A LOW FLOW-HIGH FLOW INVESTIGATION INTO THE EFFECT OF SELECTED MICRONUTRIENTS ON PRE-DETERMINED INNATE IMMUNE BIOMARKERS IN TWO FRESHWATER FISH SPECIES FROM WITBANK DAM, MPUMALANGA.

A DISSERTATION SUBMITTED FOR THE DEGREE OF MASTER OF SCIENCE IN PHYSIOLOGY IN THE DEPARTMENT OF PHYSIOLOGY AND ENVIRONMENTAL HEALTH, SCHOOL OF MOLECULAR AND LIFE SCIENCES, FACULTY OF SCIENCE AND AGRICULTURE, UNIVERSITY OF LIMPOPO, SOUTH AFRICA.

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APRIL, 2025

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BY

MURENDENI UNARINE SANDRA RADZUMA

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APRIL, 2025

DECLARATION

I, Murendeni Unarine Sandra Radzuma, declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science in Physiology has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

Student signature	3 DECEMBER 2024 Date

DEDICATION

This dissertation work is dedicated to my parents,

Nditsheni Thomas Radzuma
and
Langanani Jeanet Radzuma,

who have been my source of love, support and encouragement throughout my pursuit for education. I hope this achievement has made you proud of me.

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ABSTRACT

The aquatic environment is constantly in jeopardy since it is the ultimate recipient of waste disposal. Mining, industrial and agricultural developments have led to a significant increase in, amongst others, heavy metal contamination in aquatic systems. The aim of this study was to use seasonal surveys to investigate the impact of Fe, Cu, and Zn levels on immune modulation in *Cyprinus carpio* and *Micropterus salmoides* collected from Witbank Dam, Mpumalanga. This study consisted of two surveys that were conducted during low flow periods in June and high flow periods in November. Temperature, pH, dissolved oxygen, conductivity, total dissolved solutes (TDS) and salinity were measured using YSI multiparameter instrument. A litre of water and sediment samples were collected from three different sites, while 26 common carp and 12 largemouth bass were sampled using conventional fishing gear. For each fish, the weight and length were measured. Furthermore, epidermal mucus and blood samples were collected. Serum and mucus samples were divided, half was treated with PBS and the other with protease inhibitor cocktail and stored at -20°C until further analysis. Gills, liver, and muscle tissue were dissected, weighed, rinsed, and frozen at -85°C for later analysis of Fe, Cu, and Zn levels by Waterlab (Pty) Ltd, Pretoria. From the results, it was discovered that Witbank dam is polluted by Cu and Zn. Furthermore, Cu concentrations were only detected in the liver of both common carp and largemouth bass. Iron and zinc were detected in varying concentrations across tissues, with Fe levels being highest in the liver and gills, particularly in largemouth bass, and Zn levels being highest in the gills of common carp. The use of the protease inhibitor cocktail was found to significantly preserve CRP levels, with treated samples showing higher concentrations compared to untreated samples, indicating its effectiveness in preventing protein degradation. The study highlights the need for pre-analytical care in feral studies to ensure accurate biomarker measurements and highlights the seasonal fluctuations in metal exposure, which could influence immune responses in fish. The recommendations of the study include implementing regular monitoring of metal concentrations in aquatic ecosystems and considering the use of protease inhibitors in future studies involving immune biomarkers. The study was limited to a small sample size and one sampling area.

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LIST OF ABBREVIATIONS

Abbreviation	Description
AEV	Acute Effect Values
AMD	Acid Mine Drainage
CEV	Chronic Effect Values
CRP	C-reactive proteins
C3	Complement 3
C4	Complement 4
Cu	Copper
DEAT	Department of Environmental Affairs and Tourism
DMR	Department of Mineral Resources
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
ELISA	Enzyme-linked Immunosorbent Assay
Fe	Iron
FSH	Follicle Stimulating Hormone
GSI	Gonadosomatic Index
IgM	Immunoglobulin M
IL-6	Interleukin 6
LH	Luteinizing Hormone
MPRDA	Minerals and Petroleum Resources Development Act
NCC	Non-Specific Cytotoxic Cells
NEMA	National Environmental Management Act
NWA	National Water Act
TDS	Total Dissolved Solids
YSI	Yellow Springs Instruments
Zn	Zinc

CHAPTER 1

1. INTRODUCTION

Heavy metal contamination in aquatic environments has become a significant global concern, endangering both aquatic ecosystems and human health (Saravanan *et al.,* 2024). In recent years, metal contamination of aquatic systems has increased due to industrial, mining, and agricultural developments (Hikmat *et al.,* 2023). Mining waste, landfill leachates, municipal and industrial wastewater, urban runoff, and natural occurrences are all examples of causes of heavy metal pollution (Hikmat *et al.,* 2023). Currently, approximately 80% of the wastewater worldwide is released back into the environment untreated or repurposed (Upkeep.com, 2023). In South Africa, anthropogenic land-use activities such as coal mining have negatively impacted the Olifants River basin (DWA, 2010; DWS, 2016), which has intensified the country's freshwater demand (DWA, 2010). A study on variations in heavy metal pollution in surface and groundwater in the Mpumalanga Province and the Limpopo Province recorded a heavy metal index of over 75% in the region (Atangana and Oberholster, 2021).

The swift expansion of industrialization in cities led to the discharge of effluents contaminated with hazardous metals, such as chromium (Cr), nickel (Ni), copper (Cu), lead (Pb), iron (Fe), and zinc (Zn) (Khanam *et al.,* 2022). Due to their large atomic weight and density, heavy metals are particularly harmful to the environment and biological systems (Tchounwou *et al.,* 2012). Some heavy metals are known to play an essential role in physiological processes and are recognized as micronutrients, osmotic pressure regulators, enzyme co-factors, and molecule stabilizers in living organisms; whilst others lack biological significance and can be harmful in cases of excessive exposure (Ayangbenro and Babalola, 2017). Micronutrients such as Fe, Zn and Cu are essential to biological organisms; however, a fine balance must be maintained as their deficiencies or exposure to excessive amounts are equally undesirable for optimal physiological functions (Khanam *et al.,* 2022). Water quality and safety are significantly impacted on by heavy metals, which are recognized for their extreme toxicity, persistent presence, and bioaccumulation (Khanam *et al.,* 2022)

Heavy metals in aquatic systems can bioaccumulate in various fish tissues, either directly through their gills and/or through their diet (Bera *et al.,* 2022). These metals pose a threat when the fish's detoxification and excretion mechanisms are incapable of dealing with excessive exposure levels. This failure would ultimately increase the bioaccumulation of such metals (Balali-Mood *et al.,* 2021).

These metals have the potential to alter the haemato-biochemical profile of fish and lead to various cellular and nuclear changes in blood cells (Islam *et al.,* 2019; Islam *et al.,* 2020) as well as cause genetic damage (Suchana *et al.,* 2021). Heavy metals have the potential to significantly impact on crucial functions in fish such as reproduction and weakening of the immune systems, thereby reducing their resistance to pathogenic invasion and affecting the survival of fish (Jasim, 2017; Garai *et al.*, 2021). Ultimately, the reduced number and the quality of fish, as a protein source, will have an impact on food security in areas where the population depends on such aquatic systems to provide in their nutritional needs.

Heavy metals absorbed through the food chain in aquatic organisms can harm humans (Authman *et al.,* 2015) and potentially cause mutagenic, carcinogenic and teratogenic effects in humans (Csuros and Csuros, 2016). Even though excessive amounts of micronutrients have been reported in the Olifants River System, data is lacking regarding the impact of these elevated levels of these metals on the immunocompetence of fish species in Witbank Dam.

2. LITERATURE REVIEW

2.1 Introduction

Coal mining is important to South Africa's economy, providing the bulk of the country's basic energy needs while attracting direct foreign investment (Bench Marks Foundation, 2014). However, coal mining activities emit vast amounts of pollutants (Lakra *et al.,* 2017), that are inadvertently discharged through water draining into many of South Africa's River systems (Feris and Kotzé, 2014). Thus, coal mining has a significant environmental impact and may also impair the health and well-being of people and ecosystems in surrounding areas (Cortes-Ramirez *et al.,* 2018). In the eMalahleni area, there are approximately twenty-two active coal mines (Bench Marks Foundation, 2014).

Over two decades ago, a study revealed alarming contamination levels in Witbank dam, with metals like Cu, Fe, Aluminium and Zn exceeding safe limits for aquatic life in both water and sediment samples (Nussey *et al.,* 1999). Later, McCarthy and Pretorius (2009) attributed the increasing sulphate and total dissolved solids (TDS) levels to coal mining activities in the eMalahleni area. The situation does not seem to have improved as Mhlongo *et al.* (2018) found that the elevated sulphate levels in the Witbank Dam from its Koringspruit and Boesmankransspruit catchment areas could be linked to coal mining operations in those areas. The upper Olifants River includes mostly the area from eMalahleni (Witbank) to Middelburg. This section of the Olifants River System most probably contains the largest urban population but is also the area where most of the coal mining activities are found. Therefore, it is not surprising that Netshitungulwana *et al.* (2015) found that sediment samples collected from the upper Olifants River catchment contained high concentrations of iron, manganese, nickel, chromium and uranium. The likelihood that these untoward levels could be attributed to coal mining and ferrochrome plants in this area should not be overlooked.

Furthermore, it has been reported that the Acute Effect Values (AEV) for heavy metals like copper (Cu), iron (Fe), and especially zinc (Zn) is often exceeded at various locations in the upper catchment of the Olifants River (Dabrowski and De Klerk, 2013).

Whilst Fe, Zn and Cu are essential micronutrients, exposure to excessive amounts are harmful to aquatic organisms (Lall and Kaushik, 2021). Excessive exposure to micronutrients has the potential to disturb fish species at molecular (Cabillon and Lazado, 2019) and cellular levels (Santoso *et al.,* 2020). Such changes can compromise the fish's immune system, thereby reducing resistance to pathogenic invasion and affecting the survival of fish (Jasim, 2017; Garai *et al.*, 2021). The compromised survival of the fish could negatively impact food security for households in that area that depend on fish as a protein source. While elevated levels of micronutrients have been documented in the Olifants River System, the impact of the elevated levels of Cu, Fe and Zn on the immune competence of fish species in Witbank Dam are still unclear.

2.2 Coal mining in South Africa

South Africa began commercial coal mining in 1857, and the country has since become one of the world's leading coal producers (Hancox and Götz, 2014). The discovery of diamonds in Kimberly and gold in the Witwatersrand spurred further coal exploration to meet the growing demand for power (Eskom, 2021). Today, coal mining in South Africa is mostly concentrated in the Highveld region, with approximately 60% of the country's coal deposits located in eMalahleni (Witbank) and surrounding areas (Africa Mining IQ, 2024).

In 2022, South Africa was the fifth largest coal exporter in the world (World’s Top Export, 2023) and the seventh largest coal producer, having sold over 277 billion tons of coal (Worldometer, 2023). The coal mining industry is vital to the South African economy, contributing around 72.1% of all primary energy needs, South Africa's coal mines produce 224 million tonnes of coal annually (Eskom, 2021). It also contributes 7% to the gross domestic product and provides employment for over 400,000 people directly and 1.4 million indirectly (Chamber of Mines, 2017). In contemporary times, the coal-consuming industries have diversified, with coal serving as the primary energy source for electricity generation, petrochemicals, and steel production, along with various other industries, including brick making and cement and lime calcining (Eskom, 2021).

The Mpumalanga Province, particularly the eMalahleni area, hosts approximately 22 coal mines, including some of the largest such as the Wolwekrans

Middelburg Complex and Kriel mine (Bench Marks Foundation, 2014). These mines account for over 84% of South Africa's coal production (Marais *et al.,* 2021). Furthermore, more than 40% of South Africa's electricity is generated from the coal resources in eMalahleni (Marais *et al.,* 2021).

The coal industry not only meets a significant portion of the country's energy needs but also generates substantial revenue through exports (Marais *et al.*, 2021). Coal is responsible for over 90% of South Africa's electricity generation and 20% of its liquid fuels (Department of Energy, 2015). South Africa produces more than 90% of the coal consumed on the African continent and meets about 80% of its own energy requirements (Africa Mining IQ, 2024). Despite its low heat value and high ash content, coal remains South Africa's most abundant and affordable energy source (Jeffrey *et al.,* 2015).

2.3 Legislation of coal mining in South Africa

South Africa has had regulations that hold mine owners accountable for the negative effects of mining since 1903 (Schwab, 2002). However, due to historical gaps in these regulatory policies, many mines went out of business and had no owners (Schwab, 2002). The 1975 Fanie Botha Accord between the Minister of Water Affairs and the Chamber of Mines decided that any mines closed before 1976 would be entirely the government's responsibility, mines abandoned between 1976 and 1986 would fall under a 50/50 ownership/state split and mines that were abandoned since 1987 would entirely be that of the owner of the mine (Cochrane, 2002). Regardless of this, the South African Department of Water Affairs has spent more than R120 million to address past environmental damage; however, this sum is reportedly only a small portion of what is required (Schwab, 2002).

One significant case that exposed the effects of historical inadequate regulatory oversight is the abandoned Transvaal and Delagoa Bay Colliery. This is a huge colliery outside Witbank that posed the highest potential danger of any mine due to partially collapsed sections, big sinkholes and burning coal that caused air pollution (Munnik *et al.,* 2010). Flooded workings at the site resulted in drainage that was very acidic and salty and contained unacceptably high quantities of heavy metals. Fish and crocodile deaths at the Loskop Dam Nature Reserve downstream have been linked to poor

water quality caused by this, and other active and abandoned coal mines (Munnik *et al.,* 2010).

Since the enactment of the new constitution in 1994, the government took responsibility of all natural resources for the people of South Africa (Munnik *et al.,* 2010). Water usage, including mining, is governed by the National Water Act (NWA) (Act 36 of 1998), with an emphasis on the "Polluter Pays Principle". The latter determines that mine owners should be held accountable for their pollution, which also controls the use of mining water and the conservation of the resource. In section 19 (F) under the section "Prevention and remedying effects of pollution" it is stated "*An owner of land, a person in control of land, or a person who occupies or uses the land on which- (a) any activity or process is or was performed or undertaken; or (b) any other situation exists, which causes, has caused, or is likely to cause pollution of a water resource, must take all reasonable measures to prevent any such pollution from occurring, continuing, or recurring*" (Feris and Kotze, 2014).

The Department of Environmental Affairs and Tourism (DEAT) is responsible for enforcing the National Environmental Management Act (NEMA), Act 107 of 1998, which mandates Environmental Impact Assessments (EIAs) and Environmental Management Programmes (EMPs) for activities that have an impact on the environment (Munnik *et al.,* 2010). Its provisions are complementary to the NWA (Munnik *et al.,* 2010). In alignment with the NWA, this Act also mandates pollution prevention or correction, and if the offender fails to do so, DEAT may collect the expenses of clean-up from the offender (DFFE, 2024).

The Minerals and Petroleum Resources Development Act (MPRDA), Act 28 of 2002, administered by the National Department of Mineral Resources (DMR) provides guidelines for the prospecting, quarrying, and production of minerals in South Africa (Bench Marks Foundation and Bread for All, 2015) and requires an Environmental Management Programme Report (EMPR), which must include an EMP that specifies monitoring and assessment procedures, sufficient financial assurances for restoration as well as provisions for monitoring and auditing (Munnik *et al.,* 2010). Additionally, it should include a closure strategy with a budget that will be accessible from the beginning, during the mine's operation, and upon closure (Munnik *et al.,* 2010). The public is also expected to participate in this legislation (DFFE, 2024). Although, this legislation has made somewhat of a change, mining for coal still poses a threat to the environment and organisms surrounding it.

2.4 Ecological impact of coal mining

Even though the mining sector plays a significant role in employment and the basic energy requirements of the country, there has been some reports that have highlighted the ongoing environmental damage. Historically, coal mining has a significant environmental impact on the impaired health and well-being of people and ecosystems in surrounding areas (Cortes-Ramirez *et al.,* 2018).

Coal mining generates significant volumes of waste during mining operations (WRI, 2014) and the disposal of these waste affects the nearby land and water bodies (Mangena and Brent, 2006). Coal mining activities such as drilling, loading, unloading, and transportation create dust (Onder and Yigit, 2009; ABCDust, 2024) and emit vast amounts of metals, nitrates, as well as dissolved and suspended particulates (Lakra *et al.,* 2017). Moreover, the coal combustion process produces further pollutants such as heavy metals, poly-aromatic hydrocarbons, sulphur dioxide, and nitrous oxides (Munawer, 2018). Most of these pollutants are discharged through water draining into many of South Africa's River systems (Feris and Kotzé, 2014).

It has been pointed out that coal mining is also associated with acid mine drainage (AMD) (Prasad, 2024). Acid, sulphate ions, and soluble metal cations are produced when pyrite is exposed to oxygen, water, and naturally existing bacteria because of AMD (Vyawahre and Rai, 2016). Acid mine drainage is characterised by low pH, which results in an acidic environment and, as a result, an abundance of certain heavy metals (Luo *et al.,* 2020).

Since aquatic organisms are exposed to the complete spectrum of chemical and physical factors, including additive and synergistic effects, they are typically considered reliable and accurate indicators of the health status of an aquatic system (Chovanec *et al.,* 2003). Fish have been identified as bioindicator species in the assessment of environmental contamination, because they offer a comprehensive perspective on the state of their environment over longer periods of time (Plessl *et al.,* 2017). Thus, the use of fish as bioindicators should aid in describing the natural environment, detecting, and assessing human influences, and evaluating restoration or remediation strategies (Chovanec *et al.,* 2003; Authman *et al.,* 2015). Most heavy metals have lengthy biological half-lives, and even if the exposure is not necessarily continuous, bioaccumulation may nonetheless occur (Hofer and Lackner, 1995). In many countries, fish is also a significant source of protein for people, so it's crucial to

monitor the aquatic environment heavy and the fish health status to ensure that the meat is safe for human consumption (Plessl *et al.,* 2017). Common carp (*Cyprinus carpio*) and largemouth bass (*Micropterus salmoides*) are widely dispersed in South Africa's freshwater ecosystems and have adapted to various aquatic habitats, making them important bioindicators for studying environmental stressors and their effects on fish health. In this study, we will focus particularly on common carp and largemouth bass in the Witbank dam due to their abundance and feeding habits as well as their utility in monitoring the ecological effect of heavy metals.

2.4.1 Selected fish species

2.4.1.1 *Cyprinus carpio* (common carp)

Cyprinus carpio, universally known as common carp, is an invasive freshwater fish species from the family Cyprinidae (Lowe *et al.,* 2000; Invasive Species SA, 2021a), which originated in Europe and Asia (Winker *et al.,* 2011). It was first introduced to South Africa in 1859, primarily for recreational angling purposes (Ellender and Weyl, 2014).

Currently, common carp is the most popular species for recreational angling in South Africa (Weyl and Cowley, 2015). It is found in dams and major river systems across the country (Ellender *et al.,* 2014; Weyl *et al.,* 2020). This species contains different scales and colour, although it is often olive on the upper part and more translucent in the lower part of the body (**Figure 1**) (Invasive Species SA, 2021a). Wild carp are fully scaled, while domesticated varieties such as mirror carp (with reduced scales) and leather carp (scale-less) were selectively bred to enhance their culinary appeal (Winker *et al.,* 2011). Common carp are large fish that can grow to over 1 meter in length and weigh up to 24 kg (Winker *et al.,* 2011). Currently, the maximum length recorded is 1.2 m (Georgia Aquarium, 2024) and the maximum recorded weight is 51.2 kg (Carp Fishing App, 2024). Native to Europe and Asia, this species has been domesticated as a food source for over 2,000 years (Winker *et al.,* 2011).

Common carp is an omnivorous freshwater species and a benthic feeder (Georgia Aquarium, 2024), although its diet predominantly consists of plants (Chesapeake Bay Program, 2024). It is highly resilient to a wide range of

environmental variations (Diaz Angeriz *et al.,* 2022) such as muddy pools to slow-moving rivers and lakes (Georgia Aquarium, 2024); however, it prefers freshwater ecosystems that are vast, shallow, and covered with vegetation (Invasive Species SA, 2021a). It usually spawns during late spring (October to December) in South Africa (Invasive Species SA, 2021a) and a single female carp can lay up to 300,000 eggs (Chesapeake Bay Program, 2024).

Figure 1: *Cyprinus carpio* collected from Witbank dam, Mpumalanga [Photo credit: M.U.S. Radzuma].

Common carp has a significant detrimental effect on the ecology because of competition with other native organisms for the same food sources (Invasive Species SA, 2021a). Adult carp consume a wide range of organisms, including aquatic plants, planktonic crustaceans, insect larvae and pupae (FAO, 2024), crabs, mollusks, fish eggs, and smaller fish (FAO, 2024; Chesapeake Bay Program, 2024). Juvenile carps usually consume zooplankton, insect larvae, and ostracods (Dadebo *et al.,* 2015).

Their feeding behaviour involves sucking up and spitting mud from the sediment and eating particles that are suspended. This activity disturbs the sediment, increasing nutrient levels and water turbidity (Lougheed *et al.,* 1998; Chesapeake Bay Program,

2024). Consequently, the species negatively impacts water quality and aquatic vegetation, leading to habitat degradation (de Moor and Bruton,1988; Qiu *et al.,* 2019).

Furthermore, it is characterized by its high fecundity, short incubation time, improved fertilization, and a rapid development rate (Naik *et al.,* 2015). While common carp is a bottom-feeding omnivore that is valued for recreational angling and aquaculture, it has detrimental effects on the aquatic life and quality due to its adaptability, feeding behaviour, and high reproductive rate.

2.4.1.2 *Micropterus salmoides* (largemouth bass)

Micropterus salmoides, commonly known as largemouth bass, is considered one of the world's most invasive fish species (IUCN, 2018). It is part of the Centrarchidae family (Freshwater Fishing Advice, 2023) that originated from North America and was introduced in 1928 in South Africa (Marr *et al.,* 2017; Invasive species SA, 2021b). Initially introduced for angling purpose, it has since become widespread throughout the South Africa (Ellender *et al.,* 2014; Ellender and Weyl, 2014).

This olive-green fish is characterized by black dots forming a jagged horizontal stripe (**Figure 2**) (Invasive species SA, 2021b). In South Africa, the Largemouth bass is the most common species, reaching weights of over 4 kg (Weyl *et al.,* 2017). Currently, it has a recorded maximum weight of 11.4 kg and maximum recorded length of 75 cm (Invasive species SA, 2021b). It prefers vegetated quiet waterways and can be found in slower parts of bigger rivers and impoundments (Khosa *et al.,* 2019). In spring, males build and defend shallow-water nests to protect eggs, larvae, and fry (Khosa *et al*., 2019).

The largemouth bass is a carnivorous freshwater gamefish which feeds at any point in the water column of wetlands, streams, ponds, and lakes (Freshwater Fishing Advice, 2023). Largemouth bass, like other members of the Centrarchids genus, is an aggressive predator that feeds mostly on invertebrates as juveniles before becoming increasingly piscivorous as adults (de Moor and Bruton 1988). As the bass becomes older, it will consume larger prey such as shad, bluegill, crawfish, snails, snakes, frogs, small aquatic birds, baby alligators, and lizards (Bass Online, 2023). In larger lakes and reservoirs with deeper water, largemouth bass generally eat younger fish such shiners, sunfish, yellow perch, shad, catfish, trout, walleye, and striped bass (Bass

Online, 2023). It is a predatory fish that can lead to the extinction of endangered indigenous species (Van der Walt *et al.,* 2016).

Largemouth bass have some life-history characteristics, such as massive size, longevity, and high reproduction rates (Taylor and Weyl, 2017). Rapid rates of individual development, a high capacity for dispersion, ecological tolerance, and trophic flexibility are some of the traits that make it successful (Costantini *et al.*, 2023). The rapid spread and adaptability of *Micropterus salmoides* in South Africa highlight the significant ecological risks associated with the introduction of non-native species. Its predatory behaviour, coupled with its ability to outcompete native species, poses a serious threat to the biodiversity of freshwater ecosystems.

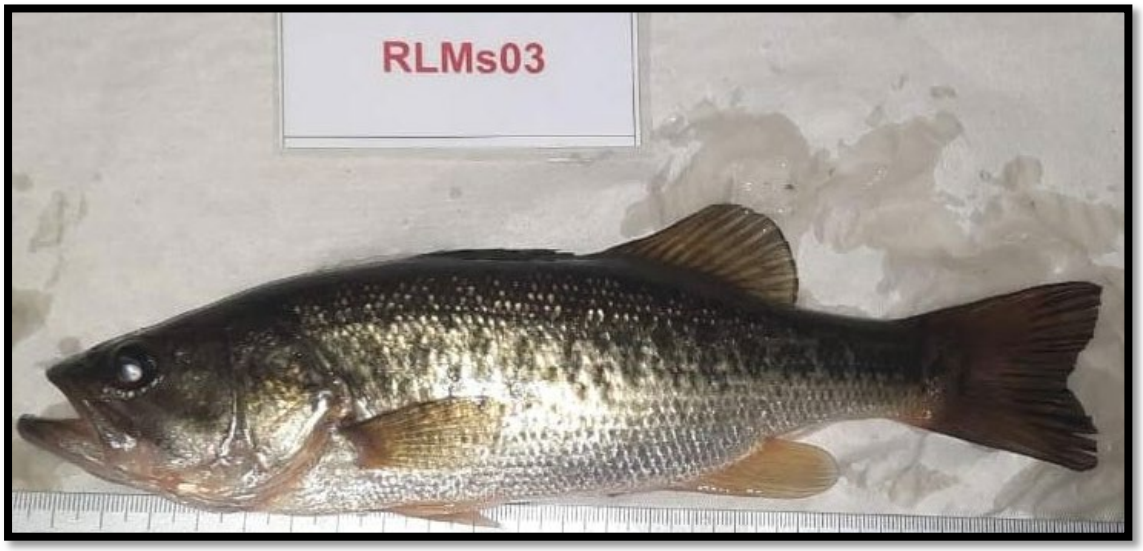

Figure 2: *Micropterus salmoides* collected from Witbank Dam, Mpumalanga [Photo credit: M.U.S. Radzuma].

2.4.2 Heavy metals

Heavy metals are a group of elements that has a relatively high atomic weight and density (Tchounwou *et al.,* 2012). Some of them are recognized as micronutrients in living organisms as they play essential roles in physiological processes. However, they can negatively affect physiological systems if they are either deficient or present in excessive quantities (Ayangbenro and Babalola, 2017). Exposure to high levels can induce histopathological alterations in several organs, including the gills, liver, kidney, and skin, resulting in the impairment of vital functions like breathing, excretion

(Gernhofer *et al.,* 2001), reproduction and immunity (Malik and Maurya, 2014; Garai *et al.,* 2021). Globally metal accumulation and contamination in aquatic bodies has increased due to industrial, mining, and agricultural developments (Castaldo *et al.,* 2020; Briffa *et al.,* 2020; Liu *et al.,* 2021). The swift expansion of industrialization in cities contributes to the discharge of effluents contaminated with hazardous metals, such as chromium (Cr), nickel (Ni), copper (Cu), lead (Pb), iron (Fe), and zinc (Zn) (Khanam *et al.,* 2022). Aquatic organisms absorb these heavy metals either directly through their gills and/or through their diets (Perera *et al.,* 2015).

The metals are concentrated in aquatic species due to their non-biodegradable nature, and they then transfer to other living things at higher trophic levels that eat these animals for nourishment (Ray and Vashishth, 2024). Thus, heavy metal pollution is a critical concern not only due to their non-biodegradability (Cevik *et al.,* 2009) and extreme carcinogenic nature (Khanam *et al.,* 2022), but also because of their inclination to bioaccumulate and in doing so contribute to the biomagnification of metals as we move up the food chain (Malik *et al.*, 2010; Authman *et al.,* 2015; Ali and Khan 2018). Coal mines are known to introduce Fe, Zn, Cu, nickel, and manganese into aquatic systems (Lakra *et al.,* 2019). The focus on Cu, Fe and Zn is of particular interest to the scope of the current study.

The lack of studies on feral species necessitates reflection on laboratory research. Controlled laboratory studies have provided valuable insights into the toxicological mechanisms and physiological impacts of metals on aquatic organisms. However, natural environments present a complex interplay of fluctuating metal concentrations, varying water chemistry, and additional stressors that modulate metal bioavailability and toxicity. These factors cannot be fully replicated in laboratory settings, making feral studies essential for understanding the ecological relevance of metal exposure and its effects on wild fish health.

2.4.2.1 Copper as micronutrient

Copper is a vital trace element that plays a crucial role in various biological activities (Garai *et al.,* 2021). It serves as a cofactor for several regulatory enzymes involved in cellular homeostasis (Belyaeva *et al.,* 2011) and is required for glycoproteins, which are essential for various biochemical processes (Garai *et al.,* 2021). Additionally, it is vital for neutralizing free radicals, facilitating cellular energy production, and supporting

the development of connective tissue (Zhao *et al.,* 2014). Copper can be found in the aquatic environment due to both natural and man-made sources. Geological deposits, volcanic activity, weathering and erosion of rocks and soils, as well as geological deposits, are all natural sources of copper in aquatic systems (USEPA, 2023). Whilst, mining operations, agriculture, metal and electrical industries, sludge from publicly owned treatment works (POTWs), and pesticide usage are examples of anthropogenic sources of copper (USEPA, 2023).

It has been reported that Cu is a key immunological element in aquatic animals. Optimal dietary Cu levels was found to improve non-specific immune responses and antioxidative abilities in Russian sturgeon (Wang *et al.,* 2016) and large yellow croaker (Cao *et al.*, 2014). However, these effects are dose-dependent and whilst an optimal amount in the diet can enhance immune function, any deficiency in/ or excesses of it may lead to adverse effects, including toxicity or compromised immunity (Tan *et al.,* 2011). Therefore, Cu is indispensable for maintaining both cellular and systemic functions in aquatic organisms, with its availability and concentration profoundly impacting health and immunity. An imbalance, particularly a deficiency, can disrupt these essential processes, leading to significant health implications.

2.4.2.1.1 The health impact of copper deficiency

In common carp, dietary copper levels below the optimal requirement of 3 mg/kg can result in Cu deficiency (Ogino and Yang, 1980). Copper deficiency in fish has been linked to significant developmental and physiological impairments. Early embryo death is a common consequence, occurring shortly after fertilization (Słomińska, 1998; Wang *et al.,* 2020), during the blastula stage (Ługowska, 2005), or body segmentation (Wang *et al.,* 2020). Additionally, copper deficiency compromises immune function, leading to reduced humoral, cell-mediated, and nonspecific immune responses, which in turn results in higher mortality rates due to infections (Stabel and Spears, 1989).

Beyond immune dysfunction, Cu deficiency can cause poor embryo pigmentation, spinal cord distortions, cranial deformities, underdeveloped jaws, reduced body length, prolonged time for yolk absorption, oedema, and opaque yolk sacs (Jezierska, 2009). Inadequate Cu levels in water also interfere with fish egg hatching by inhibiting chorionase activity. This enzymatic dysfunction causes osmotic disturbances and prevents the muscular motions required to break the eggshell

(Johnson *et al.,* 2007). While research on Cu deficiency in fish is limited, its critical roles in enzyme activity (Garai *et al.,* 2021) and iron metabolism (Belyaeva *et al.*, 2011) suggest that insufficient copper intake may severely impair these physiological functions.

2.4.2.1.2 Presentation of copper toxicity

In contrast to copper deficiency, excessive copper levels in aquatic environments pose significant toxicity risks. Copper exhibits toxicity to freshwater fish at concentrations ranging from 10–20 ppb (Woody and O'Neal, 2012). The extent of its toxicity is influenced by various environmental factors such as pH, water hardness and anions (Garai *et al.,* 2021). Low dissolved oxygen levels, reduced water hardness, and the presence of other metals can intensify copper toxicity (DWAF, 1996; Niyogi and Wood, 2004). Conversely, Cu-toxicity is mitigated by higher alkalinity, chelating agents such as humic acids and amino acids, and the presence of other elements like zinc, molybdenum, calcium, and magnesium (DWAF, 1996).

In South Africa, Chronic Effect Value (CEV) criteria for Cu range from 0.53 µg/L in soft water to 2.8 µg/L in very hard water, while the Acute Effect Value (AEV) criteria span from 1.6 µg/L in soft water to 12 µg/L in very hard water (DWAF, 1996). Excessive Cu exposure disrupts various cellular and physiological processes, impairing ionic and osmotic regulation, antioxidant defence mechanisms (Zebral *et al.,* 2019), and respiratory function (Belyaeva *et al.,* 2011). Long-term exposure to elevated Cu concentrations has also been linked to reduced body weight and reproductive issues, such as a lower gonadosomatic index (GSI) in both male and female zebrafish (Cao *et al.,* 2019).

Moreover, Cu toxicity has been observed to disrupt reproductive health in zebrafish which results to infertility. In females, it reduces hormones such as follicle-stimulating hormone (FSH), luteinizing hormone (LH) and oestrogen (E2) levels, leading to impaired oocyte maturation and vitellogenesis, which are vital for reproduction (Clelland and Peng, 2009). In males, it hinders spermatogenesis and delays gonadal maturation (Schulz *et al.,* 2010).

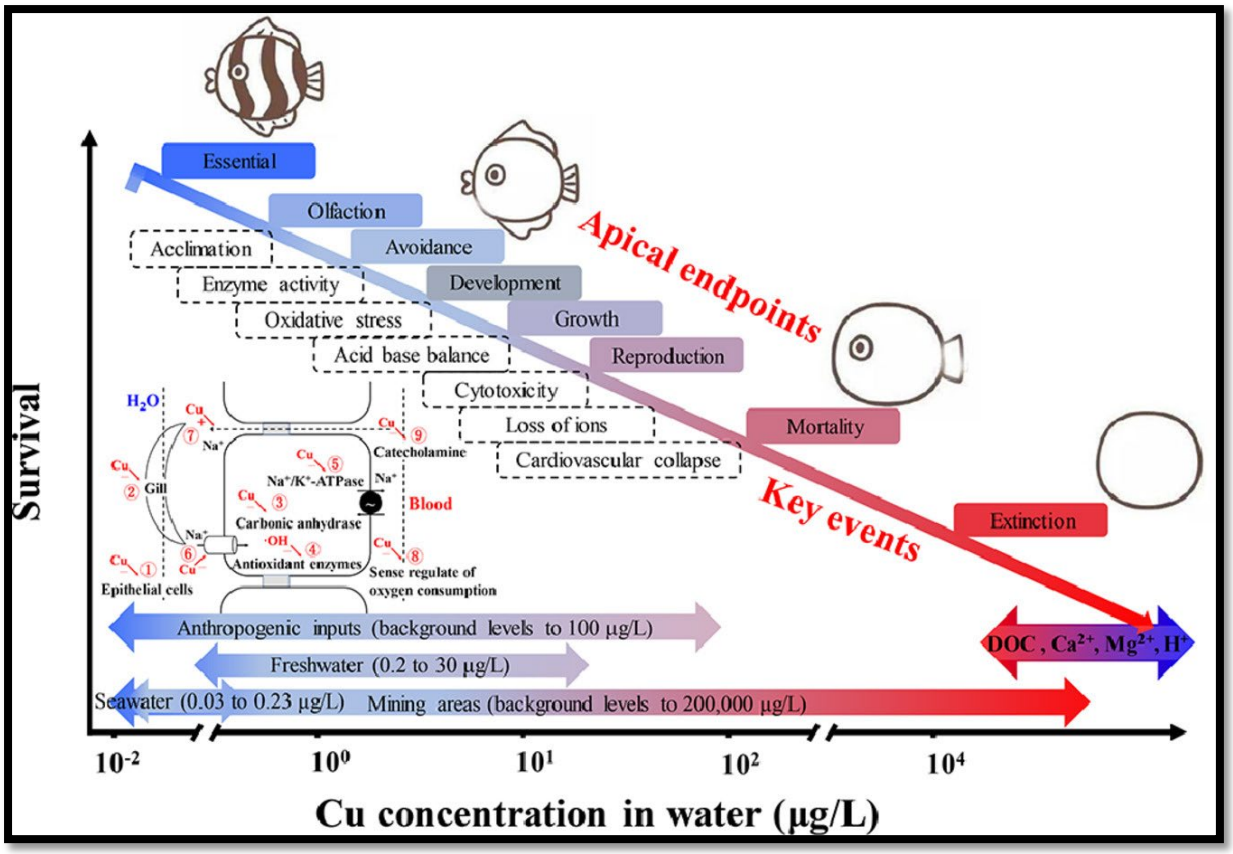

Figure 3: Graph depicting Cu concentration in water vs. apical endpoints in fish (Illustration obtained from Liao *et al.*, 2023).

Current literature indicates that the toxic effect of Cu extends across various fish species. In *Trematomus bernacchii*, Cu exposure in a laboratory-based study lead to oocyte degeneration and reduced fecundity (Motta *et al.,* 2021), while in *Collossoma macropomum*, it decreased sperm motility, fertilization rates, and hatching success (Pinto *et al.,* 2021). Copper toxicity in zebrafish negatively affects gonadal maturation and reduces GSI by impairing steroidogenesis (Cao *et al.,* 2019), while in *Pelteobagrus fulvidraco*, it alters ovarian morphology and yolk platelet structure (Zhang *et al.,* 2016). Copper toxicity has also been observed to affect mitochondrial structures in species like *Oncorhynchus mykiss* (Sappal *et al.,* 2015). In addition, it disrupts sperm mitochondrial function in *Poecilia vivipara* and contributes to premature births in the mosquito fish (*Gambusia affinis*) (Zebral *et al.,* 2019).

At the embryonic stage, Cu toxicity disrupts organogenesis in *Oryzias melastigma*, leading to a variety of organ abnormalities (Wang *et al.,* 2020). The toxic effects of Cu on late-stage embryos are linked to its increasing bioaccumulation and the declining protective barrier effect of eggshells during development (Ługowska,

2005). Over 60% of Cu-exposed embryos exhibit morphological abnormalities, such as skeletal and cardiovascular malformations, along with elevated heart rates (Wang *et al.,* 2020; Jezierska *et al.,* 2009).

Copper exposure also impairs gill function, reducing the ability of fish to regulate essential salts like sodium- and potassium-chloride (Solomon, 2009). Additionally, Cu can induce the production of reactive oxygen species (ROS), leading to oxidative stress that damages cells, organs, proteins, lipids, and DNA (Valko *et al.,* 2005) (Figure 3). Prolonged exposure to high dietary Cu levels has been associated with impaired intestinal apoptosis, reduced growth (Berntssen *et al.,* 1999; Lundebye *et al.,* 1999) and compromised immune response or antioxidation capabilities (Shariff *et al.,* 2001; Yeh *et al.,* 2004; Yacoub and Gad, 2012).

The reviewed literature highlights the multifaceted nature of copper toxicity in freshwater fish, emphasizing its dependence on both intrinsic factors and extrinsic factors. Copper, even at low concentrations of 10–20 ppb, has been shown to induce physiological disruptions, including oxidative stress and compromised metabolic functions. Copper toxicity can result increased mortality, morphological deformities, reduced hatching rates, and impaired early development (Wang *et al.,* 2020). Despite significant progress in understanding the mechanisms of copper toxicity, knowledge gaps remain regarding its chronic effects across various immunological responses.

2.4.2.2 Iron as micronutrient

Iron is a vital element required for many proteins and enzymes in fish (Thangapandiyan *et al.,* 2020). It plays a crucial role in growth, body composition and digestive enzyme activities of fish (Thangapandiyan *et al.,* 2020). It is required for the synthesis of haem, which serves as a vital prosthetic group for oxygen-binding proteins including haemoglobin and myoglobin (Zhang, 2014). It is also acts as a cofactor for iron-sulphur cluster proteins (Zhang, 2014), which play a role in electron transfer, DNA replication and repair (White and Dillingham, 2012).

Iron can directly be bound by other proteins (Zhang, 2014). For instance, ferritin, a protein which acts as reservoir for iron, which makes iron available for cellular processes (Finazzi and Arosio*,* 2014; Arosio *et al.,* 2015). Additionally, the small subunit of ribonucleotide reductase, among other members of the subfamily of proteins that possess the ferritin structure, uses iron as a direct cofactor (Andrew, 2010). The

two most prevalent states of iron in water are reduced (ferrous, Fe^{+2}) and oxidized (ferric, Fe^{+3}) (Bury and Grosell, 2003; Lall and Kaushik, 2021). Iron is naturally released into the environment by weathering of sulphide ores and igneous, sedimentary, and metamorphic rocks (DWAF, 1996). Two iron oxides and two iron hydroxides are released into the environment because of sandstone leaching (DWAF, 1996). Human activities also release iron into the environment, especially through coal combustion, acid mine drainage, mineral processing, sewage, landfill leachates, and iron and steel corrosion (DWAF, 1996). The primary source of iron absorption in fish is the intestinal mucosa, in the gastrointestinal tract, as little is absorbed by the gills (Bury and Grosell, 2003; Cooper and Bury, 2007; Chandrapalan and Kwong, 2020).

2.4.2.2.1 How does iron deficiency present?

In common carp, dietary copper levels in serum below the optimal requirement of 147.4 mg/kg can result in iron deficiency (Ling *et al.,* 2010). In fish, iron deficiency is primarily associated with anaemia and significant depletion of tissue iron stores (Zafar and Khan, 2020). This condition manifests through reduced haematocrit levels, haemoglobin concentrations, and red blood cell counts (Rios *et al.,* 2005; Zafar and Khan, 2020) as well as reduced growth and feed intake (Zafar and Khan, 2020). Beyond blood-related effects, iron deficiency is known to influence oxidative stress (Luo *et al.,* 2017; Guo *et al.,* 2023) and impairs immune function by reducing lysozyme and acid phosphatase (ACP) activities and lowering the levels of key immune proteins such as complement 3 (C3), complement 4 (C4), and immunoglobulin M (IgM) (Guo *et al.,* 2019). This deficiency also downregulates key antimicrobial peptides, including hepcidin and LEAP-2, and anti-inflammatory cytokines such as TGF-β and IL-10, increasing the fish's susceptibility to enteritis when exposed to pathogens like *Aeromonas hydrophila* (Guo *et al.,* 2019).

In contrast to this, the deficiency upregulated pro-inflammatory markers, including IL-1β, IL-8, and interferon gamma (IFN-γ), suggests that iron depletion leads to chronic inflammation in the intestines. This combination of immune suppression and increased inflammatory response indicates that iron deficiency severely impairs the intestinal immune defence, leaving the fish vulnerable to infection and inflammation.

With impaired immune defences, such fish are more vulnerable to infections and other health complications (Guo *et al.,* 2018).

2.4.2.2.2 Presentation of iron toxicity

The toxicity of Fe in fish varies depending on species and size (Rostern, 2017). In South Africa, there is insufficient data to establish Chronic Effect Value (CEV) or Acute Effect Value (AEV) criteria for iron across different water hardness levels (DWAF, 1996). The toxic effects of iron depend on whether it is present in its ferrous (Fe^{2+}) or ferric (Fe^{3+}) form, and whether it exists in suspension or solution (DWAF, 1996).

The ability of Iron to switch between the ferrous and ferric forms makes it a potent catalyst for free radical formation, posing a challenge to biological systems (Rostern, 2017). In particular, fine ferric iron particles can irritate gill tissues, leading to gill damage and increasing susceptibility to bacterial and fungal infections (Rostern, 2017). Elevated Fe levels can disrupt normal growth, feed utilization, and immune function, leading to higher mortality rates, diarrhoea, and histopathological damage to liver cells (Lall and Kaushik, 2021). Excessive Fe also disturbs iron homeostasis, resulting in Fe overload in tissues (Bury *et al.,* 2012). This Fe overload can cause DNA damage, lipid peroxidation, and protein oxidation, all of which contribute to cellular and tissue damage (Ruas *et al.,* 2008).

Iron plays a crucial role in several biochemical processes such as electron transfer, gene regulation and the binding and transport of oxygen (Lall and Kaushik, 2021). However, when iron levels become excessive, it can promote oxidative stress by facilitating the formation of reactive oxygen species, which damage proteins, lipids, and DNA. As a result, elevated iron concentrations can be toxic, leading to significant cellular and organ damage in fish (Zafar and Khan, 2020).

2.4.2.3 Zinc as micronutrient

Zinc is an essential micronutrient involved in a variety of metabolic activities (Chatterjee *et al.,* 2019) such as cell division and body growth (MacDonald, 2000). It acts as a cofactor to numerous enzymes including alkaline phosphatase (Lall and Kaushik, 2021), whose activity is critical for cell growth, apoptosis and cell migration (Sharma *et al.,* 2014; Banaee *et al.,* 2019). There is limited information on the function

of zinc in fish as most of what is known about the function of Zn comes from vertebrate studies. Zinc enters water and soil through natural processes and, anthropogenic activities such as metal manufacturing, chemical industries, home wastewater, and run-off from soil (Rostern, 2017). Zinc in aquatic environments predominantly accumulates in sediments, with only a minimal fraction remaining dissolved as fine particles in the water column (Rostern, 2017). This distribution influences the exposure pathways of fish to Zn, primarily through their feeding activities and environmental interactions. Therefore, feeding habits may play a pivotal role in determining the levels of Zn exposure among different fish species. Zinc uptake in fish typically occurs via the gills and the digestive system, with the extent of accumulation being dependent on the concentration of zinc in their immediate surroundings (Garai *et al.,* 2021).

Benthic (bottom) feeders like *C. carpio*, are more likely to encounter zinc-rich sediments during foraging, potentially leading to a higher accumulation compared to pelagic (open water) feeders such as *M. salmoides* that primarily consume other organisms in the water column. Similarly, detritivores and omnivorous species that scavenge at the bottom may also have increased exposure relative to piscivorous species. Thus, the dietary preferences and feeding behaviours of fish are crucial factors influencing their Zn intake and accumulation, highlighting the need for further research to explore these variations across different species.

2.4.2.3.1 Presentation of zinc deficiency

Zinc deficiency is associated with a significant reduction in its levels throughout the body, particularly affecting organs beyond the gut and gills (Zheng *et al.,* 2014). In zebrafish, this Zn shortage correlated with a substantial decrease in essential minerals such as calcium, potassium, and sodium after just three weeks on a low-zinc diet (Zheng *et al.,* 2014). The consequences of low dietary zinc consumption are severe; it results in suboptimal growth (Yuan *et al.,* 2023) and leads to diminished zinc concentrations in the blood, liver, scales, body, and vertebrae across various fish species (Lall and Kaushik, 2021).

Moreover, reduced Zn levels negatively impact endurance and cognitive function in adult fish (Beaver *et al.,* 2017). Insufficient maternal Zn intake has also been linked to multiple dysfunctions in juvenile fish. Parental Zn deprivation can result in a

deficiency in offspring, affecting their mineral homeostasis and embryonic development. This deficiency has been associated with a small but noticeable increase in eye and nose deformities, as well as hypoactivity in larvae (Beaver *et al.,* 2017).

At the genetic level, zinc-deficient embryos show altered expression of several zinc transporter genes (ZnT8, ZnT9) and the metal-regulatory transcription factor 1 (MTF-1), along with other genes that regulate metal homeostasis (Beaver *et al.,* 2017) potentially leading to impaired ion homeostasis (Beaver *et al.,* 2017). Additionally, zinc deficiency is linked to reduced expression of pancreatic and diabetes-related genes (Insa, Pax4, Pdx1) in developing embryos, resulting in low birth weights, poor glucose tolerance, and abnormal development and health in juvenile zebrafish fish (Beaver *et al.,* 2017).

Zinc deficiency has also been reported to decrease mRNA levels of antioxidant enzymes related to the Nrf2 pathway, while simultaneously increasing mRNA levels of apoptotic markers associated with the p38 MAPK and JNK pathways (Song *et al.,* 2017). Furthermore, it reduces the expression of tight junction proteins linked to myosin light chain kinase (MLCK), indicating potential damage to the intestinal barrier (Song *et al.,* 2017). Specifically, a low zinc diet (10.71 mg/kg) was shown to impair immune responses by decreasing antibacterial compound production and increasing pro-inflammatory cytokines, while simultaneously reducing anti-inflammatory cytokines in the intestines (Song *et al.,* 2017).

2.4.2.3.2 Presentation of zinc toxicity

Several environmental conditions, including temperature, water hardness, and dissolved oxygen concentration, affect how hazardous Zn is to aquatic organisms (Li *et al.,* 2021a). For instance, the concentration of dissolved Zn ions in water may rise when the acidity of water rises (Rostern, 2017). Total Zn concentrations in natural waterways range from one to six orders of magnitude and are substantially impacted by human activities (Rostern, 2017). The free Zn^{2+} 8 ions, a major inorganic Zn species in most natural streams, are responsible for the toxicity of waterborne Zn to fish (Rostern, 2017). Additionally species-specific, zinc toxicity varies depending on the embryonic stage of the fish (Garai *et al.,* 2021).

In South Africa, the CEV criteria for Zn in water is 3.6 µg/R. Whilst, the AEV criteria for Cu in soft water 36 µg/R (DWAF, 1996). In acute toxic concentrations of Zn, fish species are killed by damaging the gill tissue, and at chronic toxic concentrations, fish are killed by deterioration in heart, liver, kidneys, skeletal muscles, gonads and spleen (Skidmore, 1964). Zinc is thought to be a requirement for proper eye development in juvenile fish. However, excessive amounts of dietary calcium (Ca) and phosphorus in fish meal, as well as phytic acid in plant components, are known to reduce the bioavailability of Zn which then result in lens cataracts (Welker *et al.,* 2016).

Excessive dietary Zn levels may be hazardous to fish and compete for comparable binding sites in the digestive system with other bivalent minerals such as Cu, Fe, Ca, and cadmium during absorption (Clearwater *et al.,* 2002). The divalent cationic form of Zn, which interferes with Ca ion absorption in the tissue, produces hypocalcaemia, and ultimately results in fish death which is the main cause of Zn toxicity (McRae *et al.,* 2016). Sublethal Zn exposure of killifish in freshwater and seawater caused pathological changes in both calcium and sodium homeostasis and an increase in salinity exerted protective effects against sublethal and lethal Zn toxicities (Loro *et al.,* 2014).

2.5 The physico-chemical characterisation of the aquatic environment

2.5.1 Temperature

Water temperature is the most significant abiotic factor that is defined as a measurement of the average thermal energy of a substance, as the categories hot and cold are equally arbitrary (Osmond *et al.,* 1995). In South Africa, the normal range for water temperature in high and low flow is not listed. Numerous aquatic species live in various habitats, certain aquatic organisms thrive in warmer water temperatures, especially aquatic vegetation, whilst certain fish, including salmon and trout, prefer cooler temperatures (EPA, 2012a)**.**

Extreme variations in water temperature have an impact on normal physiology and metabolic processes (Lee *et al.,* 2023). It alters physiological processes like growth, metabolism, osmoregulation (Schwieterman *et al.,* 2022), heat tolerance (Bhadja and Vaghela, 2013), heart rate (Ming *et al.,* 2012), reaction times

(Schwieterman *et al.,* 2022), reproduction (Geffroy and Wedekind, 2020), internal homeostasis (Kumar *et al.,* 2019) and immunity (Kandalski *et al.,* 2018; Dawood *et al.,* 2020a). There is a clear link between water temperature and metabolic rates as higher water temperatures can increase the activity of several biological enzymes (Pearson Education, 2023). However, water temperatures that are higher than the tolerance limit induces physiological harm and homeostatic disruptions, and eventually toxicity to the fish (Kumar *et al.,* 2019).

High water temperatures can also affect aquatic life by increasing the solubility and thus the toxicity of certain chemicals such as pesticides and ammonia (USGS.gov, 2018). The temperature of the water can affect an organism's tolerance limit in addition to making harmful substances more soluble (Bhadja and Vaghela, 2013). Temperatures exceeding 25°C are associated with much greater Zn mortality rates than those below 20°C (Bhadja and Vaghela, 2013)**.** Fish that experience thermal stress have higher oxygen consumption and tissue energy metabolism, which puts more strain on their hearts and causes mitochondrial dysfunction (Ming *et al.,* 2012). Furthermore, it severely compromises the integrity of cellular membranes, disrupts cellular function (Wang *et al.,* 2022), and increases susceptibility to bacterial infection (Song *et al.,* 2018). Fish that are subjected to extreme heat stress experience alterations in their immune system, which can hinder their ability to fight off infections and ultimately result in many fish deaths in aquaculture (Ming *et al.,* 2012). Heat stress in fish can cause disrupt their immune system and trigger inflammatory reactions due to the inhibition (suppression) of immune function and impair to the antioxidant response, which alters gene expression profiles linked to immune regulation and stress adaptation (Dawood *et al.,* 2020b). Lee *et al.* (2023) reported that heat shock following pre-heat markedly increased the expression of several immune-related genes in the liver and brain of *Paralichthys olivaceus*, including interleukin 8 (IL-8), c-type lysozyme (c-lys), immunoglobulin M (IgM), Toll-like receptor 3 (tlr3), major histocompatibility complex IIα (MHCIIα), and cytotoxic T lymphocytes or CD8α. While temperature plays a significant role in shaping the physiological processes and overall health of aquatic life, another crucial abiotic factor that influences aquatic organisms is pH.

2.5.2 The pH of aquatic bodies

The pH is a value that ranges from 0 to 14 that indicates how basic or acidic a body of water is. As a logarithmic scale (OpenStax College, 2013), its value is determined by the concentration of hydrogen ions present (Nave, 2001). The normal pH range for surface water systems is between 6.5 and 8.6 (Atlas Scientific.com, 2022). In South Africa, the normal range for pH in aquatic ecosystems is between 6.00-9.00 (DWAF, 1996). The pH of water can have detrimental consequences when it rises above 9.6 or goes below 5.0 (Atlas Scientific.com, 2022). Changes in pH, especially if pH levels of the water are too high or too low can disturb the homeostatic state within animal systems which may compromise their survival (EPA, 2012b). Though some aquatic species such as *Oreochromis niloticus*, may survive in water with extreme pH levels, most aquatic life prefers a pH range of 6.5–9.0 (Fondriest.com, 2013). If the pH is below ideal ranges for a specific fish species, then that fish species become more vulnerable to fungal infections (Radke, 2006). It was also revealed that lowering to pH 4.5 reduced the levels of plasma IgM in carp (Nagae *et al.,* 2001). Furthermore, fish reproduction is often negatively impacted by pH values below 5.0, and many species (such salty fish or delicate freshwater fish like smallmouth bass) may migrate away from such regions (Lennech, 2024).

Extreme low pH values can also impact chemical reactions and heavy metal solubility (USGS.gov, 2013). It often enhances the solubility of elements and compounds, increasing the danger of aquatic life absorbing toxic chemicals and making them more mobile in addition to their biological effects (EPA, 2012b). Metal cations including aluminium, lead, and copper are released into the water when the concentration of hydrogen ions rises rather than being incorporated into the sediment (USGS.gov, 2013).

2.5.3 Dissolved oxygen in surface water

Dissolved oxygen (DO) is a crucial factor for evaluating the quality of water due to its impact on the aquatic life (Ali *et al.,* 2022). It refers to atmospheric oxygen that has diffused into water (Ali *et al.,* 2022). Dissolved oxygen is measured in mg/L or percent (Wilson, 2010). The requirement of DO differs from species to species in fish (Ali *et*

al., 2022). However, excessively high or low DO levels are known to impair aquatic life. In South Africa, the normal range for aquatic ecosystem is between 6-9 mg/L (DWAF, 1996). Dissolved oxygen below 5-6 mg/L in freshwater is known to cause hypoxia in aquatic organisms (Dong *et al.,* 2011). Low DO results in a decline in fish growth and production, and there will be a greater chance of a disease outbreak (DCCEEW, 2024). Poor water quality due to human activities might weaken the immune system's ability to fight off infections from pathogens (Hutton and Chase, 2017). Hypoxia adversely impact fish physiology, development, behaviour (Mallya, 2007; Thorarensen *et al.*, 2010). Furthermore, hypoxia can alter fish innate (Abdel-Tawwab *et al.,* 2014) and adaptive immune responses (DCCEEW, 2024). It was found that tilapia (*Oreochromis sp.*) exposed to hypoxic circumstances had a significant death rate due to streptococcal infection (Shoemaker *et al.,* 2000). Furthermore, it was discovered that as the DO level dropped, so did lysozyme activity and fish resistance to *Aeromonas hydrophila* infection (Abdel-Tawwab *et al.,* 2014; Abdel-Tawwab *et al.,* 2015).

Beyond immunological effects, hypoxia results a decrease in blood pH, partial pressure of oxygen, total oxygen content and plasma sodium and chloride (Aboagye and Allen, 2018). Furthermore, aquatic organisms like Salmon are unable to breed at concentrations lower than 6 mg/L (Wijgerde, 2012). Regions that have DO below 3.7 mg/L are avoided by coastal fish; some species leave the area entirely when DO drops below 3.5 mg/L (EPA, 2000). At 1 mg/L, benthic creatures exhibit lowered rates of survival and development (EPA, 2000).

Dissolved oxygen levels exceeding air saturation is known as hyperoxia (Ali *et al.,* 2022). Fish hyperoxia has been predominantly focused on aquaculture applications or within mechanistic frameworks (McArley *et al.,* 2020). Whilst hyperoxia may disrupt normal biochemical pathways affecting processes like metabolic regulation (McArley *et al.,* 2020); existing literature has revealed that this condition/state can be beneficial if moderate and controlled amounts such as in aquaculture (McArley *et al.,* 2020). It has been observed to enhance the resilience of coastal organisms such as fish to climate change induced problems such as elevated water temperature (Giomi *et al.,* 2019). Furthermore, it has been shown to maintain or enhance metabolic capacity during acute thermal stress (Brijs *et al.,* 2015; McArley *et al.,* 2018).

To conclude, dissolved oxygen plays a pivotal role in maintaining aquatic ecosystem health, as its levels directly influence the physiology, behaviour, and survival of aquatic organisms. While low dissolved oxygen levels (hypoxia) are widely recognized for their detrimental effects, including impaired immune responses, reduced growth, increased susceptibility to disease, and altered metabolic functions, excessively high levels (hyperoxia) can also disrupt normal biochemical processes. However, under controlled conditions, hyperoxia may offer benefits, such as improving metabolic capacity and resilience to climate change-induced stressors in aquatic species. These findings highlight the importance of maintaining dissolved oxygen levels within optimal ranges to ensure the sustainability of aquatic ecosystems and the organisms they support.

2.5.4 Total dissolved solids in the surface water

Total dissolved solids (TDS) refer to the amount of solid material dissolved in a specific volume of water, and it is normally measured in grams per litre (Christ and Wernli, 2014). In South Africa, the normal range of salinity in freshwater is not well documented; however, according to Weber-Scannell and Duffy (2007), TDS in freshwater is typically limited to 2000 mg/L though lower levels are recommended. Extreme concentrations of TDS and conductivity, whether high or low, can negatively impact various forms of aquatic life (Weber-Scannell and Jacobs, 2001). Growth and survival of aquatic organisms were significantly reduced when TDS levels exceeded 1500 mg/L (Weber-Scannell and Jacobs, 2001).

Total dissolved solids contribute to toxicity by increasing salinity, altering the ionic composition of water, and through the toxic effects of specific ions. Cells will shrink in water with an extremely high TDS content. These changes may have an impact on an organism's capacity to travel through a water column, leading it to float or sink outside of its typical range (EPA, 2012c). Total dissolved solids may also have an impact on the flavour of water and frequently denotes excessive hardness or alkalinity (Thompson *et al.,* 2006). However, TDS have been reported to be hazardous to fish and fish eggs, depending on the ionic characteristics (Fondriest.com, 2013). Reduced hatching and egg survival rates were seen in salmonids, perch, and pike when total dissolved solids were over 2200–3600 mg/L (Weber-Scannell and Jacobs, 2001). In conclusion, Total Dissolved Solids impact water quality, with concentrations above 1500 mg/L reducing aquatic life survival by increasing salinity and altering ionic

composition (Weber-Scannell and Jacobs, 2001). High TDS levels can disrupt cellular function and affect movement (EPA, 2012c), while also reducing fish egg survival (Weber-Scannell and Jacobs, 2001). The influence of salinity will be explored further in the next section.

2.5.5 Salinity of surface water

Salinity is the total concentration of all dissolved salts in water, usually measured in parts per thousand (ppt) (NOAA, 2024). It is particularly significant as it influences the solubility of dissolved oxygen, the dissolved oxygen content decreases as salinity increases (NOAA, 2024). It also is one of the most important abiotic factors that influences aquatic species' development, metabolism (Kültz, 2015) survival and immunology in aquaculture (Jeffries *et al.,* 2019). The normal range of salinity in freshwater is below 0.5 ppt (IAL and IUBS, 1958).

Water quality and aquatic life can be impacted by changes in salinity regardless of whether the cause was natural or man-made (Jeffries *et al.,* 2019). Water column stratification is a result of changes in water density brought about by temperature and salinity. A rise in salinity will have the same effect as a drop in temperature on the density of water (Liu *et al.*, 2024; NOAA, 2024). A change in the saltiness of habitat water induces salinity stress, as it disrupts physiological homeostasis and routine biological processes if not properly regulated (Kültz, 2015). While most euryhaline species fish species and some of stenohaline species can tolerate some level of salinity stress, the majority are confined to environments with relatively stable salinity levels (Kültz, 2015).

Industrial pollution is often indicated by unusual salinity values (EPA, 2012d). Most aquatic animals have evolved to tolerate varying salinities (Pawlowicz and Yerubandi, 2023). Fish deaths can occur when salinity levels exceed a certain threshold because of altered dissolved oxygen concentrations (Weber-Scannell and Jacobs, 2001), and increased TDS toxicity (Weber-Scannell and Jacobs, 2001; Pawlowicz and Yerubandi, 2023). It was discovered that salinity affected immune markers in *Scatophagus argus*, with lower levels of lysozyme, complement C3 activity, and IgM content in brackish and freshwater compared to seawater after *Aeromonas hydrophila* infection (Lu *et al.,* 2022a). In conclusion, salinity significantly affects aquatic species' development and survival by influencing water density, oxygen levels,

and physiological processes (Kütz, 2015; NOAA, 2024). While some species can tolerate salinity fluctuations, most require stable conditions. High salinity can increase toxicity and stress immune functions, leading to potential harm in aquatic life (Lu *et al*., 2022a).

2.6 The role of the innate immune system in fish health

The ability of fish to cope with pathogen invasion is mainly controlled by their immune system, which consists of both innate and adaptive components (Dash *et al.,* 2018). The innate immune system is the body's first line of defence against infections and diseases, operating without retaining memory of prior encounters (Riera Romo *et al.,* 2016). Its components include physical barriers like the skin, cellular mechanisms such as phagocytosis, and humoral elements like soluble complement proteins **(Figure 4)** (Riera Romo *et al.,* 2016). Whilst the adaptive immune system is highly specific to particular antigens and can provide long-lasting immunity (Alberts *et al.,* 2002), the innate immune system identifies pathogens through various receptors, triggering microbial defence and boosting adaptive immune responses (Kabelitz and Medzhitov, 2007). It detects pathogens via multiple receptors; thereby, activating microbial defence and stimulating the adaptive immune response (Kabelitz and Medzhitov, 2007). Pattern recognition receptors (PRRs), which are primarily expressed on the surface of immune cells, can directly recognize pathogen associated molecular patterns (PAMPs) of pathogenic microbes. Moreover, the innate immune system consists, amongst others, of vital components such as C-reactive proteins (CRP) and immunoglobulins (Salinas, 2015; Dash *et al.,* 2018).

2.6.1 The role of immunoglobulin M

Immunoglobulins, including Immunoglobulin M (IgM), are essential B-cell receptors, found either on the surface of B-cells in their membrane-bound form or in soluble form within bodily fluids (Salinas *et al.*, 2011). Structurally, they consist of two identical heavy (H) chains and two identical light (L) chains, each having variable (VH and VL) and constant (CH and CL) domains (Salinas *et al.*, 2021) for antigen recognition and mediating effector functions, such as pathogen opsonization, neutralization,

complement activation, and antibody-dependent cellular cytotoxicity (Schroeder and Cavacini, 2010).

IgM is primarily a key component of systemic immunity, yet it is also present in mucus, playing a role in mucosal immunity (Salinas *et al.*, 2015). It was noted that the composition of fish mucus changes in response to infection or immunization, emphasizing the importance of mucosal immunoglobulins in immune defence (Salinas *et al.*, 2011). IgM is the most prevalent immunoglobulin in teleost fish plasma and is involved in both systemic and mucosal immune responses (Parra *et al.*, 2013). It is significant phylogenetically, as the first immunoglobulin to emerge in evolution, and it remains the primary immunoglobulin class found in fish (Magnadóttir, 1998; Mashoof and Criscitiello, 2016). Furthermore, IgM is the first antibody produced during development in higher vertebrates, and its role in fish includes pathogen neutralization, complement activation, opsonization (Hsu and Du Pasquier, 2015), and hypersensitivity reactions (Roberts, 2001).

In addition to its involvement in systemic and mucosal immunity, IgM plays a crucial role in immunoregulation (Habte-Tsion *et al.*, 2016) and the phagocytosis of foreign organisms (Holland and Lambris, 2002). The concentration of IgM can be influenced by dietary factors such as Cu and Fe. Appropriate levels of dietary Cu can significantly increase IgM levels and activate the complement system, while excessive Cu intake lowers IgM levels, impairing immune regulation (Liang *et al.*, 2020). Similarly, elevated Fe levels have been shown to cause immune dysfunction, increasing fish susceptibility to bacterial infections. Iron overload has been linked to downregulation of key immune-related genes, such as CD83, IL-17, IL-1, Toll-like receptors, and T-cell receptors, resulting in compromised immune function (Valenzuela-Muñoz *et al.*, 2020).

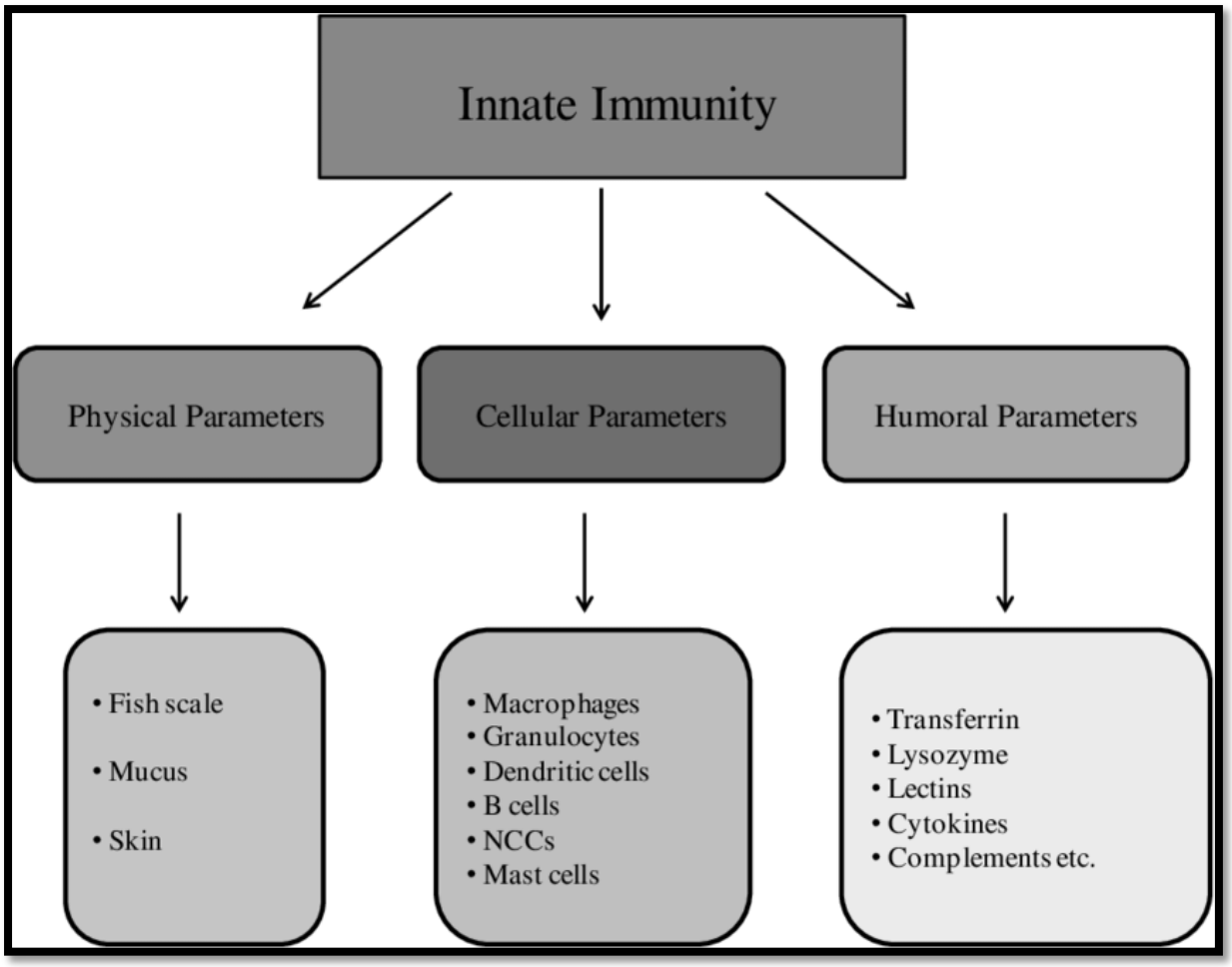

Figure 4: Components of Innate Immunity (Obtained from: https://www.researchgate.net/figure/The-components-of-the-fish-innate-immune-system-The-fish-innate-immune-system-is_fig1_331876439).

2.6.2 The role of Interleukin 6

Interleukin-6 (IL-6) is a cytokine involved in various processes such as inflammation, metabolism, tissue regeneration and neural function (Wolf *et al.,* 2014). It is produced by numerous cell types, such as T and B lymphocytes, fibroblasts, monocytes, keratinocytes, mesangial cells, endothelial cells, and several types of tumour cells (Ataie-Kachoie *et al.,* 2013). IL-6 has pro-inflammatory and anti-inflammatory properties and plays critical roles in haematopoiesis (Zou and Secombes, 2016), cellular growth, and host defence mechanisms (Eggestøl *et al.,* 2020). In fish, like in mammals, IL-6 also aids the acute phase response and its role in immunoregulation

(Metcalfe *et al.,* 2020), cytokine production, and tissue regeneration (Li *et al.,* 2021b) is well established.

The IL-6 cytokine family in fish includes IL-6, IL-11, ciliary neurotrophic factors (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-like cytokine (CLC), cardiotrophin 1 (CT-1) and IL-27 (Rose-John, 2018). While the functions of some members of the IL-6 family remain unclear, recombinant IL-6 (rIL-6) have been shown to significantly influence immune responses in rainbow trout, rIL-6 promotes macrophage proliferation, upregulates AMP gene expression, and activates important signalling pathways such as the JAK2/STAT3 axis, further cementing IL-6's role in fish immunity (Costa *et al.*, 2011).

In teleost fish, IL-6 has been shown to be highly expressed in organs such as the kidney and spleen, particularly during immune responses (Metcalfe *et al.,* 2020) and tissue regeneration (Li *et al.,* 2021b). Recombinant IL-6 (rIL-6) has demonstrated its ability to promote macrophage proliferation in rainbow trout and regulate the transient expression of SOCS1-3 and interferon regulatory factor (IRF)-1, with a sustained upregulation of antimicrobial peptides (AMPs) in primary macrophage cultures (Costa *et al.,* 2011).

IL-6 not only plays a proinflammatory role but also significantly impacts the adaptive immune system. In teleost fish, IL-6 is implicated in promoting B lymphocyte maturation and enhancing IgM production, the principal immunoglobulin involved in fish immune defence (Abós *et al.,* 2016; Metcalfe *et al.,* 2020). In salmonids two distinct IL-6 genes, IL-6A and IL-6B, each potentially serving specialized immune roles, have been identified (Veenstra *et al.,* 2017; Eggestøl *et al.,* 2020). IL-6 has also been used as a vaccine adjuvant in fish, aiding in higher serum antibody levels and enhancing protection against pathogens (Eggestøl *et al.,* 2020). Recombinant IL-6 has demonstrated its capacity to stimulate immunoglobulin production in various fish species, emphasizing its potential as a tool for improving disease resistance.

IL-6 is closely tied to haematopoiesis, acting in concert with IL-3 to induce the formation of blast cell colonies. It also supports the differentiation of macrophages and megakaryocytes, crucial components of the immune and circulatory systems (Heike and Nakahata*,* 2002). In fish, these processes are vital for maintaining a healthy population of immune cells that can respond to infection or injury. Moreover, IL-6 has a regulatory effect on cellular growth which is further demonstrated by its role in inducing vascular endothelial growth factor (VEGF). The latter promotes neo-

angiogenesis, a process that is crucial for tissue repair and regeneration in fish (Tartour *et al.,* 2011). IL-6 has been shown to play an integral role in tissue regeneration. This was observed during caudal fin regeneration after amputation where the IL-6 expression was significantly upregulated, signifying its involvement in promoting tissue repair (Li *et al.,* 2021b). This regenerative property aligns with IL-6's role in angiogenesis, where it induces the production of VEGF, facilitating the formation of new blood vessels essential for wound healing and recovery (Tartour *et al.,* 2011).

In fish species such as zebrafish and rainbow trout, IL-6 not only assists in immune defence but also contributes to overall tissue recovery following physical damage. This highlights its multifunctionality in maintaining fish health beyond its traditional role in inflammation and immune regulation. IL-6 is a pivotal mediator in both the onset and maintenance of inflammation. It assists neutrophil migration towards inflamed sites (Woodfin *et al.,* 2010), triggers the release of proinflammatory mediators such as cytokines such as IL-6, prostaglandins, reactive oxygen species (ROS), and proteases (Fielding *et al.,* 2008). This activation leads to the production of positive acute phase proteins, including serum amyloid A and C-reactive protein (CRP), known for their pyrogenic activity and role in host defence (Eggestøl *et al.,* 2020).

2.6.3 The role of C-reactive proteins

C-reactive protein (CRP) is a key acute-phase reactant protein produced whose concentration rise in response to inflammation (Kumar *et al.,* 2019). Primarily produced in the liver (Nehring *et al.,* 2023), CRP plays a pivotal role in the immune system (Kumar *et al.,* 2019). It is a non-glycosylated pentraxin (PTX) with five identical subunits and calcium binding sites, and it was first identified by its reaction with pneumococcus capsular polysaccharide (C-polysaccharide) (Du Clos and Mold, 2004). This same protein was first discovered in fish in 1973, marking its evolutionary conservation across species (Pathak and Agrawal*,* 2019).

CRP was also the first identified pattern-recognition receptor (Du Clos, 2000; Mantovani *et al.,* 2008). It has multiple immunological functions such as activating the complement system, promoting macrophage phagocytosis, interacting with FcγRI, and possibly playing a role in antigen presentation (Black *et al.,* 2004; Du Clos and

Mold, 2004), and was thought to be an inflammation marker as well as a major risk marker for cardiovascular disease (Ridker, 2009). It is considered a positive acute-phase protein, as its plasma concentration increases distinctly during acute infections, injuries, or inflammatory stimuli, often exceeding 1000-fold its normal levels (<1 mg/mL in healthy fish) (Kumar *et al.,* 2019). In common carp, basal CRP-like levels in the serum vary significantly, with average 2.9±0.15 µg/ml to 12.57±1.19 µg/ml (MacCarthy *et al.,* 2008). Once the inflammation, infection or injury subsides, CRP levels decrease drastically and return to baseline, underscoring its dynamic response to immune challenges (Sproston and Ashworth, 2018).

In arthropods and molluscs, CRP is constitutively expressed, but in humans and most fish species, it functions as an acute-phase protein, a role that likely emerged in the evolutionary development of the immune system (Pathak and Agrawal*,* 2019). This protein is distinguished by its PTX domains (HxCxS/TWxS) domains (HxCxS/TWxS) (Pepys *et al.,* 1978; Nunomura, 1992; Lee *et al.,* 2017) and binds to various ligands, in the presence of calcium, such as phosphorylcholine, phospholipids, histones, chromatin, and fibronectin (Nehring *et al.,* 2023). Structures expressed on microbial cells and disrupted eukaryotic surfaces, such as pneumococcal C-polysaccharide, also serve as ligands for CRP, aiding in pathogen recognition and clearance (Dash *et al.,* 2018; Nehring *et al.,* 2023).

CRP's primary production is induced by interleukin-6 (IL-6), which activates the transcription of the CRP gene during acute inflammatory or viral events (Nehring *et al*., 2023). C-reactive protein enhances innate immunity by activating complement pathways via Fc receptors and facilitating the recognition and clearance of apoptotic cells (Nauta *et al*., 2003). As an opsonin, it aids in agglutinating and precipitating bacteria or macromolecules with phosphorylcholine on their surfaces. These functions highlight its critical role in detecting and eliminating pathogens, as well as maintaining immune homeostasis.

Beyond its immunological functions, CRP has been recognized as a biomarker for inflammation and a major risk marker for cardiovascular diseases in humans (Ridker, 2009). This dual role underscores its significance in both acute and chronic disease contexts. The evolutionary conservation of CRP from arthropods to humans further emphasizes its importance as a fundamental component of the immune system (Pathak and Agrawal, 2019).

In summary, CRP is an ancient and highly versatile protein that plays a crucial role in innate immunity. Its ability to bind to a wide range of ligands and respond dynamically to inflammation makes it a key player in immune defence mechanisms across species. While extensive research has focussed on CRP in humans, studies on CRP in fish remain comparatively scare. Investigating CRP in fish not only sheds light on its role in non-mammalian vertebrates but also provides valuable insights into its potential applications as a biomarker in aquatic ecosystems. Understanding CRP's diverse functions across species underscores its importance as a fundamental component of immune health (Kumar *et al*., 2019).

2.7 Aim of the study

To use high flow and low flow surveys to investigate the impact of Fe, Cu, and Zn levels on innate immune modulation in *C. carpio* and *M. salmoides* collected from Witbank Dam, Mpumalanga.

2.8 Objectives

The objectives of the study are to:

I. determine the physico-chemical composition of surface water using a YSI ProQuatro Multiparameter meter (Yellow Springs Instruments, Ohio, USA) and to record how this composition changes during periods of high and low flow.
II. establish the levels of iron, copper and zinc in surface water and sediment samples.
III. use gill, muscle, liver, and blood tissue to determine the presence as well as the bio-accumulation capabilities of the selected metals.
IV. with the aid of ELISA Assays establish whether the use of the Merck: cOmplete™ Protease Inhibitor Cocktail provides better quantitative results when compared to those bio-markers measured in non-treated epidermal mucus and serum samples.
V. determine the impact of Fe, Cu and Zn on innate immune biomarkers IL-6, CRP and an adaptive biomarker IgM.

2.9 Research questions

I. What are the levels of iron, copper and zinc in surface water, sediment and tissues of fish and do they differ significantly when compared seasonally?
II. To what extent can seasonal levels of Fe, Cu and Zn be linked to innate immune modulation in selected fish species from Witbank Dam?
III. Will the use of the proteolytic enzyme inhibitor ensure better quality control over immune biomarkers than when compared to samples in which it was not used?

2.10 The composition of the dissertation

The composition consists of six distinct chapters. **Chapter 1** provides a comprehensive literature review and outlines the aims and objectives of the study. **Chapter 2** focuses on the methodology and analytical procedures employed in this research. Chapter 3 to 5 present the results and discussion of the study. **Chapter 3** delves into results and discussion of Cu, Fe and Zn concentrations in surface water and sediment samples as well as the physcio-chemical parameters. **Chapter 4** examines the pre-analytical care response of the biomarkers. **Chapter 5** investigates the bioaccumulation of Cu, Fe and Zn in tissue samples for both common carp and largemouth bass. Finally, **Chapter 6** serves as the concluding chapter followed by a reference list.

2.11 The scope of the study

This study investigates the effects of Cu, Fe and Zn on innate immune biomarkers in *Cyprinus carpio* and *Micropterus salmoides* from Witbank Dam, Mpumalanga. Witbank Dam, located in in the eMalahleni area with 22 coal mines, has been studied due to concerns over the environmental impact of mining activities on water quality and aquatic life. The study will determine the physico-chemical parameters and assess the levels of Cu, Fe and Zn in surface water, sediment sample and tissue samples of both fish species, to establish the use of the cOmplete^{TM} protease inhibitor cocktail in quantitative results in epidermal mucus and serum samples, and how to determine impact of these metals to immune biomarkers, including CRP, IL-6, and IgM in these

fish species. By focusing on the relationship between micronutrient availability and immune function, the study aims to provide insights into the health of fish in mining-impacted waters and contribute to better management of freshwater resources in the region.

3. METHODOLOGY AND ANALYTICAL PROCEDURES

3.1 Study design

This was a prospective and non-interventional study that was conducted at Witbank Dam (25.8909°S 29.3054°E), a buttress (hollow) dam located in Mpumalanga Province **(Figure 5)**, in the Olifants River System in South Africa (NSRI, 2021). It has a total capacity of 104 019 000 m^3, with a dam wall height of 42 m and length of 562 m (NSRI, 2021). This study consisted of two surveys. One was conducted during the dry season, which is typically referred to as the low flow period of this study as there will not be an influx of rainwater. This normally coincides with the Winter period that covers May to August, with the survey then conducted in June (2024). Similarly, the high flow survey was conducted during the rainy season which covers the period October to March, with November (2023) selected as the survey month. This study was approved by the University of Limpopo Animal Research Ethics Committee (AREC/48/2023:PG).

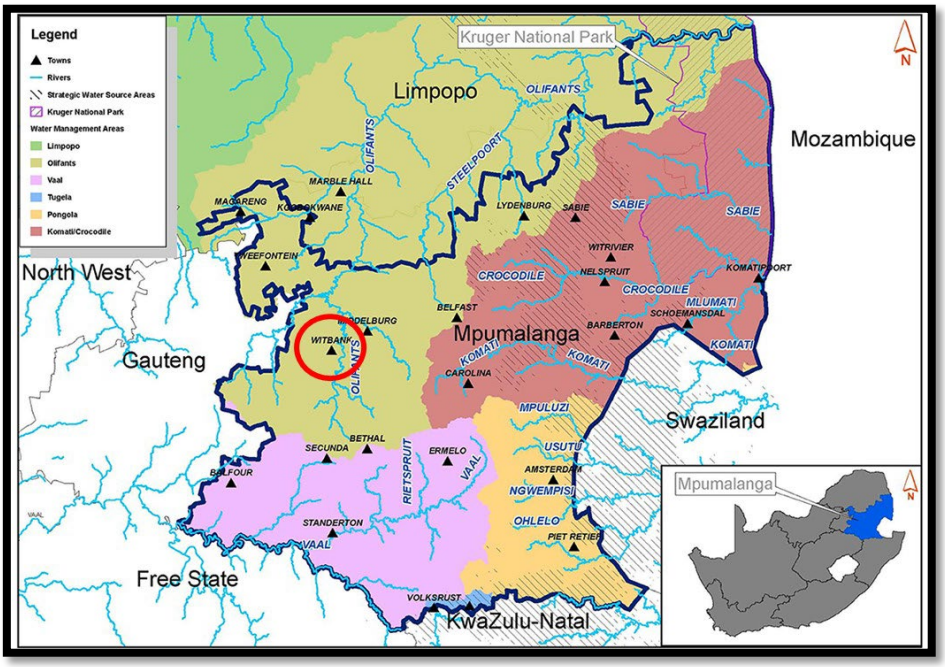

Figure 5: Location of Witbank dam in the Olifants River System (Obtained from: Simpson *et al.*, 2019).

3.2 Environmental sample collection and analysis

3.2.1 Physico-chemical characteristics of surface water samples

The YSI multiparameter instrument was used to measure physico-chemical parameters of the surface water. The parameters that were measured are pH, temperature, dissolved oxygen, conductivity, total dissolved solutes (TDS) and salinity. These measurements were taken as described by Sara *et al.* (2021).

3.2.2 Collection of water and sediment samples and the analysis of their metal contents

The water samples were collected from three different sites (**Figure 6**) around the dam (S 25° 55.216'/E29° 19.182'; S25° 56.887'/E29° 16.795'; S26° 00.608'/E29° 16.049'). Each water sample of 1L was collected, at approximately 10 cm below the surface, using pre-cleaned polypropylene containers and frozen (-85˚C) until further analysis (De Klerk *et al.*, 2012). Water samples was examined for Cu, Zn, and Fe as described by De Klerk *et al.* (2012).

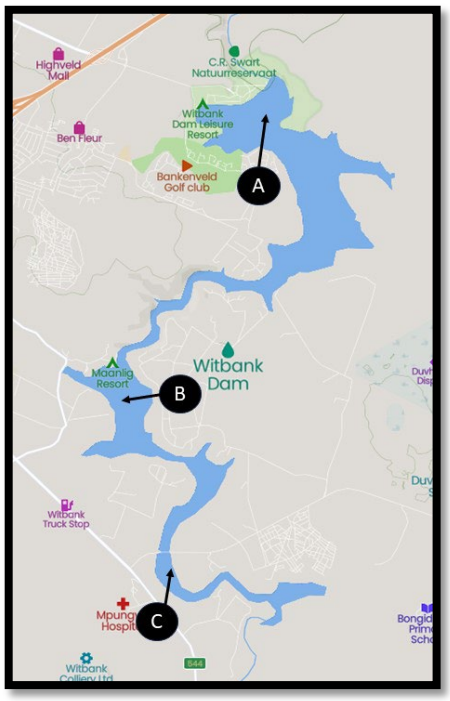

Figure 6: Sampling sites in Witbank Dam (Illustration obtained from https://www.google.com/maps).

Sediment samples were collected with a Van Veen grab sampler **(Figure 7)**, from the same sites as the surface water samples. Sediment samples were placed in pre-cleaned containers and frozen until laboratory analysis was done. Metals in the sediment samples were analysed as described by De Klerk *et al.* (2012).

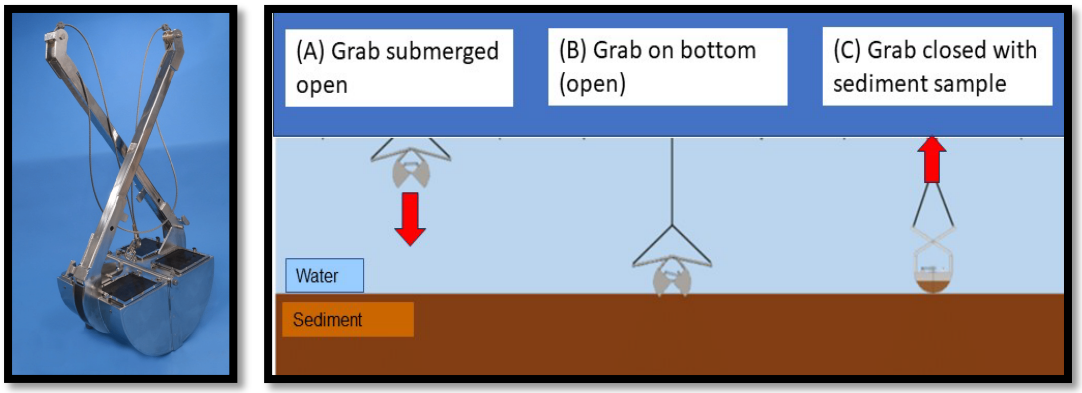

Figure 7: The Van Veen grab sampler (Illustrations obtained and adapted from: Jensen *et al.*, 2015; Bae *et al.*, 2020).

3.3 Fish species, collection, and husbandry

This study focussed on *C. carpio* (Brown *et al.*, 2005) and *M. salmoides* (Mecozzi, 2008) collected from Witbank dam. During each of the surveys, the fish was collected using conventional fishing gear (**Figure 8**). Depending on circumstances some of the fish were also be sourced from other anglers in the vicinity. A maximum number of 20 fish per species was targeted per survey. The fish was put in purposefully designed holding nets (diameter of 0.7 m and a depth of 1.0 m) that was almost entirely suspended in the dam water to allow water to freely circulate through it. Separate holding nets were used for the two species selected for this study. Care was taken not to over-crowd the fish in these holding nets as this could cause unnecessary stress. Guidelines for the ideal number of fish per volume was obtained from Aquapona (2019).

Figure 8: Conventional angling gear used in this study (Photo credits: Prof LJC Erasmus).

3.4 Animal tissue sampling, and analysis of collected samples

3.4.1 Morphometric data

The morphometric measurements total length, standard length, and standard weight for *C. carpio* and *M. salmoides* were collected as described by Sara *et al.* (2021). In brief, a purposefully designed measuring board was used to determine the fork length (FL) and total length (TL) in cm. The TL, a straight-line measure, is taken from the tip of the snout to the end of the caudal fin. Similarly the FL is a straight-line measure that runs from the tip of the snout up to the fork of the tail (caudal fin).

3.4.2 Epidermal mucus

3.4.2.1 Collection and preparation of epidermal mucus samples

Epidermal mucus samples were collected as described in Albaladejo-Riad *et al.* (2023). The skin mucus of fish was collected by gently rubbing the lateral skin of the both sides in a cephalic-caudal manner with a rubber spatula, avoiding contamination at the anal periphery. For treated samples, 50 ml of PBS was vortexed with one tablet of the Merck: cOmplete™ Protease Inhibitor Cocktail (Sigma-Aldrich), whilst for

untreated samples 1L of PBS was prepared. For every fish, half of its mucus sample was treated with an equal volume of PBS containing the cocktail, with volumes ranging between 2 to 4 ml depending on the amount of mucus collected. The remaining half of its mucus sample was treated with an equal amount of PBS. Both mucus samples were vortexed and centrifuged at 5000 rpm for 10 minutes. Supernatants, measuring between 1–2 ml each were then collected into correctly labelled Eppendorf tubes (Sigma-Aldrich) before being kept in the freezer at -20°C.

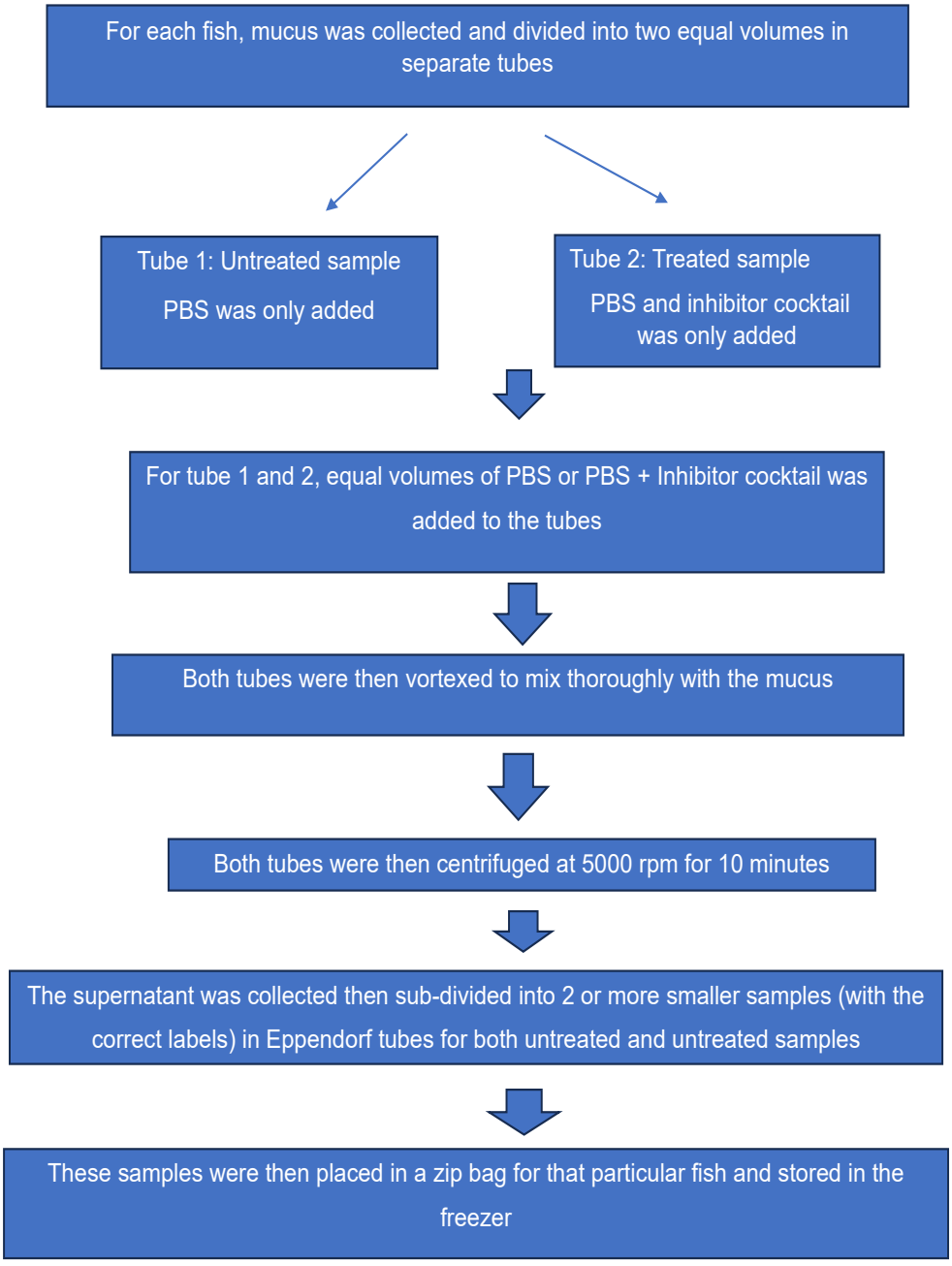

Figure 9: The preparation o mucus samples

3.4.2.2 Biochemical analysis of the epidermal mucus

Total CRP levels in mucus was measured using the FineCare FIA meter (Wondfo). For sample collection, 8.5 µL of the mucus was drawn horizontally from the cryotubes using the sample collector provided by the manufacturer. The detection buffer tube was pierced with the sample collector, and the sample was gently rotated to mix with the buffer solution. The mixture was shaken back and forth and then, three drops of the prepared sample were introduced into the test cartridge, which was then left at room temperature for thirty seconds to allow the reaction to take place. After the reaction was complete, the test result was displayed on the Finecare FIA Meter (Wondfo).

Total IL-6 levels in mucus were measured using the FineCare FIA meter (Wondfo). For sample collection, 75 µL of the sample was drawn from the cryotubes using a pipette and transferred into the buffer tube. The mixture was shaken back and forth, after which the first two drops of the prepared sample were discarded, and three drops were introduced into the test cartridge. The cartridge was then left at room temperature for 15 minutes to allow the reaction to take place. Once the reaction was complete, the test result was displayed on the Finecare FIA Meter (Wondfo).

Total IgM levels in mucus was measured using the enzyme-linked immunosorbent assay (ELISA) (Cuesta *et al.*, 2004). The reagents were prepared according to the manufacturer's instructions (Sigma-Aldrich, 2024). Wash Buffer was prepared by diluting 15 mL of Wash Buffer Concentrate (20x) with 285 mL of distilled water to make 300 mL of 1x Wash Buffer. The HRP-conjugate solution was prepared by mixing the antibody and HRP-conjugate in a 1:10 ratio. All reagents were brought to room temperature (18–25°C) prior to use. Samples were thawed and centrifuged to remove debris before use. All reagents and samples were allowed to reach room temperature to ensure consistency during the assay.

The microtiter plate was organized as follows: one blank well, six standards (run in duplicate), and wells assigned for samples. Each sample was also run in duplicate wells. Fifty microliters of standards or samples were pipetted into the assigned wells. An equal volume (50 µL) of HRP-conjugate solution was added to each well, excluding the blank. The plate was incubated at 37°C for 1 hour. Following incubation, the wells were washed three times using 200 µL of 1x Wash Buffer per well, with complete

removal of liquid after each wash to ensure assay accuracy using an automated plate washer.

After washing, 50 µL of Substrate A and 50 µL of Substrate B were added to each well, and the plate was incubated at 37°C in the dark for 15 minutes. Subsequently, 50 µL of Stop Solution was added to each well to halt the reaction. The optical density of each well was measured within 10 minutes using a microplate reader set to 450 nm.

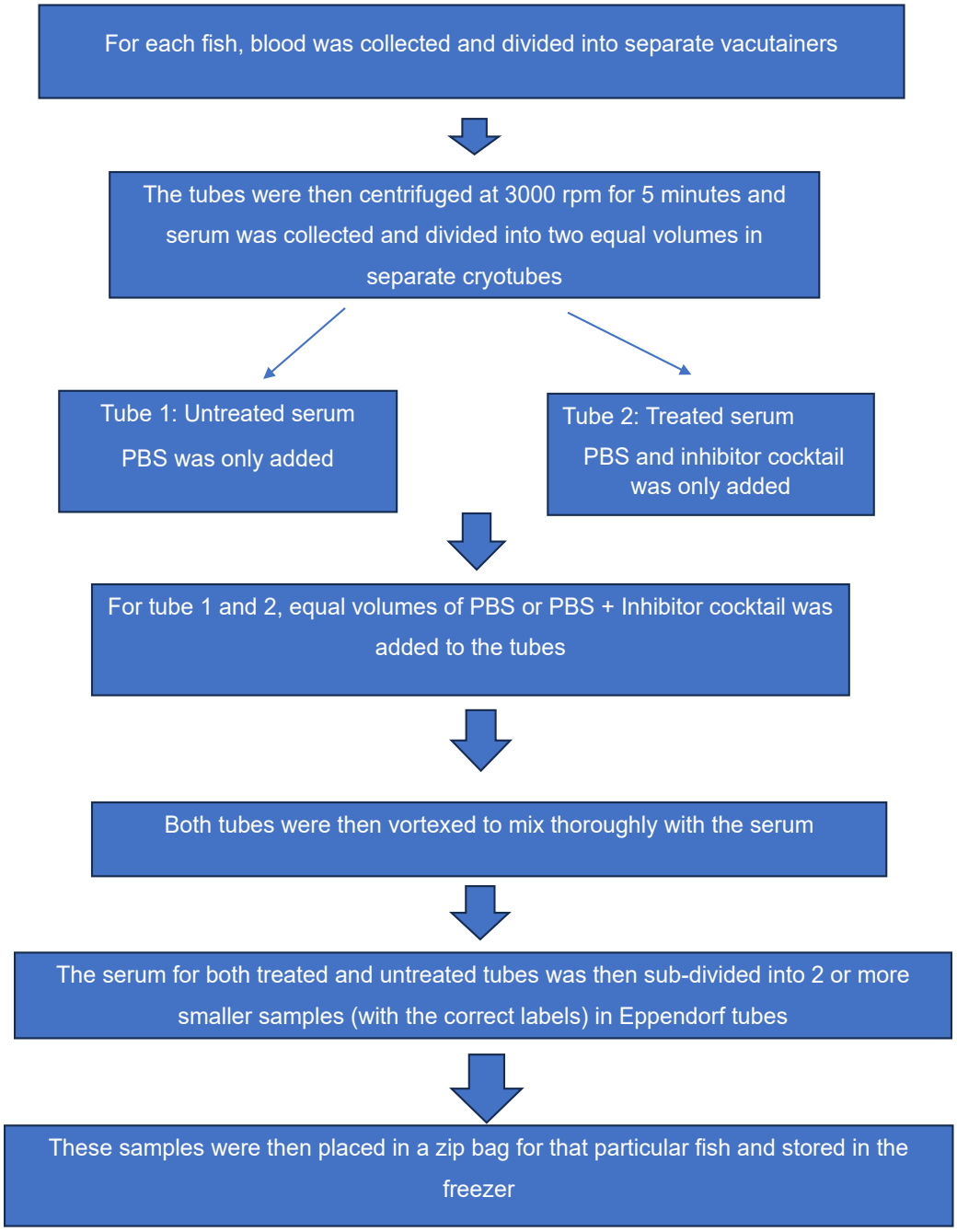

Figure 10: The preparation of serum samples

3.4.3 Haematology

3.4.3.1 Blood collection and the preparation of serum samples

Blood samples were collected from the caudal vein using a 5 mL syringe and a 14- and 21-gauge needle (Lawrence *et al.,* 2020). After collection the blood were transferred into a serum separator collecting tube (Vacutainer™). The blood was then centrifuged at 3000 rpm for 5 minutes, and the serum collected into 3 ml cryotubes. For every fish, half of its serum sample was treated with equal volumes of PBS+ cocktail as prescribed in the manufacturer's guidelines. The remaining half of its serum sample was treated with an equal amount of PBS. The samples were then sub-divided into 2 to 3 Eppendorf tubes, containing 1 ml to 2 ml, stored in the freezer at -18°C.

3.4.3.2 Biochemical analysis of serum samples

CRP, IL-6, and IgM were measured in the serum samples in a similar way as described in 3.4.2.2.

3.5 Sacrificing the fish

All fish in this study was sacrificed via severing of the spinal cord just behind the operculum. Due to the nature of this study, no fish were sedated or euthanized using clove oil or any other chemicals, as it has been reported to compromise the epidermal mucus (Albaladejo-Riad *et al.*, 2023). To sacrifice the fish in the most humane manner possible, a very sharp non-serrated knife was used, and the spinal cord was severed in a single fluent movement.

3.6 Gill, muscle, and liver tissue samples

3.6.1 Collection of tissue samples

From each fish a gill, liver, and muscle sample were dissected using a stainless-steel scalpel and tweezers. The samples were individually placed in a marked self-sealing plastic bag before being frozen in the freezer at the field laboratory. The samples were

stored at -80°C in a bio-freezer in the Department of Physiology and Environmental Health at the University of Limpopo, until further analysis.

3.6.2 Metal contents of gill, liver, and muscle tissue

The levels of Fe, Zn, and Cu in the collected tissue samples were determined at Waterlab (Pty) Ltd in Pretoria. This is a SANAS accredited facility.

3.7 Statistical Analysis

All collected data measurements were analysed using IBM SPSS Statistics version 29. The data set for the present study was non-parametric, it was presented as either mean ± standard deviation (±SD) or median ± standard error of mean (±SEM) in order to compare to other studies as it is a widely used metric. Descriptive statistics were performed for the analyses and were presented as mean ± SD. A Wilcoxon signed rank test was performed between treated and untreated serum and mucus samples. The statistical significance level for all analyses were set at $p < 0.05$.

4. RESULTS AND DISCUSSON OF ENVIRONMENTAL MONITORING

4.1 Environmental monitoring results

This study was conducted in Witbank Dam located in the eMalahleni area, Mpumalanga Province, to determine the physico-chemical composition of surface water and to record how this composition changes during periods of high and low flow. Furthermore, to assess the concentrations of copper (Cu), iron (Fe), and zinc (Zn) in water and sediment samples and to compare sediment samples under high and low flow conditions in Witbank Dam.

Table 1 presents the physico-chemical composition of surface water in Witbank Dam during the high-flow period. At Site A, the highest average values for temperature, salinity, and total dissolved solids (TDS) were recorded, with measurements of 25.3°C, 0.39 ‰, and 0.51 ppm, respectively. Site B exhibited the highest dissolved oxygen (DO) concentration and pH, with values of 10.3 mg/L and 9.79, respectively.

Table 2 shows the physico-chemical composition of surface water during the low flow period. At Site 3, the highest temperature of 12.8°C and the highest DO concentration of 7.01 mg/L were observed. Site A recorded the highest TDS value, which was slightly elevated at 0.43 ppm. Lastly, Site B showed the highest pH value of 8.29. The salinity during low flow was the same throughout all the sites with a value of 0.32 ppm.

Table 3 presents descriptive statistics for the physico-chemical parameters. During the high-flow period, the highest average values were found for temperature (24.4°C), pH (9.20), DO (8.36 mg/L), and salinity (0.33 ‰). During the low flow period, the highest TDS value was observed at 0.43 ppm.

Table 4 presents the detected surface water levels of Cu, Fe and Zn in Witbank Dam. Iron was detected at concentrations below 0.025 mg/L, Cu was detected at concentrations <0.010 mg/L, and Zn was detected at <0.0025 mg/L. Among these metals, Fe was the most abundant, followed by Cu and Zn.

Table 1: The physico-chemical composition of surface water in Witbank Dam during the high flow survey (November 2023).

PARAMETERS	SITE A			AVG	SITE B			AVG	SITE C			AVG
Temp (°C)	25.60	25.30	24.90	**25.30**	24.20	24.20	24.00	**24.10**	23.80	23.70	23.60	**23.7**
DO (mg/l)	6.69	6.65	6.32	**6.55**	9.99	10.70	10.22	**10.30**	8.43	8.10	8.14	**8.22**
pH	8.23	8.18	8.06	**8.16**	9.71	9.79	9.87	**9.79**	9.67	9.61	9.63	**9.64**
Salinity (‰)	0.39	0.39	0.38	**0.39**	0.30	0.30	0.30	**0.30**	0.29	0.29	0.29	**0.29**
TDS (ppm)	0.52	0.51	0.50	**0.51**	0.30	0.40	0.40	**0.36**	0.38	0.38	0.38	**0.38**

Table 2: The physico-chemical composition of surface water during the low flow survey (June 2024).

PARAMETERS	SITE A			AVG	SITE B			AVG	SITE C			AVG
Temp (°C)	12.71	12.51	12.3	**12.5**	12.61	12.82	12.2	**12.5**	12.80	12.71	12.92	**12.8**
DO (mg/l)	6.59	6.83	6.64	**6.69**	6.88	6.95	6.85	**6.89**	7.10	6.99	6.94	**7.01**
pH	8.33	8.21	8.11	**8.22**	8.27	8.25	8.34	**8.29**	8.30	8.24	8.29	**8.28**
Salinity (‰)	0.32	0.32	0.31	**0.32**	0.32	0.33	0.32	**0.32**	0.32	0.31	0.32	**0.32**
TDS (ppm)	0.43	0.43	0.43	**0.43**	0.42	0.42	0.42	**0.42**	0.42	0.42	0.42	**0.42**

Table 3: Descriptive statistics of the physico-chemical parameters measured during the two surveys.

PHYSICO-CHEMICAL PARAMETER	SURVEYS		TWQRS
	NOV 2023	JUN 2024	
	Mean	Mean	
Temperature (°C)	24.42	12.61	
pH	9.23	8.26	6.0 – 9.0
Total Dissolved Solids (ppm)	0.42	0.43	
Dissolved Oxygen (mg/L)	8.36	6.86	6.0 – 9.0
Salinity (‰)	0.33	0.32	

Table 4: Detected surface water levels of selected metals from Witbank Dam.

SELECTED METALS	HIGH FLOW (mg/L)	TWQR (mg/L)
Copper (Cu)	<0.010	0.0003
Iron (Fe)	<0.025	0.001 – 0.5**
Zinc (Zn)	<0.0025	0.002

**= Kempster and Van Vliet (1980) and Canadian Water Quality Guidelines (CCREM, 1987).

Values are interpreted according to DWAF (1996).

Table 5: The average concentration of metals detected in sediment collected from Witbank Dam.

SEDIMENT METAL	HIGH FLOW	LOW FLOW	SEDIMENT QUALITY GUIDELINES *(CCME 2012)*
	Mean (mg/kg dm)	Mean (mg/kg dm)	
Cu	26	13	35.7
Fe	36 899	17 527	No guidelines
Zn	40.33	32.8	123

Table 5 presents the results of Cu, Fe and Zn concentration in sediment samples collected from 3 sites in Witbank dam under high flow and low flow conditions. Copper, Fe and Zn were detected at the highest concentrations during the high-flow period, with values of 26 mg/kg dm, 36,899 mg/kg dm, and 40.33 mg/kg dm, respectively.

4.2 Discussion of results

The objective of this study was to determine the physico-chemical composition of surface water and to record how this composition changes during periods of high and low flow. Furthermore, to assess the concentrations of Cu, Fe, and Zn in water and sediment samples, and to compare sediment samples under high and low flow conditions in Witbank Dam, Mpumalanga.

The results (**Table 1**) demonstrate that Site 1 (mine inlet) had the highest temperature, salinity, and TDS, but the lowest pH and DO. This could be due to its shallow depth and close proximity to the mining area. Shallow water heats up quickly (Boyd, 2020), and elevated temperatures reduce DO due to their inverse relationship. Additionally, mining activity may also lower pH and DO levels, due to their potential discharge such as dissolved minerals, that are likely to increases salinity and TDS. Site 2 had the highest pH and DO due to its lower salinity, which enhances DO levels. Site 3 recorded the lowest temperature and salinity; this might be because site 3 is the deepest site. Deep depth prevents rapid heating due to its increased surface area. Additionally, the distance from the mine reduces the influence of pollutants, including salts and minerals.

The results (**Table 2**) show that Site 3 had the highest water temperature and DO levels. The deep depth of Site 3 likely helps maintain a consistent temperature value, while its reduced exposure to pollution allows for higher oxygen levels. In Site 1, TDS was slightly highest, with the lowest DO and pH. This might be due to its shallowness and close proximity to the mining area. The lower DO and pH levels might be due to mine-related pollution and the inverse relationship between temperature and DO. Site 2 had the highest pH, which may have been affected by the reduced salinity, resulting in increased oxygen levels and a less acidic environment.

The results from the sampled sites (**Table 3**) show significant differences in water quality parameters between high and low flow conditions. Water temperature was higher during high-flow periods compared to low flow periods likely due to seasonal fluctuations, where high flow periods are associated to warmer periods and low flow periods with colder periods (Boyd, 2020). The pH was slightly higher than the Target Water Quality Range (TWQR) during high flow, whereas pH in low flow was found within the normal range of TWQR. This could be attributed to increased salinity or elevated nutrients, particularly phosphate and nitrates which stimulates the production of carbon dioxide via high rates of respiration that then reducing localized acidic or basic influences (DESI, 2024a and 2024b). Furthermore, pH was measured in the late afternoon for both seasons and is typically higher during the day due to carbon dioxide consumption by aquatic plants and algae during photosynthesis. As a result, pH is usually highest in the late afternoon and lowest before sunrise (EPA, 2021).

Compared to Mhlongo *et al.* (2018) who conducted their surveys in the upper Olifants River (catchment of the Witbank Dam), which reported an average pH of 7.29, the observed pH levels during high and low flow in the current study indicate a notable increase. This might be due to potential seasonal or environmental changes influencing the water chemistry. The pH in high-flow conditions might raise concerns, especially if it continues to increase, as it might disrupt the tolerance limit of the aquatic species that inhabit it. Dissolved oxygen levels for both high and low flow seasons were within the normal range, moreover, the DO levels were significantly higher during high-flow conditions than in low flow. The increased water movement and aeration during high flow likely account for this trend. Higher DO levels are beneficial for aquatic ecosystems, supporting aerobic respiration and maintaining the health of aquatic organisms (Banerjee *et al.,* 2019). Conversely, the lower DO levels observed in low flow conditions could limit oxygen availability, posing risks of hypoxia for fish and other aquatic life (Hutton and Chase, 2017). Interestingly, while salinity levels remained within TWQR normal ranges, TDS and salinity concentrations were higher during low flow periods than high flow. This could be due to the concentration effect in low flow conditions, where reduced water volumes lead to an accumulation of dissolved minerals and ions (DESI, 2024a). Elevated TDS and salinity in low flow could impose osmotic stress on freshwater species, potentially affecting their survival and physiological functions.

Our results (**Table 4**) revealed that Cu and Zn concentrations in surface water exceeded the TWQR (DWAF, 1996), indicating contamination of the surface water, likely from industrial or agricultural runoff. In contrast, Fe concentration was found to be less than the TWQR of 0.5 mg/L. Comparatively, a previous study reported a mean Fe concentration of 0.05 mg/L in Witbank Dam (Mhlongo *et al.,* 2018), indicating a decrease in Fe levels over time.

In sediment samples; however, higher concentrations of these metals were observed but remained within normal sediment quality guidelines under both high and low flow conditions. This shows that while the surface water of Witbank Dam may be contaminated by Cu and Zn contamination, the sediment is not polluted by Cu, Fe, or Zn. However, the sediment acts as a sink where pollutants accumulate over time, although not presently harmful, could lead to long term impacts on benthic ecosystems if levels continue to rise (Chiaia-Hernández *et al.,* 2022).

Sediment concentrations of Cu, Fe, and Zn were higher during high flow conditions than low flow. Increased water flow can resuspend sediments, facilitating the mobilization and transport of metal contaminants into the water column (Lu *et al.,* 2022b). Moreover, the sediment concentrations of Cu, Fe, and Zn at Witbank Dam were lower than those recorded in a previous study at Flag Boshielo Dam (Sara *et al.,* 2021), located downstream in the same river basin. The average of Cu, Fe, and Zn at Flag Boshielo Dam were recorded at 31.55 mg/kg, 50 630.75 mg/kg, and 102.94 mg/kg, respectively, while at Witbank Dam, these were lower, at 26 mg/kg, 36 899 mg/kg, and 40.33 mg/kg. Flag Boshielo Dam, which serves as an important water source for irrigation, domestic, and industrial use, revealed higher metal concentrations that is likely due to increased metal concentration from upstream sources, including Witbank Dam, along with more intense anthropogenic activity. This downstream accumulation of metals is expected due to the nature of sediment transport and deposition in river systems, where metals from upstream can settle in downstream locations, contributing to a cumulative impact on water quality.

While the metal concentrations in sediment samples do not pose a threat to the aquatic ecosystem, excessive Cu and Zn levels in surface water can be highly toxic to fish, especially when exacerbated by poor water quality, such as low dissolved oxygen and low water hardness (DWAF, 1996; Niyogi and Wood, 2004). Elevated Cu disrupts essential physiological processes, impacting ionic regulation, antioxidant defence (Zebral *et al.,* 2019), and reproductive functions across various fish species (Cao *et*

al., 2019). In particular, Cu toxicity can damage gill function (Solomon, 2009), reduce growth (Berntssen *et al.,* 1999) and induce oxidative stress, which harms cells and tissues, leading to both lethal and sub-lethal effects (Valko *et al.,* 2005). High Zn concentrations damage gill tissues in fish, affect osmoregulatory functions (Welker *et al.,* 2016), and may disrupt calcium absorption (Loro *et al.,* 2014), leading to hypocalcaemia and even mortality (McRae *et al.,* 2016). Effective water management and metal regulation are vital to mitigate these toxic effects and protect fish populations (DWAF, 1996; Rostern, 2017). These toxic effects emphasize the importance of continuous monitoring and effective regulation of metal concentrations in water bodies to protect fish populations and aquatic health.

5. RESULTS AND DISCUSSION OF PRE-ANALYTICAL CARE

5.1 Pre-analytical care results

Pre-analytical care is crucial in maintaining blood and mucus sample integrity for immune biomarkers such as CRP, IgM and IL-6. Field conditions (*in situ*) often expose these biomarkers to conditions that promote protein breakdown, leading to potential underreporting of their values. A cOmplete™ Protease Inhibitor Cocktail was used in this study to attempt to counteract protein breakdown. This inhibitor cocktail effectively reduces serine, cysteine, and metalloprotease activity in bacterial, mammalian, yeast, and plant cell extracts (Sigma-Aldrich, 2024). The aim was to investigate whether the addition of cOmplete™ Protease Inhibitor Cocktail (Sigma Aldrich) to *in situ* collected serum and mucus samples could maintain sample integrity.

Table 6: Descriptive statistics of the CRP, IL-6 and IgM in treated and untreated serum expressed as mean (± Standard Deviation) and p-value.

Sample type	**Mean (±SD) (µg/L)**	***p*-value**
CRP in treated serum (n=14)	16.83 (±7.28)	0.01
CRP in untreated serum (n=14)	8.93 (±4.65)	
IL-6 in treated serum (n=18)	17.86 (±25.31)	0.02
IL-6 in untreated serum (n=18)	17.22 (±9.95)	
IgM in treated serum (n=10)	0.12 (±0.03)	0.56
IgM in untreated serum (n=10)	0.11 (±0.03)	

Table 6 presents descriptive statistics for the CRP, IL-6 and IgM in treated and untreated mucus, with the mean and standard deviation (±SD) for each. C-reactive proteins, IL-6, IgM were detected higher in treated serum with values of 16.83 µg/L, 17.86 µg/L and 0.12 µg/L respectively.

Table 7: Descriptive statistics of the CRP, IL-6 and IgM in treated and untreated mucus expressed as mean (± Standard Deviation) and p-value

Sample type	**Mean (±SD) (µg/L)**	***p*-value**
CRP in treated mucus (n=17)	15.64 (±12.26)	0.04
CRP in untreated mucus (n=17)	8.03 (±5.19)	
IL-6 in treated mucus (n=13)	5.05 (±2.34)	0.67
IL-6 in untreated mucus (n=13)	4.69 (±2.68)	
IgM in treated mucus (n=10)	0.17 (±0.01)	0.91
IgM in untreated mucus (n=10)	0.17 (±0.02)	

Table 7 presents descriptive statistics for the CRP, IL-6 and IgM in treated and untreated mucus, with the mean and standard deviation (±SD) for each. C-reactive proteins and IL-6 were detected higher in treated mucus, whilst there was no difference of means in IgM in treated and untreated mucus.

5.2 Discussion of pre-analytical care results

The results indicate that CRP levels in treated serum samples had a higher mean of 16.83 ± 7.28 µg/L compared to untreated serum which had a mean of 8.93 ± 4.65 µg/L, (p = 0.01). Similarly, in mucus, CRP levels were significantly higher in treated samples (15.64 ± 12.26 µg/L) compared to untreated samples (8.03 ± 5.19 µg/L, p = 0.04). This suggests that the protease inhibitor cocktail preserved CRP levels, preventing protein degradation and resulting in higher concentration levels in treated samples.

Moreover, the CRP in untreated serum and mucus samples were found to be within the normal reference range for *C. carpio* (2.9–12.57 µg/L). In contrast, treated serum, had elevated levels that were found outside of the normal range indicating that

untreated samples likely underwent significant protein degradation, leading to lower CRP levels. This emphasizes the importance of pre-analytical care, as untreated samples might lead to a false negative of CRP levels in fish which would then lead to masking stress responses or health issues in fish.

Unlike CRP, IL-6 levels showed less noticeable differences between treated and untreated serum samples, though the difference was still statistically significant. In treated serum, IL-6 concentration was 17.86 ± 25.31 µg/mL, while in untreated serum, it was 17.22 ± 9.95 µg/mL (p = 0.02). Although the difference is significant, the large standard deviation in treated serum indicates considerable variability, suggesting that while treatment stabilizes IL-6 to some extent, additional factors such as storage conditions or individual variability among the fish might also affect IL-6 measurements.

Similarly in mucus samples, the difference in IL-6 levels between treated (5.05 ± 2.34 µg/mL) and untreated (4.69 ± 2.68 µg/mL) samples, p= 0.67, less pronounced. This indicates that the protease inhibitor had little to no effect on IL-6 levels in both serum and mucus samples suggesting that protein degradation was not inhibited. This suggests that the treatment was unable to protect the integrity of IL-6.

The differences in IgM concentration between treated and untreated serum and mucus samples were less noticeable than CRP, though the difference was not statistically significant. IgM concentration in treated serum was 0.12 ± 0.03 µg/mL, while in untreated serum, it was 0.11 ± 0.15 µg/mL (p = 0.56). Indicating that the protein inhibitor has less of an effect on IgM than in CRP as there is not much difference in treated and untreated serum. In mucus samples, IgM levels for both treated and untreated samples were 0.17 ± 0.00 µg/mL, with a p-value of 0.91, indicating no statistically significant difference. This suggests that, unlike CRP, the protein inhibitor has no effect as both untreated and untreated IgM are the same. Similarly to IL-6, the protease inhibitor was unable to inhibit protein degradation leading to lower levels in the serum and mucus of fish. This underestimation can lead to misdiagnosing of fish. Accurate measurement of these biomarkers is critical in fish health management, as it increases in response to inflammation in fish.

6. RESULTS AND DISCUSSION OF BIOINDICATOR RESPSONSES

6.1 Bioindicator results

This section of the study consisted of sample size of 29 fish (23 common carp and 6 largemouth bass). To investigate the impact of Fe, Cu, and Zn levels on innate immune modulation in fish species this study determined the presence as well as the bio-accumulation capabilities of Fe, Cu and Zn on the tissue samples of gill, muscle, liver, and blood tissue; and also studied the relationship between the selected metals and immune biomarkers of CRP, IL-6, and Immunoglobulin M (IgM).

Table 8: The average concentrations of metals detected in tissue samples collected from Witbank Dam.

Fish species		Common carp (n= 23)			Largemouth bass (n= 6)		
Tissue Samples		Gills	Liver	Muscles	Gills	Liver	Muscles
		Mean (±SD)	**Mean (±SD)**	**Mean (±SD)**	**Mean (±SD)**	**Mean (±SD)**	**Mean (±SD)**
Metal Content (mg/kg dry weight)	Cu	0.00 (0.00)	43.43 (8.73)	0.00 (0.00)	0.00 (0.00)	81.60 (42.04)	0.00 (0.00)
	Fe	258.04 (25.91)	235.43 (39.09)	37.09 (3.28)	82.67 (10.84)	944.83 (384.47)	36.77 (14.70)
	Zn	950.83 (62.14)	670.43 (94.82)	68.87 (5.89)	67.33 (4.21)	136.50 (20.26)	25.50 (2.51)

Table 8 presents the average concentration of Cu, Fe, and Zn detected in gills, liver and muscles collect from common carp and largemouth bass in Witbank dam. In the gills of common carp, the highest metal detected was Zn (950.83 mg/kg dm), followed by Fe (258.04 mg/kg dm) whilst no Cu was detected. In largemouth bass, only Fe

(82.67 mg/kg dm) was the highest followed by Zn (67.33 mg/kg dm). In the liver of common carp, Zn (670.43 mg/kg dm) was the highest followed by Fe (235.43 mg/kg dm) and Cu (43.43 mg/kg dm). In largemouth bass, Fe (944.83 mg/kg dm) was the most abundant followed by Zn (136.50 mg/kg dm) and Cu (81.60 mg/kg dm). Lastly, in the muscles of common carp, the highest metal detected was Zn (68.87 mg/kg dm) followed by Fe (37.09 mg/kg dm). In largemouth bass, the highest concentration was Fe (36.77 mg/kg dm) followed by Zn (25.50 mg/kg dm). Copper was not detected in the muscle tissues of either fish species.

Table 9: Comparison of liver, skin, eyes, operculum and gills characteristics and parasitic presence in common carp and largemouth bass.

Fish species		Common carp (n= 23)	Largemouth bass (n= 6)
Liver (g)	Mean (±SEM)	57.67 (±8.28)	2.57 (±0.59)
	Appearance	Normal= 8.7%	Normal= 0%
		Light Red= 91.3%	Light Red= 100%
Skin		Normal= 95.7% Mild skin alterations= 4.3%	Normal= 100%
Eyes		Normal= 100%	Normal= 100%
Operculum		Normal= 95.7% Outgrown= 4.3%	Normal= 100%
Gills		Normal= 100%	Normal= 100%
Presence of Ectoparasites (%)		47.8%	0%
Presence of endoparasites (%)		0%	16.7%

The results presented in Table 9 show critical insights into the health status and parasitic presence in common carp and largemouth bass, emphasizing differences influenced by species-specific physiological and ecological characteristics.

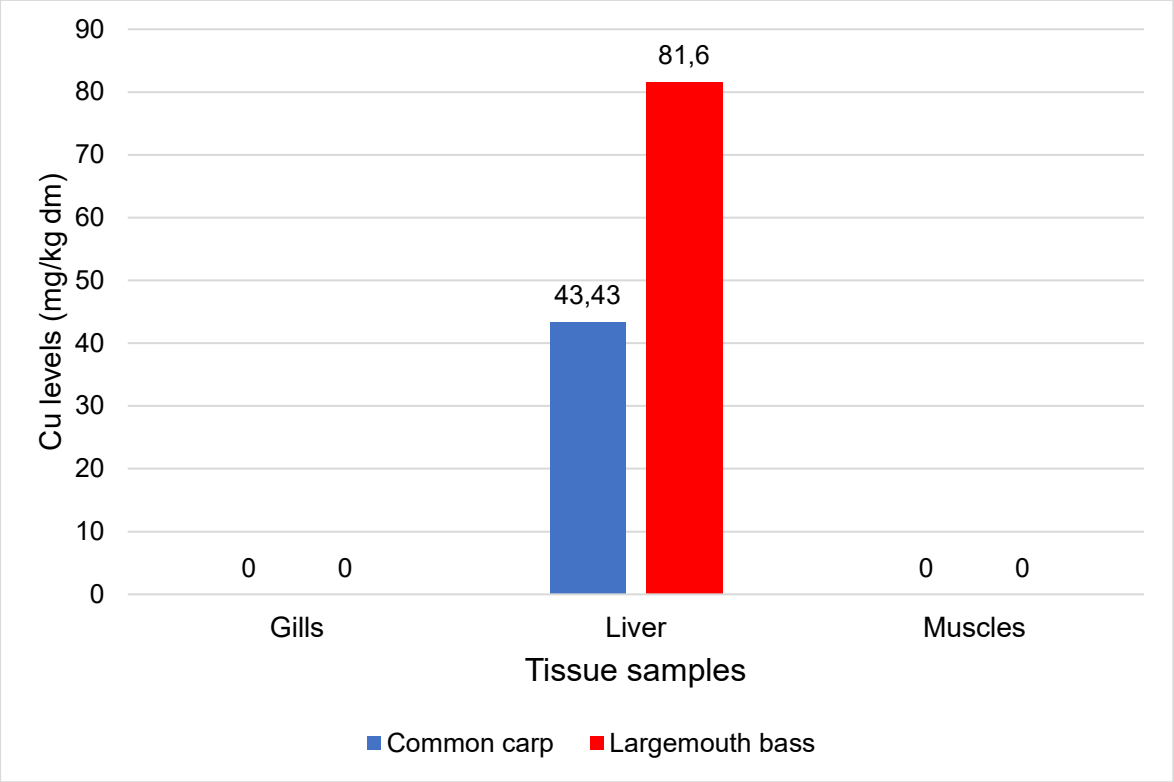

Figure 8: Comparison of Cu in gills, liver and muscles of common carp and largemouth bass.

6.2 Discussion of bio-indicator responses

The objective of this study was to use gill, muscle, liver, and blood tissue to determine the presence as well as the bio-accumulation capabilities of Cu, Fe, and Zn in common carp and largemouth bass. Our results (**Table 8**) showed that in common carp the Cu levels were detected only in the liver with a mean concentration of 43.43 ± 8.73mg/kg, whereas no Cu levels were detected in the gills and muscles. Similarly, in largemouth bass, Cu levels were detected only in the liver with a higher concentration of 81.60± 42.04 mg/kg, but none were detected in the gills nor the muscles. This shows that Cu accumulation is the highest in the liver of both these species, most probably due to the role of the liver in storing nutrients and detoxifying heavy metals (Ge *et al.,* 2019). In contrast, the absence of Cu in the gills and muscles might indicate that these tissues

are not significant sites of Cu storage or absorption in these species. Furthermore, largemouth bass had a higher accumulation of Cu in the liver than common carp. It is fair to argue in favour of species-specific differences in diet and habitat. Largemouth bass are carnivores that feed on invertebrates and a variety of small fish at any point in the water column (Freshwater fish Advice, 2013) which as a result exposes this species to copper across different trophic levels. In contrast, common carp, a bottom-feeding omnivore that primarily feeds on food found in the sediment such as plant materials, may be more limited in accumulating Cu (FAO, 2013). While this diverse diet includes some Cu sources, their exposure might be lower overall compared to that of carnivorous largemouth bass. The selective hepatic accumulation of Cu suggests that both common carp and largemouth bass exhibit effective detoxification mechanisms. These findings differ from a previous study that took place in Loskop Dam, where Cu was uniformly distributed across tissues showing that the liver's detoxification capacity had been overwhelmed, resulting in systemic toxicity (Marr *et al.,* 2017).

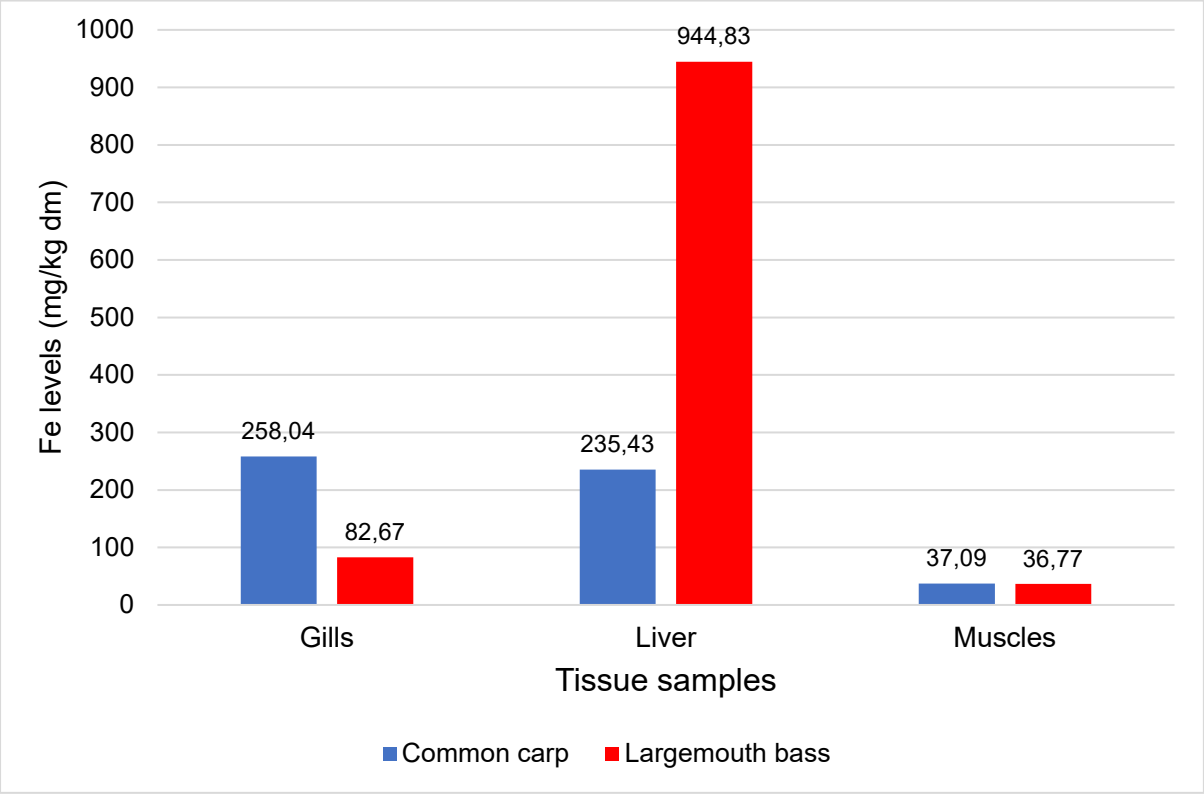

Figure 9: Comparison of Fe in gills, liver and muscles of common carp and largemouth bass.

In common carp, Fe levels were detected the highest in gills followed by the liver then muscles whereas in largemouth bass, Fe levels were the highest in liver followed by the gills then muscles. In Largemouth bass, the liver showed an elevated Fe concentration of 944.83 ± 384.47 mg/kg, compared to 235.43 ± 39.09 mg/kg in Common carp. The gills of both species also had relatively high Fe levels, with Common carp at 258.04 ± 25.91 mg/kg and Largemouth bass at 82.67 ± 10.84 mg/kg. The muscle showed the lowest Fe concentrations, 37.09 ± 3.28 mg/kg, in Common carp and 36.77 ± 14.70 mg/kg in Largemouth bass. These results follow the expected trend for Fe concentrations in largemouth bass but deviate in common carp, where the gills showed the highest Fe concentrations. The general trend of metal accumulation in fish tissues follows the pattern liver > gills > muscle (Jezierska and Witeska, 2006). In contrast, these results differ from those reported by Chandrapalan and Kwong et al. (2020) and Bury and Grosell (2003), where the gills were expected to accumulate the lowest concentrations of Fe. The elevated Fe concentrations in the liver and gills are due to the roles of these tissues in oxygen transport and filtration, which are associated with Fe-binding proteins like haemoglobin and ferritin. The higher Fe levels in the liver of Largemouth bass could imply increase exposure or differences in physiological processing between the two species.

Similarly to Fe concentrations, in common carp, Zn levels were detected the highest in gills followed by the liver then muscles whereas in largemouth bass, iron levels were the highest in liver followed by the gills then muscles. Zn concentrations were highest in the gills of common carp at 950.83 ± 62.14 mg/kg, followed by the liver with 670.43 ± 94.82 mg/kg, and lowest in the muscles at 68.87 ± 5.89 mg/kg.

For Zn bioaccumulation, these results follow the expected trend for Zn concentrations in common carp. In contrast, Zn levels in Largemouth bass were noticeably lower across all tissues compared to common carp, with the liver showing 136.50±20.26mg/kg, gills 67.33±4.21 mg/kg, and muscles 25.50 ± 2.51 mg/kg. The high Zn concentrations in the gills of common carp may indicate a greater exposure level in their environment which is evident as the surface water was discovered to be polluted by Cu and Zn concentration. Common carp, as benthic feeder, are likely to encounter Zn more as Zn settles primarily in sediments but also exists in dissolved or suspended forms (Rostern, 2017), leading to higher accumulation in their gills and liver.

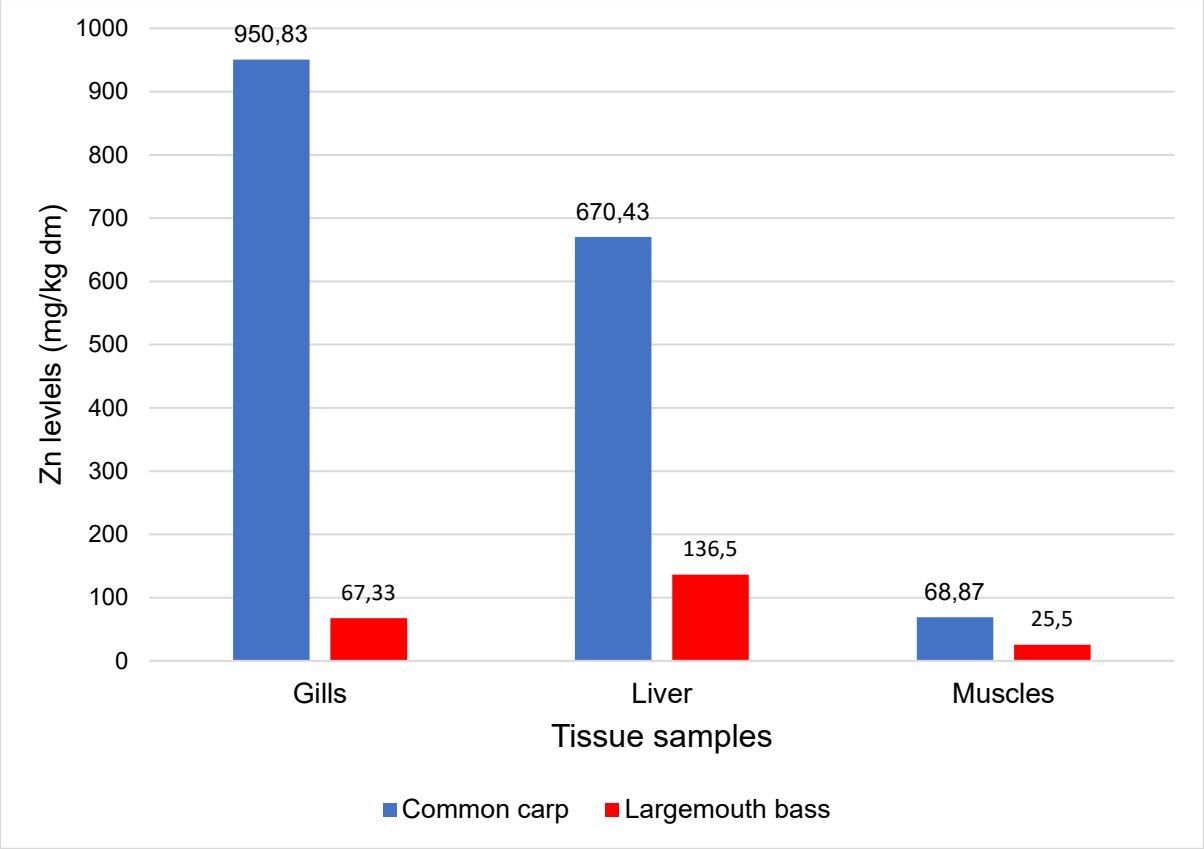

Figure 10: Comparison of Zn in gills, liver and muscles of common carp and Largemouth bass.

Whilst, Largemouth bass, which feed higher in the water column, may be less exposed to Zn-rich sediments. The high Zn levels in the gills of common carp may reflect their greater exposure to Zn in the environment, combined with active uptake through ion transport systems in the gill epithelium (Garai *et al.,* 2021).

The general appearance of both fish species indicated that the fish was in a good physical condition. The largemouth bass had no noticeable abnormalities in skin, eyes, operculum, or their gills. However, amongst the common carp in this study, two fish presented with deviations from the norm. The first had mild skin alterations and the other had an outward growth on the operculum (**Figure 11**). It is likely that the skin alterations were caused by predatory fish like *Clarias gariepinus*. The growth on the operculum depicted in Figure 11 is the subject of further histological assessment. What was noticed is that it appeared to be attached to the operculum without any deformity or damage to the underlying gills.

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Figure 11: Common carp with outward operculum

Ectoparasites were found in nearly half (47.8%) of common carp but were completely absent in largemouth bass. Whilst in largemouth bass, only a small percent (16.7%) had endoparasites but no endoparasites were detected in common carp. This significant difference is likely due to the bottom-dwelling behaviour of common carp (FAO, 2024), exposing them to sediment-rich environments where ectoparasites flourish. High ectoparasite prevalence could also indicate poor water quality or overcrowding, which are common in carp habitats. The presence of endoparasites in largemouth bass may be associated to their carnivorous diet such as smaller fish, which might act as hosts for parasites.

The liver coloration in largemouth bass showed only liver abnormalities ranging from light red to tan and focal discoloration indicating mild to moderate hepatic stress. In common carp, 91% of liver samples indicated abnormalities including light red, coffee with tan, and focal discoloration, with light red being the most common. The higher prevalence of abnormalities in common carp may result from their benthic feeding behaviour, exposing them to sediment-bound pollutants such as heavy metals, pesticides, or organic contaminants. Additionally, their omnivorous diet and constant interaction with the substrate increase their exposure to environmental toxins, leading to higher hepatic stress.

The metal concentration patterns observed between common carp and Largemouth bass suggest species-specific differences in metal accumulation, which

could be due to a difference in feeding habits, habitats, or metabolic rates. The higher metal concentrations in the liver and gills indicate that these tissues are primary sites for metal accumulation. The elevated levels of Fe and Zn in the gills and liver of common carp, compared to Largemouth bass, suggest that this species may be more susceptible to metal accumulation or has different exposure levels at Witbank Dam.

CHAPTER 7

7. CONCLUSION, RECOMMENDATIONS AND LIMITATIONS

7.1 Conclusion

Freshwater ecosystems support diverse organisms that depend on one another and their surroundings to survive. However, this aquatic environment is constantly in jeopardy, particularly due to heavy metal contamination. The aquatic life and environment are significantly impacted by these heavy metals, which are distinguished by their extreme toxicity, persistent presence, and bioaccumulation. The aim of this study was to use two seasonal surveys to investigate the impact of Fe, Cu, and Zn levels on innate immune modulation in *Cyprinus carpio* and *Micropterus salmoides* collected from Witbank Dam. The focus was: (i) to establish whether the levels of these metals in surface water and sediment samples differed significantly, (ii) to determine their bioaccumulation in these fish species, and (iii) to use the cOmplete™ protease inhibitor cocktail to evaluate its relevance in the pre-analytical care of our mucus and serum samples.

This study demonstrates significant variations in water quality parameters and metal concentrations between high and low flow conditions in Witbank dam. High flow conditions were linked with elevated water temperatures, pH, and DO, while low flow conditions showed increased TDS and salinity. Furthermore, it was discovered that Witbank dam was contaminated with Cu and Zn concentrations, yet Fe remained within acceptable levels. The sediment analysis also exhibited higher but acceptable concentrations of Cu, Fe and Zn during high flow and low flow.

The study also revealed that the use of a protease inhibitor cocktail significantly preserved CRP levels in both serum and mucus, resulting in higher and more accurate levels compared to untreated samples. While the measurements of untreated samples were lower and fell within the normal range of CRP, this suggests that protein degradation occurred. This is a concern as this leads to underreporting of fish health conditions. Whilst IL-6 concentrations in serum, demonstrated some difference between treated and untreated samples, it also indicated high variability though it were statistically significant. Moreover, IL6 in mucus, and IgM, in both serum and mucus, were stable regardless of the protease inhibitor cocktail treatment. These results

indicate that protease inhibitor cocktail was effective for only CRP in both serum and mucus.

Lastly, our results reported variations in metal accumulation in common carp and largemouth bass. This highlighted the differences in feeding habits, their preferred habitats, and physiological processes. The liver emerged as the primary site of Cu storage in both species, emphasizing its detoxification role. However, Fe and Zn displayed varied tissue distributions between species, with gills showing higher accumulation in common carp due to environmental exposure and their benthic feeding habits. These findings further support existing literature that indicate that water pollution significantly influences metal accumulation patterns in fish.

7.2 Recommendations

This study successfully established the levels of the selected metals in surface water and sediment samples. The use of the Protease inhibitor cocktail in pre-analytical care of samples did answer some questions. However, it also raised questions pertaining to current practices that spans beyond the biomarkers that we used in our study. Unlike laboratory-based studies, there is a real possibility that compromised pre-analytical care of in situ collected samples can provide false negative results. As such it is recommended that the use of protease inhibitor cocktails be further evaluated especially for biomarkers that are frequently used for diagnostic purposes.

The impact of Fe, Cu and Zn on IL-6, CRP and IgM could not be determined with certainty as the sensitivity of the analytical tests used could not provide definitive values. Future research should focus on tests that are sensitive enough to test at levels lower than what was found in our study.

Furthermore, it is recommended that future research and environmental monitoring programs be established that continuously monitor the metal concentrations. This continuous monitoring of water physico-chemical parameters and metal concentrations in sediment, water, and aquatic organisms at Witbank Dam is vital to detect, assess and address the pollution and potential risks in feral populations.

7.3 Limitations

While the study provides valuable insights into the effects of heavy metal pollution on fish species in Witbank Dam, a few limitations must be acknowledged. The present study utilized a relatively small sample size, which may limit the statistical power and the ability to detect the correlation between the selected metals and the immune biomarkers. This stemmed from the choice to use conventional angling gear rather than gill nets. During the planning stage of the study it was agreed that the use of gill nets posed an unacceptably high risk to endemic, non-targeted species and as such were not to be used.

Furthermore, the research was limited to Witbank Dam, and the results may not be fully representative of other aquatic ecosystems that are exposed to different pollution levels or environmental conditions. To enhance the generalizability of the findings, similar studies should be conducted in other water bodies with varying levels of pollution to identify whether the observed trends hold true across different geographical locations.

Future studies should increase the sample size to improve the reliability of the results, particularly when analysing correlations between metal concentrations and immune biomarker levels. The study was conducted over a limited period, focusing on specific seasons. Future studies should consider a longer sampling period to capture year-round variability and better assess seasonal and inter-annual changes.

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