and heavy metals in the Eel (*Anguilla anguilla*) at the Camargue Nature Reserve, France. *Aquatic toxicology*, 74(1):53-69.

Ritterhoff, J. & Zauke, G.-P. 1998. Potential role of metal-binding proteins in cadmium detoxification in *Themisto libellula* (Mandt) and *Themisto abyssorum* Boeck from the Greenland Sea. *Marine environmental research*, 45(2):179-191.

Robar, N., Murray, D.L. & Burness, G. 2011. Effects of parasites on host energy expenditure: the resting metabolic rate stalemate. *Canadian journal of zoology*, 89(11):1146-1155.

Roesijadi, G. & Fowler, B. 1991. Purification of invertebrate metallothioneins. *Methods in enzymology*, 205:263-273.

Romano, M.P. 1964. The water resources of the Maputo (Southern Mozambique). *South African journal of science*, 60(8):245-251.

Ruus, A., Skaare, J. & Ingebrigtsen, K. 2001. Accumulation of the lipophilic environmental contaminant lindane in metacercariae of *Bucephaloides gracilescens* (Trematoda, Bucephalidae) in the central nervous system of bullrout *Myoxocephalus scorpius*. *Diseases of aquatic organisms*, 48(1):75-77.

Saliu, J.K. & Bawa-Allah, K.A. 2012. Toxicological effects of lead and zinc on the antioxidant enzyme activities of post juvenile *Clarias gariepinus*. *Resources and environment*, 2(1):21-26.

Sanchez, M.I., Pons, I., Martinez-Haro, M., Taggart, M.A., Lenormand, T. & Green, A.J. 2016. When parasites are good for health: cestode parasitism increases resistance to arsenic in brine shrimps. *PLoS pathogens*, 12(3):e1005459.

Sancho, E., Ferrando, M. & Andreu, E. 1998. Effects of sublethal exposure to a pesticide on levels of energetic compounds in *Anguilla anguilla*. *Journal of environmental science & health. Part B. Pesticides, food contaminants, and agricultural wastes*, 33(4):411-424.

Satarug, S., Baker, J.R., Urbenjapol, S., Haswell-Elkins, M., Reilly, P.E., Williams, D.J. & Moore, M.R. 2003. A global perspective on cadmium pollution and toxicity in non-occupationally exposed population. *Toxicology letters*, 137:65-83.

Sauliutė, G. & Svecevičius, G. 2015. Heavy metal interactions during accumulation via direct route in fish: a review. *Zoology and ecology*, 25:77-86.

Schramm, M., Muller, E. & Triebskorn, R. 1998. Brown trout *Salmo trutta f. Fario* liver ultrastructure as a biomarker for assessment of small stream pollution. *Biomarkers*, 3(2):93-108.

Seixas, T.G., Kehrig, H.D. A., Fillmann, G., Di Beneditto, A.P.M., Souza, C.M., Secchi, E.R., Moreira, I. & Malm, O. 2007. Ecological and biological determinants of trace elements accumulation in liver and kidney of *Pontoporia blainvillei*. *Science of the total environment*, 385(1-3):208-220.

Sevcikova, M., Modra, H., Slaninova, A. & Svobodova, Z. 2011. Metals as a cause of oxidative stress in fish: a review. *Veterinární medicína*, 56(11):537-546.

Shaw, M.R. & Hochberg, M.E. 2001. The neglect of parasitic Hymenoptera in insect conservation strategies: the British fauna as a prime example. *Journal of Insect Conservation*, 5:253-263.

Shrivastava, A. 2009. A review on copper pollution and its removal from water bodies by pollution control technologies. *Indian journal of environmental protection*, 29(6):552-560.

Shugart, L.R., McCarthy, J.F. & Halbrook, R.S. 1992. Biological markers of environmental and ecological contamination: an overview. *Risk analysis*, 12:353-360.

Sibley, L. 2004. Intracellular parasite invasion strategies. *Science*, 304(5668):248-253.

Simpson, S.L., Pryor, I.D., Mewburn, B.R., Batley, G.E. & Jolley, D. 2002. Considerations for capping metal-contaminated sediments in dynamic estuarine environments. *Environmental science & technology*, 36(17):3772-3778.

Sindermann, C.J. 1987. Effects of parasites on fish populations: practical considerations. *International journal for parasitology*, 17(2):371-382.

Skelton, P.H. 2012. A complete guide to the freshwater fishes of Southern Africa. 2nd ed. South Africa: Penguin Random House.

Smit, N., Vlok, W., Van Vuren, J.H. J., Du Preez, L., Van Eeden, E. S., O'Brien, G. & Wepener, V. 2016. Socio-ecological system management of the lower Phongolo river and floodplain using relative risk methodology: report to the Water Research Commission. Gezina, South Africa: Water Research Commission.

Smolders, R., Bervoets, L., De Coen, W. & Blust, R. 2004. Cellular energy allocation in zebra mussels exposed along a pollution gradient: linking cellular effects to higher levels of biological organization. *Environmental pollution*, 129(1):99-112.

Smolders, R., De Boeck, G. & Blust, R. 2003. Changes in cellular energy budget as a measure of whole effluent toxicity in zebrafish (*Danio rerio*). *Environmental toxicology and chemistry*, 22(4):890-899.

Steffen, W., Crutzen, P.J. & McNeill, J.R. 2007. The Anthropocene: are humans now overwhelming the great forces of nature? *AMBIO: A journal of the human environment*, 36(8):614-622.

Stegeman, J.J., Brower, M., Di Giulio, R.T., Förlin, L., Fowler, B.A., Sanders, B.M. & Van Veld, P.A. 1992. Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. (*In* Huggett, R.J., Kimerle, R.A., Mehrle, P.P., Jr. & Bergman, H.L. *eds.* Biomarkers: biochemical, physiological, and histological markers of anthropogenic stress. Boca Raton, FL: CRC Press. p. 235-335).

Stephenson, D. 2002. Integrated flood plain management strategy for the Vaal. *Urban water*, 4(4):423-428.

Strimbu, K. & Tavel, J.A. 2010. What are biomarkers? *Current opinion in HIV and AIDS*, 5(6):463-466.

Stuart, C. & Stuart, M. 2018. Stuart's Field Guide to National Parks & Nature Reserves of South Africa. 2nd ed. Cape Town: Struik Nature,

Sunjog, K., Kolarević, S., Kračun-Kolarević, M., Višnjić-Jeftić, Ž., Gačić, Z., Lenhardt, M. & Vuković-Gačić, B. 2019. Seasonal variation in metal concentration in various tissues of the European chub (*Squalius cephalus L.*). *Environmental science and pollution research*, 26:9232-9243.

Sures, B. 1996. Untersuchungen zur Schwermetallakkumulation von Helminthen im Vergleich zu ihren aquatischen Wirten ["Studies on heavy metal accumulation in helminths as compared with their aquatic hosts"]. Karlsruhe: Universität Karlsruhe. (Dissertation – PhD).

Sures, B. 2001. The use of fish parasites as bioindicators of heavy metals in aquatic ecosystems: a review. *Aquatic ecology*, 35(2):245-255.

Sures, B. 2002. Competition for minerals between *Acanthocephalus lucii* and its definitive host perch (*Perca fluviatilis*). *International journal for parasitology*, 32(9):1117-1122.

Sures, B. 2003. Accumulation of heavy metals by intestinal helminths in fish: an overview and perspective. *Parasitology*, 126(7):S53-S60.

Sures, B. 2004. Environmental parasitology: relevancy of parasites in monitoring environmental pollution. *Trends in parasitology*, 20(4):170-177.

Sures, B. 2006. How parasitism and pollution affect the physiological homeostasis of aquatic hosts. *Journal of helminthology*, 80(2):151-157.

Sures, B. 2008. Environmental parasitology: interactions between parasites and pollutants in the aquatic environment. *Parasite*, 15(3):434-438.

Sures, B., Nachev, M., Selbach, C. & Marcogliese, D.J. 2017. Parasite responses to pollution: what we know and where we go in 'Environmental Parasitology'. *Parasites & vectors*, 10(1):65. doi:10.1186/s13071-017-2001-3.

Sures, B., Ruchter, N. & Zimmermann, S. 2015. Biological effects of PGE on aquatic organisms. (*In* Zereini, F. & Wiseman, C.L.S. *eds.* Platinum metals in the environment. Berlin: Springer. p. 383-400).

Sures, B. & Siddall, R. 1999. Pomphorhynchus laevis: the intestinal acanthocephalan as a lead sink for its fish host, chub (*Leuciscus cephalus*). *Experimental parasitology*, 93(2):66-72.

Sures, B., Steiner, W., Rydlo, M. & Taraschewski, H. 1999. Concentrations of 17 elements in the zebra mussel (*Dreissena polymorpha*), in different tissues of perch (*Perca fluviatilis*), and in perch intestinal parasites (*Acanthocephalus lucii*) from the subalpine lake Mondsee, Austria. *Environmental toxicology and chemistry*, 18(11):2574-2579.

Sures, B., Taraschewski, H. & Rokicki, J. 1997. Lead and cadmium content of two cestodes, *Monobothrium wageneri* and *Bothriocephalus scorpii*, and their fish hosts. *Parasitology research*, 83(6):618-623.

Sutherland, R. 2000. Depth variation in copper, lead, and zinc concentrations and mass enrichment ratios in soils of an urban watershed. *Journal of environmental quality*, 29:1414-1422.

Svitin, R., Truter, M., Kudlai, O., Smit, N. J. & Du Preez, L. 2019. Novel information on the morphology, phylogeny and distribution of camallanid nematodes from marine and freshwater hosts in South Africa, including the description of *Camallanus sodwanaensis* n. sp. *International journal for parasitology: parasites and wildlife*, 10:263-273.

Szefer, P., Rokicki, J., Frelek, K., Skóra, K. & Malinga, M. 1998. Bioaccumulation of selected trace elements in lung nematodes, *Pseudalius inflexus*, of harbor porpoise (*Phocoena phocoena*) in a Polish zone of the Baltic Sea. *Science of the total environment*, 220(1):19-24.

Taylor, M.P. & Lewin, J. 1996. River behaviour and Holocene alluviation: the River Severn at Welshpool, mid-Wales, UK. *Earth surface processes and landforms*, 21:77-91.

Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K. & Sutton, D.J. 2012. Heavy metal toxicity and the environment. *Molecular, clinical and environmental toxicology*, 101:133-164. doi.10.1007/978-3-7643-8340-4_6

Tendaupenyu, P. & Magadza, C.H. 2019. Enrichment and geoaccumulation of metals in the superficial sediments of Lake Chivero, Zimbabwe. *Lakes & reservoirs: research & management*, 24:275-286. doi.10.1111/lre.12282

Ter Braak, C.J. & Smilauer, P. 2012. Canoco reference manual and user's guide: software for ordination, version 5.0. https://library.wur.nl/WebQuery/wurpubs/431861 Date of access: 30 Nov. 2019.

Terra, B.F., Araújo, F.G., Calza, C. F., Lopes, R.T. & Teixeira, T.P. 2008. Heavy metal in tissues of three fish species from different trophic levels in a tropical Brazilian river. *Water, air, and soil pollution*, 187(1-4):275-284.

Thiele, D.J. 1992. Metal-regulated transcription in eukaryotes. *Nucleic acids research*, 20(6):1183.

Thielen, F., Zimmermann, S., Baska, F., Taraschewski, H. & Sures, B. 2004. The intestinal parasite Pomphorhynchus laevis (Acanthocephala) from barbel as a bioindicator for metal pollution in the Danube River near Budapest, Hungary. *Environmental pollution*, 129:421-429.

Thomann, R.V. 1989. Bioaccumulation model of organic chemical distribution in aquatic food chains. *Environmental science & technology*, 23(6):699-707.

Thornton, J., McComb, A. & Ryding, S. 1995. The role of sediments. (*In* McComb, A. *ed.* Eutrophic shallow estuaries and lagoons. Boca Raton: CRC Press. p. 205-233).

Tilzer, M. & Khondker, M. 1993. Hypertrophic and polluted freshwater ecosystems: ecological basis for water resource management. (*In* Proceedings of an international symposium on limnology held in Dhaka, Bangladesh, 25- 28 November 1991. Bangladesh: Dept. of Botany, Dhaka University).

Tria, J., Butler, E.C., Haddad, P.R. & Bowie, A.R. 2007. Determination of aluminium in natural water samples. *Analytica chimica acta*, 588:153-165.

Trombulak, S.C. & Frissell, C.A. 2000. Review of ecological effects of roads on terrestrial and aquatic communities. *Conservation biology*, 14:18-30.

UNESCO (United Nations Educational, Scientific and Cultural Organization). 1998. World water resources: a new appraisal and assessment for the 21st century: a summary of the monograph World water resources. Milton-Keyes, UK: RPL Design. https://unesdoc.unesco.org/ark:/48223/pf0000112671 Date of access: 30 Nov. 2019.

Valavanidis, A., Vlahogianni, T., Dassenakis, M. & Scoullos, M. 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and environmental safety*, 64(2):178-189.

Van der Oost, R., Beyer, J. & Vermeulen, N.P. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental toxicology and pharmacology*, 13:57-149.

Vannote, R.L., Minshall, G.W., Cummins, K.W., Sedell, J.R. & Cushing, C.E. 1980. The river continuum concept. *Canadian journal of fisheries and aquatic sciences*, 37:130-137.

Vázquez, D.P., Poulin, R., Krasnov, B.R. & Shenbrot, G.I. 2005. Species abundance and the distribution of specialization in host–parasite interaction networks. *Journal of animal ecology*, 74:946-955.

Vedolin, M., Teophilo, C., Turra, A. & Figueira, R. 2018. Spatial variability in the concentrations of metals in beached microplastics. *Marine pollution bulletin*, 129(2):487-493.

Venugopal, B. & Luckey, T.D. 1978. Metal toxicity in mammals, vol. 2: chemical toxicity of metals and metalloids. New York: Plenum Press.

Viarengo, A., Burlando, B., Dondero, F., Marro, A. & Fabbri, R. 1999. Metallothionein as a tool in biomonitoring programmes. *Biomarkers*, 4(6):455-466.

Viarengo, A., Ponzano, E., Dondero, F. & Fabbri, R. 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. *Marine environmental research*, 44(1):69-84.

Vidal-Martinez, V.M., Pech, D., Sures, B., Purucker, S.T. & Poulin, R. 2010. Can parasites really reveal environmental impact? *Trends in parasitology*, 26(1):44-51.

Vinodhini, R. & Narayanan, M. 2008. Bioaccumulation of heavy metals in organs of fresh water fish *Cyprinus carpio* (Common carp). *International journal of environmental science & technology*, 5(2):179-182.

Vörösmarty, C.J., McIntyre, P.B., Gessner, M.O., Dudgeon, D., Prusevich, A., Green, P., Glidden, S., Bunn, S.E., Sullivan, C.A., Liermann, C.R. & Davies, P.M. 2010. Global threats to human water security and river biodiversity. *Nature*, 467(7315):555-561.

Walkey, M. & Meakins, R. 1970. An attempt to balance the energy budget of a host-parasite system. *Journal of fish biology*, 2(4):361-372.

Wang, X., Wang, L., Yao, C., Qiu, L., Zhang, H., Zhi, Z. & Song, L. 2012. Alternation of immune parameters and cellular energy allocation of *Chlamys farreri* under ammonia-N exposure and *Vibrio anguillarum* challenge. *Fish & shellfish immunology*, 32(5):741-749.

Wepener, V.V., Van Dyk, C., Bervoets, L., O'Brien, G., Covaci, A. & Cloete, Y. 2011. An assessment of the influence of multiple stressors on the Vaal River, South Africa. *Physics and chemistry of the earth*, 36:949-962.

Whittington, M., Malan, G. & Panagos, M.D. 2013. Trends in waterbird diversity at Banzi, Shokwe and Nyamithi pans, Ndumo Game Reserve, South Africa. *Ostrich*, 84(1):47-61.

WHO (World Health Organization). 2017. Guidelines for drinking-water quality. 4th ed. https://apps.who.int/iris/bitstream/handle/10665/254637/9789241549950-eng.pdf?sequence=1 Date of access: 29 Nov. 2019.

Williams, E.H., Jr. & Bunkley-Williams, L. 2019. Life cycle and life history strategies of parasitic Crustacea. (*In* Smit, N.J., Bruce, N.L. & Hadfield, K.A., *eds.* Parasitic Crustacea. Berlin: Springer. p. 179-266).

Wright, D.A. & Zamuda, C.D. 1987. Copper accumulation by two bivalve molluscs: salinity effect is independent of cupric ion activity. *Marine environmental research*, 23(1):1-14.

Yang, D., Kanae, S., Oki, T., Koike, T. & Musiake, K. 2003. Global potential soil erosion with reference to land use and climate changes. *Hydrological processes*, 17(14):2913-2928.

Yi, Y., Yang, Z. & Zhang, S. 2011. Ecological risk assessment of heavy metals in sediment and human health risk assessment of heavy metals in fishes in the middle and lower reaches of the Yangtze River basin. *Environmental pollution*, 159(10):2575-2585.

Yu, K.-C., Tsai, L.-J., Chen, S.-H. & Ho, S.-T. 2001. Correlation analyses on binding behavior of heavy metals with sediment matrices. *Water research*, 35(10):2417-2428.

Zadnipryany, I.Z., Tretiakova, O.T. & Zukow, W. 2017. Experimental review of cobalt induced cardiomyopathy. *Russian open medical journal*, 6(1). doi:10.15275/rusomj.2017.0103

Zhang, G., Bai, J., Xiao, R., Zhao, Q., Jia, J., Cui, B. & Liu, X. 2017. Heavy metal fractions and ecological risk assessment in sediments from urban, rural and reclamation-affected rivers of the Pearl River Estuary, China. *Chemosphere*, 184:278-288.

Zhou, Q., Zhang, J., Fu, J., Shi, J. & Jiang, G. 2008. Biomonitoring: an appealing tool for assessment of metal pollution in the aquatic ecosystem. *Analytica chimica acta*, 606(2):135-150.

Appendices

Appendix 1: Usuthu River host biometric data as well as parasite infection numbers

Species	Sex	Total Mass	Gutted mass	Total length	STD length	*Contracaecum* sp.	*P. pseudolaeviconchus*	*T. ciliotheca*	*Pa. cyathopgharynx*	*Prote*
US 01	M	680.00	580.00	450.00	410.00	9	0	3	1	
US 02	M	1250.00	1200.00	560.00	500.00	10	2	0	10	
US 03	M	870.00	800.00	500.00	455.00	3	0	1	0	
US 04	M	330.00	305.00	335.00	287.00	0	1	1	4	
US 05	M	260.00	230.00	350.00	310.00	0	0	0	7	
US 06	F	470.00	430.00	415.00	370.00	0	3	0	2	
US 07	Juv	70.00	60.00	209.00	178.00	0	1	0	1	
US 08	M	220.00	210.00	330.00	300.00	0	0	0	7	
US 09	M	640.00	610.00	447.00	405.00	5	0	0	0	
US 010	F	1700.00	1390.00	654.00	578.00	2	0	0	10	
US 011	F	310.00	200.00	335.00	320.00	0	0	0	1	
US 012	M	1060.00	983.00	531.00	470.00	1	0	1	4	
US 013	F	1290.00	950.00	540.00	477.00	8	0	0	12	
US 014	F	1140.00	910.00	540.00	470.00	1	0	2	17	
US 015	M	701.00	650.00	435.00	385.00	1	0	0	0	
US 016	M	570.00	550.00	460.00	415.00	0	0	1	7	
US 017	F	850.00	540.00	480.00	435.00	5	1	0	7	
US 018	F	870.00	640.00	455.00	390.00	3	0	1	0	
US 019	F	940.00	880.00	555.00	445.00	0	3	1	3	
US 020	M	600.00	460.00	415.00	365.00	0	0	0	0	
US 021	F	290.00	250.00	360.00	320.00	0	0	0	7	
US 022	M	560.00	520.00	445.00	390.00	0	0	2	5	
US 023	F	1370.00	1070.00	580.00	520.00	3	0	5	13	
US 024	M	570.00	490.00	435.00	375.00	0	0	2	1	

Appendix 2: Shokwe Pans host biometric data and parasite infection numbers

Species	Sex	Total Mass	Gutted mass	Total length	STD length	*Contracaecum* sp.	*P. pseudolaeviconchus*	*T. ciliotheca*	*Pa. cyathopgharynx*	*Prote*
SH 2(1)	F	2120.00	1950.00	640.00	565.00	43	8	2	30	
SH 2(2)	F	3840.00	3500.00	775.00	705.00	25	5	5	1	
SH 2(3)	F	630.00	600.00	441.00	395.00	12	0	0	0	
SH 2(4)	M	150.00	140.00	305.00	270.00	2	0	0	2	
SH 2(5)	F	740.00	700.00	490.00	432.00	3	10	10	1	
SH 2(6)	F	1270.00	1200.00	590.00	530.00	12	3	3	2	
SH 2(7)	F	230.00	210.00	313.00	278.00	2	0	0	4	
SH 2(8)	M	160.00	150.00	298.00	265.00	0	1	1	3	
SH 2(9)	F	1150.00	990.00	560.00	495.00	19	3	3	6	

SH 2(10)	M	230.00	220.00	335.00	305.00	2	0	0	1	
SH 2(11)	F	240.00	220.00	320.00	282.00	0	0	0	0	
SH 2(12)	F	530.00	470.00	430.00	380.00	6	2	2	3	
SH 2(13)	F	640.00	610.00	460.00	430.00	17	5	5	1	
SH 2(14)	F	200.00	140.00	323.00	300.00	0	1	1	2	
SH 2(15)	F	100.00	90.00	290.00	255.00	0	0	0	3	
SH 2(16)	F	1030.00	920.00	529.00	465.00	16	0	0	12	
SH 2(17)	F	2250.00	2120.00	680.00	605.00	0	3	3	59	
SH 2(18)	F	1160.00	910.00	576.00	495.00	18	0	0	13	
SH 2(19)	F	700.00	590.00	468.00	425.00	15	1	1	1	
SH 2(20)	F	320.00	270.00	352.00	315.00	1	0	0	5	
SH 2(21)	M	870.00	720.00	535.00	465.00	0	0	0	3	
SH 2(22)	F	350.00	320.00	375.00	335.00	3	6	6	9	
SH 2(23)	F	810.00	670.00	470.00	415.00	1	2	2	1	
SH 2(24)	M	380.00	360.00	390.00	345.00	0	2	2	8	
SH 2(25)	F	670.00	600.00	460.00	400.00	8	0	0	2	

Appendix 3: Nyamithi Pans host biometric data and parasite infection numbers:

Species	**Sex**	**Total Mass**	**Gutted mass**	**Total length**	**STD length**	***Contracaecum* sp.**	***P. pseudolaeviconchus***	***T. ciliotheca***	***Pa. cyathopgharynx***	***Proteocephalus* sp.**
NY 1	M	1920.00	1650.00	640.00	580.00	186	6	6	0	
NY 2	F	2070.00	1670.00	645.00	585.00	499	1	1	1	
NY 3	M	3050.00	2360.00	710.00	625.00	378	3	3	0	
NY 4	M	2230.00	2000.00	665.00	605.00	227	5	5	0	
NY 5	M	1800.00	1620.00	620.00	550.00	213	4	4	0	
NY 6	F	2620.00	1790.00	642.00	575.00	521	0	0	0	
NY 7	F	712.00	490.00	450.00	410.00	231	0	0	5	
NY 8	M	1160.00	1000.00	560.00	510.00	303	5	5	0	
NY 9	M	2000.00	1700.00	550.00	530.00	314	0	0	1	
NY 10	M	1040.00	810.00	497.00	450.00	228	3	3	1	
NY 11	F	630.00	550.00	420.00	385.00	228	0	0	2	
NY 12	M	3700.00	3130.00	730.00	665.00	524	15	15	2	
NY 13	F	1030.00	870.00	555.00	490.00	234	2	2	1	
NY 14	M	4280.00	4090.00	790.00	710.00	235	9	9	0	
NY 15	F	702.00	510.00	458.00	417.00	145	0	0	0	
NY 16	M	560.00	490.00	470.00	415.00	165	0	0	0	
NY 17	M	580.00	490.00	450.00	409.00	156	3	3	1	
NY 18	M	680.00	600.00	470.00	420.00	210	0	0	1	
NY 19	F	3400.00	2940.00	720.00	640.00	121	4	4	1	
NY 20	M	550.00	300.00	430.00	380.00	135	0	0	0	
NY 21	F	1170.00	860.00	502.00	447.00	212	3	3	0	
NY 22	M	3650.00	2860.00	790.00	710.00	475	13	13	0	

NY 23	F	810.00	600.00	470.00	430.00	245	2	2	0	
NY 24	F	690.00	540.00	470.00	430.00	382	1	1	0	
NY 25	M	170.00	140.00	391.00	259.00	42	0	0	1	

Appendix 4:Usuthu River host metal concentrations in the muscle tissue

Species	Al	Mn	Fe	Co	Ni	Cu	Zn	As	Se	Ag	Cd	Pb
US 01	17.89007	1.472644	47.26444	0.064134	0.028141	5.995441	24.04509	0.230091	1.958207	0.037994	0.099037	1.489868
US 02	72.2821	2.863168	108.3646	0.078468	0.688144	6.487816	16.33786	0.19201	1.897142	0.052882	0.037629	0.586457
US 03	16.91819	1.520833	36.68699	0.039837	0.53125	8.912602	15.66819	0.202287	1.461128	0.040523	0.10907	1.045986
US 04	20.45667	1.588759	36.41686	0.084215	0.079906	1.967213	12.61593	0.204262	1.817564	0.013934	0.075363	0.735831
US 05	11.70947	1.322759	28.04632	0.048036	0.060498	2.195368	12.67372	0.209869	1.592145	0.01931	0.09144	0.953424
US 06	11.19332	1.185203	25.3222	0.041241	0.022148	8.093079	12.47494	0.225919	1.710501	0.05031	0.089499	0.911695
US 07	26.58811	1.632684	39.67725	0.082505	0.197208	6.565061	17.90727	0.18937	1.766906	0.058581	0.07582	0.942367
US 08	12.14855	1.217846	27.81655	0.037911	0.099751	2.372134	12.42772	0.197956	2.113659	0.009372	0.20162	1.655284
US 09	15.09245	1.20065	40.30485	0.053448	0.200075	2.477761	13.45077	0.174888	1.594203	0.063943	0.034958	0.434533
US 010	12.31076	1.310508	29.85558	0.07739	0.861803	2.464392	10.64492	0.15244	0.943974	0.006823	0.040214	0.501494
US 011	17.24171	1.238827	33.56559	0.082148	0.055142	2.000721	13.14272	0.179938	2.657376	0.02309	0.090942	0.788563
US 012	12.67963	1.187992	32.18504	0.054454	0.014493	2.15625	12.91339	0.219537	2.435039	0.009892	0.045866	0.596949
US 013	16.06183	0.961733	31.42423	0.090598	0.110137	6.517993	12.09833	0.163685	2.633046	0.047871	0.045641	0.540294
US 014	9.216837	1.104592	29.71939	0.096224	0.167449	7.696429	12.05102	0.172806	2.039541	0.037423	0.123852	1.044898
US 015	11.58677	1.079578	31.80729	0.099736	0.15489	4.448706	13.84947	0.160786	2.662991	0.048634	0.186242	1.815436
US 016	11.50024	1.14491	42.27959	0.080248		2.630297	13.0565	0.157209	2.20263	0.018753	0.070458	0.743059
US 017	15.65109	1.053181	31.21272	0.045452	0.025398	2.552187	10.333	0.164438	2.04175	0.063991	0.08089	0.98335
US 018	9.520514	1.076619	26.84132	0.040929		2.125803	10.0519	0.187148	2.478992	0.004548	0.092734	1.154968
US 019	19.08934	1.127838	40.89339	0.07848	0.026061	3.205824	9.600197	0.173124	3.168806	0.026579	0.05728	0.675962
US 020	8.725538	1.154354	30.50391	0.046208	0.015215	3.441781	11.83464	0.105259	2.047701	0.011864	0.040509	0.492172
US 021	7.090009	1.028982	24.57587	0.04279	0.03942	1.986334	11.18049	0.153935	3.60509	0.016376	0.110368	1.099434
US 022	10.72277	0.929703	36.78218	0.076856	0.099084	5.282178	17.2401	0.154109	2.132921	0.018243	0.248762	2.068564
US 023	16.81211	1.34001	42.0124	0.046137	0.107034	3.359561	17.53457	0.192799	1.681211	0.025155	0.093634	0.807344
US 024	16.10915	1.482646	38.25453	0.06494	0.082143	3.138833	15.37726	0.191097	1.964034	0.014864	0.052465	0.526408

Appendix 5: Shokwe Pans host metal concentration in the muscle tissue

Species	Al	Mn	Fe	Co	Ni	Cu	Zn	As	Se	Ag	Cd	Pb
SH 2(1)	23.98775	1.897304	53.70098	0.078725	0.235123	2.995098	17.39951	0.179926	1.729657	0.001951	0.404412	3.801471
SH 2(2)	31.17207	2.150374	41.77057	0.070224	0.171596	3.832918	23.12718	0.179202	1.722444	0.003367	0.928429	9.01995
SH 2(3)	25.14526	1.838685	38.50663	0.044037	0.278033	2.525739	14.06218	0.164551	1.49159	0.002574	0.128313	1.420999
SH 2(4)	27.11101	1.570209	38.85199	0.044687	0.214469	3.726281	20.2704	0.158871	1.828985	0.001741	0.195849	2.14777
SH 2(5)	96.53367	1.721696	53.36658	0.065611	0.185287	3.413965	17.82045	0.18399	2.423441	0.002793	0.323441	4.044888
SH 2(6)	127.0717	1.900864	76.74123	0.091866	0.223106	2.171581	16.58109	0.171479	1.79334	0.010447	0.19939	2.184037

SH 2(7)	22.58435	1.417359	31.29584	0.050611	0.323472	3.149144	15.74083	0.160318	1.787775	0.002076	0.384352	3.826406
SH 2(8)	25.66483	1.572253	54.01405	0.090517	0.396889	4.064225	26.84395	0.153813	1.978926	0.002163	0.340191	3.266433
SH 2(9)	22.18498	1.624748	30.09073	0.08256	0.108165	3.061996	20.06048	0.168095	2.02999	0.02684	0.782762	6.24244
SH 2(10)	20.18547	1.736789	35.18801	0.075534	0.042124	3.142785	19.8501	0.176728	1.841463	0.003532	0.481453	5.063516
SH 2(11)	16.2729	1.166345	34.86017	0.056823	0.02987	2.909836	16.72613	0.16661	2.090164	0.001967	0.152965	1.725651
SH 2(12)	16.50147	1.259814	32.70363	0.048209	0.01634	3.527969	12.84102	0.176717	2.430324	0.004907	0.108611	1.285819
SH 2(13)	22.42028	1.820086	34.10281	0.063256	0.11247	3.460257	25.60685	0.166445	1.828415	0.014564	1.063779	8.672061
SH 2(14)	17.58213	1.647343	28.98551	0.044469	0.055894	2.408937	17.55556	0.173237	1.847343	0.002169	0.668599	5.44686
SH 2(15)	21.67899	1.560404	30.71992	0.042308	0.80498	2.741617	15.80868	0.163856	1.816568	0.016667	0.321746	3.123767
SH 2(16)	19.64827	1.237909	29.31119	0.042599	0.000332	2.117489	12.50366	0.138935	1.987054	0.034416	0.07318	1.400098
SH 2(17)	24.65631	1.515275	33.9613	0.063518	0.365326	3.113544	15.46079	0.183987	1.725305	0.014868	0.589613	5.906314
SH 2(18)	24.52956	1.669951	36.4532	0.063227	0.178621	7.004926	18.44581	0.182069	2.591133	0.00936	0.542857	4.820197
SH 2(19)	16.53112	1.242972	33.48394	0.056727	0.225326	2.728414	17.91918	0.184588	2.044679	0.001978	0.049473	0.689006
SH 2(20)	16.93806	1.509491	35.28971	0.06039	0.094555	2.470529	16.75075	0.211738	2.388362	0.001896	0.573926	4.6004
SH 2(21)	17.59048	1.460238	36.57143	0.056024	2.864286	3.852381	23.88095	0.170524	1.871667	0.00581	0.649286	5.707143
SH 2(22)	19.58537	1.769512	29.90244	0.052341	0.137439	3.597561	16.17805	0.19639	1.932927	0.006976	0.416585	5.085366
SH 2(23)	14.68289	1.433874	31.44051	0.051967	0.074361	2.531957	49.85251	0.186111	2.322517	0.001603	0.210079	1.7559
SH 2(24)	20.44325	2.180955	36.77545	0.056527		2.85923	26.93619	0.089893	2	0.002309	0.096931	1.187043
SH 2(25)	11.81327	1.121376	25.25799	0.03828	0.062826	2.513514	13.11794	0.159238	1.825061	0.017764	0.075971	0.732187

Appendix 6: Nyamithi Pans host metal concentrations in the muscle tissue

Species	Al	Mn	Fe	Co	Ni	Cu	Zn	As	Se	Ag	Cd	Pb
NY 1	17.14615	1.259567	35.52328	0.054172	0.057699	1.927847	13.22729	0.235592	2.47349	0.019756	0.119318	1.333333
NY 2	32.51301	1.732371	53.64411	0.07513	1.037861	2.510648	12.11074	0.238523	2.334122	0.005892	0.060838	0.720303
NY 3	13.3411	1.217391	36.74948	0.053571	0.005254	2.97619	30.95238	0.253002	2.771739	0.003054	0.265269	1.901915
NY 4	14.20387	1.282986	30.25794	0.046032	0.348958	3.102679	21.30952	0.212698	2.524802	0.00246	0.174802	1.793403
NY 5	18.36686	1.090278	35.15326	0.066619	0.006274	2.195642	16.32423	0.217122	1.79909	0.012261	0.037093	0.501676
NY 6	11.2506	0.921988	22.38838	0.040711	0.084325	1.962554	11.2218	0.235694	2.394863	0.001954	0.12482	1.085934
NY 7	11.49221	1.088121	23.80477	0.040604	0.001741	3.52483	10.98832	0.221616	1.724197	0.002327	0.032425	0.362463
NY 8	12.69766	1.073098	38.0905	0.048359		2.834411	29.16459	0.180433	1.582795	0.003182	0.048782	0.56365
NY 9	17.25827	1.055534	28.67763	0.0449	1.677087	2.692989	11.20766	0.161998	1.573946	0.003569	0.113652	1.029933
NY 10	11.63704	1.07679	23.48889	0.044148		1.519259	11.31605	0.174173	2.231605	0.00178	0.075679	0.674568
NY 11	16.2321	1.22716	35.75309	0.192988	0.206741	2.292593	16.78025	0.161062	1.667407	0.012222	0.064691	0.446173
NY 12	16.47484	1.031639	35.55057	0.063328	0.410812	3.435476	20.61286	0.174315	2.867464	0.003737	0.034579	0.531639
NY 13	14.34009	1.228868	26.91547	0.049234		1.933762	10.74889	0.168191	1.541028	0.051013	0.058725	0.765447
NY 14	48.35821	1.656965	70.37313	0.111393	0.028458	2.279602	10.97512	0.194005	2.008458	0.003308	0.067114	0.644776
NY 15	14.17656	1.254698	25.04946	0.04911	1.354105	2.645895	9.695846	0.189713	2.079377	0.017433	0.046909	0.591741
NY 16	14.01493	0.911443	31.61692	0.04005	0.214478	2.624378	9.5	0.176194	1.014428	0.017239	0.1501	1.270398
NY 17	14.97264	1.415672	33.85572	0.057413	0.126617	2.433831	14.301	0.217114	2.358209	0.019428	0.107935	1.187313
NY 18	24.32325	1.284581	44.4472	0.073798	0.145364	2.746653	13.24988	0.183019	1.573624	0.004313	0.028136	0.387209
NY 19	14.4321	1.160741	30.59259	0.068173	0.203975	3.392593	10.83951	0.177309	2.755556	0.002716	0.07884	0.913827

NY 20	14.32212	1.237259	26.42256	0.046512		2.330777	17.93667	0.194112	2.445324	0.002672	0.091415	0.965116
NY 21	10.76468	0.846325	24.93833	0.041959		4.467193	9.795264	0.180957	1.683276	0.001468	0.047854	0.412432
NY 22	10.05707	0.936973	24.1464	0.053772	6.315136	2.811414	11.14144	0.174069	2.125558	0.008065	0.059702	0.607444
NY 23	13.78614	1.214855	34.72084	0.068918	0.335743	2.918744	15.4985	0.211316	2.16675	0.015753	0.030334	0.437438
NY 24	9.853525	1.117676	25	0.044886		2.200844	11.74528	0.185452	1.963257	0.016385	0.030685	0.399454
NY 25	14.89526	1.379052	33.99002	0.071646	0.067955	7.381546	15.09476	0.204589	1.605237	0.00783	0.031746	0.426933

Appendix 7: Usuthu Rivers host metal concentration in the liver tissue

Species	Al	Mn	Fe	Co	Ni	Cu	Zn	As	Se	Ag	Cd	Pb
US 01	21.6018	5.411677	1750.25	1.283433	0.200998	219.4611	145.8583	0.3498	46.70659	0.073578	0.891218	0.534182
US 02	29.53523	3.50075	3675.662	0.702399		33.4083	54.72264	0.251374	23.04848	0.021629	0.869565	0.369815
US 03	22.16142	4.390305	1528.236	1.196902		53.32334	59.44528	0.345077	21.02949	0.022589	0.496252	0.646677
US 04	32.94439	6.142006	879.5929	1.161619	0.166212	113.7537	96.64846	0.387537	22.41559	0.163878	0.793942	0.628103
US 05	15.63366	5.881188	2477.723	0.789851		50.54455	83.36634	0.27203	10.55446	0.12354	0.911881	0.495545
US 06	20.66451	4.77103	349.1787	0.644848	0.038203	87.10801	82.85217	0.269288	9.773519	0.09562	0.788452	1.039572
US 07	92.20472	10.51181	715.748	0.788189		101.6929	81.88976	1.433465	8.125984	0.766732	0.782283	5.401575
US 08	19.44045	5.854058	1673.429	0.688809	3.465314	84.84948	105.5301	0.343259	14.15576	0.056512	0.60962	0.80301
US 09	23.28921	3.046953	2959.54	0.833417	0.967033	76.82318	74.72527	0.146179	21.6009	0.033292	0.488761	0.281968
US 010	93.02956	6.08867	4534.483	0.984236		23.2734	78.84236	0.171502	7.903941	0.004579	1.034975	0.607389
US 011	32.4088	7.441279	537.4813	0.996252		51.54923	54.97251	0.384808	11.07446	0.024825	0.428036	0.924788
US 012	20.48539	4.378405	2639.921	1.199108		74.91332	82.8628	0.307083	30.01486	0.063918	0.347697	0.719663
US 013	67.78607	8.216418	633.5821	1.012935		46.64179	47.61194	0.346517	13.26866	0.027065	0.779104	0.83806
US 014	20.9824	5.718475	194.2326	0.981672	0.086241	66.27566	65.29814	0.260508	11.78886	0.024365	0.59824	0.463343
US 015	16.51874	2.377465	944.2801	0.339004	0.271203	17.66765	29.73373	0.28575	11.81953	0.011612	0.408037	0.955868
US 016	29.793	4.248398	3568.26	1.509857		25.11089	46.40217	0.32208	13.98965	0.015421	0.45934	0.824298
US 017	39.60688	4.36855	153.5872	0.710811		23.79607	49.72973	0.252334	5.614251	0.006125	0.869779	0.600737
US 018	25	5.044031	3515.166	0.740705		39.77495	53.54697	0.347847	21.02495	0.021018	0.244129	0.902642
US 019	38.48425	7.682087	2221.949	0.881398	0.17126	89.34547	82.23425	0.365157	39.17323	0.013873	0.482283	0.585384
US 020	17.4788	3.214464	3007.481	0.821197		39.37656	60.32419	0.310723	13.42643	0.026434	0.495761	0.88404
US 021	24.38398	4.08282	3040.041	1.039014		94.04517	88.50103	0.381588	22.91239	0.046749	1.042094	1.739904
US 022	30.28942	3.433134	4802.894	0.992515		29.26647	45.38423	0.206512	16.23004	0.023862	0.510978	0.434132
US 023	17.80457	4.664346	834.4107	0.708603		44.97762	57.5087	0.203879	14.50273	0.013386	0.73272	0.380408
US 024	20.665	3.9375	181.35	0.913		76.75	64.575	0.238075	8.275	0.06415	0.66275	0.210525

Appendix 8: Shokwe Pans host metal concentrations in the liver tissue

Species	Al	Mn	Fe	Co	Ni	Cu	Zn	As	Se	Ag	Cd	Pb
SH 2(1)	20.75848	7.838694	556.6367	0.374513	0.127339	23.42105	64.52242	0.212378	14.77827	0.009508	0.441764	0.269737
SH 2(2)	33.00194	4.706453	4573.23	0.349345	0.634401	23.787	59.53421	0.157035	15.54585	0.018852	0.083091	0.637555
SH 2(3)	9.238001	7.35353	2146.71	0.347772	0.159614	29.56935	76.01402	0.182774	16.96294	0.036755	0.055133	0.390336

SH 2(4)	12.84223	6.276096	2110.19	0.356472	0.324635	20.43058	86.61273	0.235465	7.163361	0.04786	0.293319	0.959812
SH 2(5)	342.3115	7.00716	1905.975	0.456563	0.244869	21.84248	88.18616	0.157661	6.918854	0.039165	0.441289	0.86611
SH 2(6)	12.99357	10.09278	1301.583	0.333763	1.578866	18.6134	88.50515	0.420464	5.340206	0.037613	0.546907	1.691237
SH 2(7)	16.85399	9.604283	1257.685	0.304469	0.194088	25.69367	87.84916	0.521415	6.061453	0.06648	0.47067	2.216015
SH 2(8)	28.97585	7.996973	2764.363	0.46443	0.082619	27.11907	78.0777	0.246039	8.9556	0.062967	0.153835	0.640515
SH 2(9)	23.51155	7.871238	879.0161	0.849285	0.710903	48.71732	97.3113	0.654662	18.71238	0.061963	0.384065	0.931179
SH 2(10)	17.84431	8.653374	1361.028	0.552147	1.582822	37.17791	91.71779	0.256779	13.14417	0.070521	0.425767	0.903067
SH 2(11)	19.49739	8.875872	869.1323	0.452393	5.476072	34.17248	83.34995	0.232777	11.44068	0.04509	0.277916	0.583749
SH 2(12)	15.63085	7.121891	3178.906	0.928856	0.777861	45.77114	87.46269	0.239527	12.33582	0.051567	0.695274	0.642786
SH 2(13)	25.70189	7.690406	1824.655	0.557864	0.769288	54.99505	102.1019	0.171538	10.55143	0.104575	0.486647	2.312315
SH 2(14)	19.00243	6.748286	937.8641	0.314398	0.733595	13.29579	65.15671	0.393242	11.54995	0.026053	0.264202	0.641038
SH 2(15)	25.06324	8.442744	1104.132	0.392646	0.515301	27.51728	76.85094	0.189684	7.050839	0.04272	0.382527	1.147828
SH 2(16)	29.09364	6.409363	1591.137	0.809761	0.897908	59.11355	105.6524	0.248108	23.8745	0.084711	0.771414	0.831673
SH 2(17)	32.55474	6.860298	241.5085	0.444071	0.296928	29.47672	87.18195	0.208617	13.31013	0.053265	0.397264	0.583533
SH 2(18)	27.84372	6.557864	428.2888	0.307369	0.363007	14.68101	143.546	0.312315	8.363007	0.057888	0.216716	1.190406
SH 2(19)	17.88198	6.860639	1457.15	0.443057	0.441558	32.41758	91.98302	0.427073	7.495005	0.092108	0.336663	1.136114
SH 2(20)	16.04333	7.487648	1140.189	0.280138	0.501976	37.72233	92.56423	0.250494	10.54101	0.126161	0.297431	0.924654
SH 2(21)	26.0966	7.603448	192.8043	0.393103	0.211946	40.17241	85.14778	0.280296	7.391626	0.076724	0.190222	0.566502
SH 2(22)	15.03131	6.617575	759.6339	0.413598	0.141286	40.23073	92.68532	0.215096	13.38733	0.05432	0.365488	0.576829
SH 2(23)	15.57505	7.510981	376.2183	0.646413	0.785749	61.12738	114.4217	0.244265	14.98536	0.50122	0.344558	1.216447
SH 2(24)	13.5541	7.026829	365.114	0.402927	0.305122	27.39024	88.07317	0.607561	11.20244	0.263171	0.220732	0.513659
SH 2(25)	20.65348	10.09495	2179.77	0.377561	1.76037	37.58121	104.9975	0.150225	10.08746	0.074013	0.526237	1.918541

Appendix 9: Nyamithi Pans metal concentrations in the liver tissue

Species	Al	Mn	Fe	Co	Ni	Cu	Zn	As	Se	Ag	Cd	Pb
NY 1	18.71324	4.977941	2382.598	0.414216	0.611765	7.715686	49.65686	0.154314	20.32108	0.018468	0.117034	0.825
NY 2	17.29226	10.11461	1657.116	0.618911	0.669532	7.970392	60.43457	0.174236	12.74117	0.003534	0.100836	0.321633
NY 3	17.84634	4.492683	1370.488	0.433659	0.16022	8.22439	60.12195	0.145146	25.17073	0.021793	0.055488	0.468293
NY 4	18.87189	6.23088	1445.029	0.376673	0.206405	9.916348	58.2696	0.156668	26.07553	0.012945	0.106429	0.304254
NY 5	18.55206	3.874092	1334.383	0.334625	0.39201	6.138015	50.46005	0.14368	15.86441	0.017201	0.050484	0.335835
NY 6	20.3644	13.96381	2380.02	0.694348	0.13468	8.946455	84.38275	0.178979	22.00793	0.006019	0.081755	0.35176
NY 7	17.54612	9.832524	2104.369	0.663592	0.12534	8.184466	80	0.113786	17.06553	0.004653	0.135267	0.386893
NY 8	19.72236	4.584792	916.9585	0.381441	0.487744	5.990495	49.3997	0.144297	16.27314	0.013764	0.046373	0.288394
NY 9	18.31748	5.380571	2946.92	0.677016	0.113145	8.557837	73.48523	0.161292	45.74362	0.015008	0.096745	0.271157
NY 10	20.2459	4.572777	1167.163	0.351217	0.138649	9.264779	61.52509	0.154098	22.76205	0.015842	0.074665	0.198882
NY 11	15.40098	4.567237	1961.125	0.387286	0.131443	7.124694	59.2665	0.165477	21.2665	0.008897	0.073521	0.561614
NY 12	15.61224	3.115804	2695.776	0.604177	0.150759	17.14048	61.46179	0.128002	34.29046	0.071998	0.045871	0.208994
NY 13	15.80733	9.920753	1452.699	0.700594	0.110327	7.327885	72.75879	0.173576	19.2843	0.004846	0.202922	0.770679
NY 14	35.40646	4.67189	1353.82	0.535749	0.361655	8.432909	73.21254	0.138908	39.86288	0.021334	0.488492	4.884917
NY 15	22.23462	11.46577	2666.171	0.766865	0.31994	8.807044	79.96032	0.14494	15.52579	0.005171	0.567708	4.246032
NY 16	18.04741	4.992669	930.5963	0.280547	3.88563	6.818182	51.88172	0.133822	26.78397	0.006938	0.233113	2.037879
NY 17	18.09345	6.596702	2446.027	0.408796	0.147851	7.166417	58.69565	0.148001	18.73563	0.006674	0.198276	1.585207

NY 18	17.7228	3.483506	1405.219	0.373708	0.158469	6.395864	51.57558	0.117701	19.72427	0.010286	0.644756	3.865091
NY 19	19.01195	9.163763	1263.564	0.72001	0.193081	10.45047	78.09856	0.141239	26.25684	0.016125	0.285217	1.781981
NY 20	40.04447	7.067688	1506.176	0.418972	0.233671	23.43379	102.1245	0.153187	30.68182	0.038019	0.803854	5.748518
NY 21	19.30739	7.104748	1779.001	0.622369	0.182256	8.634361	69.21194	0.155188	18.35047	0.00815	0.180739	1.601322
NY 22	15.9606	2.850195	1532.344	0.565418	1.423881	5.654183	48.68677	0.12162	22.55107	0.011911	0.229426	2.005107
NY 23	18.23314	7.123656	2712.61	0.499267	0.130645	8.164712	65.51808	0.155816	19.82893	0.006065	0.304985	1.393695
NY 24	16.71982	8.589303	688.6654	0.4605	0.172498	7.05103	67.07556	0.133121	12.16389	0.008798	0.24632	1.816241
NY 25	27.86517	6.928839	2913.483	0.549064	0.38764	12.18352	82.3221	0.242959	7.958801	0.015697	1.198127	9.11985

NORTH-WEST UNIVERSITY ®
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

School of Biological Sciences

ETHICS OFFICE	Standard Operating Procedure		
Title	**Standard operating procedure for euthanasia/humane killing of aquatic ectotherm vertebrates**		
SOP no	**NWU-00267-17-S5**	**Version no**	02
Date of approval		**Revision date**	13 June 2017
Web address		**Page no**	1 - 7

COMPILATION AND AUTHORISATION

Action	Designated person	Signature	Date
Compiled by:	David Huchzermeyer		10/7/17
Authorised by:			

DISTRIBUTION

Department/Unit	Name	Signature	Date
Zoology subject chair	Mark Maboeta		
Laboratory manager	Lieb Venter		

DOCUMENT HISTORY

Date	Version no	Reason for revision
28/03/2017	1	First issue of SOP

PURPOSE OF THE SOP

There frequently arises a need to euthanize aquatic ectotherm vertebrates in research. It is the ethical obligation of all personnel involved with the handling of aquatic ectotherms to restrain them in a way that will reduce or eliminate pain and distress until unconsciousness is induced and they become insensible to pain, a condition that must persist until they are dead.

SCOPE

To designate responsibility and set the welfare standards for euthanizing aquatic ectotherm vertebrates being collected as museum specimens, being processed to obtain data needed for environmental, ecological, parasite and disease studies or following experimental research. Humane endpoints are an

accepted integral part of research protocols involving live animals. The parameters underlying the establishment of the humane endpoint must be clearly defined in each relevant protocol. Humane endpoints are study-specific criteria. They indicate or predict pain, distress or death and in many cases are used as signals to end a study early to avoid or terminate pain and/or distress. Where the nature of a study requires lethal sampling the principal of humane endpoint is applied but does not signal the end of the study. When an animal reaches a specified humane endpoint the animal is put to death by recognized euthanasia methods.

ABBREVIATIONS AND/OR DEFINITIONS

Abbreviation/definition	**Description**
PI	Principle investigator
NWU	North-West University
N/A	Not applicable

RESPONSIBILITIES

The key points for fulfilling the responsibilities of both the PI and all personnel involved with the handling of aquatic ectotherms (including other NWU staff, collaborators and students) are:

- Understanding the legal requirements as laid out in the relevant legislation and internally by the research animal ethics committees.
- To establish the correct animal welfare conditions for restraining and euthanizing aquatic ectotherm vertebrates.
- Being able to distinguish pain and distress in aquatic ectotherm vertebrates from their normal state.
- To relieve or minimize the pain and distress appropriately.

PROCEDURE/S

1. Handling aquatic ectotherm vertebrates:

- Disposable gloves (vinyl or latex without powder) should always be worn when handling aquatic ectotherm vertebrates. The smooth texture of the gloves will minimize the potential for injury to the animal's integument. The gloves should be premoistened with dechlorinated water.

2. Manual restraint:

In many cases the euthanasia of aquatic ectotherms requires some degree of handling that has the potential of causing distress. Correct handling is therefore important to minimize pain and distress and to ensure the safety of persons performing the procedure

veterinarian. The use of scheduled and highly scheduled medicines is governed by the Medicines and Related Substances Act, Act 101 of 1965 and restricts the use of such substances for euthanasia of animals to veterinarians. Researchers must make prior arrangement with a suitably situated veterinarian to be present during end-point euthanasia of research animals and during emergency situations that may arise in the course of a research project. Alternative options to follow exist as follows:

- Persons who are not registered veterinarians who wish to use scheduled medicines may apply for permits from the Director General of the Department of Health; and
- Persons who motivate the need to perform a veterinary and /or para-veterinary procedure may apply for authorisation ito section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act. Council has authorised several scientists involved in research who are performing both veterinary and para-veterinary functions in the research environment. The conditions of authorisation include supervision by a veterinary or para veterinary professional.

There are a number of accepted chemical agents used in the form of an immersion bath to humanely kill fish or applied to the skin in the case of amphibians. The choice of agent will depend on availability, cost, regulatory constraints, the species and numbers, and the purpose to which the animal is to be euthanized. Early stages in the lives of fish, including embryos and larvae, may require higher concentrations of immersion anaesthetics or a longer duration of exposure than older fish.

3.1. Benzocaine – white crystalline powder, almost insoluble in water. Benzocaine hydrochloride is a relatively fast-acting and effective euthanasia agent for fish and amphibians and is of low toxicity to humans and the environment. Stock solutions are prepared in acetone or ethanol which act as irritant to the fish. The salt, benzocaine hydrochloride, is more water soluble than benzocaine but stock solutions need to be buffered. Benzocaine is broken down by sunlight and stock solutions should be stored in amber bottles. Standard stock solutions are made containing 100 gram per liter. The anaesthetic concentration is variable and depends on species but is usually in the range of 25-45 mg/L but may in some species be as high as 100-200 mg/L. The anaesthetic is absorbed with cumulative effect eventually leading to medullary collapse and death. Fish must be kept immersed for 10 minutes after opercular movement has ceased. The mode of action is similar to that of MS222. Benzocaine breaks down in water in less than 4 hours and can also readily be removed from water by filtration through activated charcoal.

For euthanasia of amphibians, benzocaine hydrochloride is administered, as a 20% concentration gel, to the ventral abdomen (20 x 10 mm application area, alternatively at a dose of 182 mg/kg). Unconsciousness in *Xaenopus laevis* is induced after 7 minutes and death occurrs after 5 hours.

3.2. Tricaine methane sulphonate (MS222) is a sulphonated analogue of benzocaine with high solubility in water. It is registered for veterinary use in Canada, the UK, Italy, Spain and Norway. MS222 can be used to euthanize, aquatic reptiles, amphibians and teleosts. Solutions of tricaine are acidic and need to be buffered. Stock solutions containing 10 g/L tricaine are prepared in a saturated solution of sodium bicarbonate that stabilizes the pH at 7 to 7.5. The anaesthetic is absorbed with cumulative effect eventually leading to medullary collapse and death. The anaesthetic concentration for induction of most adult fish lies between 100 and 200 mg/L and a 10 minute immersion is sufficient to kill most species of fish but species sensitivity is variable and an

resulting in circulatory collapse. Intraceolomic injection of MS222 is unacceptable for euthanizing fish as the chemical is rapidly excreted via the gills. Carcasses of animals euthanized with MS222 are unsuitable for consumption by humans and other animals.

Aquatic reptiles can be euthanized in 2 stages using MS222. An intracoelomic injection of 250-500 mg/L of neutralized MS222 is administered. Consciousness is lost rapidly (from <30 seconds to 4 minutes). This is followed by a second intracoelomic injection of a 50% unbuffered solution of MS222 which ensures death of the animal.

Frogs immersed in a 5g/L solution go into deep anaesthesia within 4 minutes but must remain immersed in the solution for at least one an hour to ensure death. A secondary method of euthanasia must be applied where solutions < 5gL are used. Intraceolomic injection of MS222 is ineffective and considered an unacceptable method for euthanizing frogs.

3.3. Quinaldine sulphate – light yellow crystalline powder, highly soluble in water. Stock solutions, usually at 10 g/L, are acidic and need to be buffered with sodium bicarbonate as for tricaine. They are light sensitive and need to be protected from light. Induction and recovery are rapid and no avoidance or coughing responses are observed. Quinaldine sulphate acts through depression sensory centers of the CNS and is an acceptable chemical for euthanasia of fish. The effective concentration for euthanasia should exceed 100 mg/L. Fish should remain immersed for 10 minutes after opercular movement has ceased. Carcasses of animals euthanized with quinaldine sulphate are unsuitable for consumption by humans and other animals.

3.4. 2 Phenoxyethanol – moderately water soluble clear or straw-coloured oily liquid. Solubility is reduced in cold water. Can be used to euthanize fish at concentrations of 0.5 - 0.6 mL/L or 0.3 to 0.4 mg/L or higher but has long induction times. Fish must be kept immersed for 10 minutes after opercular movement has ceased. Death is thought to occur from hypoxia following CNS depression. Can be used in sea water as it causes no pH change. With repeated exposure, may cause neuropsychological syndrome in handlers of the chemical. Carcasses of animals euthanized with 2 phenoxyethanol are not suitable for consumption by humans and other animals.

3.5. Eugenol – clove oil is a mixture of phenolic eugenol (accounts for 85-95% of the active ingredients), iso-eugenol and methyleugenol. Iso-eugenol has been shown to be carcinogenic. Clove oil is effective as an anaesthetic agent at a wide range of temperature and can be used to euthanize freshwater and marine fish. Eugenol is incompletely soluble in water. A 1:10 mixture in 95% ethanol yields a stock solution containing 100 mg/ml. Ventilatory failure leading to death is rapid following immersion in doses exceeding 400 mg/L.

3.6. Metomidate – azaperone is an imidazole-based nonbarbiturate hypnotic that has been used to anaesthetize fish. It is used at an anaesthetic concentration of 5 mg/L. Induction is rapid. Leaving fish in the anaesthetic solution for long periods results in death. Although an acceptable agent for euthanasia of some fish, use of metomidate for this purpose is illegal in the USA.

3.7. Sodium hypochlorite (bleach) can be used at 1-10% strength to euthanize embryonic zebra fish and hatchlings up to 7 days of age.

95% ethanol/L induces anaesthesia rapidly in zebra fish and prolonged immersion results in respiratory depression and death from hypoxia.

3.9. Pentabarbital. Barbiturates are widely used by veterinarians for the euthanasia of pet animals and can also be used to euthanize fish. They result in depression of the cerebral cortex and the CNS in descending order, resulting in loss of consciousness progressing to anaesthesia. With an overdose, deep anaesthesia progresses to medullary collapse, cessation of respiratory movement and cardiac arrest. An injection of pentobarbital (60 to 100 mg/kg) can be administered by the intravenous or intracoelomic route. Fish must first be anesthetized by some other method before pentobarbital is injected intracardially. Two-step injection procedures may also be used, including ketamine (IM) followed by a lethal dose of pentobarbital; a combination of ketamine and medetomidine (IM) followed by a lethal dose of pentobarbital; and propofol (IV) followed by a lethal dose of pentobarbital. The potential adverse effects of drug residues and proper disposal of carcasses should be considered when using any of these drugs.

4. **Secondary forms of euthanasia:**

Secondary forms of euthanasia may only follow primary induction of unconsciousness

4.1. Exsanguination may be used to collect blood. The procedure requires the animal (fish and other aquatic ectotherms) to be sedated, stunned or anaesthetized.

4.2. Pithing may be used to destroy the brain stem and spinal cord in animals that have been rendered unconscious by some other means and results in loss of involuntary movements. Pithing of frogs and other amphibians is discouraged unless preceded by anaesthesia.

4.3. Blunt force trauma to the head followed by pithing.

4.4. Captive bolt (non-penetrating) applied where fish are exceptionally large.

4.5. Decapitation followed by pithing.

4.6. Cervical transection followed by pithing.

4.7. Maceration. Depending on size of fish and design of macerator can result in instantaneous death.

4.8. Rapid chilling to 2-4°C, applied for 10 minutes has been used for euthanasia of adult zebra fish. For fry the chilling must extend to at least 20 minutes. This method is also accepted for other small-bodied tropical and subtropical stenothermic species. Rapid chilling in water associated with ice slurry is a suitable killing method for tropical and subtropical fish species smaller than 3.8 cm in length (tip of the snout to the posterior end of the last vertebra).

5. **Indicators of death:**

- initial flaccidity (prior to rigor mortis) may serve as indicators of death for fish
- respiratory arrest (cessation of rhythmic opercular activity) for a minimum of 10 minutes and
- loss of eyeroll (vestibulo-ocular reflex, the movement of the eye when the fish is rocked from side to side).
- Absence of a sustained heartbeat. The heart of fish may continue contracting after brain death and removal from the body, and presence of a heartbeat should not be misinterpreted.

6. Unacceptable forms of euthanasia:

- Immersing fish directly into preservative concentrations of ethanol (70%).
- Immersing fish in fixative strengths of formalin (10% formalin).
- Decapitation without pithing.
- Cervical transection without pithing.
- Immersion in ice slurry of temperate, cool, or cold-water-tolerant fish, such as carp, koi, goldfish, or other species that can survive at 4°C and below.
- Flushing of fish into a sewer, septic, or other types of outflow systems.
- Slow chilling or freezing of unanaesthetised animals. Placing fish into a freezer without prior anaesthesia.
- Death by anoxia and desiccation after removal from the water or by anoxia in water.
- Exposure to caustic chemicals.
- Prolonged traumatic injury prior to unconsciousness

7. Disposal of the remains of euthanized animals:

Residues of euthanizing chemicals have potential to harm the environment and other animals consuming the remains of euthanized animals. Careful consideration must be given to disposal methods that render both carcasses and effluents harmless. Carcasses should ideally be disposed of through incineration. Water that may contain euthanizing chemicals or residues should not be discharged into natural water. Some chemicals can be removed from the water by filtration through activated charcoal.

8. Recognition of pain and distress:

- Animals must be monitored by trained individuals as appropriate for the species, condition and procedure.
- The ability to distinguish between normal and abnormal behaviour is critical to the accurate assessment of pain and distress.

- Fishes need to be monitored as per the protocol for the relevant study.

9. **Intervention strategy**:

- If pain or distress is observed as a direct result of handling or keeping animals in temporary enclosures the staff/researchers should immediately report all incidences verbally, directly to the PI.

- Immediately return the animal to its enclosure if pain and distress resulted from handling/physical restraint, and notify the PI.

- For handling prior to euthanasia. If pain or distress is observed from animals inside the enclosures, the following steps should be taken, while observing for relief of the symptoms: cover the enclosure/ holding container/ bag with a dark cloth or place holding container/ bag in a shaded spot; monitor the water temperature in the holding container/ bag; after prolonged periods in bagged water, do not untie the knot of the bag to aerate (this will result in a potentially lethal sudden rise in pH and conversion of accumulated non-toxic to toxic ammonia); if the fish can be released into a larger container or tank, float the bag until the water temperature in the bag equals that of the surrounding water; ensure that the temperature does not increase more than 3°C per hour; make sure water level is appropriate for species; cover the tank to provide shelter and shade from above and to prevent fish from jumping out of the container until they have settled.

REFERENCE DOCUMENTS

Guidelines for Methods of Restraint and Anaesthesia for Teleost Fishes

Guidelines on Ethics and Animal Welfare of Fish Used for Research Purposes

N/A

ADDENDA

No	Document name
N/A	N/A