# EFFECTS OF POLLUTION AND METAZOAN PARASITES ON THE HEALTH AND OXIDATIVE STRESS BIOMARKERS OF TWO CYPRINID FISH SPECIES IN THE OLIFANTS RIVER SYSTEM, SOUTH AFRICA

by

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# DECLARATION

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

…….. …….

T.P. Ramalepe Date

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# ABSTRACT

The unprecedented expansion in human population and industry, since the industrial revolution in the late 1700s, has led to increased anthropogenic activities which have indisputably impacted freshwater ecosystems and biological communities therein, including fish. Although this has understandably been the focus, under natural aquatic conditions, no organism is only affected by pollution. Parasites have also been shown in a number of interdisciplinary studies to affect the health of aquatic hosts (amphibians, crustaceans, fish, and mammals). This is illustrated in a number of comprehensive studies the detrimental effects parasites exacerbate when their hosts (fish) are stressed. Therefore, the ability of parasites to interact with anthropogenic stressors, as well as effects they have on the genetic, cellular or tissue level of their host is crucial in conservation and sustaining aquatic biodiversity. As such, the present study examined the combined effects of pollution and metazoan parasites on the health and oxidative stress biomarkers, evaluated for the first time for silver carp, *Hypophthalmichthys molitrix* (Valenciennes, 1844) and rednose mudfish, *Labeo rosae* Steindachner, 1894, in one of South Africa's impacted freshwater ecosystems, Flag Boshielo Dam, Olifants River System, Limpopo Province.

Seasonal surveys were conducted from February 2012–January 2013. A total of 111 *H. molitrix* and 116 *L. rosae* fish specimens were collected using conventional angling gear, scoop and gill nets with stretched mesh sizes of 30–110 mm. The two selected cyprinid fish species were assessed for oxidative stress biomarkers [Glutathione S-transferase (GST), lipid peroxidation (MDA) and Total Antioxidant Capacity (TAC)] and parasitism of metazoan parasites. Concentrations of biomarkers of oxidative damage and antioxidant defense in the gill and liver tissue were measured to assess how these major organs of the immune system responded to oxidative stress associated with parasitic infections. In addition, water quality analyses were carried out by testing an assay of physico-chemical parameters to establish the level of contamination. Fish health was assessed using the Health Assessment Index (HAI), refined Parasite Index (PI), Inverted Parasite Index (IPI) and Condition Factor (K) protocols.

Relative to previous studies at Flag Boshielo Dam, water quality results showed an increase of nutrients, major ions and several metals which may have adverse effects that may comprise fish health; however, this dam remains moderately polluted in a mesotrophic state. The fish health assessment results indicated that *H. molitrix* was more affected in terms of the necropsy and parasite based assessments (HAI, IPI and K) with mean±SD of 65.68±35.51; 68.29±25; 0.82±0.20, respectively, as compared to 39.14±22.44; 28.79±18.33; 1.17±0.21 for *L. rosae* during the study. In addition, significantly higher parasitic infections (mean prevalence of infection with any species of parasite = 45.3±0.13) were observed for *H. molitrix* than *L. rosae* (12.0±0.05). Furthermore, there was considerable variation in biomarker concentration between highly infected and non-infected fish, for and between each species and tissues with regard to parasite infection, suggesting that the specific functions of each tissue are associated with their susceptibility to oxidative stress, as well as their ability to defend against oxidative damage.

These results illustrate that although fish are affected by aquatic contaminants they are to an extent affected by parasites, which may act synergistically on the health of the two fish species. Most importantly, it was suggested that knowledge on the parasites of alien *H. molitrix* when compared to indigenous *L. rosae* may give an indication of how adaptive this fish are to new localities as well as expands the information on the rarely studied biology, epizootiology and ecological interactions of these two cyprinid species.

**Keywords**: Health Assessment Index, refined Parasite Index, Inverted Parasite Index, Condition Factor, water quality, lipid peroxidation, Glutathione S-transferase, Total Antioxidant Capacity, *Hypophthalmichthys molitrix*, *Labeo rosae*, Flag Boshielo Dam.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AEV | Acute Effect Values |
| ANOVA | Analysis of variance |
| CCME | Canadian Council of Ministers of the Environment |
| CEV | Chronic Effect Values |
| CV | Coefficient of Variation |
| DWA | Department of Water Affairs |
| DWAF | Department of Water Affairs and Forestry |
| GST | Glutathione S-transferase |
| HAI | Health Assessment Index |
| Hct | Haematocrit |
| ICP-OES | Inductively Coupled Plasma-Optical Emission Spectrometry |
| IPI | Inverted Parasite Index |
| IUCN | International Union for Conservation of Nature |
| IWRM | Integrated Water Resource Management |
| K | Condition Factor |
| KNP | Kruger National Park |
| MDA | Melondialdehyde |
| PI | refined Parasite Index |
| PCA | Principal Component Analysis |
| RHP | River Health Programme |
| ROS | Reactive Oxygen Species |
| SANAS | South African National Accreditation System |
| SAWQG | South African Water Quality Guidelines |
| SD | Standard Deviation |
| TAC | Total Antioxidant Capacity |
| TBARS | Thiobarbituric Acid Reactive Substances |
| TWQR | Target Water Quality Range |
| USEPA | United States Environmental Protection Agency |
| WDCS | Waste Discharge Charge System |
| WHO | World Health Organization |

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# CHAPTER 1 - GENERAL INTRODUCTION

## 1.1 INTRODUCTION

For centuries, since the industrial revolution in the late 1700s, human population began to exhibit unprecedented sustainable growth. The industrial revolution marked the transition from a stable agrarian society to a modern industrial society relying on complex machinery rather than simple tools. Consequently, new technological innovations and inventions led to an increase in the use of freshwater ecosystems in the industrial sectors; as such these ecosystems became impacted in some way or the other. Freshwater ecosystems are now articulated to be among the most altered ecosystems worldwide due to continued human activities (Malmqvist & Rundle 2002).

In South Africa, many of the river systems are among the most severely degraded aquatic ecosystems altered profoundly by industrial, agriculture and urban pollution, introduction of alien species, and alteration of riparian habitat. These environmental perturbations have led to extensive ecological degradation of many rivers that resulted in the decline of water quality and quantity, changes in the distribution and structure of the aquatic biota as well as biodiversity loss (Dallas & Day 2004). One such example includes the Olifants River System, situated in the northern parts of South Africa (Heath *et al*. 2010; Madanire-Moyo *et al*. 2012a).

The Olifants River System is one of South Africa's most pivotal freshwater ecosystems involved in shaping the country's economic prospects, through power generation, irrigation, tourism, industrial production, mining and fisheries (Madanire-Moyo *et al*. 2012a). In addition, this river is regarded as one of the largest rivers flowing through South Africa's renowned Kruger National Park (KNP), inhabiting a diversity of fauna and flora (Heath *et al*. 2010). However, recently concerns have been raised over the integrity of this system, with signs of pollution, pansteatitis in fish and crocodiles, progressive and, occasional dramatic reduction in the number and abundance of several sensitive species of amphibians, fish, crocodiles and aquatic mammals (Darwall *et al*. 2009; Oberholster 2009; Ashton 2010; Heath *et al*.

2010; Botha *et al*. 2011; Huchzermeyer *et al*. 2011; Dabrowski *et al*. 2013). The demise and reduction of these organisms is according to Oberholster *et al*. (2010) a reflection of the cumulative effects of slightly more than a century of ecosystem stress due to anthropogenic activities.

## 1.2 THE USE OF FISH IN BIOLOGICAL MONITORING

The former Department of Water Affairs and Forestry (DWAF), now Department of Water Affairs (DWA), initiated the River Health Programme (RHP) in a bid to monitor selected aquatic communities by incorporating several components of the biota, including fish, macroinvertebrates and physico-chemical components to characterise the response of the aquatic environment to multiple disturbances through the use of biological monitoring programmes. Biomonitoring programmes involve the use of a wide range of bioindicators and biomarkers, from subcellular, organisms, communities to population levels (Adams 1990). Fish have received attention as indicator organisms in assessing environmental health (Simon 1999), considering their relative sensitivity to changes in the environment. Changes occurring in fish may be as a result of biochemical, histological and physical alterations and thus can give a relative rapid indication of how environmental conditions affect fish populations. Fish health may thus reflect, and give a good indication of the health status of a specific ecosystem (Van Dyk 2003), based on the assumption that the biotic integrity of an ecological system is often reflected by the health of the organisms that reside therein (Poulin 1992; Adams *et al*. 1993).

Health can be defined as the dynamic condition of an organism resulting from the body's constant adaptations and adjustments in response to stress and changes in an environment for maintenance of its vital functions. Therefore, the health of any population depends on the control of disease and maintenance of a healthy relationship between living organisms and their environment (Snieszko 1983). While antropogenic pollution of the aquatic ecosystem is an important factor contributing to the decline in many animal populations, the past years has however, seen an upsurge in the population aspects of infection by natural stressors such as parasites in aquatic ecosystems. Although recently there have been comprehensive studies on the parasite biology, distribution and ecology in a number of South African rivers, e.g. the Limpopo, Olifants, and Vaal rivers (Avenant-Oldewage 1998; Madanire-

Moyo *et al*. 2010; Madanire-Moyo *et al*. 2012b), limited information still exists on the detrimental effects exacerbated by these natural stressors when their hosts (fish) are stressed.

### 1.2.1 Effects of parasitic infections on fish health

Parasites are ubiquitous organisms occurring in almost all trophic levels in an ecosystem (Marcogliese 2005). While the presence or absence of these organisms serve as indicative tools of ecosystem health, and are referred to as indigenous components of healthy ecosystems (Sures 2001), what costs do they exert on their hosts (fish). Many parasites have long been recognised to have the potential to affect the growth, fecundity, and modulate a wide range of physiological processes, for example, their resistance to other stressors (Dautremepuits *et al*. 2003).

Fish serve as definitive, intermediate or transport host in the life cycle of many metazoan parasite species. Metazoan parasites of fish include the helminths (worms) and arthropods. For instance, larval or adult forms of metazoan parasites can be found in almost every tissue of fish. Besides direct losses due to mortality, parasites may interact with environmental contaminants and destruct antioxidant metabolisms (induce oxidative stress) in fish (Woo 1995); thereby, compromising the immune integrity of the host (Dautremepuits *et al*. 2003; Marcogliese *et al*. 2005; Marcogliese & Pietrock 2011). In this regard, monitoring the effect those parasite infections in conjuction with anthropogenic stressors has on biomarkers profiles can provide a crucial means of ensuring the conservation and sustainability of aquatic biodiversity.

### 1.2.2 Effects of parasitic infections on oxidative stress biomarkers

Parasite infections, diseases and the overall health of fish are of concern in both natural and aquaculture environments and as such have recently intensified the demand for research on host-parasite interactions including the immune response of fish (Alvarez-Pellitero 2008; Sures 2008). In the natural environment, parasites can affect the ecology and evolution of their host if infections have harmful consequences, especially where the introduction and subsequent dispersal of alien species may bring alien parasites to indigenous fish species (Howe *et al*. 1997).

Parasite infections can lead to synergistic effects resulting in interference of several metabolic pathways of cells such as destructed antioxidant metabolism (Neves *et al*. 2000; Dautremepuits *et al*. 2003; Frank *et al*. 2011). For example several studies (Belló *et al*. 2000; Dautremepuits *et al*. 2003; Marcogliese *et al*. 2005) have demonstrated that metazoan parasites such as monogeneans, digeneans, nematodes, cestodes and crustaceans infecting fish and invertebrates can affect antioxidant metabolism, consequently induce oxidative stress. In addition, several enzymatic and non-enzymatic defense systems with antioxidant capacities are presented by fish as an immune response mechanism (Del Maestro 1980; Sies 1993).

Oxidative stress occurs as a result of the excess production of reactive oxygen species (ROS) in cells and supressed defense capacity of the cell (Storey 1996; Tagliari *et al*. 2004). Reactive oxygen species include free radicals such as superoxide anion radical ($O^{-}_{2}$), hydroxyl radical ($OH^{-}$) and hydrogen peroxide ($H_2O_2$), which in excess may generate DNA alterations and damage to polyunsaturated lipids (lipid peroxidation) initiating scavenger compounds like enzymatic antioxidant defenses, catalase (CAT), superoxide dismutase (SOD), total antioxidant capacity (TAC) and glutathione S-transferase (GST), amongst others (Storey 1996). These cell defense systems can be measured as biomarkers of xenobiotic mediated oxidative stress.

Biomarkers are one of the standard approaches used to quantify the impact of environmental stressors on animal health (Sures 2008; Marcogliese *et al*. 2009). They are defined as alterations in biological responses ranging from molecular, biochemical, physiological to behavioral changes, which can be related to exposure or effects of environmental contaminants. Therefore biomarkers can measure the degree of environmental constrain on molecular, biochemical and physiological endpoints (Adams 2001; Marcogliese *et al*. 2010). In essence, biomarkers integrate the effects of multiple stressors in aquatic ecosystems and assist in elucidating particular mechanisms associated with those stressors. Attril and Bepledge (1997) asserts that, due to the complexity of natural systems, an adequate set of endpoints is required in environmental monitoring to determine the biological significance of stress and the underlying mechanistic basis of observed effects.

Biological monitoring in aquatic ecosystems is regarded as one of the methods employed to evaluate biological responses as well as adverse effects of environmental stress on aquatic biota. In that regard, a variety of chemical, physical and biological indicators need to be included in aquatic monitoring programmes each used in their respective roles as environmental stressors (i.e. xenobiotics), exposure response (i.e. biomarkers) and effects response (i.e. bioindicators) to reflect the effects of multiple stressors on the integrity of aquatic ecosystems (Xenopolus & Lodge 2006). In this context, parasites have been used recently in a number of interdisciplinary studies (Sures 2006; Thilakaratne *et al*. 2007; Vidal-Martinez 2007; Dautremepuits *et al*. 2009; Marcogliese & Pietrock 2011) as bioindicators of pollution in addition to examining their effects on physiological biomarkers of pollution.

## 1.3 FISH HEALTH ASSESSMENT

One of the introduced biomonitoring tools in South Africa is the use of a fish Health Assessment Index (HAI). The index was originally developed as a field necropsy-based method by Goede and Barton (1990) in the United States of America, to evaluate the effect of stress on the health of fish populations. This method was developed and described as a rapid and inexpensive field necropsy-based method that provided fish health assessments based on percentages of anomalies observed in tissues and organs of individuals sampled from a population (Adams *et al*. 1993). This approach profiles fish health by allocating a percentage score to anomalies observed on various external features and internal organs and some blood parameters of specimens sampled (Goede 1992). The major limitations of this method were that it did not provide quantitative results that are agreeable to statistical comparisons of data among species, sites or years. In an effort to minimise the limitations of this necropsy-based method, Adams *et al*. (1993) refined and modified it by developing the HAI.

The HAI is a quantitative index that allows statistical comparisons of fish health among datasets and includes variables that are assigned numerical values based on the severity or damage incurred by an organ or tissue as a result of environmental stressors (Adams *et al*. 1993). The index value for a specific fish is the sum total of values of all examined tissues and organs, and the mean calculated for all fish in the sample is the index value for that locality (Crafford & Avenant-Oldewage 2009). A

high index value corresponds with poor water quality, thus an increase in physiological stress; consequently poor fish health and a low value represent good water quality and a healthy fish population (Adams *et al*. 1993; Crafford & Avenant-Oldewage 2009).

The HAI, developed to monitor lotic systems in the United States of America, was introduced in South Africa by Avenant-Oldewage and Swanepoel (1993). Since then it has been tested and adapted for local conditions through various studies in the Olifants, Phongola and Limpopo River systems (Avenant-Oldewage *et al*. 1995; Jooste *et al*. 2005; McHugh *et al*. 2011; Madanire-Moyo *et al*. 2012a; Sara *et al*. 2014) as well as in the Vaal River System (Crafford & Avenant-Oldewage 2009) using different fish species as environmental indicators. The HAI proved to be useful in bioassessment surveys and was thus incorporated in the Integrated Ecological Assessment (IEA) by DWAF (Killian *et al*. 1997).

One of the variables in the HAI is the presence or absence of parasites; these received assigned values based on the severity of damage on the host. However, due to parasite diversity and life stages, parasites have been recognised as ecological indicators of their fish host life conditions and environmental health (Avenant-Oldewage 1998; Marcogliese 2004; Blanar *et al*. 2009). In South Africa, the original HAI notation with regards to parasites was reviewed by a number of authors (Marx 1996; Robinson 1996; Luus-Powell 1997), based on the general concept that contaminants have different influences on ecto- and endoparasites. The notation was thus expanded and developed into a parasite index tested in conjunction with the HAI (Marx 1996). Crafford and Avenant-Oldewage (2009) referred to this as the Original Parasite Index (OPI). This index has since been modified and refined with the introduction of the refined Parasite Index (PI) to distinguish between the number of ecto- and endoparasites present. The PI was further revised to the Inverted Parasite Index (IPI), which is based on the premises that ectoparasites are more exposed to the effects of water quality than endoparasites. Larger numbers of ectoparasites are indicative of better water quality and should be given a lower score for this correlation to be reflected in the HAI value (Crafford & Avenant-Oldewage 2009). Nevertheless, both the HAI and PI indices of fish health assessment are

necessary in environmental monitoring since they complement with each other and can provide meaningful environmental information (Jooste *et al*. 2005).

Taking the aforementioned into consideration, it is clear that the influence of pollution levels on fish and their parasites are complex. In that regard, the ability of parasites to conflate with anthropogenic stressors, as well as effects they have on the genetic, cellular or tissue level is crucial in conserving the sustainability of aquatic biodiversity. Hence, to manage healthy fish populations, it is necessary to identify early detectable warning signs of damage on cellular and tissue level before metabolic and physiological processes are affected (Van Dyk 2003). In South Africa limited information is available on the combined effect of parasite infections and other environmental stressors on fish response to oxidative stress and oxidative enzyme production to manage these health affecting factors. Thus, the present study is to our knowledge the first to evaluate the effects of pollution and metazoan parasites on the oxidative stress biomarkers and health of two Cyprinidae species, the alien fish *Hypophthalmichthys molitrix* (Valenciennes, 1844) and an indigenous species *Labeo rosae* Steindachner, 1894, inhabiting Flag Boshielo Dam, Olifants River, South Africa. These fish are enlisted by the International Union for Conservation of Nature (IUCN) as threatened and least concern, respectively.

In the present study, fish health parameters; HAI, refined by Adams *et al*. (1993), PI suggested by Jooste *et al*. (2005), IPI evaluated by Crafford and Avenant-Oldewage (2009) and Condition Factor (K) according to method of Bagenal and Tesch (1978) were applied in conjunction with water quality to assess the health of the two cyprinds. Furthermore, the study investigated and measured concentrations of the oxidative damage (lipid peroxidation) and antioxidant defense (GST and TAC) in the liver and gills between highly infected fish and non-infected fish of the two selected fish species to assess how these major organs of the immune system respond to oxidative stress associated with parasitic infections.

### 1.4 AIM

The study aims to seasonally record the quality of water and analyse the effect of metazoan parasite infections on selected oxidative stress biomarkers as well as assess the health of two cyprinid fish at Flag Boshielo Dam, by applying the HAI, PI, IPI and K protocols.

## 1.5 OBJECTIVES

The objectives of the study were to:

i) Evaluate water quality by measuring different water parameters.
ii) Determine the HAI, PI, IPI and K of *H. molitrix* and *L. rosae* populations.
iii) Determine and compare the prevalence, mean abundance and mean intensity of ecto- and endoparasites of the two selected fish species.
iv) Assess the effects of metazoan parasite infections on oxidative stress biomarkers (GST, lipid peroxidation and TAC) in the liver and gill tissue of the two fish species.
v) Determine the correlation between the parasite abundance, health of fish, water quality and oxidative stress biomarkers.

## 1.6 RESEARCH QUESTIONS

i) Can the ecological state of Flag Boshielo Dam at present be considered moderately polluted?
ii) Can the HAI, PI, IPI and K protocols be used to effectively assess the fish health status of *H. molitrix* and *L. rosae* at Flag Boshielo Dam?
iii) Can oxidative stress biomarkers comprehend the effect of metazoan parasite infections on the two selected fish species?
iv) Is there a correlation between parasite abundance, the health of fish, water quality and oxidative stress biomarkers at Flag Boshielo Dam?

## 1.7 DISSERTATION OUTLINE

To achieve the aim of the study, the dissertation consists of five chapters to address the specific objectives outlined in 1.5 and a concluding chapter containing overall remarks that highlight the main findings of the study as well as recommendations for future research. The chapters are outlined as follows: **Chapter 2** provides a detailed description of the study area, selected fish species, various indices, materials and methods used throughout the study. From **Chapter 3** through to **Chapter 5** an introduction was given on the aspects to be investigated and brief summary of methods and materials used, results obtained were discussed and conclusions were drawn to answer and respond to research questions posed in the study. Therefore, **Chapter 3** contains water quality results obtained for the selected water constituents

determined during four seasonal surveys and discussion on each of these variables and their possible influence on the aquatic environment and the health of the two fish species at Flag Boshielo Dam. **Chapter 4** contains the results and discussion of the HAI and all other health indices associated with the HAI used in determining the health of *H. molitrix* and *L. rosae* populations. **Chapter 5** provides results and discussion on oxidative stress biomarkers of the two fish species and a summary for the overall results as well as the correlation between the parasites abundance, health of fish, water quality and oxidative stress biomarkers. **Chapter 6** contains concluding overall remarks that highlight the main findings of the study and recommendations for future research. Finally, all the references used throughout the study are listed followed by an appendix containing raw data at the end of the dissertation. Please note that as every chapter is an entity, the abbreviations will be repeated for a specific chapter.

# CHAPTER 2 - MATERIALS AND METHODS

## 2.1 BACKGROUND OF THE STUDY AREA

### 2.1.1 Olifants River Basin

The Olifants River is the largest of several rivers flowing through South Africa's renowned Kruger National Park (KNP) (Coetzee *et al*. 2002; Van Vuuren 2009). At present, the Olifants River System falls within three of the country's provinces; these include the Limpopo, Mpumalanga and Gauteng provinces (De Lange *et al*. 2003). The Olifants River has its origins in the east of Johannesburg in the Gauteng Province, and flows northwards before curving eastwards towards the KNP into Mozambique, where it joins the Limpopo River before discharging into the Indian Ocean (Figure 2.1) (Coetzee *et al*. 2002; Heath *et al*. 2010).

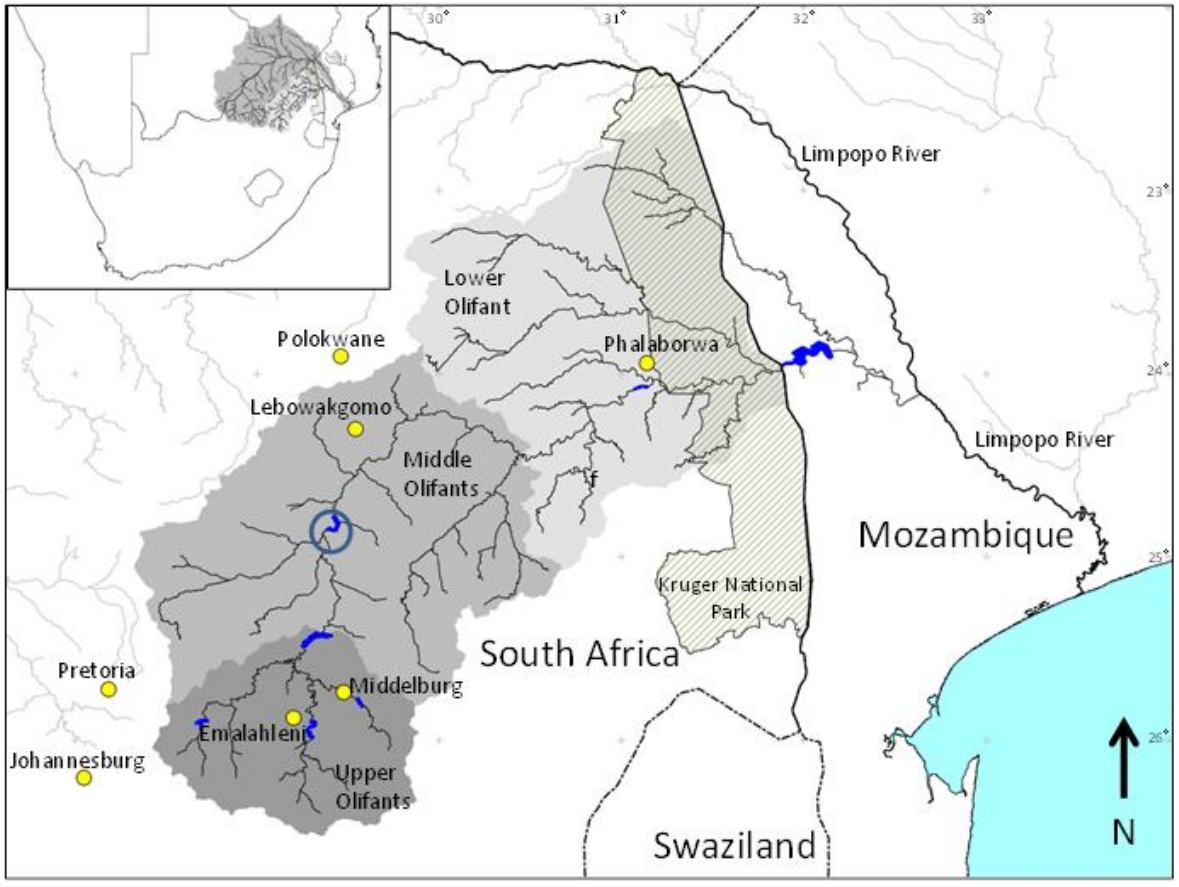


Figure 2.1: Sketch map of the Olifants River catchment showing the upper, middle and lower river reaches. The inset illustrating the position of the mapped area within southern Africa and the circled area, Flag Boshielo Dam (http://www.DWAF.gov.za).

The Olifants River System is divided into the upper, middle and lower reaches (Ashton 2010) and has a catchment area of 54 750 $km^2$, which constitutes approximately 4.3% of the total surface area of South Africa (Heath *et al*. 2010). Many of the natural rivers and streams have been extensively dammed in this system with about 202 storage dams (38 of which have volumes larger than 1 $Mm^3$) (Ashton 2010; Heath *et al*. 2010). The combined capacity of all the dams (4 688 $Mm^3$) exceeds the mean annual runoff of the system (Middleton & Bailey 2009). As such, the stream flow is now highly regulated.

The climate of the Olifants River catchment is described as predominantly semi-arid, dry and hot with an average annual rainfall of 2 400 × $10^6$ $m^3$ (FAO 2004; De Villiers & Mkwelo 2009). Ichthyofauna of the Olifants River comprises of at least 124 fish species, most of which are cichlids, cyprinids, gobies and mochokid catfishes (Thieme *et al*. 2005). According to Skelton *et al*. (2001) this is due to the diversity of southern temperate and tropical faunas in the region.

The mineral potential of this basin is significant, considering mining activities around the Olifants River, i.e. iron, chromium, magnesium, coal, phosphate and copper as well as complex coal power generations. These activities have been reported to generate high concentrations of heavy metals in key reservoirs in the Olifants River System (Ashton *et al*. 2001; Oberholster 2009). These pollutants have an influence on the pH (lower pH), total dissolved solids (TDS) concentrations (higher TDS), resulting in decreased water quality which can be fatal to aquatic life in the system (Luus-Powell 1997; De Villiers & Mkwelo 2009). For instance, the decline in piscivorous bird (especially heron) populations which is according to Myburgh and Botha (2009) most likely linked to the ecological deterioration of the river.

**The upper reaches** of the Olifants River catchment is characterised by intensive irrigated agriculture, large-scale coal mining, most of which occurs in the Witbank and Highveld coalfields (Oberholster 2009), and industrial developments as well as several towns and smaller urban centres such as Bronkhorstspruit, Kinross, Kriel, Klipspruit, Hendrina and Trichardt (Heath *et al*. 2010). The upper reaches of the Olifants River catchment comprise the drainage areas of the Olifants, Klein Olifants and Wilge rivers with tributaries down to Loskop Dam. This dam is the largest in the catchment and incorporates the most industrialised region of the Olifants River.

**The middle reaches** catchment contains extensive areas of irrigated agriculture (Figure 2.2) of diverse crops, the largest of which is the Elands River Irrigation Scheme. The middle Olifants River catchment comprises the drainage areas of the Olifants River downstream of Loskop Dam to Flag Boshielo Dam. Several small platinum, chrome and vanadium mines are found in the catchments of Klipspruit, Moses and Loopspruit rivers as well as the area east of Marble Hall (Heath *et al*. 2010), and this region has numerous smaller urban centres such as Groblersdal and Marble Hall (Ashton *et al*. 2001). Flag Boshielo Dam is regarded as one of the key water reservoirs of the Olifants River (Ashton 2010).

**The lower reaches** of the catchment is characterised by several small mines of rare minerals, including phlogopite, platinium and europium and an important copper and phosphate mining complex around the town of Phalaborwa and municipal sewage treatments plants as well as industrial activities. The impact of the industrial effluent on the quality of water entering the KNP is of major concern to conservationists, even more so to the aquatic biota (Ashton 2010). The KNP is situated along the lower reaches of the Olifants River, and is one of the conservation areas in the system. This park represents a highly unique conservation area preserving most of South Africa's natural resources (Heath *et al*. 2010). It is therefore imperative that the effects of all activities on the water quality and aquatic biota of this river be determined in order to sustain species diversity and secure the ecosystem for future generations.

### 2.1.2 Flag Boshielo Dam

The present study was conducted at Flag Boshielo Dam (24°49′ 57″ S; 29°24′ 59″ E), formerly known as Arabie Dam. It is located in the middle reaches of the Olifants River (Clark 1997) approximately 85 km downstream of Loskop Dam (in the upper reaches) and approximately 25 km north-east of Marble Hall (Dabrowski *et al*. 2014). This dam was built to provide water for irrigation, domestic and industrial supply as well as for recreational purposes (Heath *et al.* 2010). Flag Boshielo Dam was constructed in 1987, and in response to the ever increasing water demands, the dam wall (Figure 2.3) was raised by another 5 meters in 2005 (Ashton 2010). The main tributaries of the dam include Selons, Bloed, Moses and Elands rivers (Heath *et al*. 2010).

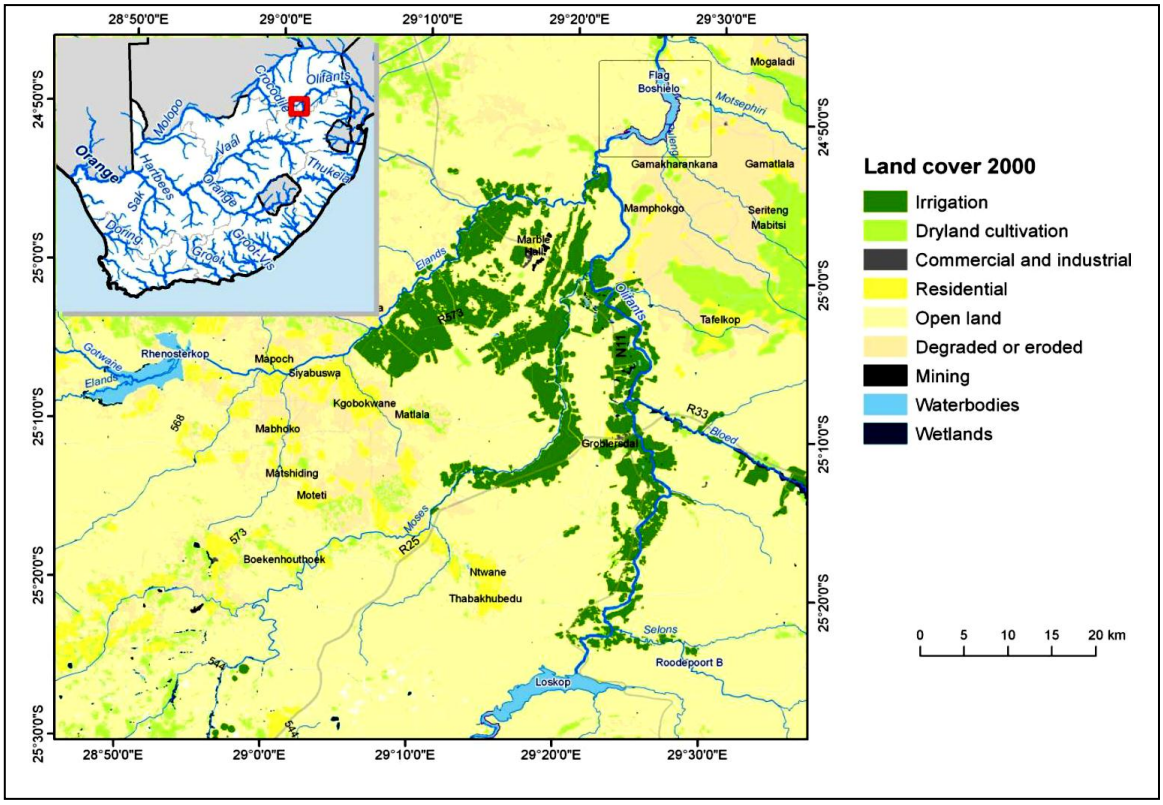


Figure 2.2: Map showing the catchment areas: rivers and urban/industrial developments around Flag Boshielo Dam, Olifants River System (http://www.DWAF.gov.za).

Flag Boshielo Dam has a catchment area of 23 712 km$^2$, a carrying capacity of 104 106 m$^3$ and an average depth of 15 m (DWAF 2003; Heath *et al*. 2010). There are commercial and subsistence agriculture (Figure 2.2) as well as numerous point and diffuse sources of industrial pollution along the Olifants River towards the dam (Heath & Claassen 1999).

This dam is a mainstream reservoir in the Olifants River and provides water supplies to numerous small towns and settlements in the area, as well as large volumes of water for irrigation schemes below the dam (De Villiers & Mkwelo 2009). This region is perhaps the most economically important agricultural area in the sub-catchment. Few industries are present in the towns of Marble Hall and Roedtan and these industries are geared specifically to meet the needs of the extensive agricultural activities in the middle Olifants River sub-catchment (De Lange *et al.* 2003; Dabrowski *et al*. 2014).

Figure 2.3: Dam wall of Flag Boshielo Dam (http://www.DWAF.gov.za).

## 2.2 WATER QUALITY PARAMETERS

Selected water quality parameters were measured *in situ* (Figure 3.1) seasonally in March (autumn), June (winter), September (spring) 2012 and January 2013 (summer), using a handheld multi parameter instrument (YSI 556 Multi Probe System). These included surface water variables such as temperature, dissolved oxygen (DO), electric conductivity (EC), pH and salinity. Subsurface water samples were collected in 500 ml polyethylene bottles pre-treated in an acidified phosphate-free bath and rinsed in deionised water, and subsequently frozen for further analysis at an accredited laboratory [Capricorn Veterinary Laboratories cc; South African National Accreditation System (SANAS)] in Polokwane. The subsurface water samples were analysed for the following constituents: turbidity, dissolved nutrients [ammonia ($NH_3$), ammonium ($NH_4$), orthophosphate ($PO_4$), nitrites ($NO_3$) and nitrates ($NO_2$)], anions [fluoride (F) and sulphate ($SO_4^{2-}$)], cations [calcium ($Ca^{2+}$), magnesium ($Mg^{2+}$), potassium ($K^{+}$) and sodium ($Na^{+}$)], non-toxic constituents (total dissolved solids (TDS) and alkalinity) and toxic constituents [aluminium (Al), arsenic (As), copper (Cu), iron (Fe), lead (Pb), manganese (Mn) and zinc (Zn)]. Results obtained for water quality during the study were compared against levels specified as permissible by the South African Target Water Quality Range (TWQR), the Chronic Effect Values (CEV) and Acute Effects Values (AEV) standards for aquatic ecosystems where applicable and available as set out by DWAF (1996a).

### 2.2.1 Target Water Quality Range

In South Africa the recognition of the need to implement measures on the usage of water in an environmentally sustainable manner led to the first edition of the South African Water Quality Guidelines (SAWQG). These guidelines are used by DWA as primary source of information provided for a specific water quality constituent and decision-support to judge the fitness of water for use, protection of the health and integrity of aquatic ecosystems as well as for other water quality management purposes. The SAWQG consist of TWQR and water quality criteria CEV and AEV for each of the constituents in the guidelines, with support information such as the occurrence of the constituent in the aquatic environment, the norms used to assess its effects on water uses. The TWQR is the range of constituents or levels within which no measurable adverse effects are expected on the health of aquatic ecosystems and should therefore ensure their protection. The CEV is defined as that concentration or level of constituent at which there is expected to be a probability of measurable chronic effects up to 5% of the species in the aquatic community. The AEV is defined as that concentration or level of a constituent above which is expected to be a significant probability of acute toxic effects of up to 5% of the species in the aquatic community (DWAF 1996a).

## 2.3 FISH SPECIES

The family Cyprinidae consists of about 275 genera, comprising of more than 1 600 species from Africa, Europe, Asia and North America. In Africa, the Cyprinidae consists of at least 24 genera and 475 species. In southern Africa cyprinid fish are widely distributed with approximately 7 genera and about 80 species (Skelton 2001). Two species of the Cyprinidae, i.e. *Hypophthalmichthys molitrix* (Valenciennes, 1844) (Figure 2.4) and *Labeo rosae* Steindachner, 1894 (Figure 2.5) were selected for this study on the basis that they have been reported to be free-ranging in the Olifants-Limpopo River Systems. In addition, because *H. molitrix* is an alien species while *L. rosae* is an indigenous species (Skelton 2001) and as mentioned previously parasite infections may proof to be problematic especially, where there are introductions and subsequent dispersals of alien species that may bring problematic parasites to infect indigenous fish species. In addition, because of numerous incidents of fish kills of *L. rosae* reported at Loskop Dam upstream of Flag Boshielo

Dam (Van Vuuren 2009; Dabrowski *et al*. 2013) and also of episodic *H. molitrix* deaths at Flag Boshielo Dam (personal observation).

### 2.3.1 *Hypophthalmichthys molitrix*

A detailed taxonomic classification of the fish host according to Skelton (2001):

Kingdom: Animalia
Phylum: Chordata
Class: Actinopterygii
Order: Cypriniformes
Family: Cyprinidae
Genus: *Hypophthalmichthys* Bleeker, 1860
Species: *Hypophthalmichthys molitrix*

*Hypophthalmichthys molitrix*, commonly known as the silver carp, is a freshwater species native to Asia (Skelton 2001), but alien to Africa, North America and South America (Singh & Kaur 2014). Silver carp was introduced into South Africa for aquaculture purposes, controlling excessive growth of phytoplankton and water quality in natural waters (Ellender & Weyl 2014). This fish species is a filter feeder with the potential to reduce native diversity by competing for and depleting plankton populations, thus altering the food web in the system (Skelton 2001; FAO 2004).

Figure 2.4: *Hypophthalmichthys molitrix* from Flag Boshielo Dam.

### 2.3.2 *Labeo rosae*

A detailed taxonomic description of the host species as described by Skelton (2001):

Kingdom: Animalia
Phylum: Chordata

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Genus: *Labeo* Cuvier, 1816

Species: *Labeo rosae*

*Labeo rosae*, commonly known as the rednose mudfish, is widely distributed in Africa, and occurs in the warmer reaches of rivers, particularly in sandy stretches (Reid 1985). *Labeo rosae* is found in the Lowveld reaches of the Limpopo, Incomati and Phongolo River systems (Skelton 2001). The rednose mudfish is a benthic feeder, feeding on detritus, algae and small invertebrates. This fish is an active fish and leaps at barriers when migrating upstream in swollen rivers to breed in summer, thus it is a good angling fish.

Figure 2.5: *Labeo rosae* from Flag Boshielo Dam.

## 2.4 SAMPLING OF FISH

Seasonal surveys were conducted from February 2012–January 2013 (December 2012–January 2013 = summer; March 2012–May 2012 = autumn; June 2012–August 2012 = winter; September 2012–November 2012 = spring). Fish were collected using conventional angling gear, scoop nets and gill nets with stretched mesh sizes of 30–110 mm (Figure 2.6B). A total of 111 *H. molitrix* specimens were collected during the four sampling seasons: summer (*n* = 32), autumn (*n* = 21), winter (*n* = 28) and spring (*n* = 30) while 116 *L. rosae* specimens were collected; these include: summer (*n* = 30), autumn (*n* = 34), winter (*n* = 29) and spring (*n* = 23). Each species collected was identified and retained in separate holding tanks filled with dam water, and aerated to keep the fish alive until they were processed.

## 2.5 FISH HEALTH ASSESSMENT

### 2.5.1 Health Assessment Index

As soon as the fish was removed from the holding tanks, mucus smears from the entire body surface were taken using microscope slides. Subsequently the entire external surface was examined for mobile ectoparasites, gross pathological changes were noted and depending on the degree of stressor-induced abnormalities, numerical values were assigned for the HAI categories as suggested by Adams *et al*. (1993) and Jooste *et al*. (2005) (Table 2.1) and recorded on HAI data sheets. Fish was subsequently measured for length, i.e. standard length (SL), total length (TL) and fork length (FL) to the nearest 0.1 cm using a calibrated measuring board (Figure 2.6C) and weighed to the nearest 0.1 g using a Salter Model 235E electronic balance (Figure 2.6D) and recorded on a HAI data sheet for condition factor calculations.

The fish were then placed on a dissecting board and approximately 2 ml of blood was extracted via venipuncture of the caudal vein using a syringe. The blood was transferred into two micro-haematocrit capillary tubes that were sealed and plugged at one end using commercial critoseal clay. The micro-haematocrit capillary tubes were centrifuged in a micro-haematocrit centrifuge (model: KHT-400) at 3 000 revolutions per minute (RPM) for 5 minutes (Figure 2.6E). A micro-haematocrit reader was used to express haematocrit (Ht) as percentage of total blood volume (Figure 2.6F) as outlined by Crafford and Avenant-Oldewage (2009) and recorded on the HAI data sheet.

Fish was sacrificed by severing the spinal cord and dissected from the anus to the head. The sex of the fish was determined after opening the viscera cavity and recorded on the HAI data sheet. The internal organs (liver, gills, kidney, hindgut, spleen, eyes, swimbladder, bile and intestine) (Figure 2.6H) were placed in separate Petri dishes and covered with distilled water to prevent dehydration and examined with the aid of a stereomicroscope then assigned HAI index values according to their condition as indicated by Adams *et al*. (1993) and Jooste *et al*. (2005) (Table 2.1). Interpretation of colour characteristics of the organs was done with the aid of a colour chart developed by Watson (2001) (Figure 2.7) and recorded on the HAI data sheet.

Table 2.1: Fish health variables with assigned characters showing the norm and deviation from the norm in the Health Assessment Index (Adams *et al*. 1993; Jooste *et al*. 2005).

| Variables | Variable condition | Original field designation | Substituted value for the HAI |
|---|---|---|---|
| **External variables** | | | |
| Length | Total length in millimetres | Mm | - |
| Weight | Weight in gram | G | - |
| Eyes | Normal | N | 0 |
| | Exopthalmia | E1/E2 | 30 |
| | Haemorrhagic | H1/H2 | 30 |
| | Blind | B1/B2 | 30 |
| | Missing | M1/M2 | 30 |
| | Other | OT | 30 |
| Fins | No active erosion or previous erosion healed over | 0 | 0 |
| | Mild active erosion with no bleeding | 1 | 10 |
| | Severe active erosion with haemorrhage / secondary infection | 2 | 20 |
| Skin | Normal, no aberrations | 0 | 0 |
| | Mild skin aberrations | 1 | 10 |
| | Moderate skin aberrations | 2 | 20 |
| | Severe skin aberrations | 3 | 30 |
| Opercules | Normal/no shortening | 0 | 0 |
| | Mild/slight shortening | 1 | 10 |
| | Severe shortening | 2 | 20 |
| Gills | Normal | N | 0 |
| | Frayed | F | 30 |
| | Clubbed | C | 30 |
| | Marginate | M | 30 |
| | Pale | P | 30 |
| | Other | OT | 30 |
| Pseudobranch | Normal | N | 0 |
| | Swollen | S | 30 |
| | Lithic | L | 30 |
| | Swollen and lithic | P | 30 |
| | Inflamed | I | 30 |
| | Other | OT | 30 |
| Thymus[a] | No haemorrhage | 0 | 0 |
| | Mild haemorrhage | 1 | 10 |
| | Moderate haemorrhage | 2 | 20 |
| | Severe haemorrhage | 3 | 30 |
| **Internal variables (necropsy)** | | | |
| Mesenteric fat | (Internal body fat expressed with regard to amount present) | | |
| | None | 0 | - |
| | Little, where less than 50% of each cecum is covered | 1 | - |
| | 50% of each cecum is covered | 2 | - |
| | More than 50% of each cecum is covered | 3 | - |
| | Cecae are completely covered by large amount of fat | 4 | - |
| Spleen | Black | B | 0 |

| Variables | Variable condition | Original field designation | Substituted value for the HAI |
|---|---|---|---|
| | Red | R | 0 |
| | Granular | G | 0 |
| | Nodular | NO | 30 |
| | Enlarge | E | 30 |
| | Other | OT | 30 |
| Hindgut | Normal, no inflammation or reddening | 0 | 0 |
| | Slight inflammation or reddening | 1 | 10 |
| | Moderate inflammation or reddening | 2 | 20 |
| | Severe inflammation or reddening | 3 | 30 |
| Kidney | Normal | N | 0 |
| | Swollen | S | 30 |
| | Mottled | M | 30 |
| | Granular | G | 30 |
| | Urolithic | U | 30 |
| | Other | OT | 30 |
| Liver | Red | A | 0 |
| | Light red | B | 30 |
| | “Fatty” liver, “coffee with cream” colour | C | 30 |
| | Nodules in liver | D | 30 |
| | Focal discolouration | E | 30 |
| | General discolouration | F | 30 |
| | Other | OT | 30 |
| Bile[a] | Yellow or straw colour, bladder empty or partially full | 0 | - |
| | Yellow or straw colour, bladder full, distended | 1 | - |
| | Light green to “grass” green | 2 | - |
| | Dark green to dark blue-green | 3 | - |
| Blood (Haematocrit) | Normal range | 30 – 45% | 0 |
| | Above normal range | >45% | 10 |
| | Below normal range | 19–29% | 20 |
| | Below normal range | <18% | 30 |
| Blood (plasma protein) | Normal range | 30 – 69mg/dL | 0 |
| | Above normal range | >70mg/dL | 10 |
| | Below normal range | <30mg/dL | 30 |
| Parasites | No observed parasites | 0 | 0 |
| | Few observed parasites | 1 | 10 |
| Endoparasites[b] | No observed endoparasites | 0 | 0 |
| | Observed endoparasites < 100 | 0 | 10 |
| | 101 – 1000 | 1 | 20 |
| | > 1000 | 3 | 30 |
| Ectoparasites[b] | No observed ectoparasites | 0 | 0 |
| | Observed ectoparasites 1 – 10 | 1 | 10 |
| | 11 – 20 | 2 | 20 |
| | > 20 | 3 | 30 |

a = no values were assigned to these values in the original HAI;

b = refinement of the HAI by Jooste *et al*. (2005)


Figure 2.6: Health assessment. A- conventional angling gear and scoop nets; B- gill nets; C- calibrated measuring board; D- Salter Model 235E electronic balance; E- micro-haematocrit centrifuge (model: KHT-400); F- micro-haematocrit reader; G- aerated holding tanks; H- examination of fish organs in a field laboratory.

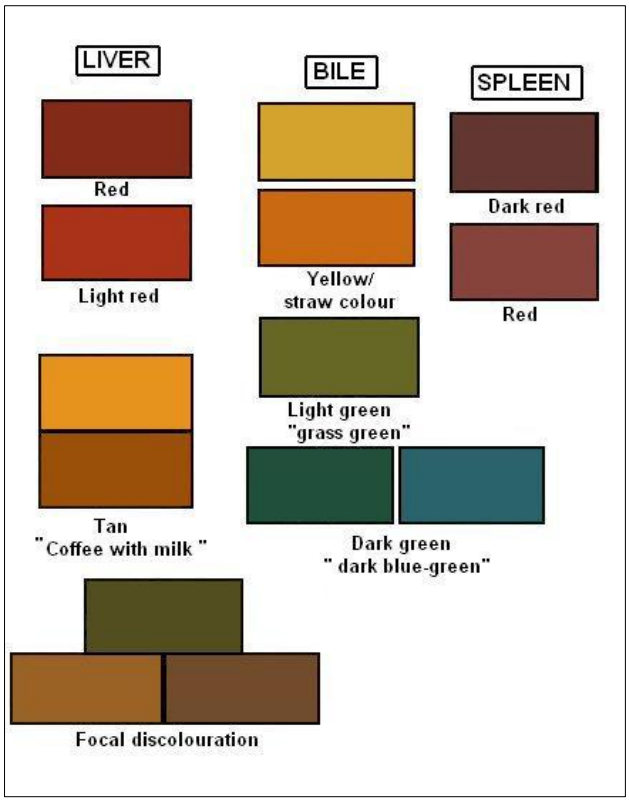


Figure 2.7: Colour chart used to compare the colour of liver, bile and spleen (Watson 2001).

### 2.5.2 Calculation of Health Assessment Index

Following examination of the fish in the field, the categories as adapted by Adams *et al*. (1993) and Jooste *et al*. (2005) were used to assign numerical values to account for the state of each organ. The index of Adams *et al*. (1993) was used with the modification or insertion of a few parameters which are indicated by letter (b) in Table 2.1. Original field designations of all variables from the necropsy-based method were substituted with comparable numerical values into the HAI. All the variables of the HAI are presented with a value ranging from 0–30, depending on the condition of the organs tested. Any abnormalities in the eyes, gills, pseudobranch, kidney, liver, and spleen are given values of 30.

The skin and fin abnormalities are rated 0, 10, 20, or 30 depending on the degree of abnormality, with the greatest abnormality given a value of 30. To calculate the HAI value for each fish within a sample, numerical values for all variables summed and metrics were recorded. All the individual fish health index values were added and

divided by the total number of fish examined; to calculate the average HAIs for a sample population.

The standard deviation (SD) and coefficient of variation (CV) were determined as proposed by Adams *et al.* (1993). The latter indicates the level of degree of stress experienced by a fish population. The SD as outlined by Adams *et al*. (1993) for each sample was calculated as follows:

$$SD = \frac{\sum_{i-1}^{N} (X_i - X)^2}{N - 1}$$

Where: N = number of fish per season

$X$ = average index for each season

$X_i$ = index value for fish $i$

The CV was calculated as proposed by Adams *et al.* (1993) where:

$$CV = 100 \times SD/X$$

SD = standard deviation

$X$ = average index for each season

2.5.3 Condition factor

The condition factor (K) of fish is based on the analysis of length-weight data; it indicates the physical condition of fish in a habitat (Dirican *et al*. 2012). As mentioned above, length and weight measurements were done and K was determined for the two fish populations to ascertain if there was a correlation between parasite burden/total number of parasites and the condition of the fish. The relationship for both endo- and ectoparasites, of each fish species was plotted following methods of Madanire-Moyo *et al*. (2010); Smit and Luus-Powell (2012) and Sara *et al*. (2014). The K of each fish was calculated using Fulton's condition index according to method of Bagenal and Tesch (1978) where:

$$K = \frac{W \times 100}{L^3}$$

Where: W = weight in g

L = total length in cm

### 2.5.4 Parasites

As soon as fish were taken out of the gill nets (Figure 2.6B), and from the conventional angling gear or scoop nets (Figure 2.6A) it was identified and scrutinised for mobile ectoparasites. If any parasites were found the fish was marked and records were kept of the parasites collected from that specific fish. The fish was then placed in its respective holding tank on the boat. On land at a field laboratory (Figure 2.6H) skin and fins were assessed for HAI and mucus smears were prepared by holding the fish firmly at the mouth and scraping the skin surface, pelvic, pectoral, anal, caudal and dorsal fins with separate appropriately marked microscope slides. These mucus smears were examined for the presence of ectoparasites with the aid of a stereomicroscope (Figure 2.6H). Subsequent to the collection of ectoparasites, fish were sacrificed by severing the spinal cord and dissected ventrally from the anus to the head. Body cavity and mesenteries were examined for helminths.

All internal organs mentioned previously, were removed, placed in separate Petri dishes, covered with distilled water and examined with the aid of a stereomicroscope (Figure 2.6H). Parasites were preserved according to the following standard methods for each group: Monogeneans collected from the gills were mounted on a microscope slide in glycerin jelly or glycerin ammonium picrate (GAP) and covered with a cover slip. When using glycerin jelly, some pressure was placed on the coverslip, while the microscope slide was heated in an open flame to melt the jelly. The coverslip were sealed using clear nail varnish and labeled. Morphological features were used for identifications of the parasite. Digeneans were placed in saline solution and shaken vigorously from time to time to dislodge debris, fixed flat in hot alcohol-formalin acetic acid for approximately 10 minutes and preserved in 70% ethanol to which 5% glycerine was added. Nematodes collected were carefully removed from the body cavity or stomach (nematodes in the stomach attached firmly when disturbed therefore they were brushed with a fine brush to help release their firm hold) and fixed in glacial acetic acid for approximately 2 minutes. When they uncoil and stretch they were preserved in 70% ethanol with 2% glycerin added. Copepods and branchiurans from the skin and gills were kept alive in dam water. Excess mucous and debris were removed from the parasite with the aid of a fine brush. They were fixed by adding 70% ethanol to the water in small quantities over a period of approximately one hour, where after they were stored in 70% ethanol.

### 2.5.5 Parasite identification and preparation of whole mounts

Monogeaneans and digeneans were stained in Horen's trichome, counterstained in acetocarmine, cleared in clove oil and mounted in Canada balsam or Entellan following standard procedures used by Douëllou (1993) and Barson *et al*. (2008). Nematodes were examined as temporary mounts in lactophenol (Anderson 1992). Copepods were cleaned and studied using the wooden slide technique (Humes & Gooding 1964) and identified with the aid of an Olympus BX50 microscope. Branchiurans were examined with lactic acid (Avenant-Oldewage & Swanepoel 1993). All parasites collected were identified based on their morphology.

### 2.5.6 Parasite Index and Inverted Parasite Index

Parasites collected for each of the two selected fish species were counted and recorded on HAI data sheets. Numerical values for refined Parasite Index (PI) were assigned as suggested by Jooste *et al.* (2005) while the scoring system for the Inverted Parasite Index (IPI), were applied as described by Crafford and Avenant-Oldewage (2009). The ecto- and endoparasites are categorised as presented in Table 2.2.

Table 2.2: The numerical systems in use with the refined Parasite Index (PI) (Jooste *et al*. 2005) and Inverted Parasite Index (IPI) (Crafford & Avenant-Oldewage 2009).

| **Ectoparasites** | **PI** | **IPI** | **Endoparasites** | **PI** |
|---|---|---|---|---|
| **Numbers present** | **Score** | **Score** | **Numbers present** | **Score** |
| **Zero parasites observed** | **0** | **30** | **Zero parasites observed** | **0** |
| 1 – 10 | **10** | **20** | <100 | **10** |
| 11 – 20 | **20** | **10** | 101 – 1000 | **20** |
| >20 | **30** | **0** | >1000 | **30** |

## 2.6 BIOMARKER ANALYSIS

The liver and gill tissue (0.5 g) of each of the selected fish were placed in cryotubes and frozen in liquid nitrogen, subsequent to health assessment and examination of parasites at the field laboratory. The tissues samples were later frozen at −80°C at the University of Limpopo (UL) and kept frozen until analysis of different oxidative stress biomarkers at Stellenbosch University (SU). Tissue samples of each fish species were weighed to the nearest 0.1 g and washed 3 times with 1× Phosphate

Buffer Saline (PBS), (pH 7.4) and centrifuged at 10 000 × g for 2 minutes at 4°C to remove nuclei, mucus and cell debris. Subsequently, they were stored at −80°C until examination of the oxidative stress biomarkers. All protein analyses were completed according to standard methods using various assay kits. As a measure of lipid peroxidation, its reactive compound malondialdehyde (MDA) were determined with the use of OxiSelect™ Thiobarbituric Acid Reactive Substances (TBARS) assay kit as described by Ohkawa *et al*. (1979). The activity of antioxidant enzyme glutathione S-transferase (GST) was measured using a GST assay kit as suggested by Habig *et al*. (1974). While CellBiolabs' OxiSelect™ TAC assay kit were used to measure the total antioxidant capacity of biomolecules for the samples as described by Frei *et al*. (1992). All the samples were assayed in triplicate unless indicated otherwise.

2.6.1 Glutathione S-transferase

The tissue samples were homogenised in 5 ml of cold (4°C) GST buffer, (i.e. 100 mM potassium phosphate, containing 2 mM EDTA) at pH 7.0. The samples were sonicated with the use of Omni-Ruptor 400 (OMNI International Inc.) (Figure 2.8B). Homogenates were centrifuged at 10 000 × g for 10 minutes at 4°C and the resulting supernatants were collected and placed on ice for protein determination with the aid of a NanoDrop® (ND-1000 Spectrophotometer) (Figure 2.8C) and subsequently assayed for GST activity. The supernatant was aliquoted at −80°C until examination of the GST antioxidant enzyme activities. The samples were assayed in triplicates.

The GST activity was evaluated using 1-chloro-2,4 dinitrobenzene (CDNB) (Cayman, USA) as substrate, as previously described by Habig *et al*. (1974). The assay was commenced by adding 170 µl of assay buffer and 20 µl of glutathione in the non-enzymatic three wells. In the positive control wells, 150 µl of assay buffer, 20 µl of glutathione and 20 µl of diluted GST (control) were added to three wells. The 150 µl of assay buffer, 20 µl of Glutathione, and 20 µl of the sample were added to three wells. Therefore, the reaction was initiated by the addition of 10 µl of 1-chloro-2,4-dinitrobenzene (CDNB) to all of the wells. The starting time for the reaction was recorded and the CDNB was added soon thereafter. The 96-well was vortexed for a few seconds and the absorbance readings were measured with a spectrophotometer at 340 nm. The absorbance was read using Glomax ®- Multi Microplate Multimode Reader (Promega Instruments, France) (Figure 2.8A) to obtain at least 5 time points. All measurements were performed in triplicate for each sample and in duplicate for

the standards. One unit of the GST was defined as the quantity of enzyme that catalyses the formation of 1.0 nmol of CDNB with reduced glutathione per minute at 25°C. The GST activity was expressed as nmol/ min/ ml. The following calculation was used to determine GST activity:

$$\frac{\Delta A340/min}{0.00503\ \mu M^{-1}} \times \frac{0.2\ ml}{0.02\ ml} \times \text{sample dilution} = \text{nmol/ min/ ml}$$

Where: 0.00503 $\mu M^{-1}$ = the extinction coefficient for the CDNB conjugate at 340 nm.

2.6.2 Lipid peroxidation

The TBARS test for malondialdehyde (MDA) was used to measure and quantify lipid peroxidation for the liver and gill samples of each fish. This assay is the most widely employed method to measure lipid peroxidation (Di Giulio *et al*. 1989). The MDA levels were determined according to the method of Ohkawa *et al*. (1979). The tissue samples were homogenised on 1× PBS, containing 1× BHT, sonicated with the use of Omni-Ruptor 400 (OMNI International Inc.). Homogenates were centrifuged at 10 000 × g for 10 minutes at 4°C to collect the supernatants. The supernatant were collected and placed on ice for protein concentration with the aid of the NanoDrop® (ND-1000 Spectrophotometer) and subsequently assayed for TBARS levels.

The liver and gill samples were assayed in duplicates, adding 100 µl of the sample and MDA standards to separate microcentrifuge tubes. Furthermore 100 µl of sodium Dodenyl Sulphate (SDS) lysis solution was added to both the samples and standards, mixed thoroughly and incubated for 5 minutes at room temperature. Thereafter, the samples and standards were diluted with 250 µl of TBA reagent and upon the addition tubes, the reaction proceeds for 45–60 minutes at 95°C. The tubes were removed and cooled to room temperature in an ice bath for 5 minutes. The sample tubes were centrifuged at 3000 rpm for 15 minutes and the supernatant was removed for further analysis. For spectrophotometer measurements 200 µl of the MDA standards and samples were transferred to a 96 well microplate and absorbance were measured at 532 nm on a Glomax ®- Multi Microplate Multimode Reader (Promega, France). All samples and standards were measured in duplicate.

2.6.3 Total Antioxidant Capacity

Although the products of ROS-induced oxidative stress are extensively used to monitor their biological effects on organisms, it is also important to evaluate the antioxidant capacity of biological fluids, cells, tissues and extracts. As such, the liver and gill samples of each fish were assessed with the use of single electron transfer (SET) because antioxidants tend to neutralise radicals via this mechanism. The TAC was assessed using the method of Frei *et al*. (1992). The tissue samples were homogenised on cold 1× PBS (pH 7.4) and sonicated with the use of Omni-Ruptor 400 (OMNI International Inc.) (Figure 2.8B). Homogenates were centrifuged at 10 000 × g for 10 minutes at 4°C and the resulting supernatants were collected and placed on ice for protein determination with the aid of the NanoDrop® (ND-1000 Spectrophotometer) (Figure 2.8C) and subsequently TAC assay.


Figure 2.8: Oxidative stress biomarker analysis. A- Glomax ® Multi Microplate Multimode Reader; B- Omni-Ruptor 400; C- NanoDrop.

Following this, 20 µl of the diluted Uric Acid Standards or homogenates were added to the 96-well microtiter plate. In addition, 180 µl of the 1× reaction buffer was added to each well on the plate, mixing thoroughly. The initial absorbance was obtained by reading the plate at 490 nm. Thereafter, the reaction was initiated upon the addition of 50 µl of the 1× copper ion reagent into each well, incubating for 5 minutes on an orbital shaker, whereafter the reaction preceded. The reaction was terminated by addition of 50 µl of 1× stop solution to each well. The plate was read again at 490 nm with a standard 96-well spectrophotometric microplate reader, Glomax ®- Multi Microplate Multimode Reader (Promega, France) (Figure 2.8A). All measurements were performed in triplicate for each sample and in duplicate for the standards. The net absorbance values of antioxidants were compared with a known uric acid standard curve. Absorbance values were proportional to the sample's total reductive capacity. The results were expressed as mM Uric Acid equivalents.

## 2.7 DATA ANALYSIS

### 2.7.1 Analysis of variance

Data were tested for normality and homogeneity of variance using Levene's tests. The fish health parameters indices i.e. HAI and K were calculated and presented as mean±SD. Seasonal variations in HAI values for a given species were tested using one-way ANOVA; this model was also used to test for inter-species difference for K. Two-way ANOVA was used to test seasonal variation between the two species, where ANOVA revealed significant differences, Tukey's *post-hoc* test multiple comparisons were performed to establish where differences occurred. Regression analysis was used to determine any significant correlation between K of each fish species and parasite burden. The analysis were performed using Statistical Package for Social Scientists (SPSS Statistics version 21) and the significance of results was ascertained at $p<0.05$.

### 2.7.2 Infection statistics

Infection parameters (mean abundance, mean intensity and prevalence) were calculated according to Bush *et al*. (1997). Mean abundance was calculated as the total number of individuals of a particular parasite species in a sample of hosts divided by the total number of individuals of the host species in a sample. Mean intensity was calculated as the total number of individuals of a particular parasite species in a sample of a host species, divided by the number of infected individuals of the host species in a sample. Prevalence was calculated as number of individuals of a host species infected with a particular parasite species divided by the number of host examined (expressed in %).

### 2.7.3 Multivariable Analysis

Multivariable analysis [i.e. SIMPER analysis and Principal Component Analysis (PCA)] were used during the present study. SIMPER analysis was performed to determine the water quality variables contributing to the differences between the selected sites. The PCA was performed to determine the correlation of the different water quality parameters, parasite abundance, oxidative stress biomarkers and health parameters used for the two fish species between the four seasons. These analyses were done using R statistical package (R Development core team 2012). All the data were considered statistically significant for the 95% level ($p<0.05$).

# CHAPTER 3 - WATER QUALITY

## 3.1 INTRODUCTION

Water is a vital source for all life forms, covering about 70% of the earth's surface. However, 97% of this water is salty seawater whereas only 3% of the world's water is freshwater. Of the 3% of freshwater, 2% is frozen in polar glaciers and icecaps, and more than half of the remaining 1% (about 0.6%) is in the form of groundwater in aquifers (Shiklomanov 1993) while the remainder is surface water in swamps, lakes and rivers. This surface water is essential for the world's unique biodiversity (its inhabitants) and human communities. Despite this, freshwater ecosystems are subjected to environmental perturbations due to increased anthropogenic activities. Most of these activities have led to the deterioration of water quality resulting in aquatic environmental changes.

Water quality refers to the physical, biological and aesthetic properties of water that determine its fitness for a variety of uses and for the protection of the health and integrity of aquatic ecosystems (DWAF 1996a). Many of these properties are controlled or influenced by constituents that are either dissolved or suspended in water (DWAF 1996a). In South Africa the principal legal instrument relating to water resource management, the National Water Act (Act 36 of 1998), and other legislation such as the Water Services Act (Act 108 of 1997) and the National Environment Management Act (Act 107 of 1998) are implemented incrementally to recognise usage of water in an environmentally sustainable manner and for long term protection. As such, DWA published the first edition of South African Water Quality Guidelines (SAWQG) in 1996, comprising of eight volumes dealing with the following water uses: domestic, industrial, agricultural water use (irrigation and aquaculture) and aquatic ecosystems (DWAF 1996b).

The eighth volume of the SAWQG is a field guide listing all Target Water Quality Range (TWQR) values of water quality constituents, for the above mentioned water uses. The guidelines were developed to set a derived set of water quality criteria for

safeguarding freshwater ecosystems (DWAF 1996a). These guidelines are referred to as scientific and technical information provided for a particular water quality constituent in the form of numerical data or narrative descriptions of its effects on the fitness of water for a particular use or on the health of aquatic ecosystems (Hohls *et al*. 2002).

The water quality guidelines consist of the TWQR and water quality criteria, the Chronic Effect Values (CEV) and Acute Effect Values (AEV) together with the support information which includes the occurrence of the constituents in the aquatic ecosystem. The TWQR is a management objective derived from quantitative and qualitative criteria. The TWQR is the range of constituents or levels within which no measurable adverse effects are expected on the health of aquatic ecosystem and should therefore ensure their protection (DWAF 1996a). Subsequently as a matter of policy the Department of Water Affairs (DWA) strives to protect South Africa's water resources by maintaining water quality within the TWQR. However, due to the lack/absence of TWQR's for some consituents considered in this study, the SAWQGs were supplemented by the existing international guidelines the World Health Organization (WHO 2006) and Canadian (CCME 2012) for aquatic ecosystems. The SAWQGs were in this study given first preference because they are primarily aimed at local water resourses.

Water quality constituents can be divided into four categories based on the effects that the constituents may have on the aquatic biota (DWAF 1996a). These include the following: **System variables**; including temperature, pH and dissolved oxygen (DO), which regulate the essential ecosystem processes such as spawning and migration of aquatic fauna. These variables depend on abiotic factors such as the geomorphology, geological formations, climatic fluctuations and physico-chemical quality of the water (DWAF 1996a; Poff *et al*. 1997). **Non-toxic constituents** are defined as constituents that may cause toxic effects on aquatic life at high concentrations, i.e. total dissolved solids (TDS), electric conductivity (EC), total water hardness and salinity. Their natural concentrations depend on localised physical, geochemical and hydrological processes (DWAF 1996a; Leske & Buckley 2003). Criteria for these constituents are given as numerical ranges or as proportional changes from local background conditions.

**Nutrients**; include all major inorganic nitrogen compounds ($NO_3$, $NO_2$, $NH_4$ and $NH_3$) and phosphorus ($PO_4$); these are generally not toxic but can stimulate eutrophication if present in excess (Dallas & Day 2004). **Major ions** are divided into two categories; anions ($Cl^-$, F and $SO_4^{2-}$) and cations ($Ca^{2+}$, $Mg^{2+}$, $Na^+$ and $K^+$). The major ions of inland waters are derived from the dissolution of rocks, as well as the atmosphere (Dallas & Day 2004). For instance, in South Africa the waters of the Highveld, including the Olifants River, tend to be dominated by calcium, magnesium and bicarbonate ions, whereas those in the coastal regions and the arid west tend to be dominated by sodium and chloride ions (Day & King 1995). **Toxic constituents**; (organic and inorganic) seldom occur in high concentrations in unimpacted systems. Various toxic constituents accumulate in aquatic ecosystems as a result of various anthropogenic activities (mining, agriculture, power generation and sewage treatments). Toxic constituents include; inorganic (Al, Cu, Pb and Mn) and organic constituents, (phenol and atrazine). The toxicity of these variables depends highly on physico-chemical properties of the water i.e. pH, temperature and TDS (Svobodóva *et al.* 1993; DWAF 1996a).

The primary objective of this section was to determine the concentration of selected water quality variables in the middle Olifants River System, Flag Boshielo Dam, at selected sampling sites (Figure 3.1). As mentioned in Chapter 1, in order to obtain reliable and general assessment of the physical and chemical as well as metal pollution of the water, it is important to correlate this with biological monitoring, which is presented in Chapter 4. Thus, in this chapter, the physical and chemical characteristics of the water, as well as certain metal concentrations in the water were investigated to access the overall water quality of Flag Boshielo Dam.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Field procedures

Water samples were collected seasonally (March 2012–January 2013) from three selected sampling sites in the dam; this includes the inflow, middle of the dam and the dam wall (Figure 3.1). Selected water quality parameters were measured *in situ* using a handheld multi parameter instrument (YSI 556 Multi Probe System). These include surface water variables such as temperature, pH, DO, TDS and salinity.

### 3.2.2 Laboratory procedures

Subsurface water samples were also collected seasonally (March 2012–January 2013) at approximately 10 cm below the water surface in 500 ml polyethylene bottles and frozen immediately for further analysis at an accredited laboratory [Capricorn Veterinary Laboratories cc; South African National Accreditation System (SANAS)]. The samples were analysed for the following water constituents: dissolved nutrients ($NO_3$, $NO_2$, $NH_4$, $NH_3$ and $PO_4$), anions (F and $SO_4^{2-}$), cations ($Ca^{2+}$, $Mg^{2+}$, $Na^{+}$ and $K^{+}$), non-toxic constituents (TDS, EC and salinity) and toxic constituents (Al, Ar, Cu, Fe, Pb, Mn and Zn). These were analyzed using sequential inductively coupled plasma-optimal emusion spectrometry (ICP-OES).

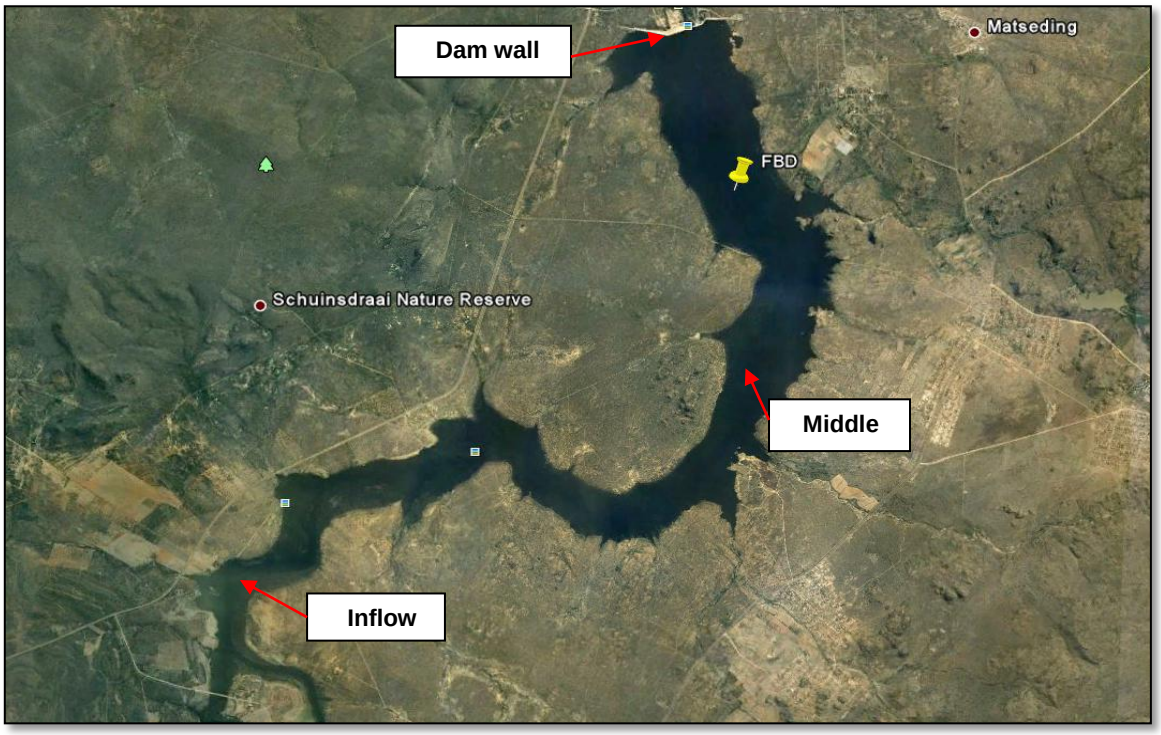


Figure 3.1: Satellite image of Flag Boshielo Dam showing the three sampling sites (Google Earth).

### 3.2.3 Data analysis

All raw data of water quality constituents measured during the study are included in the appendices (Appendix A: Table 1). The seasonal values for the selected water quality parameters were compared to World Health Organization (WHO) and Canadian (CCME) guidelines and TWQR set out by SAWQG for aquatic ecosystems for relevant variables where available and applicable. These values are presented

graphically in Figures 3.2–3.5. In addition, SIMPER analysis was performed to determine the water quality variables contributing the differences between the three sampling sites. A non-metric dimensional scaling (NMDS) plot was used to illustrate limnological differences amongst the three sampling sites at Flag Boshielo Dam. Mean seasonal values and descriptives, i.e. SD, minimum (min) and maximum (max) values are summarised in Table 3.2 to show variability of the different water constituents. The significant differences between the selected sites and seasons for the different water quality constituents were considered statistically significant for *p*-values less than 5% ($p<0.05$; ANOVA). The TWQR, AEV, CEV and acceptable limits for aquatic ecosystems by WHO and the CCME guidelines are illustrated by different lines were applicable using aov () function in the R 3.0.3.6 statistical package (R Development core team 2012).

## 3.3 RESULTS AND DISCUSSIONS

### 3.3.1 System variables

#### 3.3.1.1 Temperature

In aquatic ecosystems temperature plays a major role in water quality, affecting the rates of chemical reactions, metabolic rates and distribution of aquatic organisms. For instance, aquatic organisms have a specific temperature or range of temperatures at which optimal growth, reproduction and general fitness occurs (Palmer *et al.* 2004). In South Africa, the Department of Water Affairs stipulated that the TWQR for aquatic ecosystems should not be allowed to vary from the background average daily water temperature considered to be normal for that specific site and time of day by >2°C, or by >10% (DWAF 1996a).

Water temperature recorded during the study ranged from 14.82–28.9°C with expected higher temperatures recorded in summer and lower in winter (Figure 3.2A). Summer temperatures were all above 25°C at all the sites of the dam, while winter temperatures did not exceed 16°C. Statistically, there was significant variation of surface water temperature between different seasons ($p = 0.000$) and the three sampling sites ($p = 0.000$). The latter is evident, whereby water temperature at the inflow and the dam wall were somewhat similar but differed by approximately 1°C to water temperature recorded in the middle of the dam. In South Africa the inland water bodies have temperatures that range from 5–30°C (DWAF 1996a), thus the

temperature were within normal limits. In addition, the two fish species selected were consistently collected throughout the sampling periods, an indication that fish can easily tolerate natural fluctuation of water temperature within the water body (Svobodóva *et al.* 1993).

#### 3.3.1.2 pH

pH is a measure of the acidic or basic character of a solution. The pH value in an aquatic ecosystem is a measurement of the hydrogen ion activity. The ionization constant of water is dependent on temperature; therefore, the exact point of neutrality varies with water temperature. The pH of natural water ranges from <4 to >12, but usually falls between 6 and 9 at 25°C; and neutrality is represented by a pH value of 7. The TWQR for pH set for aquatic ecosystem is 6.5–9 (DWAF 1996a). In addition, DWAF (1996a) stipulated that the pH of all aquatic ecosystems should not be allowed to vary from the range of the background pH values for a specific site and time of day by >0.5 of a pH unit, or by >5%.

The pH value at Flag Boshielo Dam ranged from 6.6–9.15, thus the surface water was alkaline and within TWQR throughout the study with the exception of the middle of the dam during summer (Figure 3.2B; Appendix A: Table 1). Overall the highest pH values recorded during this study were at the middle of the dam and showed a decreasing trend towards the dam wall. The lowest pH values were recorded at the dam wall and inflow in spring with 6.75 and 6.62, respectively. Significant variation in the seasonal water pH was found ($p$ = 0.001); however, no variation was observed between the sampling sites ($p$>0.05). Alabaster and Lloyd (1980) asserts that, a pH value between 6.5 and 9 is harmless to fish. However, this depends on the fish species, i.e. some cyprinids have been reported to prefer an alkaline pH of <10.8 (Svobodóva *et al.* 1993), while some *Tilapia* are fairly robust with regard to this variable, surviving at pH of 3.5, and even at pH 11 (Yada & Ito 1997). Therefore, the pH recorded during the present study can be considered ideal for the two selected cyprinids.

#### 3.3.1.3 Dissolved oxygen

Dissolved oxygen (DO) is the amount of oxygen dissolved in the water at a given temperature, atmospheric pressure and salinity (Dallas & Day 2004). This variable is expressed as dissolved oxygen saturation (%) and as concentration of dissolved oxygen (mg/l) at the time of sampling (DWAF 1996a). Concentrations of <6 and >9 mg/l may adversely affect the functioning and survival of aquatic biological communities, as such TWQR for DO for South African aquatic ecosystems is 6–9 mg/l (DWAF 1996a). The oxygen saturation TWQR is between 80 and 120%. These numerical ranges are considered suitable for protection of all life stages of most southern Africa aquatic biota endemic to or adapted to aerobic warm water habitats (DWAF 1996a).

Dissolved oxygen percentage saturation recorded during the study ranged from 65.9–123.5%. Consequently this constituent was below and above the TWQR during certain periods of sampling (Figure 3.2C). The DO saturation above the TWQR was in summer at the middle of the dam (Figure 3.2C; Appendix A: Table 1). This could be due to the high pH value and temperatures recorded at this site (Figure 3.2A–B); such levels are indicative of active primary production in the surface water during the sampling period (DWAF 1996a).

Dissolved oxygen concentration recorded during the study ranged from 7.14–10.08 mg/l (Figure 3.2D). Dissolved oxygen concentrations were recorded within the TWQR throughout the study at all the sampling sites, with an exception of high concentrations recorded at the inflow and dam wall in spring (Figure 3.2D; Appendix A: Table 1). Statistically, no significant variation was found between sites ($p>0.05$), but seasonal variation was observed ($p<0.05$) (Appendix A: Tables 2, 3). According to Svobodóva *et al.* (1993), cyprinid fish can thrive in water containing 6–8 mg/l and at concentrations below 1.5–2 mg/l will show signs of suffocation. The concentrations recorded during the present study can thus be considered acceptable for the two cyprinids, with the exception of the high levels recorded during spring.

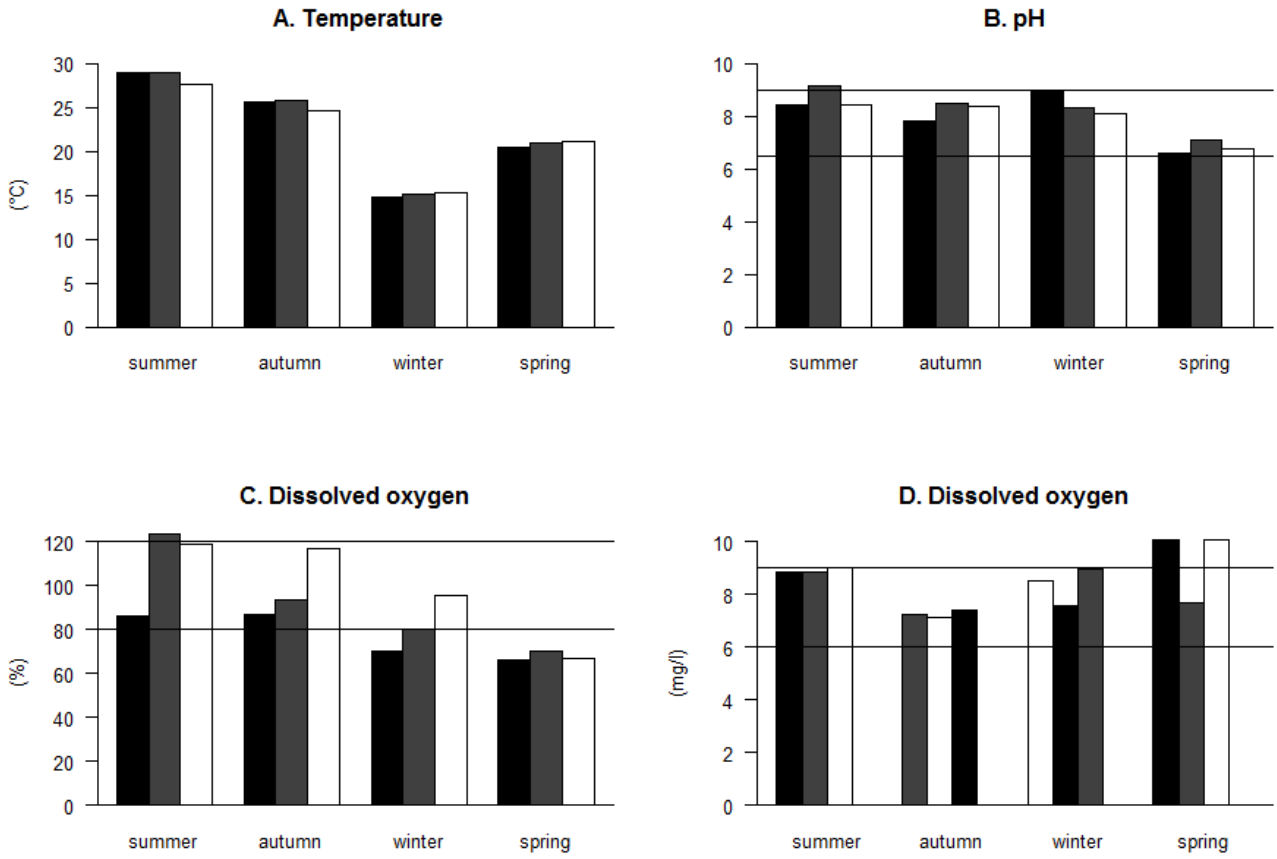


Figure 3.2: The seasonal variation in the, A- surface water temperature; B- pH; C- dissolved oxygen saturation; D- dissolved oxygen concentration at three selected sites inflow (black bars), middle (grey bars) and dam wall (white bars), at Flag Boshielo Dam (March 2012–January 2013). Solid lines indicate the TWQR (DWAF 1996a).

### 3.3.2 Non-toxic constituents

#### 3.3.2.1 Total dissolved solids

Total dissolved solids (TDS), is defined as a composite measure of the total amount of soluble materials dissolved in the water. The ionic component of TDS in natural aquatic ecosystems is often pH related and determined by the degree of weathering, the chemical composition of rocks, relative influences of evaporation and rainfall in the catchment (Davies & Day 1998; Van Vuren *et al*. 1999). There is no TWQR for TDS for South African aquatic ecosystems, but DWAF (1996a) suggested that the concentrations should not be changed by >15% from the normal cycles of the water body under un-impacted conditions at any time of the year. Whereas, the acceptable limit stipulated by the WHO guidelines is 1000 mg/l.

Total dissolved solid concentrations recorded during this study ranged from 245.1–386.8 mg/l (Appendix A: Table 1). Throughout the study higher TDS concentrations were recorded during autumn at all the sampling sites (Figure 3.3A). This spike is likely due to a noticeable trend in the relative composition of high $SO_4^{2-}$ concentrations recorded in autumn (Figure 3.5; Appendix A: Table 1). Sulphate has been reported to influnce TDS concentrations in a system (Heath *et al*. 2010). Lower TDS concentrations were recorded at the middle of the dam and dam wall in spring. TDS concentrations recorded during this study were within the acceptable limit stipulated by the WHO guideline (Figure 3.3A).

Statistically, no significant variation was found for TDS levels between the sampling sites and seasons ($p>0.05$). Dallas and Day (2004) reported that TDS concentrations that are too high or too low may limit growth and may lead to the death of many aquatic organisms. However, concentrations recorded during the study can be considered moderate. Nevertheless, these TDS concentrations need to be intensely monitored, as an increase of these suspended solids in the water can result in increased effluent inflow into the downsteam region of Flag Boshielo Dam.

#### 3.3.2.2 Electric conductivity

Electric conductivity (EC) is a numerical expression of the ability of water to conduct an electric current, expressed in millisiemens per meter (mS/m) (DWAF 1996a; Dallas & Day 2004). This ability is a result of the presence of ions such as sodium ($Na^+$), potassium ($K^+$), calcium ($Ca^{2+}$), magnesium ($Mg^{2+}$), chlorine ($Cl^-$), sulphate ($SO_4^2$), nitrate ($NO^{3-}$), bicarbonate ($HCO^{3-}$), and carbonate ($CO_3^{2-}$) all of which carry electric charge (DWAF 1996a; Daniel *et al*. 2002; WHO 2006). TWQR of EC is not available. However, WHO guidelines suggested an acceptable limit of 100 mS/m.

The electric conductivity recorded during the study ranged from 34.8–59.5 mS/m. The highest EC records were observed at the inflow in autumn and lowest at the dam wall in spring (Figure 3.3B). The range was thus within acceptable limits during all the sampling periods at all the sites. Statistically, significant variation was found seasonally ($p<0.05$), but no variation between the sampling sites ($p>0.05$).

### 3.3.2.3 Salinity

Salinity is defined as the mass (in grams) of the dissolved inorganic solids in 1 kg of sea water and the unit is often given as parts per thousand (‰) (DWAF 1996b). Salinity have been highlighted by Jooste *et al*. (2005) to be of vital importance to fish health as it can impact directly on the metabolic and physiological processes. However, the tolerance of fish species to variations in salinity depends on their physiological adaptation. Species capable of tolerating wide salinity ranges are defined as euryhaline while those tolerating only limited ranges are referred to as stenohaline (DWAF 1996b; Skelton 2001). The limit for salinity in freshwater ecosystems is according to DWAF (1996a) <0.5 ‰, or should not change by <0.05% from normal cycles.

In the present study, salinity concentrations of 0.26 ‰ were recorded at all the sites throughout the study, thus it fell within the TWQR acceptable limit (Table 3.2; Figure 3.3C). Statistically, there was no significant variation in surface water among sites and seasons ($p$>0.05). In general, it seems that many species are able to survive and even flourish at relatively high salinities (Dallas & Day 2004). However, the tolerance limits of the selected fish species are unknown.

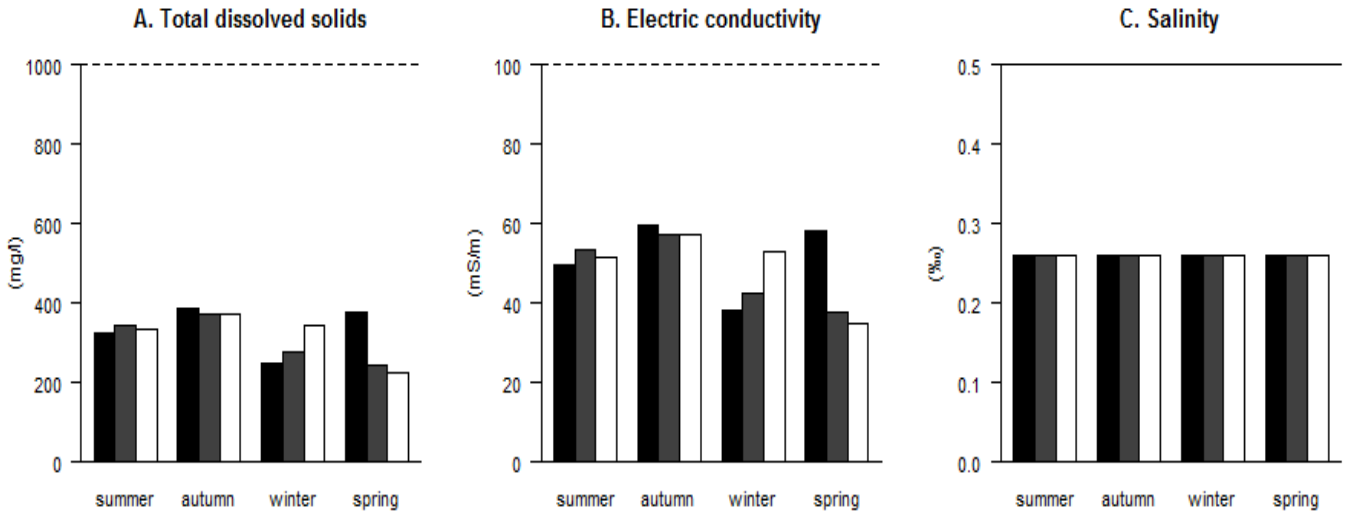


Figure 3.3: The seasonal variation in the mean A- total dissolved solids; B- electric conductivity; C- salinity at the three selected sites inflow (black bars), middle (grey bars), dam wall (white bars) at Flag Boshielo Dam (March 2012–January 2013). Dashed lines indicate the acceptable limit by the WHO guidelines for aquatic ecosystems (WHO 2006), and solid line indicate the TWQR (DWAF 1996a).

### 3.3.3 Nutrients

#### 3.3.3.1 Ammonia

Ammonia ($NH_3$) is produced by heterotrophic bacteria as the primary end product of biodegradation of nitrogenous organic matter. Ammonia may be present in a free, un-ionised form ($NH_3$) or ionised form as ammonium ion ($NH_4^+$). The toxicity of ammonia is directly related to the undissociated amount of free ammonia in the solution. The potential effects of ammonia in the aquatic environment is modified by chemical species present, as well as the relative proportions of each, and other factors such as pH, temperature and DO concentration (Svobodóva *et al*. 1993; Dallas & Day 2004; Palmer *et al*. 2004). For instance at a pH value of 9.5 and temperature of 25°C, $NH_3$ will contribute 64% of the total ammonia concentrations (Table 3.1). DWAF (1996a) stipulated that 90% of all free ammonia estimates should be within the TWQR (<0.007 mg/l) and that all free ammonia estimates should be below the CEV (0.015 mg/l) and AEV (0.1 mg/l).

Table 3.1: Contribution of un-ionised $NH_3$ to total ammonia (expressed as a %), as a function of pH value and water temperature (DWAF 1996a).

| **pH** | **Water temperature (°C)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 5 | 19 | 15 | 20 | 25 | 30 | 35 |
| **6** | 0.0083 | 0.012 | 0.019 | 0.027 | 0.039 | 0.056 | 0.079 | 0.11 |
| **6.5** | 0.026 | 0.039 | 0.059 | 0.086 | 0.12 | 0.18 | 0.25 | 0.35 |
| **7** | 0.083 | 0.12 | 0.18 | 0.27 | 0.39 | 0.56 | 0.79 | 1.1 |
| **7.5** | 0.26 | 0.39 | 0.58 | 0.85 | 1.2 | 1.7 | 2.4 | 3.4 |
| **8** | 0.82 | 1.2 | 1.8 | 2.6 | 3.8 | 5.3 | 7.3 | 9.9 |
| **8.5** | 2.6 | 3.8 | 5.5 | 7.9 | 11 | 15 | 20 | 26 |
| **9** | 7.6 | 11 | 16 | 21 | 28 | 36 | 44 | 52 |
| **9.5** | 21 | 28 | 37 | 46 | 55 | 64 | 71 | 78 |

In the study, ammonia concentrations recorded ranged from 0.11–0.41 mg/l. The highest value was recorded in spring at the dam wall and the lowest at the middle of the dam in autumn (Figure 3.4A). $NH_3$ concentrations were recorded above the TWQR, CEV and AEV throughout the study at all the sampling sites with an exception of the middle of the dam in autumn (Figure 3.4A). $NH_3$ displayed no significant variation between the sampling sites as well as seasons ($p$>0.05) at Flag Boshielo Dam. Elevated concentrations at this part of the Olifants River are likely due to the inadequate management by the Riverview Wastewater Treatment Works (WWTW) in the upper reaches of the Olifants River scoring very poorly in the annual

Green Drop Report (DWA 2011; Dabrowski & De Klerk 2013; Dabrowski *et al*. 2014). Elevated concentrations of 0.3–0.8 mg/l for ammonia affect the respiratory system of many organisms, either by inhibiting metabolism or decreasing oxygen permeability of cell membrane (Lee *et al*. 2008). Thus, the observed high concetrations could result in possible sub-lethal effects for the selected fish species.

#### 3.3.3.2 Ammonium

Ammonium ion ($NH_4^+$) is the reduced form of inorganic nitrogen derived mostly from aerobic and anaerobic decomposition of organic material. Ammonium in aquatic ecosystems may be present in a form of ionised ammonia. This variable is pH depended, at low to medium pH values, the ammonium ion dominates, but as pH increases ammonia is formed. Ammonium is as compared to ammonia, non-toxic (DWAF 1996a; Dallas & Day 2004), but contributes significantly to eutrophication in aquatic ecosystems. The TWQR for ammonium stipulated by DWAF (1996a) is 0.2 mg/l for aquatic ecosystems.

The ammonium concentrations recorded during the present study ranged from 0.09−0.43 mg/l. The highest value was recorded in spring at the dam wall, and the lowest in autumn at the middle of the dam (Figure 3.4B). Overall the concentrations of $NH_4^+$ were above the TWQR during all the seasons with the exception of middle of the dam in autumn and inflow in winter and spring (Figure 3.4B; Appendix A: Table 1). Significant variation was observed between sampling sites and seasons ($p$<0.05). Therefore, monitoring the water quality at Flag Boshielo Dam is crucial as increase of these variables can lead to eutrophication.

#### 3.3.3.3 Nitrate

Nitrate ($NO_3$) is the end product of two bacterially mediated processes in the nitrification of ammonia, derived mainly from the oxidation of plant, animal debris and excrements (DWAF 1996a). Nitrate is seldom abundant in natural surface waters because it is incorporated into cells or is chemically reduced by microbes and converted into atmospheric nitrogen (Davies & Day 1998). In South Africa, inorganic nitrogen concentrations in un-impacted, aerobic surface waters usually is below 0.5 mg/l but may increase to above 5–10 mg/l in highly enriched waters (DWAF 1996a). In contrast, in un-impacted and well oxygenated waters (DO concentration 80–120%

saturation), most (>80%) of the inorganic nitrogen should be present as nitrate; typically, $NH_3$ concentrations will be below 0.1 mg/l, or less than 20% of the inorganic nitrogen present (DWAF 1996a). A TWQR for aquatic ecosystems is unavailable; however, the Canadian guidelines suggested an acceptable limit of 13.0 mg/l.

Nitrate concentrations recorded were consistently <1.4 mg/l throughout the study at all sites (Appendix A: Table 1). Thus Flag Boshielo Dam can still be referred to as unimpacted, with values above 0.5 mg/l but <5–10 mg/l with <80% DO and $NH_3$ concentrations of above 0.1 mg/l. Though Loskop Dam, upstream of Flag Boshielo Dam, has been reported to be polluted as a result industrial and agricultural waters in its catchment with >2.5 mg/l of inorganic nitrogen (Dabrowski *et al*. 2013), the current study site fell within the mesotrophic range of 0.5–2.5 mg/l (DWAF 1996a). This finding illustrates that agriculture and sewage effluents from the upper reaches and within the Flag Boshielo Dam catchment may be playing a prominent role in increasing $NO_3$ levels in the dam.

#### 3.3.3.4 Nitrite

Nitrite ($NO_2$) is the intermediate product of inorganic oxidation of ammonia and of the bacteria mediated processes, nitrification and denitrification. Nitrites are usually found at low concentration because they are readily oxidised to nitrate or reduced to ammonia, both chemically and biochemically by mainly two groups of highly aerobic, autotrophic bacteria, i.e. *Nitrosomonas* spp. and *Nitrobacter* spp. (DWAF 1996a). Nitrites TWQR is unavailable for South African aquatic ecosystems (DWAF 1996a). The Canadian guidelines suggested an acceptable limit of 0.06 mg/l.

Nitrite concentrations during this study were consistently recorded at <0.01 mg/l and well within the acceptable limits at all the sampling sites throughout the study (Appendix A: Table 1). The low concentration of nitrite might be due to the fact that nitrite is readily oxidised to nitrate or is reduced to ammonia, recorded above the TWQR in this study (Figure 3.4A). The recorded nitrogen levels are to be expected as dams act as “sinks”, collecting and reducing the nutrient content via utilisation by microphytes and micro-organisms present in this dam (Heath *et al*. 2010).

#### 3.3.3.5 Orthophosphate

Orthophosphate is an important macro-nutrient which plays a major role in the formation of nuclide acids. In aquatic ecosystems, it can occur in numerous organic and inorganic forms, as a dissolved and particulate species. Orthophosphate species $H_2PO_4$ and $HPO_4^{-2}$ are the only forms of soluble inorganic phosphorus directly utilised by aquatic biota and exert a strong influence on rates of primary production (DWAF 1996a). Orthophosphate ($PO_4$) is according to DWAF (1996a) the only oxy-anion in natural waters derived from weathering rocks and decomposition of organic matter. Anthropogenic sources include agricultural runoff, mining, industrial, and sewage wastewater effluents. The SAWQG for aquatic ecosystems indicate that $PO_4$ ranges are associated with the resulting trophic conditions of a water body. Oligotrophic conditions occur at <0.005 mg/l, mesotrophic conditions between 0.025–0.25 mg/l, eutrophic conditions between 0.25–0.250 mg/l, and hypertrophic conditions occurs at >0.250 mg/l (DWAF 1996a).

Orthophosphate concentrations recorded were consistently <0.05 mg/l at all the sampling sites throughout the study. These values were within the TWQR of 0.025–0.25 mg/l, indicating mesotrophic conditions. The findings of this study are dissimilar to those by Madanire-Moyo *et al*. (2012a), describing Flag Boshielo Dam with oligotrophic conditions. However, similar to those in a study conducted by Jooste *et al*. (2014) and Dabrowski *et al*. (2014), illustrating Flag Boshielo Dam in a mesotrophic state with respect to this variable. The findings in this study are in agreement with the analyses by Dabrowski and De Klerk (2013), whereby it was highlighted that this part of the Olifants River is subjected to significant increasing levels of $PO_4$ concentrations. Whilst phosphate reflects changes in the trophic status of Flag Boshielo Dam, it can still be considered moderately polluted as compared to Loskop Dam upstream of Flag Boshielo Dam with eutrophic conditions (Oberholster *et al*. 2010; Madanire-Moyo *et al*. 2012a; Dabrowski *et al*. 2013; Dabrowski *et al*. 2014).

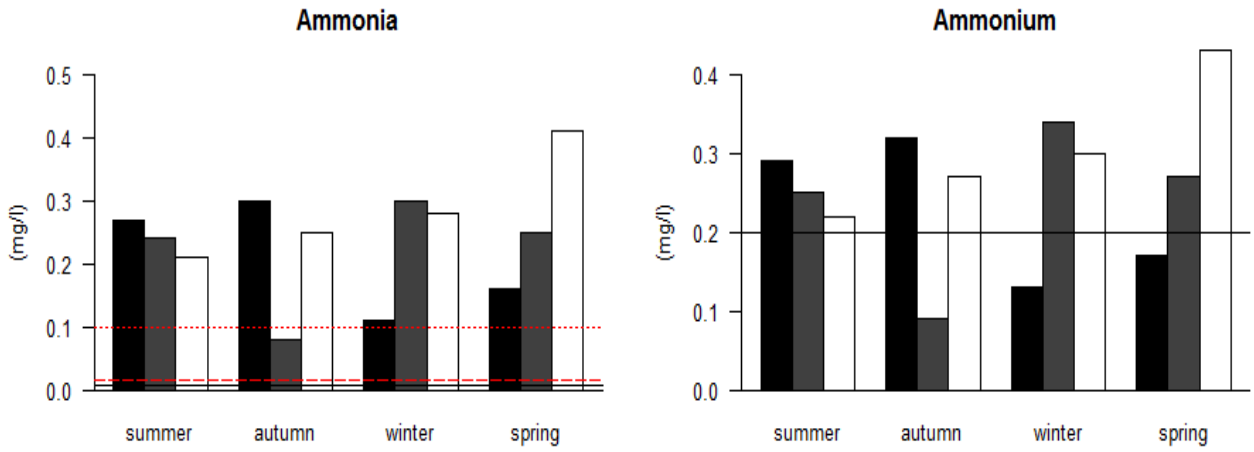


Figure 3.4: The seasonal variation in the mean A- ammonia; B- ammonium at the three selected sites inflow (black bars), middle (grey bars), dam wall (white bars) at Flag Boshielo Dam (March 2012–January 2013). Solid, red dashed, red dotted lines represent the TWQR, CEV and AEV, respectively (DWAF 1996a).

### 3.3.4 Major ions

#### 3.3.4.1 Cations

##### 3.3.4.1.1 Calcium

Calcium ($Ca^{+}$) is one of the major elements essential for living organisms, vital for muscle contractions, energy metabolism and other biochemical interactions (Dallas & Day 2004; Dalesman & Lukowiak 2010). In most natural freshwater systems calcium is often the predominant cation, derived mainly from geological formations in the catchment area. This variable has the capacity to decrease the direct uptake of cationic metals by competing for binding sites on the gill surface. However, very little is known about the actual effects of changes in its concentration on aquatic biota (DWAF 1996a). A TWQR of calcium is unavailable, but DWAF (1996a) asserts that a concentration of >80 mg/l have no severe effects on the aquatic biota.

In the present study, calcium concentrations ranged from 15.52–31.34 mg/l. The highest $Ca^{+}$ concentration was recorded at the middle of the dam in autumn and the lowest value was recorded at the dam wall in spring (Appendix A: Table 1). Statistically, no significant variation was found for $Ca^{+}$ between the seasons and sites ($p$>0.05). The concentrations recorded during the study are within acceptable limits, despite the fact that waters of the Highveld, i.e. Upper Olifants River, are dominated by calcium, magnesium and bicarbonate ions (Day & King 1995).

#### 3.3.4.1.2 Magnesium

Magnesium ($Mg^{2+}$) is an essential element for cellular metabolism and other physiological functions in the diet of vertebrates. This element is relatively abundant in the earth's crust and therefore a common element of natural waters (Bartram & Ballance 1996). The main source of $Mg^{2+}$ is the weathering of rocks containing ferromagnesium and some carbonate rocks. Natural concentrations of magnesium in freshwaters may range from 1 to >100 mg/l, depending on the rock types within the catchment. The TWQR for $Mg^{2+}$ suggested for aquatic ecosystems is 70 mg/l (DWAF 1996a).

Magnesium concentrations recorded in this study ranged from 12.57–23.59 mg/l. The highest $Mg^{2+}$ concentration were recorded in spring at the inflow and the lowest in spring at the dam wall (Appendix A: Table 1). Statistically, no significant variation was found between the seasons and sites ($p$>0.05). The $Mg^{2+}$ concentrations recorded during the study were within the TWQR, regardless of the presence of a common magnesium mineral dolomite, the type of rock formation within the Flag Boshielo Dam catchment (Ashton *et al.* 2001).

#### 3.3.4.1.3 Potassium

Potassium (K) is an alkali metal which reacts with water to form positively-charged potassium ions ($K^{+}$) (DWAF 1996a). This variable is involved in transmission of nervous impulses, muscle contractions and ionic balance in all organisms (DWAF 1996a). No criteria is available for potassium in aquatic ecosystems. The typical concentration of potassium in freshwater is within the range of 2–5 mg/l (DWAF 1996a). However, Chapman (1996) asserts that $K^{+}$ is a relatively abundant element but its concentration in natural freshwater is usually <10 mg/l. This concentration is mainly due to the fact that the rocks which contain $K^{+}$ are relatively resistant to weathering. Anthropogenic sources of $K^{+}$, is attributed by potassium salts widely used in industries and as fertilisers in agriculture; therefore, may enter freshwaters with industrial discharges and agricultural run-off (Chapman 1996).

In the present study, potassium concentrations recorded ranged from 3.11–6.5 mg/l. The concentrations were the highest at the dam wall during summer and lowest at the inflow in spring (Appendix A: Table 1). The $K^{+}$ concentrations recorded were

above those stipulated for South African aquatic ecosystems (DWAF 1996a). Statistically, no significant variation was found between the sampling sites and seasons ($p$>0.05). The recorded $K^+$ concentrations indicate inputs or leeching of potassium salts from agricultural and industry effluents evident in the water from the upstream Olifants catchment (Oberholster *et al*. 2011).

#### 3.3.4.1.3 Sodium

Sodium ($Na^+$) is the least toxic metal cation in aquatic systems (Hellawell 1986), its important occurrence is the contribution to TDS (Dallas & Day 2004). Sodium occurs widely in natural waters and its concentration in natural surface waters depends on the geology of the catchment area. The anthropogenic sources include sewage and industrial effluents (Chapman 1996). Sodium is involved in ionic, osmotic and water balance in all organisms and is also necessary in the transmission of nervous impulses and in muscle contractions. The TWQR for $Na^+$ is 100 mg/l (DWAF 1996a).

Sodium concentrations recorded in the present study ranged from 23.71–59.67 mg/l. The highest concentrations of $Na^+$ were recorded during spring, lowest in winter (Appendix A: Table 1). Overall $Na^+$ concentrations were highest at the inflow as compared to the other two sites (Figure 3.6). Statistically, no significant variation was found between the sampling sites and seasons ($p$>0.05). The concentration of $Na^+$ exhibit an increasing trend in comparison to concentrations reported in a study by Madanire-Moyo *et al*. (2012a) at Flag Boshielo Dam with a maximum value of 0.4 mg/l. Therefore, this illustrates that mining and industrial activities at the upper catchment as well as the agricultural activities at the middle catchment may have attributed to the elevated concentration of $Na^+$ at Flag Boshielo Dam. The concentrations were nonetheless within the acceptable TWQR for aquatic ecosystems.

### 3.3.4.2 Anions

#### 3.3.4.2.1 Fluoride

Fluoride (F) is a halogen gas, seldom in nature as free fluoride gas but highly reactive with a variety of substances, i.e. calcium, potassium or phosphates (DWAF 1996a). Anthropogenic sources of fluoride in aquatic ecosystems include its release from WWTW and most public water supplies. Fluoride values in aquatic ecosystems should ideally range between 0.75–1.5 mg/l (DWAF 1996a). The TWQR for fluoride

is stipulated to be ≤0.75 mg/l. The toxicity of this variable to some freshwater fish ranges between 3–4 mg/l (WHO 2006). However, Smith *et al*. (1985) investigated the effect of F on three fish species and found $LC_{50}$ values ranging between 51–460 mg/l.

In the present study, fluoride concentrations recorded ranged from 0.53–0.92 mg/l. The highest average levels of F were recorded during autumn and above the TWQR at all the sampling sites during this period as compared to the other seasons (Appendix A: Table 1). This finding is corroborated by the level of calcium that were significantly high during autumn at the study site. Dissolved fluoride reacts with calcium and phosphate to form insoluble complexes with certain ions ($Mg^{2+}$), which tend to settle out of the water column (Smith-Hopkins 1964). Nonetheless, fluoride values were within the acceptable TWQR throughout the study (Table 3.2) with the exception of autumn. Statistically, significant variation was found between the seasons ($p$<0.05), but no variation between the sampling sites ($p$>0.05). The concentrations recorded during the study, can thus be considered unacceptable for the two selected fish species.

#### 3.3.4.2.2 Sulphate

Sulphate ($SO_4^{2-}$) is an oxy-anion of sulphur in the +VI oxidative state and forms salts with various cations such as potassium, barium, sodium, magnesium and calcium. Sulphate occurs naturally in freshwaters arising from the dissolution of mineral sulphates in soil and rock, particularly calcium sulphate (gypsum) and other partially soluble sulphate minerals (DWAF 1996a). It is an abundant ion in the earth's crust and its concentration in water can range from a few milligrams to several thousand milligrams per litre (Bartram & Ballance 1996).

Sulphate is not toxic in natural waters. In excess, however, they form sulphuric acid, which is a strong acid that reduces pH, enhance algal growth and can have devastating effects on aquatic ecosystem. There is no prescribed TWQR value for sulphate for the aquatic ecosystem in the SAWQG and WHO guidelines, however maximum dissolved sulphate value of 100 mg/l have been proposed by the Canadian guidelines for aquatic ecosystems (CCME 2012).

Sulphate concentrations recorded ranged from 46.95–122.83 mg/l during the study. The highest sulphate concentrations were recorded at the inflow and the lowest at the dam wall (Appendix A: Table 1). The highest $SO_4^{2-}$ concentrations were recorded in autumn at all the sampling sites as compared to the other seasons. Overall, $SO_4^{2-}$ concentrations exceeded the maximum acceptable limits. Statistically, no significant variation was found between the sampling sites and seasons ($p$>0.05). The findings in the study are related to high levels of sulphate reported at Loskop Dam (Dabrowski *et al*. 2013) caused by acid mine drainage from the Upper Olifants River sub-catchment (De Villiers & Mkwelo 2009; Dabrowski *et al*. 2014). In addition, $SO_4^{2-}$ concentrations showed an increase, from values recorded by Madanire-Moyo *et al*. (2012a). Consequently, this signals the importance of effective and critical monitoring of eutrophication at this part of the Olifants River System.

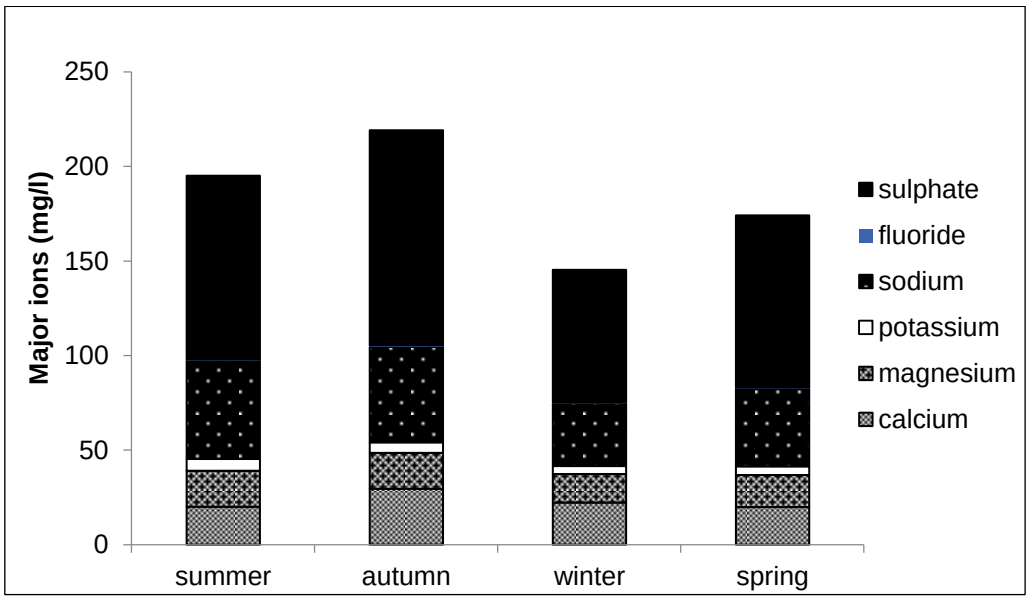


Figure 3.5: Seasonal relative composition of cations and anions in milliequivalents per litre with the mean of all the sampling sites at Flag Boshielo Dam.

### 3.3.5 Toxic constituents

#### 3.3.5.1 Aluminium

Aluminium (Al) is the third most abundant element in the earth's crust but it is present in trace concentrations in natural waters (DWAF 1996a). It occurs primarily as alumina-silicate minerals which are too insoluble to participate readily in bio-geochemical reactions. The solubility of aluminium in water is strongly pH dependent (Dickson 1983; DWAF 1996a); it becomes more soluble and toxic at acidic pH levels

(Buergel & Soltero 1983; Norrgreen *et al*. 1991). At intermediate pH values, aluminium is partially soluble, while at alkaline pH values it is present as soluble but biologically inaccessible (DWAF 1996a).

Aluminium concentrations in summer and autumn were recorded below the detection level (<0.01 mg/l) of the inductively coupled plasma-optical emission spectrometry (ICP-OES), except the winter and spring measurements at the inflow (Appendix A: Table 1). Overall, aluminium concentrations were above the TWQR (0.001 mg/l) but within the CEV (0.02 mg/l) and below AEV (0.15 mg/l) suggested by DWAF (1996a). These elevated aluminium concentrations can be linked to acid mine drainage decanting from the abandoned coal mines in the upper Olifants River. Statistically, significant variation of aluminium concentrations was found between seasons ($p$<0.05), but no variation between the sites ($p$>0.05). The findings in this study are in agreement with reports that the Olifants River is impacted by mining and agriculture, high levels of heavy metals of aluminium (from Klipspruit; Mpumalanga) (Heath & Claassen 1999) and Loskop Dam (Oberholster *et al*. 2010).

#### 3.3.5.2 Arsenic

Arsenic (As) is a metalloid element toxic to both marine and freshwater aquatic life. The United States Environmental Protection Agency (2007), highlight arsenic as one of the Endocrine Disruptive Metals (EDM) known to be carcinogenic. pH plays a major role in determining the form of arsenic in freshwater, and thus its toxic effects (DWAF 1996a). The presence of dissolved and particulate organic matter, suspended solids and sediments are also important, since arsenic adsorbs readily to suspended material and combines with dissolved organic carbon (DWAF 1996a).

Arsenic concentrations during the study were consistently recorded at <0.03 mg/l, thus above the TWQR (0.01 mg/l) and non-detectable concentrations <0.001 mg/l and above the detectable level 0.006 mg/l throughout the study at all the sampling sites. Statistically, no significant variation of arsenic concentrations was found between sites and seasons ($p$>0.05). The high arsenic concentrations are likely generated by agricultural and industrial pollutants, via using or discharging arsenic compounds (pesticides and fertilizers) (DWAF 1996a) in the catchment. Thus, certain carcinogenic sub-lethal effects would be expected on the endocrine system of the two selected fish species at Flag Boshielo Dam.

#### 3.3.5.3 Copper

Copper (Cu) is a common metal occurring in three oxidative states in aquatic ecosystems as metallic copper, cuprous copper and cupric copper (DWAF 1996a). This variable is rapidly accumulated by plants and animals, as an essential component of enzymes involved in redox reactions (Sorensen 1991; Galvin 1996). The toxicity, mobility and solubility depend on pH, water hardness and the presence of Zn and Mn (Dallas & Day 2004). A TWQR stipulated for copper is 0.05 mg/l (DWAF 1996a).

Copper concentrations of <0.01 mg/l were recorded throughout the study at all the selected sampling sites, this concentration was recorded above detection level (<0.001 mg/l), but within the TWQR (DWAF 1996a). Statistically, no significant variation of Cu concentration was found between sites and seasons ($p$>0.05). DWAF (1996a) asserts that, the solubility of copper is high in acidic pH water and precipitates in alkaline water. In that regard, low concentrations recorded may be attenuated by the alkaline pH water during the sampling periods.

#### 3.3.5.4 Iron

Iron (Fe) is the fourth most abundant element in the earth's crust, occurring in two common states, as reduced ferrous ($Fe^{2+}$) and oxidised ferric ($Fe^{3+}$). In natural ecosystems, iron occurs due to weathering of sulphide ores, igneous, sedimentary (sandstone) and metamorphic rocks. For instance, leaching from sandstone releases iron oxides and hydroxides to the environment. The form and solubility of iron in natural waters are strongly dependent upon pH and the oxidation-reduction potential of the water. Ferric iron is found in solution only at a pH of <3 (Bartram & Ballance 1996). The iron concentration in unpolluted surface waters ranges from 0.001–0.5 mg/l (DWAF 1996a). However, these concentrations are site specific and while no TWQR is available, DWAF (1996a) suggests this variable should not exceed 10% of the background dissolved iron concentration.

Iron concentrations recorded during the study ranged from <0.01–0.01 mg/l (Appendix A: Table 1). The values recorded were within the acceptable limits DWAF (1996a). Statistically, no significant variation was found for Fe concentrations between seasons and among sites ($p$>0.05). Though, Fe is not known to be

hazardous to aquatic biota, it is generally accepted that the concentration of soluble ionised forms of iron should not exceeds 0.2 mg/l in cyprinid fish (Svobodóva *et al*. 1993). Thus, Fe concentrations can at present be regarded as acceptable for the two selected cyprinids.

#### 3.3.5.5 Lead

Lead (Pb) is a common toxic trace metal which readily accumulates in living tissue and is considered carcinogenic, an EDM, potentially hazardous to most forms of life and relatively accessible to aquatic organisms (DWAF 1996a). The water solubility and toxicity of lead compounds depend on water hardness and pH, i.e. the toxicity of lead is reduced with increasing alkalinity, while decreasing pH increases the bioavailability of divalent lead, fostering accumulation by aquatic biota (Dallas & Day 2004). The TWQR suggested by DWAF (1996a) for lead is 0.012 mg/l, the CEV is 0.001 mg/l and AEV is 0.007 mg/l.

Lead concentrations recorded during the study were consistently <0.09 mg/l throughout the study at all the sampling sites (Appendix A: Table1), thus above detectable concentrations (0.001 mg/l), the TWQR, CEV and AEV (DWAF 1996a). Statistically, no significant variation was found between seasons and sites ($p$>0.05). Pb has been reported to be potentially toxic at low concentrations (Dallas & Day 2004). The availability of this constituent in the dam might be due to accumulated lead in the sediments over time from anthropogenic sources such as industrial and municipal wastewater discharge; mining, milling and smelting of lead and metals associated with lead, i.e. zinc, copper, silver and arsenic (DWAF 1996a), evident in this water system. For instance, Jooste *et al*. (2014) recorded lead concentrations of 50 mg/kg, thus above the CCME (1999) acceptable limit of 35 mg/kg.

#### 3.3.5.6 Manganese

Manganese (Mn) is the eighth most abundant metal in nature. This metal is a functional component of nitrate assimilation, and a catalyst of numerous enzyme systems in animals (DWAF 1996a). In aquatic ecosystems, manganese may exist in the form of soluble manganous ($Mn^{2+}$). Toxicity effects of manganese to aquatic biota are very limited, but Dallas and Day (2004) and USEPA (2004) reported that high concentrations of manganese are toxic and may lead to disturbances in various

metabolic pathways in vertebrates, in particular disturbances of the central nervous system caused by the inhibition of the formation of dopamine (a neurotransmitter). Similar to iron, the concentration of dissolved manganese is influenced by changes in redox potential, DO, pH and organic matter. i.e. $Mn^{2+}$ occurs at low pH values. DWAF (1996a) stipulates acceptable manganese concentration of 0.0002–0.13 mg/l and TWQR of 0.18 mg/l in aquatic ecosystems.

Manganese concentrations recorded during the study ranged from <0.01–0.01 mg/l (Appendix A: Table 1). Mn concentrations were below detection levels in summer and autumn, including at the inflow in spring and low during the other seasons, at the respective sites. In general, these concentrations are within the TWQR (DWAF 1996a). Statistically, no significant variation was found between sites and seasons ($p$>0.05).

#### 3.3.5.7 Zinc

Zinc is an essential nutritional trace element, widely used in nucleic acid synthesis and occurs in many enzymes. In aquatic ecosystems, this variable occurs in two oxidative states, namely as the metal zinc (I), and as zinc (II). Zinc enters aquatic ecosystems through both natural processes such as weathering or through industrial activity. Zinc (II) ion is toxic to aquatic ecosystems particularly to fish even at relatively low concentration (DWAF 1996a). The toxicity of zinc to fish is influenced by the chemical characteristics of water, e.g. increasing calcium concentrations reduce the toxicity of zinc (Svobodóva *et al*. 1993). The concentration of zinc in inland waters is usually low, typically about 0.015 mg/l. The TWQR for zinc in the aquatic ecosystem is <0.002 mg/l; CEV is 0.0036 mg/l; AEV is 0.036 mg/l (DWAF 1996a).

Zinc concentrations recorded during the study ranged from <0.01–0.01 mg/l, thus it was above the TWQR and CEV but below the AEV throughout the study at all sites. The intensity of mining in the catchment and the direct input of point source pollution from the WWTWs (releasing partially treated and untreated sewage) in the upper region of the Olifants River can be linked to the high zinc concentrations recorded during the present study at Flag Boshielo Dam.

### 3.3.6 Integrated water quality analysis

The results of the different water quality variables at the three selected sites at Flag Boshielo Dam are presented in Figure 3.6, to illustrate limnological differences amongst the three sampling sites. During the study concentrations of metals were recorded below the instrument detection levels throughtout the period of sampling (Table 3.1) and were thus excluded from the SIMPER analysis. The SIMPER analysis of the detected water quality variables showed that 68.6% of total variation of the environmental characteristics, between the three sampling sites where attributed to sulphate (22.4%), ammonia (17.8%), ammonium (19%) and sodium concentration (9.45%).

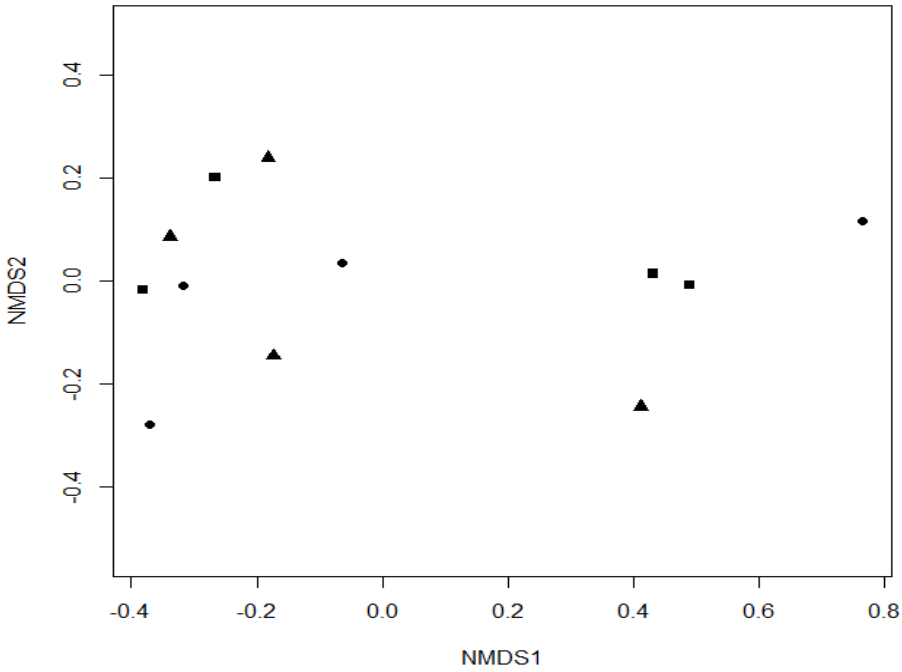


Figure 3.6: A non-metric dimensional scaling (NMDS) plot for the physico-chemical parameters of the water quality at Flag Boshielo Dam. The data for the three selected sampling sites are represented by the shape of the symbols: inflow (●), middle (■), dam wall (▲).

During the study, it was evident that the sampling sites dispersed in terms of the water quality (Figure 3.6). The inflow and dam wall were found with a clear separation of the assessed physico-chemical parameters. In contrast, the middle of the dam showed cluster of some water quality variables. Thus, the NMDS plot of the water quality data at Flag Boshielo Dam demonstrates the distinct water quality signatures of the three selected sites according to their degree of impact.

Table 3.2: Summary of seasonal water quality variables measured at the three selected sites in the middle region of the Olifants River, at Flag Boshielo Dam during March 2012–January 2013. Unless indicated, units are mg/l; dashes denote unavailability of data. The target water quality ranges (TWQR) for all the water use are as stipulated by DWAF (1996a). Values above and below the South African TWQR are highlighted in bold.

| | Seasons | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | summer | | | | autumn | | | | Winter | | | | spring | | | | |
| Parameters | Mean | SD | Min | Max | Mean | SD | Min | Max | Mean | SD | Min | Max | Mean | SD | Min | Max | TWQR |
| Temperature (°c) | 28.47 | 0.751 | 28 | 29 | 25.37 | 0.67 | 25 | 26 | 15.08 | 0.23 | 15 | 15 | 20.87 | 0.28 | 21 | 21 | – |
| pH | | | 8 | **9.15** | | | 8 | 9 | | | 8 | 9 | | | 6.2 | 7 | 6.6–9 |
| DO (%) | 109.5 | 20.45 | 86 | **124** | 99.07 | 15.61 | 87 | 117 | **60.73** | 8.17 | **55** | **70** | **67.7** | 2.4 | **65.9** | **70.4** | 80–120 |
| DO (mg/l) | 8.89 | 0.095 | 9 | 9 | 7.25 | 0.12 | 7 | 7 | 8.36 | 0.69 | 8 | 9 | **9.29** | 1.37 | 8 | **10** | 6.0–9 |
| TDS | 335.2 | 11.06 | 324 | 247 | 381.23 | 4.83 | 378 | 387 | 290.3 | 50.12 | 248 | 346 | 323.3 | 69.5 | 245 | 378 | – |
| Conductivity (EC) $mSm^{-1}$ | 51.57 | 1.701 | 50 | 53 | 58.13 | 1.18 | 57 | 60 | 44.67 | 7.71 | 38 | 53 | 43.67 | 12.76 | 35 | 58 | – |
| Salinity | 0.26 | 0 | 0.26 | 0.26 | 0.26 | 0 | 0.26 | 0.26 | 0.26 | 0 | 0.26 | 0.26 | 0.26 | 0 | 0.26 | 0.26 | – |
| Ammonia | **0.24** | **0.031** | **0.021** | **0.27** | **0.21** | **0.12** | **0.08** | **0.25** | **0.23** | **0.1** | **0.11** | **0.3** | **0.27** | **0.12** | **0.16** | **0.41** | <0.007 |
| Ammonium | **0.25** | 0.04 | **0.22** | **0.29** | **0.23** | 0.12 | **0.27** | **0.32** | **0.26** | 0.11 | 0.13 | **0.34** | **0.29** | 0.13 | 0.17 | **0.43** | 0.2* |
| Nitrates | **<1.4** | 0 | **<1.4** | **<1.4** | **<1.4** | 0 | **<1.4** | **<1.4** | **<1.4** | 0 | **<1.4** | **<1.4** | **<1.4** | 0 | **<1.4** | **<1.4** | 0.5* |
| Nitrites | <0.01 | 0 | <0.01 | <0.01 | <0.01 | 0 | <0.01 | <0.01 | <0.01 | 0 | <0.01 | <0.01 | <0.01 | 0 | <0.01 | <0.01 | 0.2* |
| Ortho-phosphate | <0.05 | 0 | <0.05 | <0.05 | <0.05 | 0 | <0.05 | <0.05 | <0.05 | 0 | <0.05 | <0.05 | <0.05 | 0 | <0.05 | <0.05 | 0.1* |
| Sulphate | 97.18 | 9.24 | 87 | **104** | **113.73** | 4.22 | **110** | **118** | 69.88 | 27.33 | 47 | 100 | 91.65 | 27.03 | 75 | **123** | 100** |
| Fluoride | 0.72 | 0.04 | 0.67 | 0.75 | **0.87** | 0.04 | **0.85** | **0.92** | 0.69 | 0.03 | 0.67 | 0.73 | 0.59 | 0.12 | 0.53 | 0.73 | ≤0.75 |
| Calcium | 20.04 | 3.17 | 16 | 33 | 29.63 | 2.73 | 27 | 31 | 22.19 | 7.63 | 17 | 31 | 19.94 | 6.1 | 16 | 27 | – |
| Magnesium | 18.97 | 1.2 | 18 | 20 | 18.87 | 0.26 | 19 | 19 | 15.12 | 5.79 | 11 | 22 | 16.87 | 5.89 | 13 | 24 | 70 |
| Potassium | 6.26 | 0.32 | 7 | 7 | 5.62 | 0.23 | 5 | 6 | 4.28 | 1.36 | 3 | 6 | 4.58 | 1.38 | 4 | 6 | 200 |
| Sodium | 51.67 | 1.24 | 51 | 53 | 50.38 | 1.79 | 49 | 32 | 33.2 | 11.6 | 24 | 46 | 40.99 | 16.2 | 31 | 60 | 100 |
| Aluminium | <0.01 | 0 | <0.01 | <0.01 | <0.01 | 0 | <0.01 | <0.01 | 0.013 | 0.006 | 0.01 | 0.01 | 0.01 | 0 | 0.01 | 0.01 | 0.01 |
| Arsenic | <0.03 | 0 | <0.03 | <0.03 | <0.03 | 0 | <0.03 | <0.03 | <0.03 | 0 | <0.03 | <0.03 | <0.03 | 0 | <0.03 | <0.03 | 100 |
| Copper | <0.01 | 0 | <0.01 | <0.01 | <0.01 | 0 | <0.01 | <0.01 | <0.01 | 0 | <0.01 | <0.01 | <0.01 | 0 | <0.01 | <0.01 | 0.5 |
| Iron | <0.01 | 0 | <0.01 | <0.01 | <0.01 | 0 | <0.01 | <0.01 | <0.01 | 0 | <0.01 | <0.01 | <0.01 | 0 | <0.01 | <0.01 | 0.2 |
| Lead | **<0.09** | 0 | **<0.09** | **<0.09** | **<0.09** | 0 | **<0.09** | **<0.09** | **<0.09** | 0 | **<0.09** | **<0.09** | **<0.09** | 0 | **<0.09** | **<0.09** | 0.012 |
| Manganese | <0.01 | 0 | <0.01 | <0.01 | <0.01 | 0 | <0.01 | <0.01 | <0.01 | 0 | <0.01 | <0.01 | <0.01 | 0 | <0.01 | <0.01 | 0.18 |
| Zinc | **<0.01** | 0 | **<0.01** | **<0.01** | **<0.01** | 0 | **<0.01** | **<0.01** | **<0.01** | 0 | **<0.01** | **<0.01** | **<0.01** | 0 | **<0.01** | **<0.01** | 0.002 |

**Key:** mean = mean seasonal values, SD = standard deviation, Min = minimum, Max = maximum, TWQR = target water quality range for South African Water Quality Guidelines for aquatic ecosystems (DWAF 1996a), * = World Health Organization Guidelines (1984), ** = Canadian Guidelines 2012.

## 3.4 CONCLUSIONS

Recent research in the upper region of the Olifants River has illustrated that the "cocktail' of pollutants has manifested in a number of critical ecological health concerns downstream to the middle and lower region of the river. Thus, the research question posed in Chapter 1; 1.6 (i), was addressed in response to the degrading ecological state of the Olifants River System, to evaluate whether Flag Boshielo Dam can at present be considered moderately polluted.

Analysis of different physico-chemical parameters from the selected sites at Flag Boshielo Dam provided an overview of the water quality at this part of the Olifants River System. Relative to the SAWQG, WHO and Canadian guidelines of aquatic waters, most nutrients, cations i.e. sulphate and fluoride as well as few metals such as lead and zinc were found to be above acceptable limits stipulated. This is evident from historical data available regarding the heavy metals, nutrients and major ions inputs into the dam that the elevated levels can attribute to urban non-point contamination, geochemistry, sewage, industrial and mining discharge from the surrounding catchments.

Despite the fact that the upper region of the Olifants River receives industrial sewage, discharge of treated and untreated domestic effluents and acid mine drainage emanating from a number of abandoned mines, Flag Boshielo Dam is presently noted as being in an mesotrophic state. Thus, Flag Boshielo Dam can still be regarded as moderately polluted, compared to Loskop Dam (eutrophic state) in the upper region of the Olifants River. Dams high up in the Olifants River (particularly in the middle reaches) have been reported to improve the water, entering the KNP. However, the impact of mining and agriculture evident at the study site shows that there is an increasing trend of some constituents towards the middle region of the Olifants River System. This trend is of concern as the deterioration of the quality of South Africa's water resources is one of the major threats to the country's capability to provide sufficient water of appropriate quality while ensuring environmental sustainability. Therefore the regulatory tools (Integrated Water Resource Management (IWRM), Waste Discharge Charge System (WDCS) and Load reduction) implemented by the Department of Water Affairs for the upper and middle regions of the Olifants River needs to be applied in an effective and consistent manner to mitigate and control the ongoing pollution of the Olifants River System.

# CHAPTER 4 - FISH HEALTH ASSESSMENT

## 4.1 INTRODUCTION

The use of fish and their attributes have long been applied to assess the integrity or ecological state of freshwater aquatic ecosystems (Barbour *et al*. 1999). In South Africa, particularly in the Olifants River, it is only with the occurance of fish kills in the upper and lower Olifants River regions during the past fifteen years that attention has been drawn towards ecological deterioration of this river, subsequently the use of fish in biological assessments.

One of the biological assessment approaches, the Health Assessment Index (HAI), developed in the United States of America by Adams *et al*. (1993), has been introduced (Avenant-Oldewage & Swanepoel 1993), applied and adapted for local conditions (Crafford & Avenant-Oldewage 2009; McHugh *et al*. 2011; Madanire-Moyo *et al*. 2012a; Watson *et al*. 2012). This approach was initially developed by Goede and Barton (1990) as a field necropsy method, which provides fish health assessments based on percentages of anomalies observed in tissues and organs of individuals sampled from a population. However the methods' limitation to provide quantitative results that were agreeable to statistical comparisons of data among species, sites or years promped Adams *et al.* (1993) to enhance the necropsy method by developing the HAI, in order to curtail the limitations of the former.

The HAI is an extension and refinement of a field necropsy method, designed to complement each other in the evaluation of fish health (Crafford & Avenant-Oldewage 2009). The HAI is a quantitative index that allows statistical comparisons of fish health among datasets and includes index variables that are assigned numerical values based on the severity or damage incurred by an organ or tissue as a result of environmental stressors (Adams *et al*. 1993). A value of zero represents normal condition while values of 10, 20 and 30 are assumed to be abnormal conditions (Table 2.1). The HAI involves a series of simple, ordered observations, measurements of external characteristics, internal organs and some blood parameters. These variables are grouped into four components: 1) Blood parameters

(haematocrit percentage, white blood cell counts); 2) Length, weight and condition factor (K); 3) Percentage of fish with normal and abnormal eyes, gills, pseudobranch, fins, spleen, kidney and liver; and 4) Index values of damage to skin, fins, thymus and hindgut inflammation. However, according to Adams *et al*. (1993) and Klemm *et al*. (1995), when applying a necropsy-based method such as the HAI, some assumptions are required: i) When all organs and tissues appear normal according to the autopsy criteria, there is a good probability that the fish is normal; ii) When fish are exposed to elevated levels of contaminants, tissue and organ function will change in order to maintain homeostasis; iii) If a change in function persists in response to continuing stress, there will be gross change in the structure of organs and tissues; and iv) If the appearance of an organ or tissue system departs from the normal condition or control condition, the fish is responding to changes brought about by the environmental stressor. Internal variables, bile colour and mesenteric fat, are excluded in the HAI calculations because these are subjective and can differ widely depending on fish size, sex, reproductive status, stress, nutritional status, feeding regimes and aquatic ecosystems (Adams *et al.* 1993).

One of the variables in the HAI is the presence or absence of parasites, and these received assigned values based on the severity of damage on the host. No parasites observed were given a value of zero, while a few parasites were given a numerical value of 10. Given the relevance of parasite data in environmental monitoring, the original HAI notation was thus expanded and a parasite index was developed and tested in conjunction with the HAI (Marx 1996; Robinson 1996; Luus-Powell 1997). Crafford and Avenant-Oldewage (2009) referred to these as the Original Parasite Index (OPI). This index was revised with an introduction of the Inverted Parasite Index (IPI), which is based on the premises that ectoparasites are more exposed to the effects of water quality than endoparasites. Larger numbers of ectoparasites are indicative of better water quality thus healthy fish population and should be given a lower score for this correlation to be reflected in the HAI value (Jooste *et al*. 2005; Crafford & Avenant-Oldewage 2009; Madanire-Moyo *et al*. 2012a; Watson *et al*. 2012). Nevertheless, the HAI and associated PI will test differently in every aquatic system, depending primarily on the fish species used, parasite species and type of pollution (Jooste *et al*. 2004; Sures 2006).

Most importantly it should be noted that the HAI is not a diagnostic tool but rather provide means of establishing a database for detecting trends in the health and condition of a fish population (Adams *et al*. 1993). This index is not a substitute but cumulative with other health measures and can detect gross changes in the health of fish early for effective and critical monitoring.

As such, the main objective of this section was to use fish health parameters, HAI, PI, IPI and K to access the health status of two species of Cyprinidae (Actinopterygii: Cypriniformes) i.e., *Hypophthalmichthys molitrix* and *Labeo rosae* in the middle region of the Olifants River, Flag Boshielo Dam. The HAI is used in conjuction with physical and chemical characterisation of the water (water quality), to access the overall health of this ecosystem and its inhabitants.

## 4.2 METHODS AND MATERIALS

### 4.2.1 Field procedures

The two selected fish species, *H. molitrix* and *L. rosae*, were sampled seasonally using a variety of collection methods such as conventional angling gear, scoop nets and gill nets with stretched mesh sizes of 30–110 mm (Figure 2.6A–B) depending on the species' habitat preferences. Each species collected was identified and scrutinised for any mobile ectoparasites and if any were encountered the fish was marked and the parasites recorded on the HAI data sheet and retained in its respective holding tanks filled with dam water, and aerated to keep the fish alive until they were processed in the field laboratory (Figure 2.6H).

### 4.2.2 Laboratory procedures

At the field laboratory, fish was removed from the holding tanks, and the entire body surface was examined. Gross pathological changes were noted and depending on the degree of stressor-induced anomalies, numerical values were assigned for the HAI variables as suggested by Adams *et al*. (1993) and Jooste *et al*. (2005), subsequently recorded on HAI data sheets. Fish was measured for standard length (SL), total length (TL), weighed (g) and recorded on a HAI data sheet for K calculations. Blood was drawn for blood analysis (haematocrit). Fish were then sacrificed by severing the spinal cord and dissected. All internal organs were placed in separate petri dishes and assigned HAI values according to their condition with the aid of a colour chart developed by Watson (2001) (Figure 2.7). All the organs

were examined for helmith parasites, and numerical values were assigned according to the methods of Jooste *et al*. (2005) and Crafford and Avenant-Oldewage (2009). The standard deviation (SD) and coefficient of variance (CV) for each sample were calculated in accordance with Adams *et al*. (1993). A detailed account of the sampling procedures, calculations, the examination of fish and parasites is given in Chapter 2.

### 4.2.3 Data analysis

Data were tested for normality and homogeneity of variance using Levene's tests. The indices i.e. HAI, PI, IPI and K were calculated and presented as mean±SD. Seasonal variations in HAI values for a given species were tested using one-way ANOVA; this model was also used to test for inter-species difference for K. Two-way ANOVA was used to test seasonal variation between the two species. Where ANOVA revealed significant differences, Tukey's *post-hoc* test multiple comparisons were performed to establish where differences occurred. Regression analysis was used to determine any significant correlation between K of each fish species and parasite load. These analyses were performed using SPSS Statistics version 21 and the significance of results was ascertained at $p<0.05$. Infection statistics of all the parasites were calculated according to Bush *et al*. (1997). These parameters are described in detailed in Chapter 2.

## 4.3 RESULTS AND DISCUSSION

### 4.3.1 Health Assessment Index

The two selected fish *H. molitrix* and *L. rosae* species had higher HAI values during autumn and spring as compared to the other two seasons. These spikes coincide with several water parameters recorded during these periods, which were found to exceed TWQR. Evidently, many studies (Adams *et al*. 1993; Jooste *et al*. 2005; Crafford & Avenant-Oldewage 2009; Van Dyk *et al*. 2009; Madanire-Moyo *et al*. 2010; McHugh *et al*. 2011; Madanire-Moyo *et al*. 2012a; Watson *et al*. 2012; Sara *et al*. 2014) substantiate that a high HAI value corresponds with decreased water quality and a increased physiological stress, thus a fish population with poorer health. *Hypophthalmichthys molitrix* had reported the highest HAI value (Figure 4.1) throughout the study with mean values that ranged from 55.3–74.8 compared to 25.5–47.7 for *L. rosae* (Table 4.1). The necropsy related anomalies of *H. molitrix*

included discoloured liver (Figure 4.4G), white patched, frayed, clubbed and deformed gills (Figure 4.4D–E), lesions on the skin (Figure 4.4 A–B), missing fins (Figure 4.4C) and abnormal haematocrit levels that were below the normal ranges as defined by Adams *et al*. (1993). Anomalies observed for *L. rosae* included; discoloured liver (Figure 4.4H), and abnormal haematocrit levels. Overall, the variables that contributed most to the HAI values of the two fish include; abnormal haematocrit values, discoloured liver, and the presence of ecto- and endoparasites. An analysis of the factors used in calculating the HAI values indicated that these necropsy anomalies was higher during autumn and spring (Figure 4.3A–B). The CV for HAI, for the two fish species were higher in winter and summer indicating a lower level of stress experienced by those fish during these sampling periods (Table 4.1). Overall, higher CV was found for *L. rosae* compared to *H. molitrix.* Statistically, seasonal variations between HAI values were observed for *H. molitrix* ($p$<0.05) but there was no seasonal significant differences found for *L. rosae* ($p$>0.05). A two-way ANOVA was performed to determine whether there was seasonal variation in HAI between the two species. The analysis revealed that there was significant difference between species ($p$<0.05), but no significant difference in seasons between the two species ($p$>0.05).

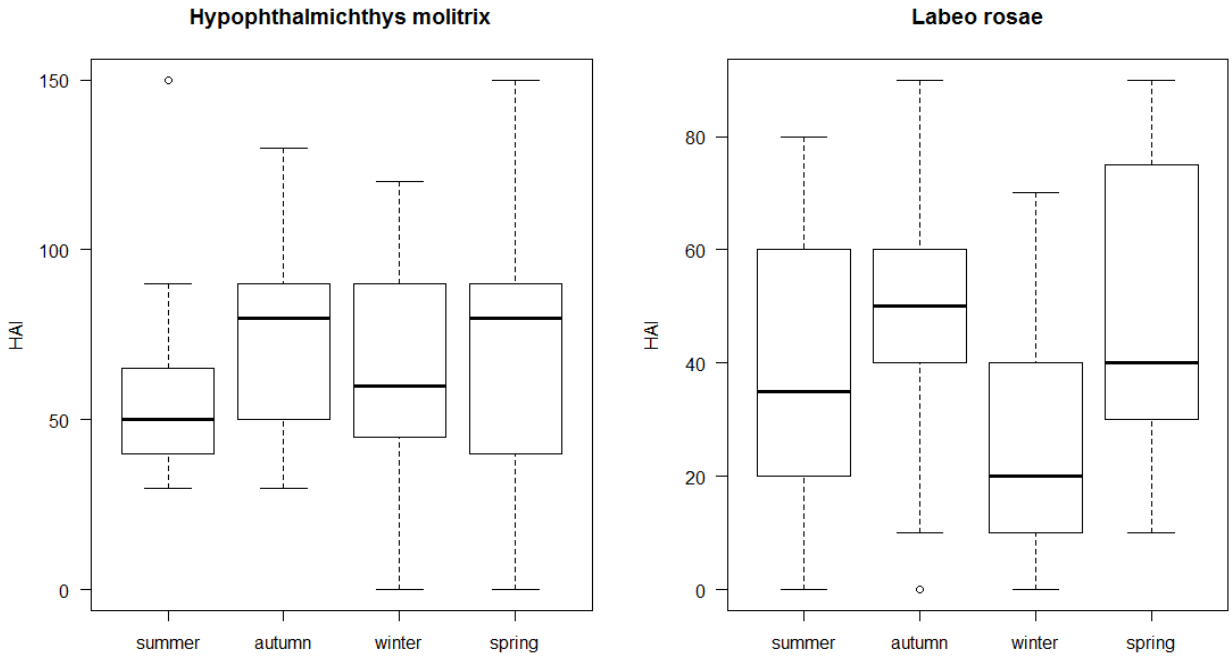


Figure 4.1: Box and whisker plots of Health Assessment Index (HAI) values, without IPI of *Hypophthalmichthys molitrix* and *Labeo rosae* at Flag Boshielo Dam during February 2012–January 2013.

The HAI values with the inclusion of inverted parasite index (IPI) similarly revealed that *H. molitrix* had the highest HAI values that ranged from 60.9–77.1, while *L. rosae* had HAI values ranging from 24.4–33.7 (Table 4.1). In contrast to the HAI not using IPI, significant seasonal variations were observed between HAI values for the two fish species ($p$<0.05). However, similar to the HAI not using IPI, the two way ANOVA analysis revealed that there was no significant difference ($p$>0.05) between species with regard to seasonality.

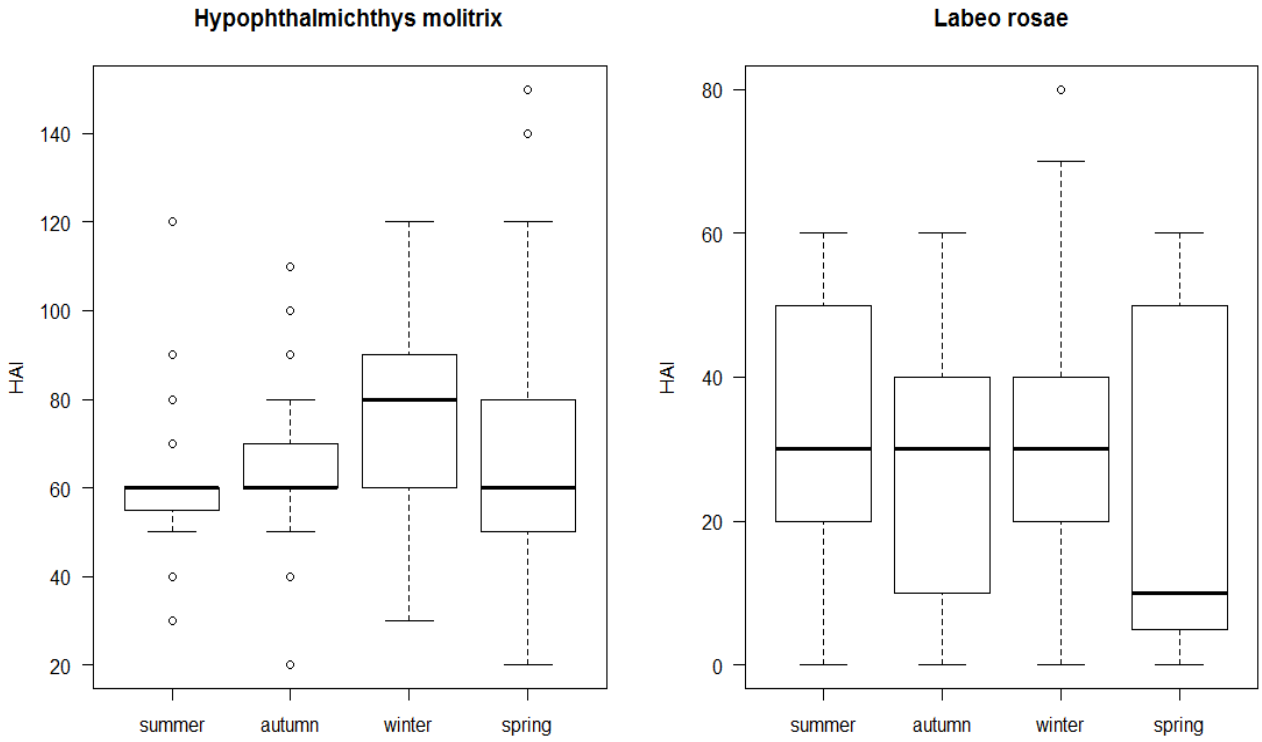


Figure 4.2: Box and whisker plots of Health Assessment Index (HAI) values, using IPI of *Hypophthalmichthys molitrix* and *Labeo rosae* at Flag Boshielo Dam during February 2012–January 2013.

As mentioned in Chapter 1, the present study is the first to assess the health status of *H. molitrix* using the HAI and IPI protocols, thus making it difficult to compare the health status of this species with other fish from different systems for subjectivity. However, the HAI values recorded for *L. rosae* during this study are considerably lower than the 43.5–73.5 recorded for the same species at Loskop Dam and 20–55.3 at Flag Boshielo Dam (Lebepe 2012; Jooste *et al*. 2014). The quality of water entering Loskop Dam is considered polluted, due to the mining and industrial activity within its catchment area (Oberholster *et al*. 2010). In another study, Madanire-Moyo *et al*. (2012a) recorded HAI values that ranged from 42.7–93.3 from three dams in

the Limpopo and Olifants River Systems using one of the generally considered hardiest freshwater fish species in South Africa, *Clarias gariepinus* (Skelton 2001). In that study, mean HAI values varied across the sampling sites with 42.7 recorded from the unpolluted site (Nwanedi-Luphephe Dams), 84 for a moderately impacted site (Flag Boshielo Dam) and 93.3 recorded from a highly impacted site (Return Water Dam). While in another study in the Vaal Dam (unpolluted site) and Vaal River Barrage (polluted site), the HAI scores ranged from 93.0–97.0 and 115.2–117.7, respectively, for the same species (Crafford & Avenant-Oldewage 2009). Although fish species used in the present study and *C. gariepinus* are different, the former is considered a good indicator of species of chronic environmental stress, reflecting cumulative effects of both past and recent water quality conditions. The HAI values in the present study were notably lower than the mean HAI recorded from the above mentioned sites, thus can be considered indicative of a moderately polluted site.

Table 4.1: Seasonal mean Health Assessment Index (HAI) values without using the Inverted parasite Index (IPI) and mean HAI values with IPI of *Hypophthalmichthys molitrix* and *Labeo rosae* at Flag Boshielo Dam during February 2012–January 2013.

| | **Fish species** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Hypophthalmichthys molitrix* | | | | *Labeo rosae* | | | |
| **Variables** | summer | autumn | winter | spring | summer | autumn | winter | spring |
| | | | | **Population HAI without IPI** | | | | |
| **Sample size** | 32 | 21 | 28 | 30 | 30 | 34 | 29 | 23 |
| **HAI** | 55.3 | 74.8 | 63.2 | 72.7 | 36.3 | 47.7 | 25.5 | 47.4 |
| **SD** | 26.03 | 32.19 | 33.12 | 39.56 | 20.25 | 18.59 | 19.38 | 25.62 |
| **CV %** | 47.05 | 43.06 | 52.39 | 54.06 | 54.44 | 39.02 | 75.95 | 55.75 |
| | | | | **Population HAI with IPI** | | | | |
| **Sample size** | 32 | 21 | 28 | 30 | 30 | 34 | 29 | 23 |
| **HAI** | 60.9 | 65.2 | 77.1 | 70 | 33.7 | 25.6 | 31 | 24.4 |
| **SD** | 15.10 | 19.39 | 22.42 | 35.43 | 14.26 | 17.79 | 17.79 | 23.13 |
| **CV %** | 24.79 | 29.73 | 29.06 | 50.61 | 42.35 | 69.51 | 57.35 | 94.97 |

Key: HAI = Health Assessment Index, SD = standard deviation, CV % = coefficient of variation.

#### 4.3.1.1 Percentage of fish with anomalies

During the four seasonal surveys conducted, the HAI results indicated that *H. molitrix* was more affected in terms of abnormal haematocrit values and necropsy-based anomalies, compared to *L. rosae*. The HAI variables that were predominantly responsible for the high prevalence of anomalies were recorded for liver, haematocrit values, gills, ecto- and endoparasites during all seasons (Figure 4.3A).

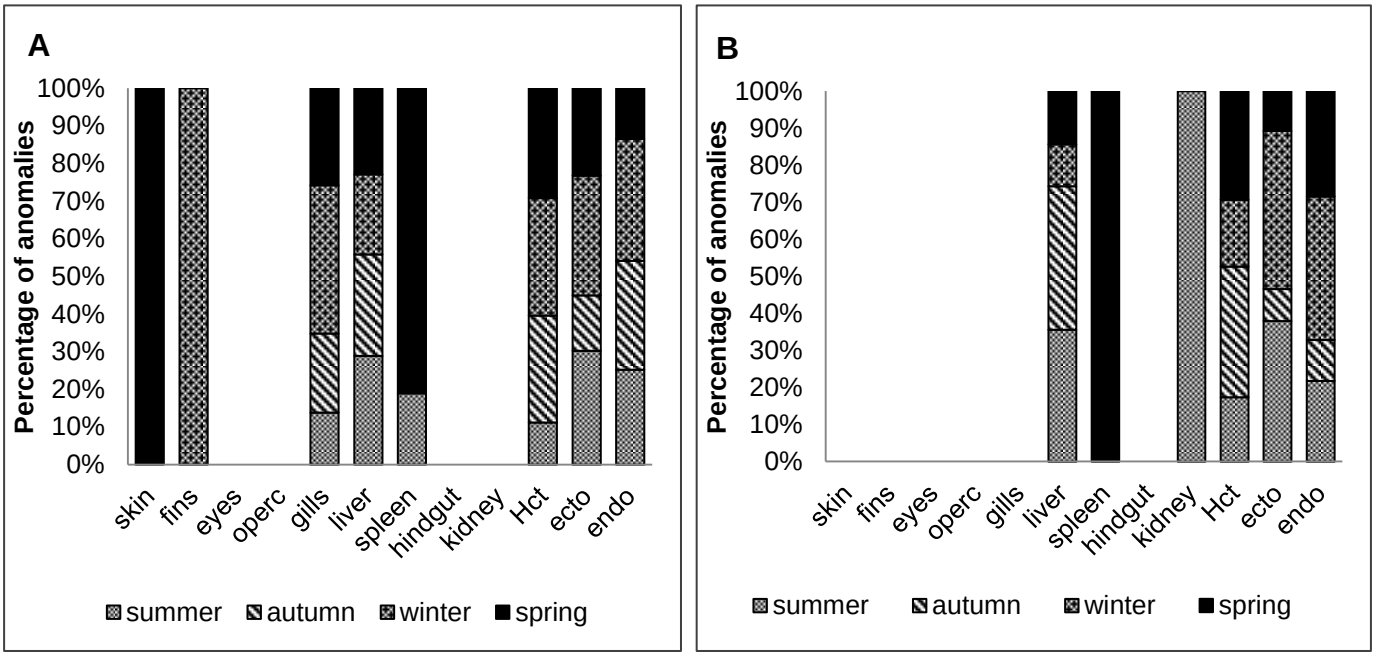


Figure 4.3: Percentage of fish with organ, haematocrit (Hct), endoparasite and inverted ectoparasite abnormalities in samples collected during the four seasons from two selected fish species A- *Hypophthalmichthys molitrix* and B- *Labeo rosae*.

*A*nomalies in the liver of *H. molitrix* included focal discoloration and inflammation. These anomalies were the highest in 96.9% of fish in summer and the lowest 71.4% in winter. Liver anomalies recorded for *L. rosae* included fatty and red liver in 47.1% of fish in autumn and in 13.8% fish specimens during winter. *Hypophthalmichthys molitrix* had the most abnormal haematocrit readings with 78.6% during winter and 28.1% in summer. For *L. rosae* examined, the most abnormal haematoctrit values were recorded during autumn (47.1%) and the lowest (24.1%) during winter. Only *H. molitrix* had gill anomalies during the study. The highest prevalence of gill anomalies was recorded in winter (35.7%) and lowest during autumn (19%), while 3.3% of *H. molitrix* were recorded with skin aberrations and fin anomalies. Both *H. molitrix* and *L. rosae* had no hindgut, opercule, and eyes anomalies. The percentages of fish specimens with the highest number of ectoparasites were in winter and lowest in autumn for both fish species (Figure 4.3A–B). A lower number of endoparasites were retrieved from *H. molitrix* than for *L. rosae*. It is only during autumn that 28.6% of *H. molitrix* were recorded with more endoparasites than 11.8% *L. rosae*. Overall, the two selected cyprinid fish had lower endoparasite numbers than ectoparasites during all the surveys conducted, thus, this parasite data congruent with the IPI principle.

#### 4.3.1.2 External variables

##### 4.3.1.2.1 Skin

The skin is an important organ of fish; it maintains proper osmoregulatory function and act as the first limiting barrier to a wide range of environmental stressors (Tort *et al*. 2003). As such, skin can be subjected to pollution, predation and parasite infections which have been reported in numerous studies (Hinton & Laurén 1990; Madanire-Moyo & Barson 2010; Watson *et al*. 2012) to cause skin lesions and aberrations, leading to secondary infections by bacteria (Tort *et al*. 2003). In the present study only 3.3% of *H. molitrix* during spring showed severe skin aberrations (Figure 4.4A–B). No other aberrations were recorded during the other seasons. These lesions may be due to the intensity of a parasitic copepod (*Ergasilus* sp.) infection. In contrast, no skin aberrations were observed for *L. rosae* during the study even whilst some host were infected with *Ergasilus* sp. recorded attached to the skin, although in much lower numbers than recorded from *H. molitrix*.

##### 4.3.1.2.2 Fins

The fins of fish function in balance, propulsion and paddling. However, several factors such as environmental contaminants, water quality, parasites and bacterial infections can affect the fins of fish (Ziskowski & Murchelano 1975). During the present study only 3.3% of *H. molitrix* during winter were recorded with deformed pectoral fins (Figure 4.4C). Forked and abnormal fins may be a result of genetic variation or they may originate from environmental influences (Reichenbach-Klinke 1973). No fin anomalies were recorded for *L. rosae*.

##### 4.3.1.2.3 Eyes

The eyes are regarded as one of the most important organs of fish as impaired vision could led to increased predation by fish-eating birds and other predators inhabiting that ecosystem (Bush *et al.* 2001). According to Reichenbach-Klinke (1973), invasion of eyes by parasites and bacteria usually results in lesions of the peri-orbital tissue. A digenean metacercaria *Diplostomum* sp. (Figure 4.6E) was recorded in the eye of some specimens of *H. molitrix* during all seasons. While a digenean larvae *Nematobothrium* sp. (Figure 4.6G) were observed in the eye orbit and *Diplostomum* sp. (Figure 4.6F) in the eye of some *L. rosae* collected during the study. No eye anomalies were observed for the two cyprinids, eventhough infection

with *Diplostomum* sp. is know to induce cataracts through mechanical destruction of the lens structure.

#### 4.3.1.2.4 Opercules

The operculum is the hard bony flap covering and protecting the gills. Operculum reacts to vitamin and calcium deficiencies resulting in edge necrosis and deformation (Reichenbach-Klinke 1973). During the study none of the 227 specimens collected exhibited anomalies of the opercula and therefore a value of zero was recorded for all fish (Appendix B: Tables 1–8).

#### 4.3.1.2.5 Gills

Gills are respiratory organs found in many aquatic organisms including fish. Gills are referred to by Heath and Heath (1995) as multipurpose organs directly involved in a variety of functions including respiratory, osmoregulation, acid-base balance and nitrogenous waste excretion. Considering their close contact with the external environment, gills are particularly vulnerable to environmental stressors and parasitic infections (Marcogliese & Pietrock 2011). During the present study gill anomalies were observed only for *H. molitrix*; these included pale-gill, white patches, clubbed, frayed (Figure 4.4D–E) and marginated. No gill lesions or other anomalies were observed for *L. rosae*, despite a high prevalence of monogeneans, *Dactylogyrus pienaari* (Figure 4.6C) found on the gills of most of the host specimens.

### 4.3.1.3 Internal variables

#### 4.3.1.3.1 Liver

The liver is generally considered the largest organ in teleosts, and is composed of hepatocytes and a large mass of glandular tissue (Klaassen 1976). The liver is important in many aspects; it is involved in the uptake, biotransformation and excretion of toxicants (Hinton & Laurén 1990). Thus, the liver is susceptible to a vast number of toxic and metabolic disturbances. Abnormal manifestation of the liver includes fatty liver, tan colour, liver nodules and focal discoloration (Table 2.1). Fatty liver may result from the inability to convert stored fat in hepatocytes to phospholipids or they may be diet-related (Runnells *et al*. 1965). Anomalies noted in the current study included fatty liver and focal discolouration for *H. molitrix* (Figure 4.4G). These anomalies may be an indication of aberration at a specific part of the liver, induced by focal infections, inflammation or necrosis (Goede & Barton 1990).

Similarly, the liver of *L. rosae* exhibited some fatty and focal discoloration anomalies (Figure 4.4H) throughout the study although not as severe as in *H. molitrix*. During the present study, the liver was notably the most affected organ for both selected fish (Appendix B: Table 1–8).

#### 4.3.1.3.2 Hindgut

The hindgut is the final site of digestion and absorption prior to defecation of waste products. Inflammation of the hind portion of the gut may indicate a stress-related infection from facultative pathogens or parasites, but this condition may also be induced by a variety of irritants and toxicants (Goede & Barton 1990). However, in some fish species the cells of the intestine become shrunken and dark, during cyclical periods of starvation, spawning and migration. Thus, some physiological changes may take place in the digestive tract (Ellis *et al*. 1978). During the present study no parasites or pollutant induced anomalies were recorded for the hindgut of the two selected fish species.

#### 4.3.1.3.3 Spleen

The spleen is the only lymph-node like organ in teleost fish and is primarily responsible for blood-cell production and storage (Ellis *et al*. 1978). The enlargement of the spleen and increased vacuolation of hemoblasts may suggest physiological stress exerted on the immune system or could be indicative of disease (Adams *et al*. 1993). Such changes could, presumably, be reflected by change in the gross appearance of the spleen. However, during this study no spleen anomalies were recorded for the two cyprinids at Flag Boshielo Dam.

#### 4.3.1.3.4 Kidney

The kidney of fish is a primary hemopoietic organ responsible for excretion of water (Tort *et al*. 2003) and as such, this organ is often subjected to contaminants in the water. This organ is regarded as one of the major organs involved in immune responses of fish (Dautremepuits *et al*. 2003; Alvarez-Pellitero 2008; Marcogliese & Pietrock 2011). Ellis *et al*. (1978) and Adams *et al*. (1993) assert abnormal conditions of the kidney to be swollen, granular or mottled. During the present study, no kidney anomalies were recorded for both *H. molitrix* and *L. rosae* during all the surveys.


Figure 4.4: Health conditions recorded for the selected fish species at Flag Boshielo Dam. A & B- skin aberrations of *H. molitrix*; C- deformed fins of *H. molitrix*; D- deformed fins of *H. molitrix* and E- white patches on gills of *H. molitrix*; F- gills of *L. rosae*; G- focal discoloration of the liver of *H. molitrix* and H- fatty *L. rosae* liver.

#### 4.3.1.4 Blood analysis

##### 4.3.1.4.1 Haematocrit

Blood is recognised in many studies as an indicator of the physiological state of animals, with no exception to fish. Fish are closely associated with the aquatic environment and blood parameters will reflect the health conditions of the fish prior pathological changes as well as outward manifestation of disease, given that change in the hematological profile may indicate infections or even environmental changes (Blaxhall 1972; Fernandes & Mazon 2003).

Haematocrit is the ratio of the cellular fraction to the total blood volume; therefore, it reflects the percentage of red blood cells to total blood volume (Schuett *et al.* 1997). Haematocrit values vary depending on the health and physiological condition of the individual fish. Adams *et al.* (1993) and Jooste *et al.* (2005) suggested that the normal values range from 30–45% (Table 2.1). However, a species range should be established for each species or groups of similar species for major geographical areas. In the necropsy based study by Goede and Barton (1990) it is assumed that elevated levels of haematocrit may represent a population under stress while low levels indicate the presence of disease. In the present study the haematocrit value of most *H. molitrix* were <18% and as low as 3% for some fish specimens throughout the study (Appendix B: Tables 1–4). In contrast, haematocrit values for *L. rosae* were within the normal range for most of the fish during all the seasons (Appendix B: Tables 5–8). The lower haematocrit values of *H. molitrix* may be a result of acute stress caused by gill damage (Figure 4.4D–E) and impaired osmoregulation (Larsson *et al*. 1985). Subsequently, this anaemic state contributes to a reduced oxygen carrying capacity of the blood, which may result in hypoxia (Madanire-Moyo *et al*. 2012a).

### 4.3.2 Parasites

Parasites are defined as organisms that lives in or on another organism (the host), commonly exhibiting some degree of adaptive structural modification, and for all or some part of its existence deriving food from it (Bush *et al*. 2001). Parasites are ubiquitous and diverse organisms parasitising all organisms, including vertebrates and invertebrates in almost all food webs at all trophic levels and environments (Marcogliese 2005). In other words parasitism is a way of life that transcends all

phylogenetic boundaries. Some researchers refer to this mode of life as the oldest on earth. Parasites usually exist in equilibrium with their hosts as a survival strategy (Bush *et al*. 2001), and this has been supported by a number of studies which indicated that there exists a close and highly susceptible link between environmental conditions and parasitism (Marcogliese 2005; Marcogliese *et al*. 2006; Sures 2008; Madanire-Moyo & Barson 2010; Madanire-Moyo *et al*. 2012b).

For heteroxenous (indirect life cycle) metazoan parasites, environmental conditions must be favourable for all host levels (i.e. intermediate and final hosts) and for free-living stages of the parasites (Madanire-Moyo *et al*. 2012b). Monoxenous (direct life cycle) metazoans, which are normally ectoparasites, are in constant contact with water, suggesting that poor water quality may adversely affect their diversity (Avenant-Oldewage 2001; Madanire-Moyo *et al*. 2012b). The species composition of parasite communities is clearly impacted by environmental stress, and species richness tends to decrease under degraded conditions. Therefore, populations of both heteroxenous and monoxenous parasites are expected to be affected by changing environmental conditions. Due to the great diversity of parasites as well as their trophic relations, fish parasites have been used in many studies (Poulin 1992; Marcogliese & Cone 1997; Marcogliese 2005; Sures 2006) as bioindicators of their hosts' life conditions, integrating the adverse effects as well as reflecting the difference in the composition of their hosts. Furthermore, fish parasites have been reported to thrive in healthy ecosystems and their presence or absence can serve as an indicator tool of ecosystem health (Sures 2001).

#### 4.3.2.1 Parasites collected during the parasitological survey

During this study, seven metazoan parasite species were identified from 8181 parasites specimens collected from the two cyprinid fish species. Of the seven parasite species retrieved, a total number of 4601 parasites were collected from 111 *H. molitrix* fish hosts and 3580 from the 116 *L. rosae* hosts examined. Three ectoparsites were collected from *H. molitrix*, these include; a branchiuran and copepod [*Argulus japonicus* (Figure 4.6A) and *Ergasilus* sp. (Figure 4.6B)] and one digenean endoparasite [*Diplostomum* sp. (Figure 4.6E)]. The *Ergasilus* sp. (Figure 4.6B), was the most abundant and dominant parasite with a total number of 4505 encountered, infecting the skin, gills and fins of 64.1% of *H. molitrix* hosts. The

branchurian *A*. *japonicus* (Figure 4.6A) retrieved during the study was found on the skin and fins of 22% of the fish examined. A total of 4512 ectoparasites were collected from *H. molitrix* during the present study while 89 endoparasites specimens were collected. The endoparasite was a digenean metacercaria, *Diplostomum* sp. (Figure 4.6E), recorded from the eyes of 22.1% of the hosts. From all the *H. molitrix* examined, only 0.9% (*n* = 1) were infected with all three parasite species recorded (Figure 4.5A). Most importantly, *Diplostomum* sp. found in the eye of *H. molitrix* is a new host and locality record for South Africa. The *Ergasilus* sp. and *A*. *japonicus* recorded from *H. molitrix* are new host records for South Africa.

A total of 116 *L. rosae* host specimens were collected and 3580 parasites were retrieved consisting of all seven parasite species collected during the present study. These included four ectoparasite and three endoparasite species. The ectoparasites collected included monogeneans i.e. *Dactylogyrus pienaari* (Figure 4.6D) and *Paradiplozoon* sp. (Figure 4.6C) collected from the gills, a copepod (*Ergasilus* sp.) (Figure 4.6B) infecting the skin, fins and gills, and a branchurian (*A. japonicus*) (Figure 4.6A) collected from the skin and fins of the fish. Three endoparasite species included a digenean metacercaria, *Diplostomum* sp. (Figure 4.6F), recovered from the eye, a digenean, *Nematobothrium* sp. (Figure 4.6G), collected from the orbit of the eye and a nematode, *Paracamallanus cyathopharynx* (Figure 4.6H) collected from the intestine. A total of 3510 ectoparasite and 70 endoparasite specimens were collected from *L. rosae*, of which the monogenean *D. pienaari* was the most abundant parasite with a total number of 3094 specimens, infecting 96.3% of the hosts examined. During the study 92% (*n* = 89) of *L. rosae* examined were infected with one to four, but not more than five parasite species (Figure 4.5A). Moreover, none of the fish hosts examined were infected with all seven parasites recorded during the parasitological investigation at Flag Boshielo Dam (Figure 4.5B). All parasites recorded from *L. rosae* during this study have been previously recorded for by Kekana (2012) and Lebepe (2012) at Flag Boshielo Dam.

During this study a larger number of ectoparasites were recorded from *H. molitrix* than for *L. rosae*; however, a higher diversity of ectoparasite species were recorded for *L. rosae* throughout the study. In contrast, a higher number and diversity of endoparasites were recorded for *L. rosae*. It should be noted that even though *L.*

*rosae* had larger number and greater diversity of endoparasites than *H. molitrix*, both fish species exhibited more ectoparasites than endoparasites. The findings in this study are congruent with the IPI premise. In addition, the parasite component community, species richness and diversity are reasonably similar to those recorded by Madanire-Moyo *et al*. (2012a) and Jooste *et al*. (2014). These studies refer to Flag Boshielo Dam as a moderately polluted site in an oligotrophic to eutrophic state, which requires extensive monitoring and proper management to conserve and sustain biodiversity.

Parasite component community refers to the number of species present in a sample from a single locality (Sousa 1994). Species richness is defined as the actual number of different species in a single locality. Species diversity reflects the value that relates both the number of species, and their abundances, in a locality (Bush *et al*. 2001).

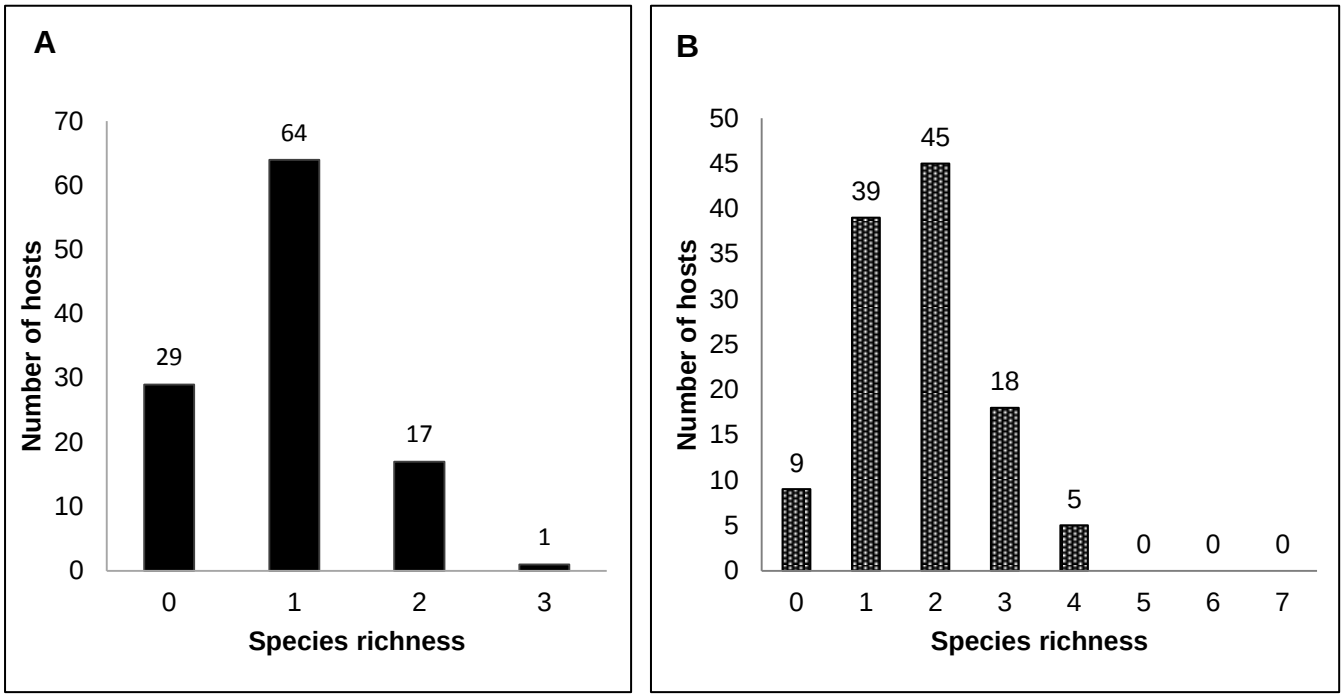


Figure 4.5: Frequency distribution of metazoan parasite species in A- 111 specimens of *Hypophthalmichthys molitrix* and B- 116 *Labeo rosae* at Flag Boshielo Dam during February 2012–January 2013.

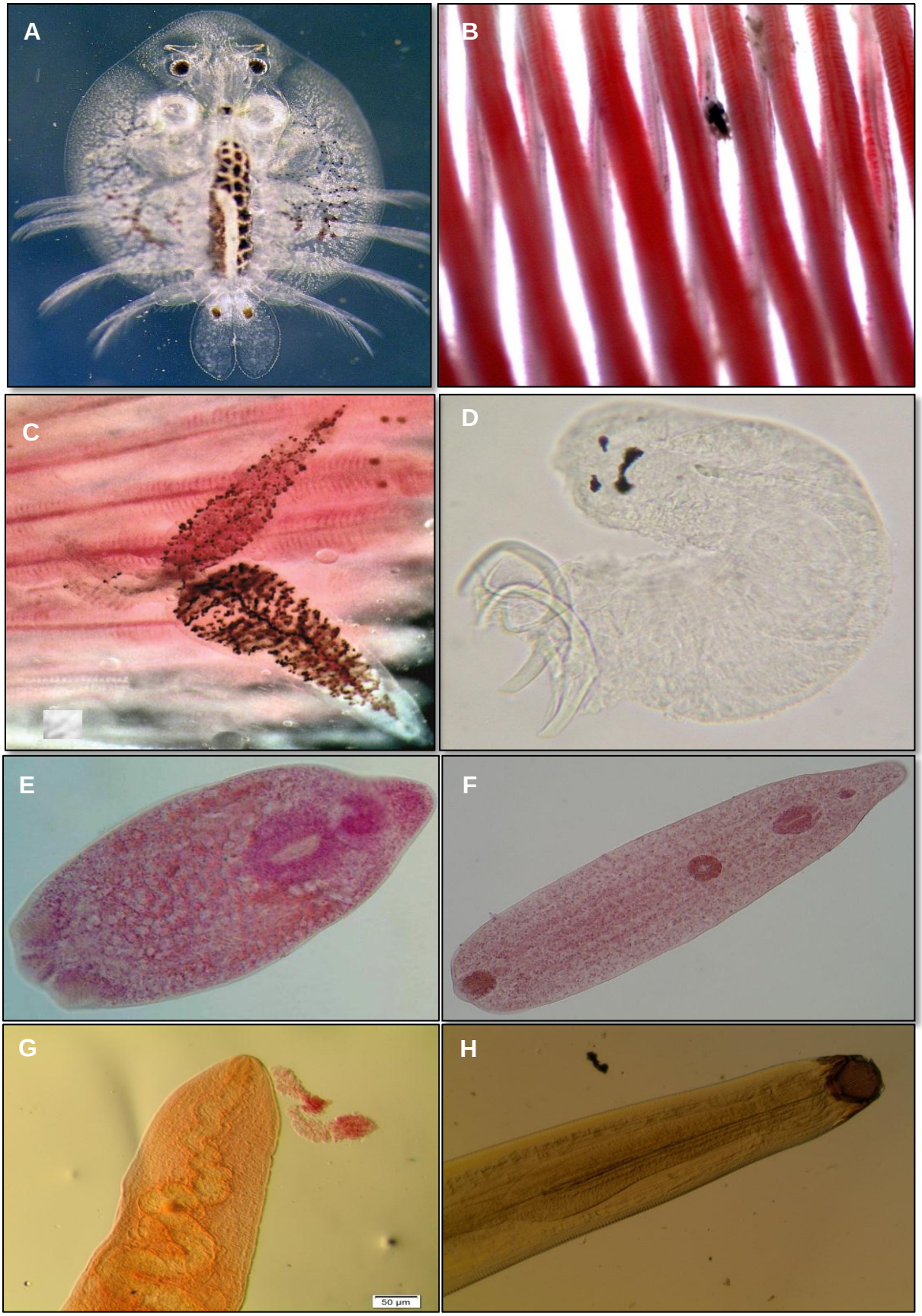


Figure 4.6: Parasites collected during the study. A- *Argulus japonicus* and B- *Ergasilus* sp. from *Hypophthalmichthys molitrix* and *Labeo rosae*; C- *Paradiplozoon* sp.; D- *Dactylogyrus pienaari* from *L. rosae*; E- *Diplostomum* sp. from *H. molitrix*; F- *Diplostomum* sp.; G- *Nematobothrium* sp.; H- *Paracamallanus cyathopharynx* from *L. rosae*.

#### 4.3.2.2 Infection statistics

The infection parameters (prevalence, mean abundance and mean intensity) for each of the parasite species recorded were calculated according to methods of Bush *et al*. (1997). In the present study comparisons between seasons and species indicated that parasite burden varied throughout the study. The infection statistics for the respective fish species are presented in Figure 4.7A–F.

For *H. molitrix*, the copepod *Ergasilus* sp. had the highest prevalence throughout the study. The high prevalence of the monoxenous *Ergasilus* sp. could be attributed to the presence of a suitable definitive host (fish) in its life cycle. The prevalence for *Ergasilus* sp. were the highest during spring (73.3%) and lowest in winter (50%). The damage caused by parasites to their host is generally related to the intensity of infection (the number of parasites within the infected host) and depth of parasite penetration within the host tissue (Howe *et al*. 1997). The mean abundance and mean intensity were the highest during autumn and the lowest in winter, with 88.9, 9.7 and 124.4, 19.4, respectively. In a number of studies, authors such as Kabata (1970) and Vinobaba (2007) highlighted that low numbers of *Ergasilus* is tolerated by most hosts unless additional factors contribute to the pathogenity. Thus, peaks of this copepod during autumn concide with the decline of water quality, subsequently high HAI, IPI and lower K recorded during this period of sampling.

The prevalence of the *Diplostomum* sp. was the highest during winter (28.6%) and the lowest in spring (16.7%). While, the mean abundance was the highest during winter (1.96) and the lowest in summer (0.05). Mean intensity was recorded highest during winter (6.9) and lowest in winter (1). *Diplostomum* sp. has been recorded for *H. molitrix* in China and Iran (Woo 1995) with lens and cataract damages noted with the presence of this digenean. In the present study no fish was observed with cataract damages caused by *Diplostomum* sp. *Argulus japonicus* was the parasite recorded in lower numbers for *H. molitrix*, with the highest prevalence of 14.3% recorded during autumn and no branchiurans recorded during winter. The mean abundance and mean intensity were highest during autumn (1.67, 0) and lowest in winter (0.24, 0). Although, Avenant-Oldewage and Everts (2010) and Madanire-Moyo *et al*. (2012a) describes *A. japonicus* as an opportunistic ectoparasite with infections that can reach severe proportions in a very short time leading to severe

skin aberrations and in some cases catastrophic kills, none of the fish examined during the present study had any skin aberrations that were caused by this parasite.

For *L. rosae*, monogenean, *D*. *pienaari* had the highest prevalence recorded during spring (100%) and lowest in winter (72.4%). The high prevalence of *D. pienaari* recorded during this study could be that this parasite species demonstrate a high degree of host specificity parasitizing a single host species (Olivier *et al*. 2009). The mean abundance was the highest during autumn (45.6) and notably lowest in winter (9.07). Similarly, the mean intensity was highest in autumn (50.1) and lowest in winter (12.5). *Ergasilus* sp. was the second most dominant parasite for *L. rosae* with the highest prevalence of 66.7% in summer and lowest in winter (34.5%). The mean abundance and mean intensity were the highest during autumn (6.65, 0.38) and the lowest in winter (10.3, 1.1), respectively. *Argulus japonicus* was recorded with the highest prevalence during summer (10%) and the lowest in spring (0%). Similarly the mean abundance and mean intensity were highest in summer (0.1, 0) and lowest in spring (1, 0). In the present study, the ectoparasite with the lowest prevalence was *Paradiplozoon* sp. with the highest prevalence 4.3% in spring and the lowest 0% in almost all the seasons for *L. rosae* (Figure 4.7D). The mean abundance and mean intensity were the highest during autumn and lowest in spring (Figure 4.7E–F).

The endoparasite recorded with the highest prevalence was *Nematobothrium* sp. during winter (55.2%) and considerably lower in autumn (8.8%). The mean abundance was the highest during spring (1.6) and the lowest in autumn (0.32). In contrast, the mean intensity for *Nematobothrium* sp. was the highest during autumn (3.7) and lowest in winter and spring (both 1.6). The nematode *Paracamallanus cyathopharynx* is the endoparasite with the lowest prevalence throughout the period of investigation with the highest prevalence of 6.7% during summer and lowest in autumn and winter (0%) for *L. rosae*. In general, it can be noted that the component community of the helminth parasites of fish from Flag Boshielo Dam indicated that there are high levels of species richness and relatively high mean abundance levels of most species, although very low mean intensity levels were recored for some metazoan parasites (e.g. *Paradiplozoon* sp. and *P. cyathopharynx* of *L. rosae*).

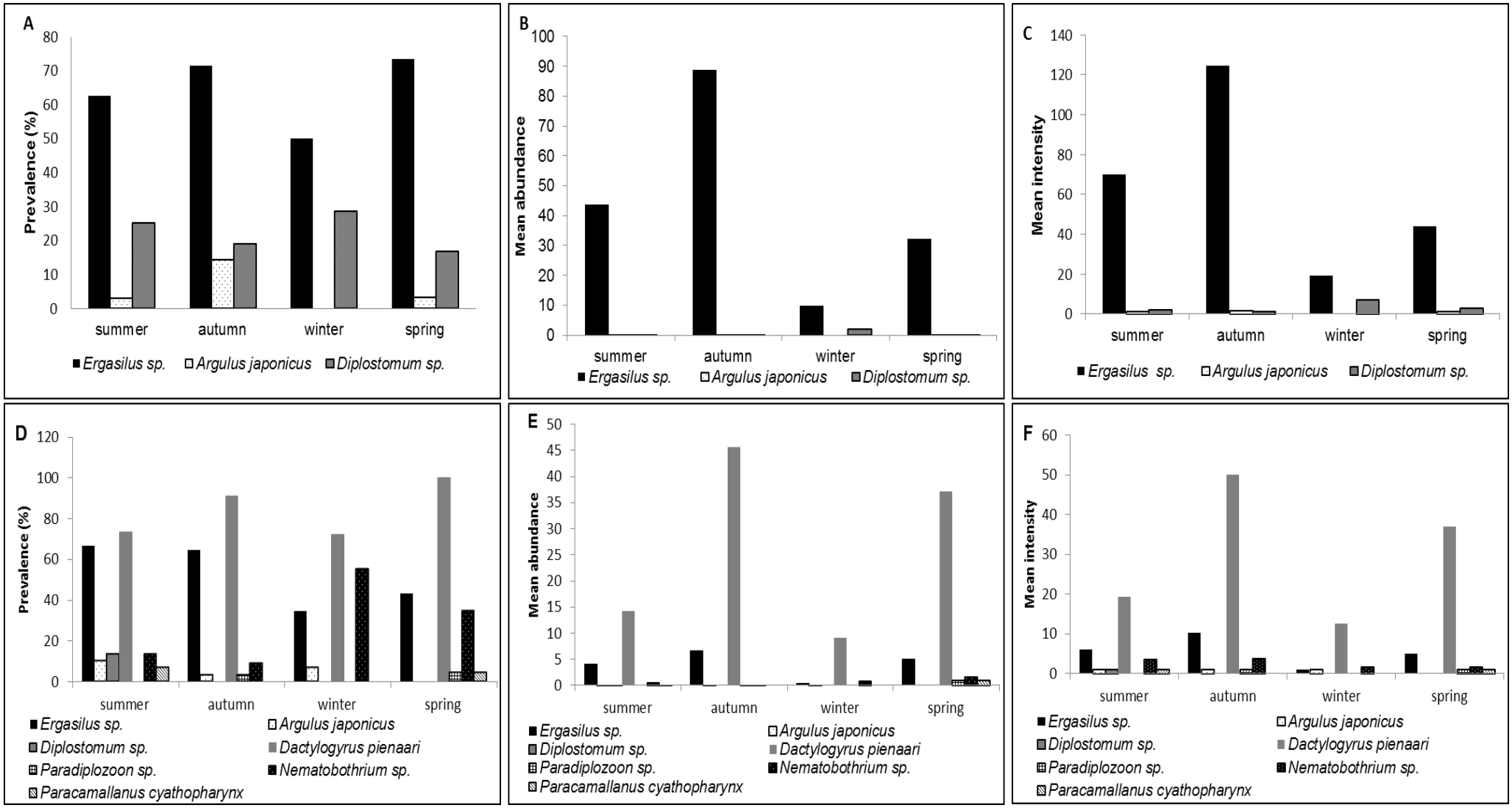


Figure 4.7: Infection parameter statistics (A- Prevalence, B- Mean abundance and C- Mean intensity) of the parasites collected for *Hypophthalmichthys molitrix* and (D- Prevalence, E- Mean abundance, F- Mean intensity) for *Labeo rosae* at Flag Boshielo Dam.

### 4.3.3 Condition factor

Condition factor (K) or Fulton's condition index represents the mass of an individual relative to its body length. In other words, it is a length-weight relationship based on the premises that heavier fish of a given length are in good energetic state and better condition (Neff & Cargnelli 2004). This index has been recognised and used as a health assessment component to evaluate bioenergetics status and the effect of stress, be it biotic or abiotic on the health of fish populations (Klemm *et al*. 1995; Adams *et al*. 1993; Dirican *et al*. 2012). However, fish exhibit natural fluctuations in K due to a variety of factors such as age, sex, species, stage of maturation, degree of muscular development, fat reserves and season (Barnhan & Baxter 1998; Jenkins 2004). A K value of 1.00 is classified as ideal and indicates fish with good health conditions (Doyon *et al*. 1988). This value is; however, dependent on the fish species due to variation in length-weight relationship of different species. Thus, K cannot be compared between different species; as such K of the two selected fish species in this study will be compared individually between different seasons.

The length-weight relationship calculated for the two fish species (Table 4.2), indicated that *L. rosae* was found with positive allometric growth (b>3), while *H. molitrix* was found with negative allometric growth (b<3). The K values closest to the ideal value (1.00) for the two fish were recorded for *L. rosae*, with the mean value of 1.17, as compared to 0.82 for *H. molitrix* (Table 4.2). The K values for *L. rosae* during the study are reasonably similar to values recorded by Lebepe (2012) and Jooste *et al*. (2014) for the same species at Flag Boshielo Dam, with mean values of 1.02 and 1.05, respectively. The K value ranged from 1.00–1.10, with the highest value recorded during winter and the lowest in autumn. For *H. molitrix*, K values were found to be considerably less than 1.00 throughout the study (Figure 4.8A) at Flag Boshielo Dam. The highest K value was recorded during winter (0.86) and the lowest in autumn (0.66). However, due to the lack of health assessment studies done on *H. molitrix* in South Africa, K values were compared to those of *H. molitrix* from the Amu Darya River, Canada, with mean values of 2.29 (Naseka & Bogutskaya 2011) and 1.96 in Iran (Jalali 1997). Since these studies are undertaken in other countries, the geographical differences of the host species are also likely to have an influence in the K.

Statistically, no significant difference was found for both species in relation to seasonality ($p$>0.05). Seasonal fluctuations of K reflect of changes in feeding activity, nutrient availability or an increase in metabolic rate in response to certain stressors (Adams 1976). The decline and spike of K values in autumn and winter of the two fish species appear to indicate that there is a relationship between K, parasites abundance, water quality and oxidative stress biomarkers and this was confirmed in Chapter 5, using principal component analysis (PCA) (Figure 5.6). Given the intensity of infection (the number of parasites within the infected hosts) during the present study at Flag Boshielo Dam, regression analysis was used to determine the effect of parasite burden on K of the two fish species (Figures 4.9–4.10).

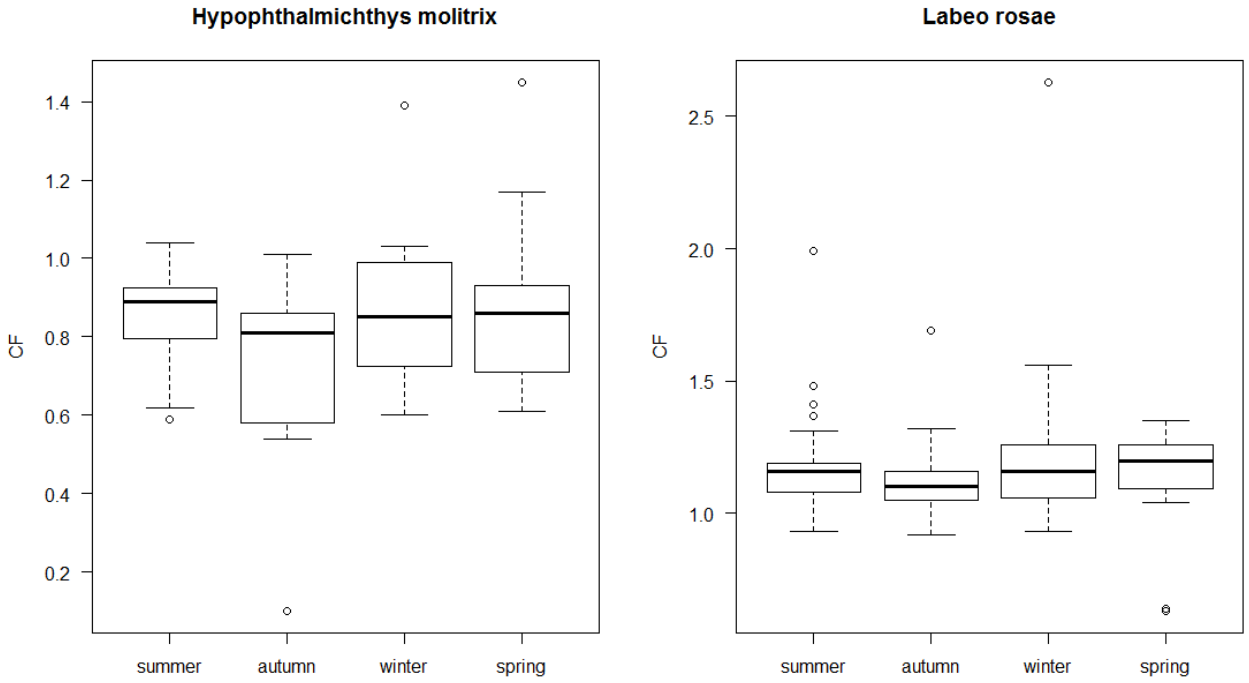


Figure 4.8: Box and whisker plots illustrating seasonal variations in condition factor of the two cyprinid fish of Flag Boshielo Dam. A- *Hypophthalmichthys molitrix*, B- *Labeo rosae*.

This analysis indicated that parasite burden was negatively related with K for both *H. molitrix* and *L. rosae*. For *H. molitrix* the effect of parasites on K was $y = -0.0005\times + 0.8403$ and contributed 5% to the variance in K (Figure 4.9). The effect of parasite on K for *L. rosae* was $y = -0.0004\times + 1.1812$ with the contribution of 0.4% to the variance in K (Figure 4.10). In general there was no significant variation ($p$>0.05) between parasite burden and K. However, it is important to note that the effect of

parasites on *H. molitrix* was higher compared to those for *L. rosae*, with slightly more negative relation and lower K.

Table 4.2: Mean body length, mean body mass and mean condition factor of *Hypophthalmichthys molitrix* and *Labeo rosae* at Flag Boshielo Dam during February 2012–January 2013.

| **Variable/ Parameter** | **Fish species** | |
|---|---|---|
| | *Hypophthalmichthys molitrix* | *Labeo rosae* |
| **Sample size (*n*)** | 111 | 116 |
| **Mean sex ratio (F%: M%)** | 84:27 | 83:33 |
| **Mean body length (mm)** | 63.69±22.83 | 29.41±3.99 |
| **Mean body mass (g)** | 2695.33±2241.52 | 309.76±129.99 |
| **Condition factor (K)** | 0.82±0.20 | 1.17±0.21 |
| **Length-weight relationship** | **Weight** = 0.0283 ***SL** $^{2.6919}$ | **Weight** = 0.0107 ***SL** $^{3.0184}$ |
| **Adjusted $R^2$** | 0.9356 | 0.8895 |

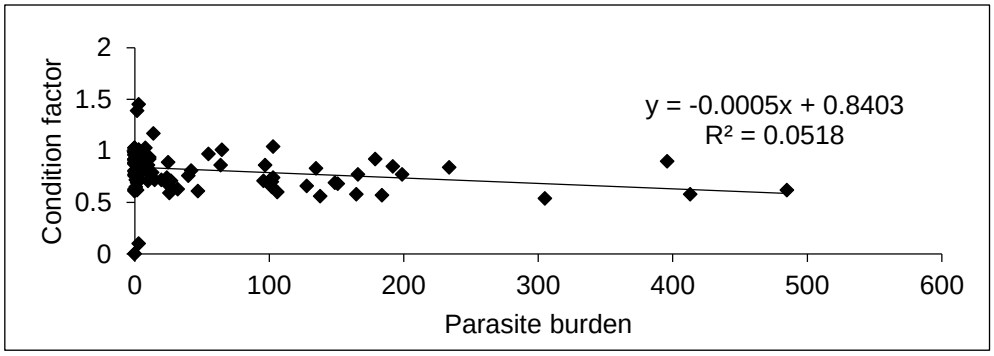


Figure 4.9: Regression analysis showing the effect of parasite burden on condition factor of *Hypophthalmichthys molitrix*.

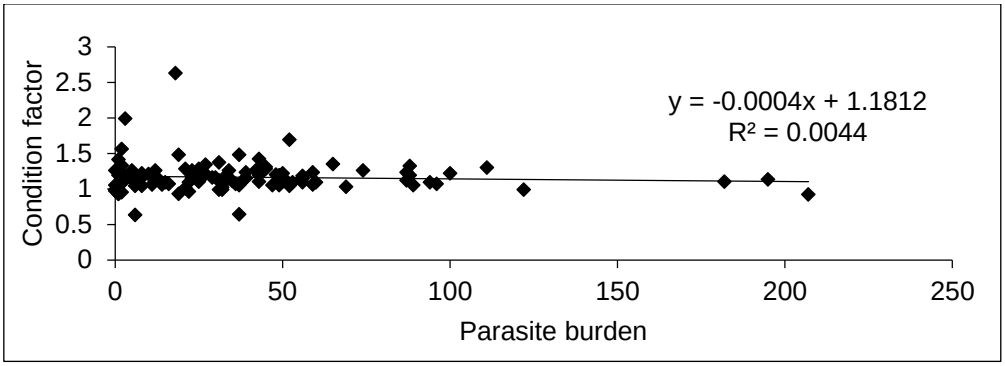


Figure 4.10: Regression analysis showing the effect of parasite burden on condition factor of *Labeo rosae.*

## 4.4 CONCLUSION

This component of the study addressed the health status of the two fish species at Flag Boshielo Dam as determined by using the fish HAI, PI and IPI and K indices of fish health. Although there is substantial evidence of progressive and sporadic fish kills in the Olifants River in the past decade, particularly die-offs of Mozambique tilapia (*Oreochromis mossambicus*) and the reported 14 tons of predominantly *L. rosae* fish in Loskop Dam during 2007–2008. The two selected fish species in the present study, *H*. *molitrix* and *L. rosae* appear to be in overall good health with only relatively high HAI, IPI and low K recorded for *H. molitrix* compared to *L. rosae*.

Fish health assessment parameters used in conjuction with the water quality analysis indicate that the health of the two selected cyprinids is comprised to a certain extent by the relatively high levels of xenobiotics (slightly above the TWQR) during the summer and spring seasons. Compared to other comprehensive studies conducted previously (2009–2011) at Flag Boshielo Dam, *L. rosae* is exhibiting lower HAI values. Although no studies exist in South Africa that highlight the health profile of *H. molitrix*, the health of this fish remains of concern with low K and necropsy anomalies (liver, gill and skin aberrations and abnormal haematocrit levels). Moreover, the changing trophic status at Flag Boshielo Dam from oligotrophic to mesotrophic conditions signals continuous monitoring of the vast majority of the fish populations in this dam to assess the health and evaluate impacts of environmental disturbances on the aquatic biota.

# CHAPTER 5 - BIOMARKERS

## 5.1 INTRODUCTION

The deleterious effects of pollutants are often difficult to detect since many manifest after prolonged exposure (chronic toxicity), thus promped research to establish early-warning signals, or biomarkers (Van Der Oost *et al*. 2003). Biomarkers are measurements of biological materials ranging from biomolecules (i.e. enzymes) to organelles, cells, tissues and whole organisms (Venter *et al*. 2004). A biomarker can thus be defined as any biological response (from molecular, cellular, physiological to behavioural changes) measured inside an organism reflecting an interaction between a biological system and a stressor, which may be chemical, physical or biological (WHO 1993; Van Der Oost *et al*. 2003).

In aquatic ecosystems, especially in natural conditions, no organism will only be affected by either pollution or parasites (Sures 2008). Pollution and parasites affect the health of organisms (fish, amphibians, crustacens, and mammals), with some similarly responses (defense mechanisms) against both stressors. Immune response biomarkers have been used as one of the standard approaches that can quantify the effects and assist in elucidating particular mechanisms associated with these stressors. In fish, parasites particularly, metazoan parasites have been shown in a number of comprehensive studies (Thilakaratne *et al*. 2007; Dautremepuits *et al*. 2009; Marcogliese & Pietrock 2011) to exacerbate detrimental effects when their hosts are stressed. Parasites usually exist in equilibrium with their hosts as a survival strategy (Bush *et al*. 2001); however, as hosts evolve to fight off these organisms, so do parasites, by using different strategies in order to penetrate and evade their host's immune defenses for the uptake of nutrients (Sitjà-Bobadilla *et al*. 2008; Kiron 2012). Thereby, impact the biochemical, physiological and metabolic activities of their host, consequently, affecting the health of their hosts (directly or indirectly).

Innate immunity, compared to adaptive immunity is recognised as the non-specific immune meachanism, independent upon previous recognision of the surface structure of the invader (Tort *et al*. 2003; Alvarez-Pellitero 2008). The innate

response is generally divided into cellular and humoral, and provides the first line of immune defense (Aoki *et al*. 2008). Recent enhanced interest in these defense mechanisms led to recognition of its suitability as a biomarker for assessing adverse biological effects of certain environmental stressors (Alvarez-Pellitero 2008; Marcoglise & Pietrock 2011). Inflammatory reactions involving cellular reactions (including oxidative mechanisms) are modulated by many parasites. Parasite infections can lead to synergistic effects resulting in interference of several metabolic pathways of cells such as destructed antioxidant metabolism (Neves *et al*. 2000; Dautremepuits *et al*. 2003; Frank *et al*. 2011). For example, several studies (Belló *et al*. 2000; Dautremepuits *et al*. 2003; Marcogliese *et al*. 2005) have demonstrated that metazoan parasites such as monogeneans, digeneans, nematodes, cestodes and crustaceans infecting fish and invertebrates can affect antioxidant metabolisms consequently induce oxidative stress. Nonetheless, several enzymatic and non-enzymatic defense systems commonly referred to as oxidative stress biomarkers, with antioxidant capacities are presented by fish as an immune response mechanism to parasite infections (Del Maestro 1980; Sies 1993).

Oxidative stress is a physiological condition that occurs as a result of an imbalance between concentrations of reactive oxygen species (ROS) and depletion of antioxidant defenses (Storey 1996; Tagliari *et al*. 2004). Reactive oxygen species include free radicals such as superoxide formation anion ($O^{-}_{2}$), hydroxyl radical ($OH^{-}$) and hydrogen peroxide ($H_2O_2$), which may generate DNA alterations and lipid peroxidation initiating scavenger compounds like enzymatic antioxidant defenses; i.e. catalase (CAT), superoxide dismutase (SOD), total antioxidant capacity (TAC) and glutathione S-transferase (GST), among others (Storey 1996). These cell defense systems can be measured as biomarkers of xenobiotic mediated oxidative stress. Thus, the health of aquatic organisms such as fish can be linked to production of ROS and antioxidant compounds, such as antioxidant enzymes. For instance, in a study by Dautremepuits *et al*. (2003), an increase in antioxidant enzyme (GST, CAT and GPx) levels were reported in common carp (*Cyprinus carpio*) infected with a cestode (*Ptychobothrium* sp.) compared to uninfected fish. While in another study, Marcogliese *et al*. (2005) recorded higher levels of lipid peroxidation in the liver of *Perca flavescens* infected with *Raphidascaris acus* and *Apophallus brevis* than in an uninfected fish collected at the same locality.

The primary objective of this section is to evaluate, and obtain insights on the oxidative stress biomarkers (lipid peroxidation, GST and TAC) in the gill and liver tissue of *H. molitrix* and *L. rosae*, to assess how these major organs (and immune system) respond to oxidative stress associated with parasitic infections. In addition, the last component of this section is composed of results on correlation of the different water quality parameters, parasites abundance, oxidative stress biomarkers and health parameters used for the two fish species, illustrated as an ordination pattern.

## 5.2 METHODS AND MATERIALS

### 5.2.1 Field procedures

Following health assessments and examination of parasites; gill and liver tissue samples of *H. molitrix* and *L. rosae* were placed in cryotubes and frozen in liquid nitrogen at the field laboratory, and later placed at −80°C in a bio-freezer in the laboratory at the University of Limpopo prior to further analysis of different oxidative stress biomarkers at the Stellenbosch University. All oxidative stress biomarkers analyses were done according to standard methods using various assay kits.

### 5.2.2 Laboratory procedures

In the laboratory, the tissue samples of each fish species were weighed to 0.1 g and washed 3 times with 1× Phosphate Buffer Saline (PBS), (pH 7.4) and centrifuged at 10 000 × g for 2 minutes at 4°C to remove nuclei, mucus and cell debris. All protein analyses were completed according to standard methods using various assay kits. As a measure of lipid peroxidation, its reactive compound malondialdehyde (MDA) were determined with the use of OxiSelect™ Thiobarbituric Acid Reactive Substances (TBARS) assay kit according to the method of Ohkawa *et al*. (1979). The activity of antioxidant enzyme GST was measured using a GST assay kit as suggested by Habig *et al*. (1974). While CellBiolabs' OxiSelect™ TAC assay kit was used to measure the total antioxidant of biomolecules for the samples as described by Frei *et al*. (1992). The samples were assayed in triplicates unless indicated otherwise. The biomarker analysis procedures are given in detail in Chapter 2.

### 5.2.3 Data analysis

All raw data of oxidative stress biomarkers is presented in Appendix C. To determine the effect of metazoan parasites on oxidative stress biomarkers, during all four seasons, each fish species were grouped into two categories depending upon the frequency distribution of infection intensity of parasites: uninfected/infected. The cut-off intensity was that which divided all fish into relatively equal sample sizes. Oxidative stress data were presented as mean±standard deviation (SD). Differences in the means between species and seasons were subjected to student *t*-test and analysed. The data were considered statistically significant for the 95% level ($p<0.05$). Regression analysis was performed to determine the potential correlations between parasite abundance (number of parasites per host) of the two fish species and all the oxidative stress variables of the data sets. Lastly, principal component analysis (PCA) was used on all the data to express the results of the correlation of the different health parameters for the two fish species as an ordination pattern to show certain (dis)similarities between each other in terms of the changes of the health status and response to oxidative stress biomarkers, and the different water quality parameters assessed. These analysis were conducted using vegan aov () function in the R statistical package (R Development core team 2012).

## 5.3 RESULTS AND DISCUSSION

### 5.3.1 Glutathione S- transferase

Glutathione S-transferase is a group of antioxidant enzymes important in the detoxication of many different xenobiotics. These are antioxidant compounds produced as a mechanism to counteract an imbalance in redox state, thus tissue and DNA damage, to protect against oxidative stress (Dautremepuits *et al*. 2009; Frank *et al*. 2011). Enzyme activity changes are used as indicators of tissue damage and exposure of animal biomarkers to elevated concentrations of pollution (Ozmen & Gungordu 2006). However, under natural conditions aquatic organisms are equally affected by pollution as they are by parasites (Marcogliese *et al*. 2005; Sures 2008). These stressors may interact and act additively or synergestically on the health of an organism rendering poor health. For instance, Dautremepuits *et al*. (2003) reported an increase of GST activities in *C. carpio* infected with *Ptychobothrium* sp. compared to uninfected fish of the same species at a contaminated site than at a reference site.

The results of the GST activities recorded during the present study were higher in autumn for both fish as compared to the other seasons (Figure 5.1A–B). These findings correlates with numerous water quality variables (Figure 5.6), most of which in excess have the ability to cause adversely affect the health of the fish and lead to altered biochemical and metabolic activities. Statistically, significant difference was not found for *H. molitrix* in relation to seasonality, and the two assessed organs (liver and gill) with an exception of the gill tissue during autumn. However, significant difference was found between infected and non-infected fish during autumn and spring as compared to the other three seasons ($p$<0.05).

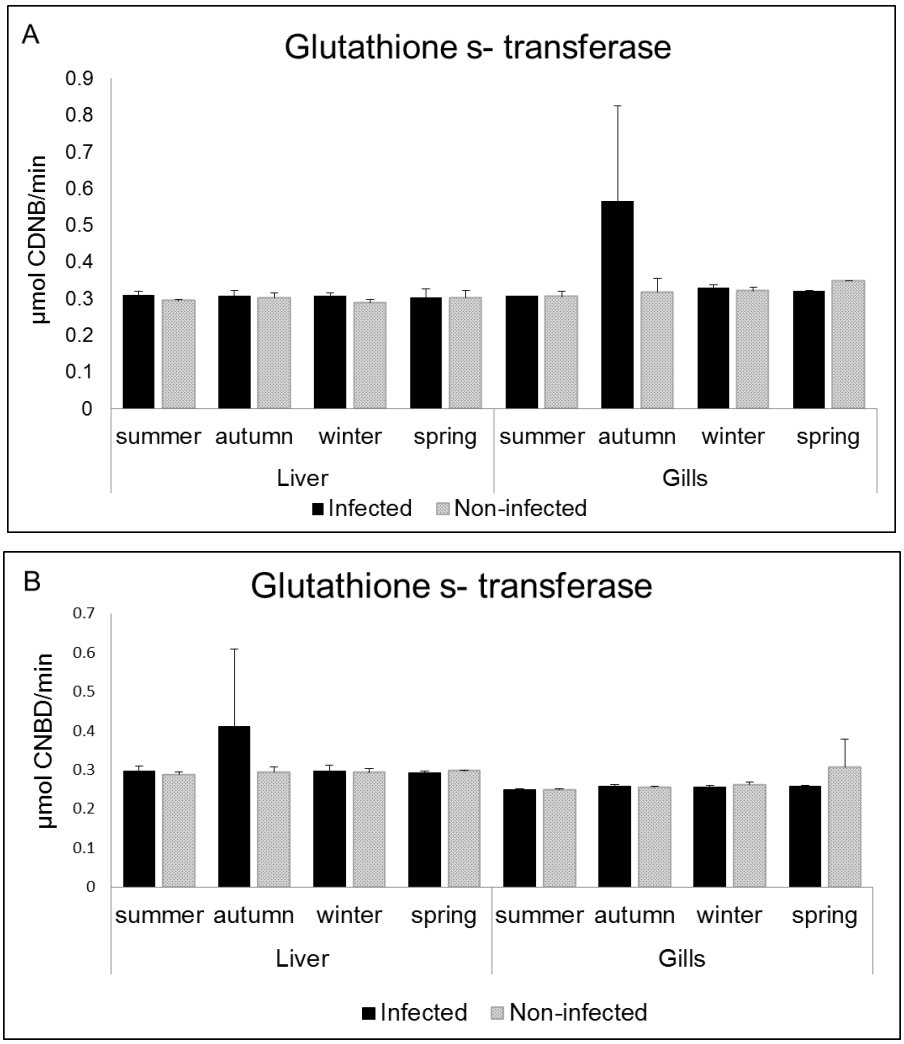


Figure 5.1: Seasonal variation of glutathione S-transferase (GST) activities in the liver and gills tissue of A- *Hypophthalmichthys molitrix* and B- *Labeo rosae* at Flag Boshielo Dam. Data are expressed as mean±SD.

In *L. rosae* significant differences were observed between seasons and the two organs, and similar to *H. molitrix*, differences was found between the infected and

non-infected fish, particularly the spike during autumn for the infected fish in liver. The two-way ANOVA analysis performed to determine the GST activity differences between the two fish species revealed that there was no significant difference observed. In contrast to the few studies by Dautremepuits *et al*. (2003); Marcogliese *et al*. (2005) and Marcogliese and Pietrock (2011) reporting on the modulation of GST activity by parasites with values of 0.9–1.2. In this study, the antioxidant response remained considerably low (0.24–0.32) with the exception of autumn (0.43–0.6). This increased antioxidant response in parasitised fish might be due to the striking impact reported for the copepod *Ergasilus* sp. on the gills of *H. molitrix* and other food fish (Jalali 1997; Vinobaba *et al*. 2007; Singh & Kaur 2014). The increased GST activity in the liver of *L. rosae* can be that the liver of this fish is efficient to counteract the oxidative stress experienced. The results denote different patterns of antioxidant enzyme response, suggesting that the spike of parasites and pollutants during autumn may have induced pro-oxidant responses and each fish responded depending on its ability to produce ROS and antioxidant enzymes detoxifying them.

### 5.3.2 Lipid peroxidation

Lipid peroxidation refers to the process in which oxidative degradation of lipids occurs as a result of excess free radicals in the cell membrane. Parasite infections and pollution have been reported to induce oxidative stress and lipid peroxidation in fish as a result of an imbalance between pro-oxidants and non-enzymatic antioxidants (Bellό *et al*. 2000; Marcogliese *et al*. 2010). In a study by Bellό *et al*. (2000) it was indicated that *Clinostomum detruncatum* infection reduces the non-enzymatic antioxidant defenses with respect to pro-oxidant production in the muscle of the freshwater teleost *Rhamdia quelen* and resulted in lipid peroxidation. In addition, Marcogliese *et al*. (2005) indicated that at a contaminated sites, levels of lipid peroxidation in the liver of *Perca flavescens* infected with *Raphidascaris acus* and *Apophallus brevis* were higher than in uninfected fish; whereas, no differences was observed between infected and uninfected fish collected at the reference site. Lipid peroxidation has been widely used in a number of comprehensive studies to demonstrate the general effects of environmental and natural stressors on fish (Shugart *et al*. 1992; Dautremepuits *et al*. 2009; Marcogliese & Pietrock 2011).

During the present study, the liver and gill tissue of *H. molitrix* displayed significantly higher lipid peroxidation induction in the liver and gill tissues than *L. rosae*, independent of parasite infections (Figure 5.2A–B). Malondialdehyde (MDA), a measure of lipid peroxidation was considerably higher in the liver as compared to gills. For *H. molitrix* significant variations was found seasonally, with infestation and site (liver and gill) ($p$<0.05). These results suggest that lipid peroxidation can be used as a biomarker of pathological effects caused by parasites. In contrast, higher MDA levels were recorded in the gills of *L. rosae* as compared to the liver. Significant difference was observed seasonally and between the infected and non-infected fish ($p$<0.05), however no difference was found between the tissue types.

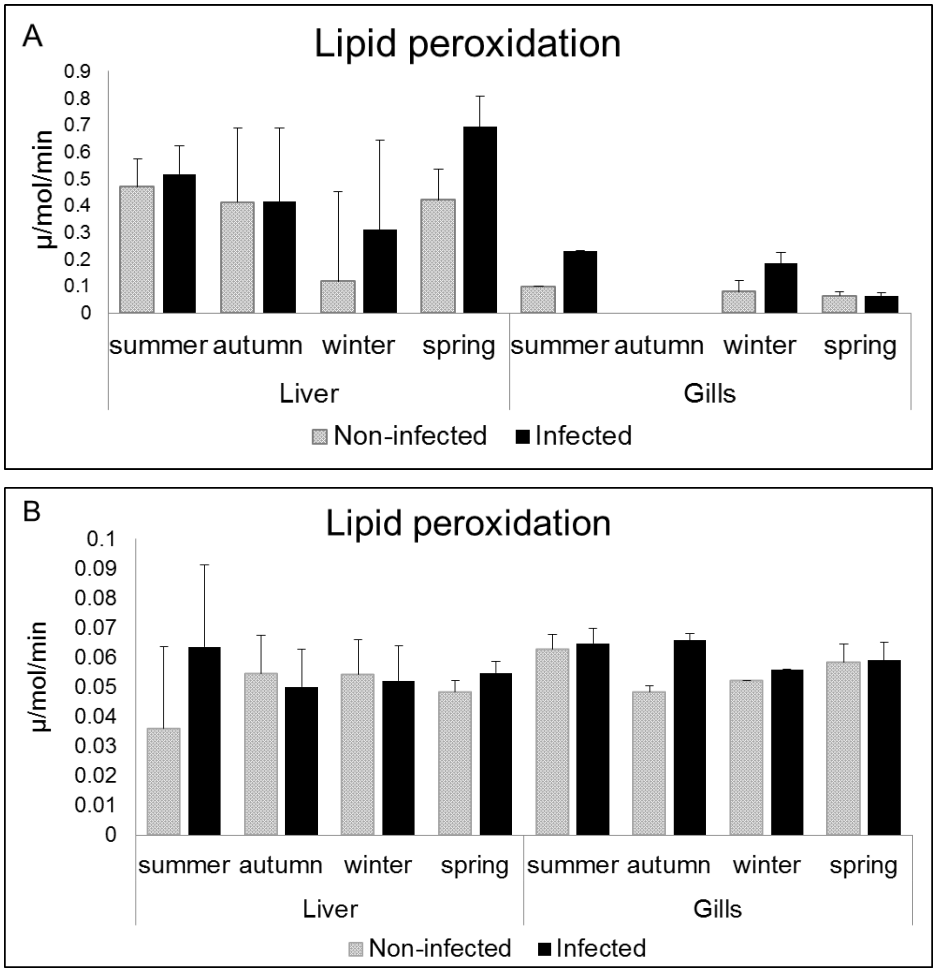


Figure 5.2: Seasonal variation of lipid peroxidase (MDA) in the liver and gills tissue of A- *Hypophthalmichthys molitrix* and B- *Labeo rosae* at Flag Boshielo Dam. Data are expressed as mean±SD.

The two selected fish had relatively lower lipid peroxidase levels compared to *Pagrus major* collected in the Meizhou (contaminated site) in China with mean±SD value of 0.836±0.453 in the liver and 0.168±0.116 in the gills (Gopolokrishnan *et al*. 2011) and at the reference site in the same study. Results from the present study showed the one fish species (*H. molitrix*) collected had higher lipid peroxidase levels, thus induced oxidative stress. However, an expected higher lipid peroxidase levels were recorded for both fish species in spring for the non-infected fish. This might be due to the increased lead and zinc concentrations recorded in the bioaccumulation study in the gills of *L. rosae,* collected from the same locality in spring (Mogashoa 2014, MSc dissertation submitted).

### 5.3.3 Total Antioxidant Capacity

Total Antioxidant Capacity can be defined as the measure of biomolecules from a variety of samples via a single electron transfer (SET) mechanism. These are mainly as a result of the imbalance between concentrations of ROS and antioxidants that resulting in the physiological condition referred to as oxidative stress (Diaz-Jaramillo *et al*. 2010). Excessive ROS accumulation has been reported to lead to cellular injury, such as damage to proteins and lipid membranes. The former has been implicated in the development of many disease states, such as cancer and apoptosis in mammals (Alvarez-Pellitero 2008).

The results of total antioxidant competence indicated lower levels in the gill tissue of *H. molitrix* as compared to the liver tissue of this species, and the gills and liver tissue of *L. rosae* (Figure 5.3A–B). This variation indicates a potential higher susceptibility of gills to oxidative stress resulting due to specific oxyradicals, a similar situation which has been demonstrated in a study by Diaz-Jaramillo *et al*. (2010). Althought significant difference was observed between the liver and gill tissue, no significant variations was found between seasons and infected and non-infected fish. For *L. rosae* the results on TAC levels concurred with the results obtained for lipid peroxidase recorded during this study (Figure 5.2B). Therefore, it can be concluded that this response was sufficient to prevent oxidative damage. In *L. rosae* no significant difference was found with regard to seasonality, site (liver and gills) as well as variations between the infected and non-infected fish.

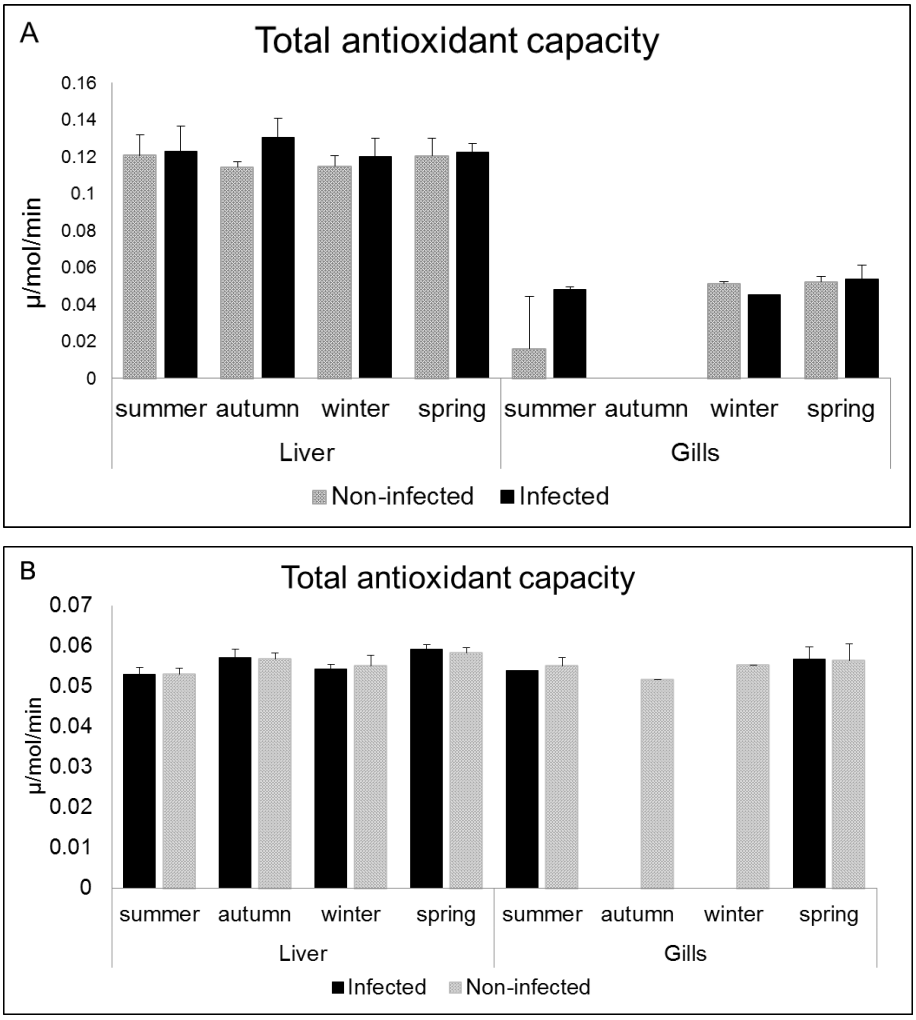


Figure 5.3: Seasonal variation of Total Antioxidant Capacity (TAC) activities in the liver and gill tissue of A- *Hypophthalmichthys molitrix* and B- *Labeo rosae* at Flag Boshielo Dam. Data are expressed as mean±SD.

### 5.3.4 Relationship between parasites and oxidative stress biomarkers

The parasitological investigation during this study revealed that from the *n* = 111, *H. molitrix* hosts examined, 77% were infected by metazoan parasites and the *n* = 116 *L. rosae* collected during this study, 80% were infected by metazoan parasites recovered. K of the two fish in correlation with parasite burden was analysed in Chapter 4, and only slight positive correlation were revealed for *H. molitrix* but negative correlation for *L. rosae*. Subsequently, this section of the study analyse the relationship between oxidative stress biomarkers assessed and parasite abundance of the two fish species.

Regression analysis was performed to explore the relationship between the three oxidative stress biomarkers (GST, LPO, TAC) and abundance of metazoan parasites

collected during the study for *H. molitrix* and *L. rosae*. In *H. molitrix*, the analysis showed a positive correlation between parasite abundance and lipid peroxidation in the gill tissue (Figure 5.4C), and significant results were observed ($R^2$>0.48). These results indicated that fish with a high parasite abundance; and low K, can be associated with elevated lipid peroxidation levels. Negative correlations were observed for other oxidative stress biomarkers in both the liver and gill tissue (Figure 5.4), with $R^2$≤0.008. Among the correlations observed for *H. molitrix* at Flag Boshielo Dam only the effects of metazoan parasites seemed to be related to oxidative stress.

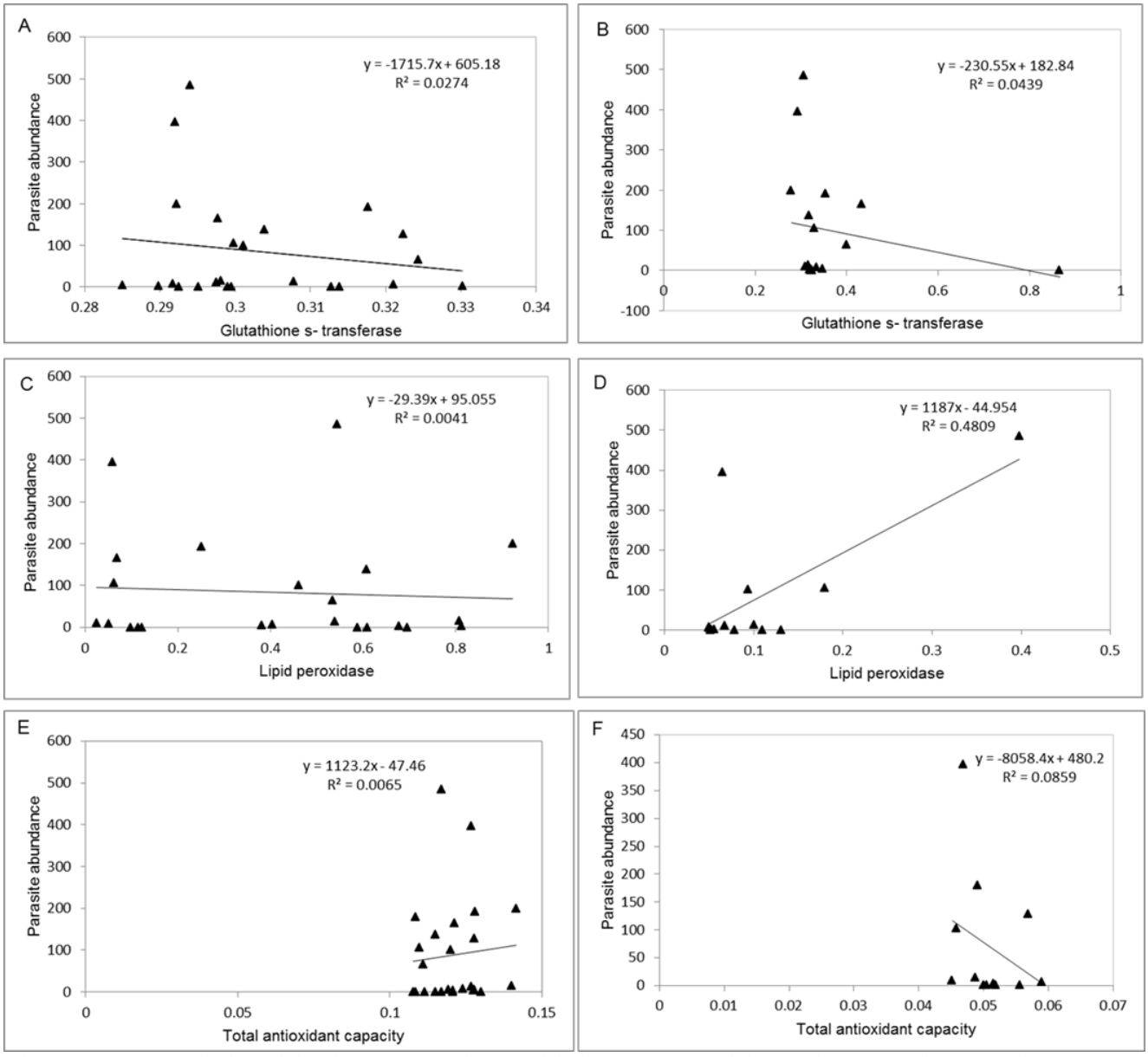


Figure 5.4: Relationship between the oxidative stress biomarkers and parasite abundance (parasites per host) of *Hypophthalmichthys molitrix* in the liver (A, C, E) and gills (B,D,F) at Flag Boshielo Dam collected during February 2012–Janury 2013.

For *L. rosae*, two of the oxidative stress biomarkers were positively correlated with $R^2$>0.15 (Figure 5.5B, E, F). Thus, this demonstrates that parasites affect the expression of these biomarkers. Negative correlations were found for lipid peroxidase and GST (Figure 5.5C, D, A). Various studies have demonstrated that parasitic infections may lead to an increase in the number of antioxidant enzymes and thus rendering oxidative damage in various tissues of fish. It has been clearly observed in the present study that there was considerable variation in biomarker concentration between tissues with regard to parasite infection, suggesting that the specific functions of each tissue are associated with their susceptibility to oxidative stress as well as their ability to defend against oxidative damage.

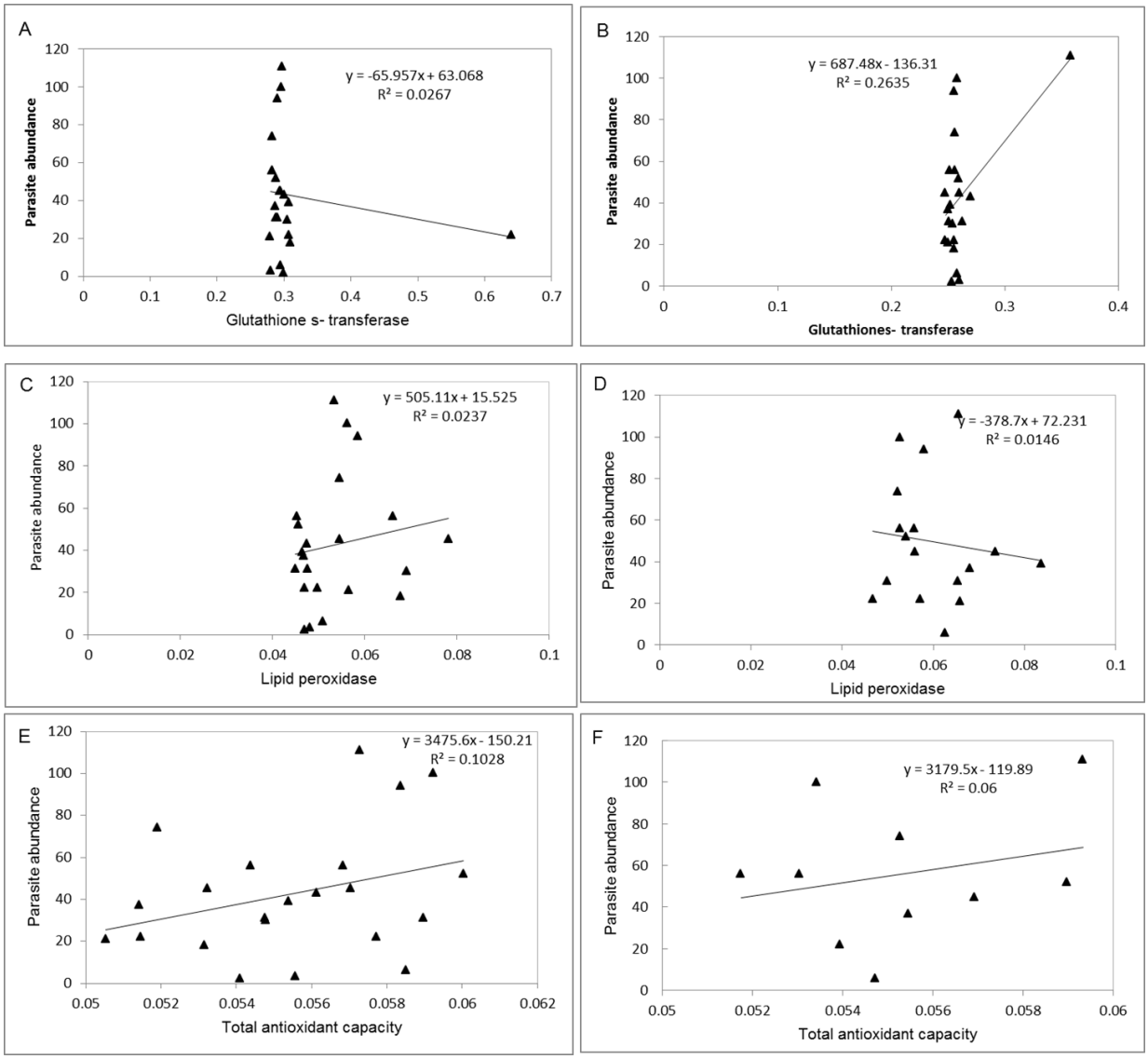


Figure 5.5: Relationship between the oxidative stress biomarkers and parasite abundance (parasites per host) of *Labeo rosae* in the liver (A, C, E) and gills (B, D, F) at Flag Boshielo Dam collected during February 2012–January 2013.

## 5.4 PRINCIPAL COMPONENT ANALYSIS

The results of the PCA biplot ordination illustrating the correlation of the different water quality and fish health parameters, parasite abundance and oxidative stress biomarkers for the two selected fish species between the four seasons at Flag Boshielo Dam are presented in Figure 5.6. The PCA plot showed proportion of variance accounted for by the first three components is additive, they accounted for 64.2% of total variation. The first component (PC1) accounted for 35.4%, the second component (PC2) 15.6% and the third component (PC3) contributed 13.2%. These three components loadings (correlation coefficients) and variances have been calculated and presented in Table 5.1. The correlation coefficient was strongly positive during summer for most of the variables for the two fish species. Negative correlations are associated with the winter season for most of the variables.

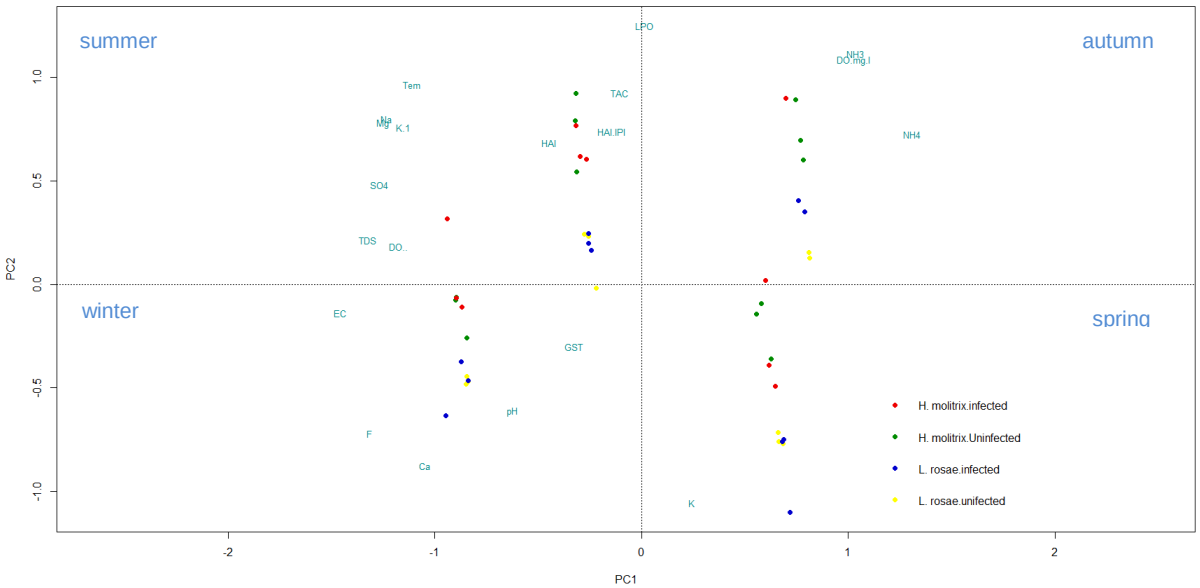


Figure 5.6: Principal component analysis (PCA) ordination biplot illustrating the correlation of water quality, parasite abundance, fish health and oxidative stress biomarkers at Flag Boshielo Dam during February 2012–January 2013.

Table 5.1: Factor loadings of the variables from the PCA in Figure 5.6, shown in eigenvalues of the correlation matrix.

| Eigenvalues of the correlation matrix | | | |
|---|---|---|---|
| PCA axes | Eigenvalue | Proportion | Cumulative |
| PC1 | 10.269 | 5.574 | 0.354 |
| PC2 | 4.5144 | 0.674 | 0.509 |
| PC3 | 1.0101 | 0.1324 | 0.6422 |

## 5.5 CONCLUSION

This component of the study on the oxidative stress biomarkers implicated in the response to metazoan parasites revealed that while there was slight variation in biomarker concentration of the highly infected and non-infected fish (for each species and between tissues) with regard to parasite infection. The biochemical functions of each tissue are associated with their susceptibility to oxidative stress as well as their ability to defend against oxidative damage.

Although, the present study, is to our knowledge the first in a South African freshwater ecosystem designed to provide a more co-ordinated response to pollution and parasitism to ensure conservation and sustainability of biodiversity. The findings of the study clearly indicate there are still important gaps in the knowledge of numerous immune mechanisms and the available information is scant and varies among fish species. Although it is not possible to give a detailed picture of all the toxic effects of various pollutants and individual parasites, this study forms a baseline on the use of immune defense biomarkers, as tools for environmental assessments in South African freshwater ecosystems.

# CHAPTER 6 - CONCLUSION AND RECOMMENDATIONS

## 6.1 GENERAL CONCLUSION

Given the unprecedented expansion in human population and industry since the industrial revolution, freshwater ecosystems have seen an upsurge in an effort to maintain sustainability of the growing human population. It remains indisputable, the impact that the increased anthropogenic activities (introduction of alien taxa; alteration of riparian habitat; habitat fragmentation; mining, agriculture and water abstraction) have had in the last century on aquatic biodiversity. Until recently, little attention was given to anthropogenic influences on the distribution and abundance of aquatic fauna and flora. Although this has understandably been the focus, under natural conditions no organism is only affected by pollution. Parasites have also been shown in a number of interdisciplinary studies to affect the health of aquatic hosts (amphibians, crustaceans, fish, and mammals). This is illustrated in a number of comprehensive studies the detrimental effects parasites exacerbate when their hosts (fish) are stressed. Therefore, the ability of parasites to interact with anthropogenic stressors, as well as effects they have on the biochemical, physiological and metabolic activities is crucial in conservating and sustaining aquatic biodiversity.

The present study is to our knowledge the first in South Africa to assess the effects of metazoan parasites on the health and oxidative stress biomarkers of two cyprinid species in correlation with water quality in one of South Africa's most pivotal rivers, the Olifants River, as an attempt to monitor and converse biodiversity, subsequently the overall ecosystem health. This river has received increased attention in the past few years due to ecological degradation caused by most of the above mentioned factors. Biodiversity loss is particularly of great concern in this river system with reports of progressive and occasional dramatic reduction in the number and abundance of several sensitive species of insects, amphibians, fish, crocodiles and aquatic mammals. Thus, the study is comprised of three components addressed in

individual chapters to suffice the research questions posed in response to the current health of the Olifants River System.

In summary, the findings with regard to water quality indicated that the quality of water at Flag Boshielo Dam was strongly influenced by nutrients (nitrates, nitrites, ammonium and ammonia); cations (i.e. sulphate and fluoride) as well as a few metals such as lead and zinc which were found to be above the South African TWQR for aquatic ecosystems. The increased levels and concentrations of these variables as well as comparison with previous studies (2009–2011) indicated that there have been changes in the trophic status of the dam from oligotrophic to mesotrophic. The changes are however slight, subsequently, Flag Boshielo Dam can still be considered moderately polluted, compared to Loskop Dam reported with eutrophic conditions in the upstream of this region of the Olifants River. Dams in the middle reaches of the Olifants River have been reported to improve the water, entering the Kruger National Park (KNP). However, the impact of mining and agriculture evident at the study site shows that there is an increasing trend (higher levels) of some constituents towards the middle region of the Olifants River System. This trend is of concern as the deterioration of the quality of South Africa's water resources is one of the major threats to the country's capability to provide sufficient water of appropriate quality while ensuring environmental sustainability. Therefore, the regulatory tools the (i.e. Integrated Water Resource Management (IWRM), Waste Discharge Charge System (WDCS) and load reduction) implemented by the Department of Water Affairs for the upper and middle regions of the Olifants River need to be applied in an effective and consistent manner to mitigate and control the ongoing pollution of the Olifants River System.

Fish health assessment parameters used in conjunction with the water quality analysis during the present study illustrates that it is likely that the health of the two fish are comprised to a certain extent by the relatively high xenobiotics which were above the TWQR during the summer and spring months. However, compared to other studies in other systems, i.e. Okavango Delta, Vaal and Phongolo rivers the two selected fish species in the present study, *H. molitrix* and *L. rosae*, appear to be in overall good health with relatively lower HAI values. In addition, *L. rosae* exhibited lower HAI, ideal K values compared to previous health assessment studies (2009–

2011) at Flag Boshielo Dam. However, high HAI, IPI and low K were recorded for *H. molitrix* compared to *L. rosae*. Although no studies exist that highlight the health profile of *H. molitrix* in South Africa, the health of this fish species remains of concern with low K, higher parasite mean intensity levels and severe necropsy anomalies (liver and gill anomalies and abnormal haematocrit levels). Thus, comparative studies using the same selected fish species are required, i.e. in Massingir Dam situated in the lower region of the Olifants River for subjectivity. The changing trophic status at Flag Boshielo Dam signals continuous monitoring of the health of fish species, macroinvertebrates and the Riparian Vegetation Index (RPI) to assess the health and evaluate impacts of environmental disturbances on the aquatic biota.

High ectoparasites numbers and diversity were recorded during the study, and this is regarded as an indication of good water quality, thus a healthy system. The copepod *Ergasilus* sp. which was the most abundant parasite during the study is suspected to be one of the factors which may have induced lesions on the skin, and atrophy of gills, thus exhibit abnormal haematocrit concentrations for most of the *H. molitrix* specimens examined at Flag Boshielo Dam. Thereby, exerts adverse effects compromising the health of this fish by inducing oxidative stress, consequently decreasing immune competence, thus fostering susceptibility to other environmental stressors. The gills and liver, which were the most affected organs during the study; and are regarded as major organs of immune response, were assessed for different oxidative stress biomarkers (GST, LPO and TAC). Highly infected and non-infected fish were selected and the activities of the biomarkers were assessed simultaneously in both organs. There was slight variation in biomarker concentration of the highly infected and non-infected fish, for each species and between tissues with regard to parasite infection, suggesting that the specific functions of each tissue are associated with their susceptibility to oxidative stress as well as their ability to defend against oxidative damage. These results illustrate that although fish are affected by aquatic contaminants they are to an extent affected by parasites, which may act addictively or synergistically on the health of the two fish species.

## 6.2 RECOMMENDATIONS

Although, this study to our knowledge the first in a South African freshwater ecosystem, designed to provide a more co-ordinated response to pollution and parasitism to ensure conservation and sustainability of the aquatic biodiversity. The findings of the study clearly indicate there are still important gaps in the knowledge of numerous immune mechanisms and the available information is scant and varies among fish species. Whilst it is not possible to give a detailed picture of all the toxic effects of various pollutants and individual parasites, this study serve as a baseline on the use of immune defense biomarkers, as tools for environmental assessments in South African freshwater ecosystems for future biomonitoring. In order to validate the role of pollution and parasites, more investigations on the use of different biological assessments is essential to describe pollution-parasite and host interactions, to realisticly describe scenarios organisms are confronted with.

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## APPENDIX A

Table 1: The seasonal variations of water quality parameters from the three sampling sites at Flag Boshielo Dam.

| Parameters | Summer | | | Autumn | | | Winter | | | Spring | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Inflow | Middle | Dam wall | Inflow | Middle | Dam wall | Inflow | Middle | Dam wall | Inflow | Middle | Dam wall |
| Temperature | 28.9 | 28.9 | 27.6 | 25.7 | 25.8 | 24.6 | 14.82 | 15.17 | 15.26 | 20.55 | 20.95 | 21.1 |
| pH | 8.45 | 9.15 | 8.45 | 7.8 | 8.5 | 8.4 | 9 | 8.35 | 8.1 | 6.62 | 7.09 | 6.75 |
| Dissolved oxygen % | 86 | 123.5 | 118.9 | 87 | 93.5 | 116.7 | 70.1 | 79.9 | 95.3 | 65.9 | 70.4 | 66.7 |
| Dissolved oxygen mg/l | 8.83 | 8.84 | 9 | 7.23 | 7.14 | 7.38 | 8.5 | 7.6 | 8.97 | 10.08 | 7.71 | 10.08 |
| Total dissolved solids | 324.4 | 346.5 | 334.8 | 386.8 | 378.8 | 378.1 | 248.3 | 276.9 | 345.8 | 378.3 | 245.1 | 346.5 |
| Electric conductivity | 49.9 | 53.3 | 51.5 | 59.5 | 57.5 | 57.4 | 38.2 | 42.6 | 53.2 | 58.2 | 37.7 | 34.8 |
| Salinity | 0.26 | 0.26 | 0.26 | 0.26 | 0.26 | 0.26 | 0.26 | 0.26 | 0.26 | 0.26 | 0.26 | 0.26 |
| Nitrites | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| Nitrates | <1.4 | <1.4 | <1.4 | <1.4 | <1.4 | <1.4 | <1.4 | <1.4 | <1.4 | <1.4 | <1.4 | <1.4 |
| Sulphate | 86.66 | 100.9 | 103.99 | 118.46 | 110.34 | 112.38 | 46.95 | 62.58 | 100.12 | 122.83 | 77.5 | 74.63 |
| Ortho-phosphate | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 |
| Ammonium | 0.29 | 0.25 | 0.22 | 0.32 | 0.09 | 0.27 | 0.13 | 0.34 | 0.31 | 0.17 | 0.27 | 0.43 |
| Ammonia | 0.27 | 0.24 | 0.21 | 0.3 | 0.08 | 0.25 | 0.11 | 0.3 | 0.28 | 0.16 | 0.25 | 0.41 |
| Calcium | 21.01 | 22.61 | 16.49 | 26.49 | 31.34 | 31.07 | 16.61 | 19.06 | 30.89 | 26.9 | 17.4 | 15.52 |
| Magnesium | 17.59 | 19.5 | 19.81 | 18.63 | 19.15 | 18.84 | 10.66 | 13.03 | 21.67 | 23.59 | 14.46 | 12.57 |
| Potassium | 5.9 | 6.39 | 6.5 | 5.35 | 5.79 | 5.71 | 3.11 | 3.95 | 5.77 | 6.15 | 4.06 | 3.54 |
| Sodium | 50.56 | 52.46 | 52.89 | 52.31 | 50.05 | 48.77 | 23.71 | 29.75 | 46.13 | 59.67 | 32.56 | 30.74 |
| Fluoride | 0.75 | 0.73 | 0.67 | 0.92 | 0.85 | 0.85 | 0.73 | 0.69 | 0.67 | 0.73 | 0.53 | 0.53 |
| Aluminium | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | 0.02 | 0.01 | 0.01 | <0.01 | 0.01 | 0.01 |
| Copper | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| Arsenic | <0.03 | <0.03 | <0.03 | <0.03 | <0.03 | <0.03 | <0.03 | <0.03 | <0.03 | <0.03 | <0.03 | <0.03 |
| Cadmium | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| Iron | 0.01 | 0.01 | 0.01 | <0.01 | <0.01 | 0.01 | <0.01 | 0.01 | <0.01 | 0.01 | 0.01 | 0.01 |
| Lead | <0.09 | <0.09 | <0.09 | <0.09 | <0.09 | <0.09 | <0.09 | <0.09 | <0.09 | <0.09 | <0.09 | <0.09 |
| Manganase | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | 0.01 | 0.01 | <0.01 | 0.01 | 0.01 | 0.01 |
| Zinc | <0.01 | <0.01 | <0.01 | <0.01 | 0.01 | <0.01 | <0.01 | <0.01 | 0.01 | <0.01 | <0.01 | <0.01 |

## APPENDIX B

Table 1: Health Assessment Index of *Hypophthalmichthys molitrix* at Flag Boshielo Dam for summer.

| Fish No | Length | | Mass | Sex | Eyes | Skin | Fins | Opercule | Gills | Liver | Spleen | Hindgut | kidney | Hct | Ecto PI | Endo PI | Ecto IPI | HAI | HAI IPI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SL | TL | | | | | | | | | | | | | | | | | |
| 1 | 27.5 | 34.5 | 402.5 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 10 | 30 | 50 | 60 |
| 2 | 80 | 93 | 6342.4 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 20 | 0 | 10 | 80 | 70 |
| 3 | 38 | 44.8 | 819 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | – | 0 | 0 | 30 | 30 | 60 |
| 4 | 69 | 80 | 4709.8 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 5 | 80 | 93.5 | 5055 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | – | 30 | 0 | 0 | 90 | 60 |
| 6 | 74 | 86 | 5740.3 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | – | 30 | 0 | 0 | 90 | 60 |
| 7 | 84 | 98 | 5587 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 10 | 30 | 0 | 0 | 70 | 40 |
| 8 | 69 | 83 | 5952.3 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 9 | 77 | 88 | 5670.5 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | – | 30 | 0 | 0 | 60 | 30 |
| 10 | 32 | 39 | 543.6 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 60 | 90 |
| 11 | 79 | 92.5 | 5681.3 | Male | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | – | 20 | 0 | 10 | 80 | 70 |
| 12 | 72 | 83.6 | 4330.8 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 30 | 0 | 0 | 30 | 30 | 0 | 0 | 150 | 120 |
| 13 | 36.4 | 45 | 750 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 10 | 20 | 50 | 60 |
| 14 | 37.5 | 45 | 726.91 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 0 | 0 | 30 | 50 | 80 |
| 15 | 37 | 45.2 | 800.92 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 10 | 10 | 0 | 20 | 50 | 60 |
| 16 | 37.5 | 45 | 805.69 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| 17 | 39 | 47.5 | 976.26 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 10 | 20 | 50 | 60 |
| 18 | 36 | 43.5 | 791.16 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 10 | 20 | 50 | 60 |
| 19 | 40 | 48.5 | 1065.53 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 0 | 10 | 30 | 40 | 70 |
| 20 | 37 | 45.3 | 721.26 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 10 | 20 | 50 | 60 |
| 21 | 34 | 41 | 677.7 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 30 | 60 |
| 22 | 33.5 | 44 | 530.85 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| 23 | 33 | 41.1 | 498.73 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| 24 | 36 | 43.3 | 688.43 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 10 | 20 | 50 | 60 |
| 25 | 35 | 40.8 | 610.34 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| 26 | 34.5 | 41 | 621.4 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 10 | 20 | 50 | 60 |
| 27 | 35.3 | 42.5 | 738.3 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 30 | 60 |
| 28 | 33 | 40 | 588.4 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| 29 | 38 | 46 | 849.96 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 30 | 60 |
| 30 | 32.5 | 38.3 | 534.74 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| 31 | 36.7 | 44 | 820.3 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 30 | 60 |
| 32 | 35 | 42.5 | 673.93 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 30 | 60 |
| | | | | | | | | | | | | | | | Mean HAI | | | 55.3 | 60.9 |
| | | | | | | | | | | | | | | | Total HAI | | | 1770 | 1950 |

Table 2: Health Assessment Index of *Hypophthalmichthys molitrix* at Flag Boshielo Dam for autumn.

| Fish No | Length | | Mass | Sex | Eyes | Skin | Fins | Opercule | Gills | Liver | Spleen | Hindgut | kidney | Hct | Ecto PI | Endo PI | Ecto IPI | HAI | HAI IPI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SL | TL | | | | | | | | | | | | | | | | | |
| 1 | 37.7 | 43 | 784.21 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 30 | 60 |
| 2 | 45 | 50 | 111.7 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 10 | 10 | 0 | 20 | 50 | 60 |
| 3 | 72 | 83 | 4709.3 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | – | 0 | 0 | 30 | 30 | 60 |
| 4 | 73 | 88.7 | 5827.6 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | – | 0 | 0 | 30 | 30 | 60 |
| 5 | 33 | 40 | 571.79 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | – | 0 | 10 | 30 | 40 | 70 |
| 6 | 68.5 | 80 | 3351.76 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 10 | 0 | 100 | 70 |
| 7 | 80 | 92 | 4341.8 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | 30 | 30 | 10 | 0 | 130 | 110 |
| 8 | 32 | 38 | 528.33 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 10 | 10 | 20 | 70 | 80 |
| 9 | 75 | 87.5 | 3854.1 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 30 | 0 | 0 | 50 | 20 |
| 10 | 78 | 90 | 3939.18 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 11 | 45.5 | 50 | 1015.8 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 70 | 80 |
| 12 | 29.3 | 35.5 | 394.5 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 30 | 60 |
| 13 | 74 | 87.8 | 3861.9 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 30 | 0 | 0 | 80 | 50 |
| 14 | 70 | 85 | 4253.6 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | 30 | 30 | 10 | 0 | 130 | 100 |
| 15 | 71 | 86.5 | 5000 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 16 | 71.5 | 85 | 5218.9 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 17 | 81.5 | 93.3 | 7005.2 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 18 | 77 | 91 | 4348 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 19 | 80 | 93.5 | 5819.4 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 20 | 30 | 37 | 504.1 | Female | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 30 | 30 | 10 | 0 | 70 | 40 |
| 21 | 77 | 88.3 | 5816.4 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 120 | 90 |
| | | | | | | | | | | | | | | | Mean HAI | | | 74.8 | 65.2 |
| | | | | | | | | | | | | | | | Total HAI | | | 1570 | 1370 |

Table 3: Health Assessment Index of *Hypophthalmichthys molitrix* at Flag Boshielo Dam for winter.

| Fish No | Length | | Mass | Sex | Eyes | Skin | Fins | Opercule | Gills | Liver | Spleen | Hindgut | kidney | Hct | Ecto PI | Endo PI | Ecto IPI | HAI | HAI IPI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SL | TL | | | | | | | | | | | | | | | | | |
| 1 | 79 | 92 | 6372.4 | Male | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | 30 | 10 | 0 | 20 | 100 | 110 |
| 2 | 80.5 | 96 | 6237.4 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 120 | 90 |
| 3 | 80 | 93 | 5036.6 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 4 | 79 | 91.8 | 5742.2 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 120 | 90 |
| 5 | 42 | 51 | 1114.4 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 10 | 0 | 20 | 70 | 80 |
| 6 | 42.5 | 50.5 | 1101.4 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 10 | 0 | 20 | 60 | 70 |
| 7 | 40.5 | 48 | 990.8 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 30 | 10 | 0 | 90 | 60 |
| 8 | 33.5 | 40 | 889.4 | Female | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 30 | 10 | 0 | 20 | 70 | 80 |
| 9 | 37 | 43.5 | 820.6 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 0 | 30 | 20 | 50 |
| 10 | 34.5 | 42 | 764.8 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 0 | 0 | 30 | 50 | 80 |
| 11 | 37.5 | 44 | 859.6 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 10 | 0 | 20 | 60 | 70 |
| 12 | 36 | 43 | 605.9 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 60 | 90 |
| 13 | 73 | 89 | 4263.8 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 120 | 90 |
| 14 | 77 | 89 | 4877.5 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | 30 | 10 | 0 | 20 | 100 | 110 |
| 15 | 39.5 | 49 | 905 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 0 | 30 | 20 | 50 |
| 16 | 41 | 48 | 1019.9 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | 20 | 0 | 10 | 30 | 90 | 120 |
| 17 | 36.5 | 43.3 | 807.7 | Male | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | 0 | 0 | 10 | 30 | 70 | 100 |
| 18 | 36 | 43.3 | 758.6 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 10 | 30 | 30 | 60 |
| 19 | 39 | 47 | 1056.9 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 30 |
| 20 | 32.5 | 39.5 | 627.6 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | 0 | 0 | 10 | 30 | 70 | 90 |
| 21 | 37 | 45 | 897.6 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 30 |
| 22 | 35 | 42.5 | 718.7 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 10 | 30 | 30 | 60 |
| 23 | 36 | 43.5 | 713.4 | Male | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 20 | 0 | 10 | 30 | 60 | 90 |
| 24 | 35.5 | 42.3 | 623.1 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 0 | 10 | 30 | 40 | 70 |
| 25 | 76.5 | 89.5 | 4503.1 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 60 | 90 |
| 26 | 81.5 | 95 | 5130.5 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 60 | 90 |
| 27 | 66 | 77 | 3647.2 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 60 | 90 |
| 28 | 29 | 35.5 | 316.5 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 10 | 20 | 50 | 60 |
| | | | | | | | | | | | | | | | Mean HAI | | | 30 | 77.1 |
| | | | | | | | | | | | | | | | Total HAI | | | 840 | 2160 |

Table 4: Health Assessment Index of *Hypophthalmichthys molitrix* at Flag Boshielo Dam for spring.

| Fish No | Length | | Mass | Sex | Eyes | Skin | Fins | Opercule | Gills | Liver | Spleen | Hindgut | kidney | Hct | Ecto PI | Endo PI | Ecto IPI | HAI | HAI IPI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SL | TL | | | | | | | | | | | | | | | | | |
| 1 | 70 | 84 | 4307.3 | Female | 0 | 30 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | 30 | 10 | 0 | 20 | 130 | 140 |
| 2 | 73 | 87.5 | 5930.6 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 90 | 120 |
| 3 | 76 | 89 | 4983.1 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 10 | 0 | 20 | 70 | 80 |
| 4 | 76 | 90 | 4771.3 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | – | 10 | 0 | 20 | 40 | 50 |
| 5 | 39 | 46.5 | 875.6 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 10 | 0 | 20 | 60 | 70 |
| 6 | 40 | 47.3 | 977.4 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 0 | 0 | 30 | 50 | 70 |
| 7 | 42 | 51 | 1362.2 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 70 | 80 |
| 8 | 80 | 94 | 6381.8 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 9 | 75 | 87 | 4745.5 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 10 | 38 | 48 | 955.1 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 10 | 10 | 20 | 40 | 50 |
| 11 | 41 | 49.5 | 1243.2 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 30 | 10 | 40 |
| 12 | 35.5 | 43.5 | 742.5 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| 13 | 72 | 85.5 | 5370.2 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 14 | 70 | 84 | 4808.1 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 15 | 79.8 | 90 | 7098.2 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 16 | 34.5 | 43 | 732 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | – | 10 | 0 | 20 | 40 | 50 |
| 17 | 66 | 85 | 4302 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 18 | 35 | 38.8 | 681.1 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 10 | 10 | 30 | 20 |
| 19 | 76 | 89 | 5373.6 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | – | 30 | 0 | 0 | 60 | 30 |
| 20 | 73 | 86 | 4595.3 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 21 | 70 | 83 | 3786.5 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 22 | 74.2 | 86.4 | 3912.4 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 23 | 33 | 36.5 | 705.8 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 10 | 0 | 20 | 30 | 40 |
| 24 | 35 | 42.5 | 714.6 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 10 | 0 | 20 | 30 | 40 |
| 25 | 37 | 44.5 | 804.2 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 30 |
| 26 | 31 | 38 | 512.6 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 10 | 30 | 30 | 60 |
| 27 | 77 | 90 | 4941.2 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 30 | 0 | 0 | 30 | 30 | 0 | 0 | 150 | 120 |
| 28 | 78 | 92 | 5154.3 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 30 | 0 | 0 | 30 | 30 | 0 | 0 | 150 | 120 |
| 29 | 75 | 88 | 6405.2 | Male | 0 | 0 | 0 | 0 | 30 | 30 | 30 | 0 | 0 | 30 | 10 | 0 | 20 | 130 | 150 |
| 30 | 73 | 86.5 | 4043.2 | Female | 0 | 0 | 0 | 0 | 30 | 30 | 30 | 0 | 0 | 30 | 0 | 0 | 30 | 120 | 150 |
| | | | | | | | | | | | | | | | Mean HAI | | | 72.7 | 70 |
| | | | | | | | | | | | | | | | Total HAI | | | 2180 | 2100 |

Table 5: Health Assessment Index of *Labeo rosae* at Flag Boshielo Dam for summer.

| Fish No | Length | | Mass | Sex | Eyes | Skin | Fins | Opercule | Gills | Liver | Spleen | Hindgut | kidney | Hct | Ecto PI | Endo PI | Ecto IPI | HAI Total | HAI IPI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SL | TL | | | | | | | | | | | | | | | | | |
| 1 | 26 | 33 | 428.3 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 10 | 20 | 20 | 30 |
| 2 | 21 | 26.2 | 200.2 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 10 | 10 | 30 | 20 |
| 3 | 23.3 | 29.5 | 246.8 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 10 | 0 | 20 | 30 | 40 |
| 4 | 21 | 27 | 207.7 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 10 | 0 | 20 | 40 | 50 |
| 5 | 21 | 26 | 194 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| 6 | 21 | 26.5 | 184.8 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 10 | 20 | 20 | 30 |
| 7 | 20.5 | 26.5 | 214.3 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 10 | 10 | 30 | 20 |
| 8 | 22 | 26.5 | 202 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 10 | 10 | 20 | 40 | 50 |
| 9 | 16.5 | 21 | 85.7 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 10 | 20 |
| 10 | 17 | 21 | 91.5 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 10 | 20 | 20 | 30 |
| 11 | 23 | 28.2 | 281.7 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | – | 30 | 0 | 0 | 60 | 30 |
| 12 | 28 | 34.5 | 539.3 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 30 | 0 | 0 | 80 | 50 |
| 13 | 26 | 31 | 409.5 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | – | 30 | 0 | 0 | 60 | 30 |
| 14 | 21 | 26.5 | 222 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 30 |
| 15 | 25 | 31 | 331.2 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 60 | 30 |
| 16 | 26 | 31 | 441.8 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | – | 30 | 0 | 0 | 30 | 0 |
| 17 | 22.4 | 27 | 232.6 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 20 | 0 | 10 | 70 | 60 |
| 18 | 23 | 28 | 259.5 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 20 | 10 | 10 | 60 | 50 |
| 19 | 23 | 29 | 243.8 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 60 | 30 |
| 20 | 23.5 | 29.7 | 283.71 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 10 | 20 |
| 21 | 24 | 30 | 293.62 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 10 | 20 |
| 22 | 22.4 | 26.5 | 275.2 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 20 | 0 | 10 | 30 | 20 |
| 23 | 20.5 | 25.3 | 183.22 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 20 | 0 | 10 | 30 | 20 |
| 24 | 26 | 32.5 | 406.6 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| 25 | 26 | 31 | 351.23 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 10 | 0 | 20 | 20 | 30 |
| 26 | 23 | 29 | 232.49 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 10 | 20 |
| 27 | 21 | 25.5 | 190.98 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| 28 | 22.5 | 27.5 | 290.52 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| 29 | 27.5 | 33 | 423.5 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 60 | 30 |
| 30 | 24 | 28.5 | 271.83 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| | | | | | | | | | | | | | | | Mean HAI | | | 36.3 | 33.7 |
| | | | | | | | | | | | | | | | Total HAI | | | 1090 | 1010 |

Table 6: Health Assessment Index of *Labeo rosae* at Flag Boshielo Dam for autumn.

| Fish No | Length | | Mass | Sex | Eyes | Skin | Fins | Opercule | Gills | Liver | Spleen | Hindgut | kidney | Hct | Ecto PI | Endo PI | Ecto IPI | HAI | HAI IPI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SL | TL | | | | | | | | | | | | | | | | | |
| 1 | 22.2 | 27.9 | 255.02 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 20 | 0 | 10 | 50 | 40 |
| 2 | 20 | 25.2 | 184.25 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 60 | 30 |
| 3 | 23 | 28 | 270.2 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 30 | 0 | 0 | 50 | 20 |
| 4 | 21 | 27 | 210.01 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 30 | 0 | 0 | 50 | 20 |
| 5 | 27 | 33 | 400 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 30 | 10 | 0 | 50 | 20 |
| 6 | 21 | 27.9 | 200 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 0 |
| 7 | 26.5 | 33 | 420 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 60 | 30 |
| 8 | 24.7 | 30.1 | 300 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 30 | 0 | 0 | 40 | 10 |
| 9 | 22.9 | 28 | 230 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| 10 | 23 | 28.7 | 230 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 30 |
| 11 | 21.5 | 27.8 | 200 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 10 | 20 |
| 12 | 21.3 | 26.8 | 192.02 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 10 | 30 | 0 | 0 | 70 | 40 |
| 13 | 21.5 | 27 | 203.24 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 30 | 10 | 0 | 40 | 20 |
| 14 | 23 | 27.8 | 283.3 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 30 | 0 | 0 | 50 | 20 |
| 15 | 26 | 33 | 397.95 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 10 | 30 | 10 | 0 | 80 | 50 |
| 16 | 23 | 29 | 256.31 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 30 | 0 | 0 | 40 | 10 |
| 17 | 28 | 35.6 | 467.35 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 10 | 10 | 0 | 20 | 50 | 60 |
| 18 | 22 | 28.1 | 265.46 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 30 | 0 | 0 | 40 | 10 |
| 19 | 25 | 31.2 | 349.9 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| 20 | 27 | 33.4 | 409.1 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 0 |
| 21 | 30 | 37.5 | 609.2 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 30 | 30 | 0 | 0 | 90 | 60 |
| 22 | 24.5 | 30.5 | 347.3 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 0 |
| 23 | 27 | 34.5 | 463.8 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 30 | 10 | 0 | 70 | 40 |
| 24 | 26.4 | 33 | 396.2 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 30 | 0 | 0 | 40 | 10 |
| 25 | 27.5 | 34.3 | 466.3 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 0 |
| 26 | 24.5 | 30.3 | 304.4 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 60 | 30 |
| 27 | 23.5 | 29.5 | 281.4 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | – | 30 | 0 | 0 | 60 | 30 |
| 28 | 21.4 | 27 | 220.6 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 0 |
| 29 | 30 | 37 | 541.1 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 60 | 30 |
| 30 | 27.7 | 34.5 | 452.8 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 0 |
| 31 | 29.5 | 37 | 518.7 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 10 | 30 | 0 | 0 | 50 | 40 |
| 32 | 25.5 | 32 | 357.9 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 60 | 30 |
| 33 | 25 | 30.5 | 289.1 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 60 | 30 |
| 34 | 23 | 28 | 250.9 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 10 | 30 | 0 | 0 | 70 | 40 |
| | | | | | | | | | | | | | | | Mean HAI | | | 47.6 | 25.6 |
| | | | | | | | | | | | | | | | Total HAI | | | 1630 | 870 |

Table 7: Health Assessment Index of *Labeo rosae* at Flag Boshielo Dam for winter.

| Fish No | Length | | Mass | Sex | Eyes | Skin | Fins | Opercule | Gills | Liver | Spleen | Hindgut | kidney | Hct | Ecto PI | Endo PI | Ecto IPI | HAI | HAI IPI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SL | TL | | | | | | | | | | | | | | | | | |
| 1 | 19.5 | 24.3 | 165.2 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 10 | 20 | 20 | 30 |
| 2 | 19 | 24 | 146.4 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 10 | 10 | 20 | 50 | 60 |
| 3 | 19.2 | 24.5 | 153.9 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 10 | 0 | 40 | 10 |
| 4 | 15.3 | 18.5 | 98.9 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 30 | 10 | 40 |
| 5 | 17 | 22.4 | 104.6 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 10 | 20 |
| 6 | 17.5 | 21.5 | 114.3 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 10 | 20 |
| 7 | 19.5 | 23.5 | 155.7 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 10 | 20 |
| 8 | 19 | 24.5 | 146.2 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 30 |
| 9 | 20 | 24.5 | 152.7 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 10 | 20 |
| 10 | 18.5 | 23.4 | 160.9 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 30 |
| 11 | 25.5 | 32 | 400.1 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 10 | 20 | 20 | 30 |
| 12 | 27 | 33.3 | 423.4 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 20 | 10 | 10 | 60 | 50 |
| 13 | 26 | 33 | 416.1 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 10 | 0 | 20 | 60 | 70 |
| 14 | 25 | 31 | 395.7 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 10 | 20 |
| 15 | 26 | 32 | 414.3 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 10 | 20 |
| 16 | 21.5 | 30 | 339.3 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 10 | 20 | 20 | 30 |
| 17 | 24 | 25.5 | 436.3 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | – | 20 | 10 | 10 | 30 | 20 |
| 18 | 21.5 | 26.5 | 214.1 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 10 | 10 | 20 | 70 | 80 |
| 19 | 22.5 | 28 | 247 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 10 | 20 |
| 20 | 19.5 | 23 | 145.5 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 10 | 20 | 10 | 30 |
| 21 | 30 | 37 | 718.9 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 30 | 0 | 0 | 50 | 20 |
| 22 | 27 | 33.5 | 435 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 10 | 20 | 20 | 30 |
| 23 | 25.5 | 31.5 | 328.9 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 0 | 30 | 20 | 50 |
| 24 | 26.5 | 32.5 | 438.2 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 30 | 0 | 0 | 50 | 20 |
| 25 | 26 | 32.5 | 386.5 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 10 | 20 |
| 26 | 25 | 31.5 | 371.6 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| 27 | 17.4 | 21.5 | 104.9 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | – | 20 | 10 | 10 | 30 | 20 |
| 28 | 24 | 29.5 | 310.5 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | – | 30 | 0 | 0 | 30 | 0 |
| 29 | 19 | 23.5 | 134.5 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 10 | 0 | 20 | 30 | 40 |
| | | | | | | | | | | | | | | | Mean HAI | | | 25.5 | 31.0 |
| | | | | | | | | | | | | | | | Total HAI | | | 740 | 900 |

Table 8: Health Assessment Index of *Labeo rosae* at Flag Boshielo Dam for spring.

| Fish No | Length | | Mass | Sex | Eyes | Skin | Fins | Opercule | Gills | Liver | Spleen | Hindgut | kidney | Hct | Ecto PI | Endo PI | Ecto IPI | HAI | HAI IPI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SL | TL | | | | | | | | | | | | | | | | | |
| 1 | 25.5 | 31.2 | 321.8 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 0 |
| 2 | 28 | 34.3 | 544.3 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 0 |
| 3 | 28.3 | 35.2 | 585.1 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 30 | 0 | 0 | 80 | 50 |
| 4 | 26 | 32 | 414.3 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 20 | 10 | 20 |
| 5 | 27 | 32.5 | 423.2 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 0 |
| 6 | 27 | 33 | 460.5 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 30 | 0 | 0 | 50 | 20 |
| 7 | 25.5 | 31.5 | 349.9 | Male | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 10 | 30 | 0 | 0 | 70 | 40 |
| 8 | 19.3 | 23.5 | 163.2 | male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 10 | 0 | 40 | 10 |
| 9 | 22 | 27 | 248.3 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 30 | 0 | 0 | 80 | 50 |
| 10 | 26.5 | 33 | 392.3 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 10 | 20 | 10 |
| 11 | 25.4 | 32.3 | 361.3 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 10 | 20 | 10 |
| 12 | 26.5 | 33 | 460.5 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | – | 30 | 10 | 0 | 40 | 10 |
| 13 | 31 | 38 | 693.5 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 0 |
| 14 | 24 | 30 | 324.3 | Female | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 20 | 30 | 10 | 0 | 90 | 60 |
| 15 | 26.5 | 32 | 427.5 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 10 | 0 | 40 | 10 |
| 16 | 26 | 32.5 | 418.8 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 10 | 0 | 40 | 10 |
| 17 | 26 | 33.6 | 428.6 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 0 |
| 18 | 21.5 | 26.5 | 222.3 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 30 | 0 |
| 19 | 28.5 | 35 | 270.5 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 10 | 0 | 20 | 30 | 40 |
| 20 | 22.5 | 28 | 242.5 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 20 | 30 | 0 | 0 | 80 | 50 |
| 21 | 23.5 | 29 | 278.3 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 10 | 0 | 20 | 40 | 50 |
| 22 | 22.3 | 27.5 | 216.5 | Male | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 20 | 30 | 10 | 0 | 90 | 60 |
| 23 | 22 | 30.5 | 181.3 | Female | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 20 | 30 | 10 | 0 | 90 | 60 |
| | | | | | | | | | | | | | | | Mean HAI | | | 47.4 | 24.3 |
| | | | | | | | | | | | | | | | Total HAI | | | 1090 | 560 |

## APPENDIX C

Table1: Oxidative stress biomarkers in the liver and gill tissue of *Hypophthalmichthys molitrix* at Flag Boshielo Dam during February 2012–January 2013.

| Species | Infection | Season | Oxidative stress biomarkers | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | GST | | LPO | | TAC | |
| | | | Liver | Gill | Liver | Gill | Liver | Gill |
| *H. molitrix* 15 Feb | 0 | summer | 0.299432 | – | 0.54316 | 0.398058 | 0.117026 | – |
| *H. molitrix* 11 Jan | 15 | summer | 0.298079 | – | 0.059717 | 0.065102 | 0.127808 | – |
| *H. molitrix* 2 Jan | 0 | summer | 0.307804 | 0.316747 | 0.404479 | – | 0.107745 | – |
| *H. molitrix* 12 Feb | 0 | summer | 0.320992 | – | 0.538249 | 0.100319 | 0.10838 | 0.049178 |
| *H. molitrix* 6 Jan | 396 | summer | 0.291971 | 0.294342 | 0.807205 | – | 0.126604 | 0.046982 |
| *H. molitrix* 5 Jan | 485 | summer | 0.294034 | 0.307902 | 0.609411 | – | 0.126645 | 0.048846 |
| *H. molitrix* 5 Apr | 0 | autumn | 0.297649 | 0.43314 | 0.60738 | – | 0.114956 | – |
| *H. molitrix* 3 May | 192 | autumn | 0.317686 | 0.355138 | 0.923718 | – | 0.141468 | – |
| *H. molitrix* 2 Apr | 0 | autumn | 0.295053 | 0.865985 | 0.533916 | – | 0.12803 | – |
| *H. molitrix* 7 May | 0 | autumn | 0.324279 | 0.40118 | 0.098292 | – | 0.121094 | – |
| *H. molitrix* 2 May | 199 | autumn | 0.29217 | 0.279411 | 0.25166 | – | 0.117038 | – |
| *H. molitrix* 5 May | 138 | autumn | 0.30385 | 0.317954 | 0.068864 | – | 0.111014 | – |
| *H. molitrix* 12 Aug | 0 | winter | 0.291652 | 0.325346 | 0.061497 | 0.17975 | 0.10956 | – |
| *H. molitrix* 10 Aug | 0 | winter | 0.297466 | 0.323078 | 0.024317 | 0.067241 | 0.114907 | 0.051908 |
| *H. molitrix* 9 Jun | 0 | winter | 0.299779 | – | 0.051175 | 0.050023 | 0.123824 | 0.045147 |
| *H. molitrix* 10 Jun | 8 | winter | 0.299021 | 0.336007 | 0.121842 | 0.051528 | 0.120604 | 0.050518 |
| *H. molitrix* 2 Aug | 11 | winter | 0.313854 | 0.309926 | 0.115005 | 0.130502 | 0.108519 | – |
| *H. molitrix* 13 Jun | 106 | winter | 0.312753 | 0.330688 | 0.694411 | 0.109371 | 0.127411 | – |
| *H. molitrix* 3 Sep | 0 | spring | 0.289782 | – | 0.676942 | 0.055381 | 0.120599 | 0.051538 |
| *H. molitrix* 5 Oct | 101 | spring | 0.301048 | – | 0.460479 | 0.093586 | 0.114907 | – |
| *H. molitrix* 2 Nov | 0 | spring | 0.330172 | 0.320731 | 0.587398 | 0.078521 | 0.119908 | 0.045872 |
| *H. molitrix* 8 Oct | 5 | spring | 0.284985 | 0.347979 | 0.3816 | 0.0496 | 0.127756 | 0.056975 |
| *H. molitrix* 4 Nov | 0 | spring | – | 0.323523 | 0.81306 | 0.052349 | 0.111352 | 0.050054 |
| *H. molitrix* 7 Nov | 64 | spring | – | 0.322012 | – | – | 0.119174 | 0.059042 |
| *H. molitrix* 2 Oct | 0 | spring | 0.292488 | – | 0.81306 | 0.052349 | 0.129938 | 0.055641 |
| *H. molitrix* 6 Nov | 128 | spring | 0.322325 | – | – | – | – | – |

Table 2: Oxidative stress biomarkers in the liver and gill tissue of *Labeo rosae* at Flag Boshielo Dam during February 2012–January 2013.

| Species | Infection | Season | Oxidative stress biomarkers | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | GST | | LPO | | TAC | |
| | | | Liver | Gill | Liver | Gill | Liver | Gill |
| *L. rosae* 8 Dec | 56 | summer | 0.283352 | 0.251526 | 0.066042 | 0.052714 | 0.054373 | 0.053041 |
| *L. rosae* 3 Dec | 0 | summer | 0.308378 | 0.247745 | 0.046886 | 0.057059 | 0.051458 | 0.053951 |
| *L. rosae* 2 Jan | 45 | summer | 0.294101 | 0.247753 | 0.078119 | 0.073655 | 0.05323 | 0.056938 |
| *L. rosae* 6 Jan | 0 | summer | 0.287941 | 0.250615 | 0.046645 | 0.067971 | 0.051422 | 0.055462 |
| *L. rosae* 5 Dec | 0 | summer | 0.279525 | 0.250471 | 0.056404 | 0.065858 | 0.050531 | – |
| *L. rosae* 10 Dec | 31 | summer | 0.290863 | 0.25079 | 0.04758 | 0.065409 | 0.054753 | – |
| *L. rosae* 2 May | 0 | autumn | 0.305671 | 0.254067 | 0.0691 | – | 0.054783 | – |
| *L. rosae* 10 May | 0 | autumn | 0.288643 | 0.26319 | 0.044952 | 0.049757 | 0.058958 | 0.051743 |
| *L. rosae* 15 May | 56 | autumn | 0.283745 | 0.256431 | 0.045285 | 0.05572 | 0.056829 | – |
| *L. rosae* 8 May | 0 | autumn | 0.641018 | 0.255469 | 0.049701 | 0.0467 | 0.057715 | – |
| *L. rosae* 4 May | 39 | autumn | 0.308474 | 0.252589 | 0.04646 | 0.083619 | 0.055381 | – |
| *L. rosae* 13 May | 94 | autumn | 0.29162 | 0.255816 | 0.058456 | 0.057981 | 0.058366 | – |
| *L. rosae* 4 Aug | 45 | winter | 0.295236 | 0.260246 | 0.05457 | 0.055992 | 0.057025 | – |
| *L. rosae* 9 jul | 0 | winter | 0.300415 | 0.253714 | 0.046946 | – | 0.054095 | – |
| *L. rosae* 7 Jul | 0 | winter | 0.310367 | 0.255909 | 0.067789 | – | 0.053144 | – |
| *L. rosae* 5 Aug | 0 | winter | 0.280926 | 0.259985 | 0.047973 | – | 0.055561 | 0.055282 |
| *L. rosae* 6 Jul | 74 | winter | 0.283299 | 0.255961 | 0.054531 | 0.052068 | 0.051902 | – |
| *L. rosae* 1 Aug | 43 | winter | 0.30149 | 0.269976 | 0.047355 | – | 0.056131 | – |
| *L. rosae* 5 Oct | 100 | spring | 0.297242 | 0.258407 | 0.05618 | 0.052636 | 0.059221 | 0.05343 |
| *L. rosae* 4 Nov | 0 | spring | 0.289051 | 0.259332 | 0.04553 | 0.053924 | 0.060036 | 0.058988 |
| *L. rosae* 4 Oct | 111 | spring | 0.298314 | 0.358146 | 0.053412 | 0.065433 | 0.057276 | 0.059335 |
| *L. rosae* 1Nov | 0 | spring | 0.295208 | 0.258212 | 0.050934 | 0.062586 | 0.058492 | 0.054726 |