Pansteatitis in tilapia (*Oreochromis mossambicus*) from Loskop Dam, South Africa: links between the environment, diet and thyroid status.

By

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Date submitted: July 2014

Declaration: I declare that the thesis which I hereby submit for the degree Doctor of Philosophy at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

ACKNOWLEDGEMENTS

First and foremost, this work is dedicated to my husband and daughter. For their belief, endless support and sacrifice of time together, I am forever grateful. In particular the personal investment of time, physical labour and constructive intellectual debate from my husband has been a constant source of encouragement. My father has continually inspired me to pursue a career in science, and has supported me in more ways than can be mentioned here. I have both my parents to thank for providing excellent examples of a good work ethic, dedication to a task, a keen interest in the natural environment, and a desire to improve it. My mother in particular has instilled a caring sensitivity to the natural world and all its inhabitants.

My sincere thanks to Dr. Paul Oberholster for facilitating my studies, supporting me throughout my academic career, and his friendship. Thanks to J.M. Dabrowski, N. Lubcker and A. Hoffman for long hours of physical labour and assistance during water sampling, fish collection and dissections. A-M Oberholster is thanked for support and assistance with laboratory analyses. Interpretive assistance from J. Steyl with histology is much appreciated. N. Lubcker, S. Woodborne and G. Hall are thanked for isotopic signature determinations. D. Helsel is thanked for his advice on the analysis of trends in water quality data, and D. Phillips is thanked for assistance with the analysis of isotope data. Assistance from D. Power with the interpretation of thyroid data was highly appreciated. Both M. Silberbauer and S. Jooste from the Department of Water Affairs are thanked for assistance obtaining data and determining the best methods of analysis. M. Claassen is thanked for many insights and thought-provoking conversations.

Fish collection and dissection methods were approved by the Animal Use and Care Committee of the Faculty of Veterinary Science, University of Pretoria (Protocol V017-10). Thanks to the Olifants River Forum for providing funding for this research, and the National Research Foundation for providing personal financial support.

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SUMMARY

PANSTEATITIS IN TILAPIA (*OREOCHROMIS MOSSAMBICUS*) FROM LOSKOP DAM, SOUTH AFRICA: LINKS BETWEEN THE ENVIRONMENT, DIET AND THYROID STATUS.

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The Olifants River is recognised as one of South Africa's "hardest working rivers". Deteriorating water quality has been attributed to increased discharge of effluents from mines, industry, and wastewater treatment works, along with escalating demand for power generation and agriculture. Between the years 2005 and 2009 mortalities of Nile crocodiles, *Crocodylus niloticus* (Laurenti), and Mozambique tilapia, *Oreochromis mossambicus* (Peters), were reported at Loskop Dam and were attributed to pansteatitis. Downstream in the Kruger National Park (KNP), the disease was also reported in crocodiles and sharptooth catfish, *Clarias gariepinus* (Burchell) during the same time period. Environmental conditions were investigated in Loskop Dam, as well as Flag Boshielo Dam, located < 100 km downstream, where pansteatitis has never been reported in any species. Frequent blooms of *Microcystis aeruginosa* and *Ceratium hirundinella* at Loskop Dam were the cause of high fluctuations in physico-chemical conditions, especially dissolved oxygen and pH. The trophic state of Loskop Dam was classified as meso- to eutrophic, and analysis of historic data showed increasing trends of dissolved inorganic nitrogen and phosphorus. In contrast, Flag Boshielo Dam was classified as oligotrophic with a decreasing trend in inorganic nitrogen, which reflected in very low chlorophyll-*a* concentrations. Pansteatitis is a nutritionally mediated disease reportedly associated with a diet high in polyunsaturated fats, frequently of fish origin. In order to determine whether piscivory (opportunistic or otherwise) was a contributing factor, the diet of *O. mossambicus* from both reservoirs was studied using stable isotopes and stomach contents. There was no evidence of piscivory in fish from either reservoir, including samples collected at Loskop Dam during historic fish mortality events. Fish diets differed markedly between reservoirs. The dinoflagellate *C. hirundinella* was the dominant food source in Loskop Dam, followed by zooplankton, detritus and *M. aeruginosa*. In Flag Boshielo Dam, sediment and detritus dominated the fish diet. This suggests that the classic aetiology of pansteatitis does not apply. A distinct characteristic of the disease is an abundance of mesenteric fat. The thyroid status of *O. mossambicus* from both reservoirs was evaluated as a biomarker of exposure to xenobiotics, and to determine whether disruption of the thyroid cascade could be affecting metabolic processes leading to lipid accumulation. Fish from Loskop Dam had elevated T3 concentrations along with increased thyroid follicle size, vacuolisation and epithelial cell height. Plasma cholesterol and triglycerides, hepatocyte size, and liver fat content were all elevated in fish from Loskop Dam and were indicative of high levels of energy storage. These indicators of nutritional status were all positively correlated with elevated T3 concentrations. Positive correlations between several indicators of nutritional state and thyroid hormones showed that elevated thyroid activity in fish was partly due to high nutritional inputs, and no disruption of the thyroid axis was detected. Although no definitive aetiology of pansteatitis was determined in this study, the results provide valuable information for hypothesis building, thus facilitating future research.

Keywords: Pansteatitis; *Oreochromis mossambicus*; Loskop Dam; Flag Boshielo Dam; diet; thyroid

LIST OF ABBREVIATIONS

Al	Aluminium
AMD	Acid Mine Drainage
CSIR	Council for Scientific and Industrial Research
DWA(F)	Department of Water Affairs (and Forestry)
FBR	Flag Boshielo Reservoir*
Fe	Iron
FIA	Flow injection analyser
FSL	Full supply level
H&E	Haematoxylin and eosin stain
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
KNP	Kruger National Park
LOWESS	Locally weighted scatterplot smooth
LR	Loskop Reservoir*
Mn	Manganese
ORF	Olifants River Forum
TSI	Trophic State Index
TWQR	Target Water Quality Range
WWTW	Wastewater treatment works

* Referred to as ‘reservoir’ and not ‘dam’ in chapters 5 and 6 due to international journal requirements.

Chapter 1: INTRODUCTION

1.1 Background to this study

1.1.1 *Aquatic ecosystem degradation at Loskop Dam*

The Olifants River is widely regarded as one of South Africa's most threatened river ecosystems (Ashton, 2010; Ashton & Dabrowski, 2011). The river flows in a north-easterly direction from its source on the Highveld region down the escarpment, through the Kruger National Park (KNP), and into the Massingir Dam in Mozambique, after which it flows into the Indian Ocean. Loskop Dam is an impoundment in the upper catchment of the Olifants River, which is the main inflow to the dam. Dominant land uses that impact water quality in this catchment include coal mining, power generation, industry, agriculture and effluent from wastewater treatment works (WWTWs). There is extensive evidence of the negative impact that acid mine drainage (AMD) from working and abandoned coal mines, and nutrient-rich effluent discharged from WWTWs, in particular, are having on water quality in the upper catchment (Ashton & Dabrowski, 2011; Dabrowski & De Klerk, 2013). Loskop Dam acts as a sink for these pollutants. Coal mining related impacts include increasing sulphate concentrations which have risen seven-fold since the 1970's (De Villiers & Mkwelo, 2009). Periods of low pH (< 6) have been reported at the inflow to Loskop Dam, along with concentrations of aluminium (Al), iron (Fe), manganese (Mn) and copper (Cu) that exceeded water quality guidelines for ecosystem health (Oberholster *et al*., 2010). High inputs of phosphorous originating from WWTWs have led to increased eutrophication, and the dam has been variously classified as hyper-eutrophic (Oberholster *et al*., 2010) and meso- to eutrophic (Oberholster *et al*., 2013). The combination of these impacts, along with an extended drought period, culminated in serious environmental problems at several trophic levels during the mid-2000s at Loskop Dam.

Between the years 2002 and 2005, water levels in the dam failed to reach full supply level (FSL), with the lowest dam level of 26% recorded in early 2004. In 2007 the cyanobacterium *Microcystis aeruginosa* and the dinoflagellate *Ceratium hirundinella* started forming seasonal blooms, which were ongoing during this study. At least four large scale fish kills involving several species occurred between 2006 and 2007, and were attributed to AMD in combination with other environmental factors (Dabrowski, 2012). While investigating fish kills, veterinary pathologists from the Faculty of Veterinary Science at the University of Pretoria also observed a pattern of spring die-offs of predominantly large (> 30 cm) male *Oreochromis mossambicus* (Mozambique tilapia) in the transitional zone of the dam. This area is a mixing zone characterised by reducing flows as water enters the lacustrine environment from the river. Algal blooms are most concentrated in this area. A diagnosis of pansteatitis (yellow fat disease) was made based on autopsies and histological examination of tissues. These revealed that *O. mossambicus* were extremely fat, with extensive inflammation, and hardening of the fat caused by ceroid pigment associated with lipid peroxidation. Other fish species appeared to be unaffected by the disease. In 2005 the first Nile crocodile (*Crocodylus niloticus*) was diagnosed with pansteatitis. Mortalities have been ongoing, and the most recent estimate of the crocodile population was around 6 individuals (Botha *et al*., 2011). Prior to this, estimates of the crocodile population ranged from 20 to 80 individuals (Jacobsen, 1984; Driescher, 2008).

1.1.2 *An overview of pansteatitis in the Olifants River catchment*

The events at Loskop Dam did not occur in isolation. During 2008 and 2009, downstream from Loskop Dam, at least 180 crocodiles were found dead at the Olifants River gorge in the KNP. After performing autopsies, veterinarians from the KNP determined that pansteatitis was the cause of death. Crocodile carcasses were extremely fat with extensive inflammation and hardening of the fat. Subsequent studies have also reported pansteatitis in *Clarias gariepinus* (sharptooth catfish), but no other fish species, from the same location (Huchzermeyer *et al*., 2011). Just prior to the crocodile deaths, the level of the Massingir Dam was raised in 2007. After this event, characteristics of the river in the gorge changed from a riverine to more lacustrine environment, and reduced flow resulted in increased deposition of clay-rich sediments.

The distance along the Olifants River between Loskop Dam and the gorge is ca. 520 km, and no reports of pansteatitis in aquatic animals have been made between these two locations. Flag Boshielo Dam is located ca. 90 km downstream of Loskop Dam, and has the Olifants and Elands rivers as the main inflows. This reservoir has the largest population of crocodiles on the Olifants River outside of the KNP. To date, pansteatitis has not been reported in fish or crocodiles from this location, and fish kills are not frequently reported. Its location downstream of Loskop Dam means the potential exists for a similar range of pollutants to affect water quality. However, the combination of conditions that cause pansteatitis are not present at Flag Boshielo Dam, making it a suitable reference site for studies of the disease aetiology.

1.2 Research foundation

In 2009 the Council for Scientific and Industrial Research (CSIR) was appointed by the Olifants River Forum (ORF) to conduct a three-year multidisciplinary study to investigate pollution sources and their implications in the upper Olifants River system. As a member of the project team, the author commenced studies to evaluate various aspects of the health of *O. mossambicus,* and environmental conditions in Loskop Dam as a first stage approach to determine possible causes of pansteatitis. This formed the basis of an MSc dissertation (Dabrowski, 2012). The results of this work showed distinct patterns in surface water quality parameters at Loskop Dam (such as pH and dissolved oxygen), many of which were strongly influenced by the presence of algal blooms. One hypothesis was that the oxidative stress apparent in the fish fat may have originated from chronic exposure to dissolved metals. But there was no evidence of bioaccumulation of metals (Al, Fe, Mn, Cu and Zn) in various fish tissues (gills, liver, brain, bone or muscle). In fact, Cu was significantly depleted in the livers of fish from Loskop Dam. Parasite communities of *O. mossambicus* were evaluated as an indicator of ecosystem health, and results were indicative of ecosystem degradation. During this study, steatitis lesions were observed in 100% of the *O. mossambicus* examined from Loskop Dam, suggesting that the causes of pansteatitis are present at the population level.

Several research questions surrounding the diet of *O. mossambicus*, and origins of their abundant fat arose from this work. The preliminary assessment of water quality also required improvement to incorporate seasonal variation and physico-chemical variation within the water column at different depths, a suitable reference site, and improved analytical methods. This PhD study addresses these research questions and aims to fill knowledge gaps identified in previous work.

1.3 Study justification

Determination of the aetiology of pansteatitis in *O. mossambicus* from Loskop Dam would enable management authorities to focus directed efforts to eliminate the combination of factors that cause the disease. This would improve conservation of the aquatic ecosystem and keystone species such as the Nile crocodile. Water quantities are already strained in the catchment, a situation exacerbated by deteriorating water quality. Improving water quality would reduce time and costs associated with treatment by users for industry, irrigation, and drinking water. Pansteatitis does not occur frequently in free-ranging animals, and this is the first known case of the disease in *O. mossambicus*. To date, no study of the disease in free-ranging animals has conclusively determined the cause. Results of this study can be used to establish environmental similarities and differences in cases of pansteatitis in the KNP and the rest of the world. It is not clear whether the causes of pansteatitis in fish and crocodiles are associated. However a study of potential causes in fish may reveal links to the disease in crocodiles. Finally, their utility as an aquaculture species has resulted in *O. mossambicus* being widely distributed around the world, and in many countries they are considered to be an invasive species (Russell *et al*., 2012). The results of this work will contribute to knowledge of their biological characteristics in free-ranging populations within their native range.

Chapter 2: LITERATURE REVIEW

2.1 Pansteatitis: Established pathology and aetiology

Pansteatitis, synonymous with yellow fat disease, is nutritionally mediated and characterised by degeneration, necrosis and inflammation of fat cells, with associated accumulations of ceroid pigment (Suarez-Bonnet *et al*., 2008; Huchzermeyer *et al*., 2011; Orós *et al*., 2013). Fat tissue affected by steatitis is yellow in colour which is why the disease is often referred to as yellow fat disease. Ceroid pigment is responsible for this discoloration, and is the final product of lipid peroxidation (Danse & Steenbergen-Botterweg, 1974). The term steatitis is used to describe non-generalised lesions of fat cell necrosis, while pansteatitis is used to describe the generalised disorder (Huchzermeyer, 2012).

Mesenteric or subcutaneous fat tissues may become hardened and painful as a result of fat cell degeneration and inflammation. These areas may be palpable as subcutaneous nodular masses, and can affect mobility of the animal (Larsen *et al*., 1983, De Bruijn *et al*., 2006). In crocodiles, hardened fat in the tail may render the animal unable to swim (Huchzermeyer, 2003). Fever, malaise and cachexia frequently occur in endotherms (De Bruijn *et al*., 2006; Niza *et al*., 2003). Obesity has been reported in horses (De Bruijn *et al*., 2006; Suarez-Bonnet *et al*., 2008) but most affected species experience weight loss (German *et al*., 2003; Juan-Salles *et al*., 2003; Niza *et al*., 2003). Pansteatitis can cause acute mortalities. But observations of farmed crocodiles and alligators have shown that some animals recover and survive, continue growing, and steatitis lesions are only discovered at slaughter (Huchzermeyer, 2003; Larsen *et al*., 1983). Similarly, a great blue heron with pansteatitis was treated with vitamin E and recovered. But subsequent biopsies of subcutaneous fat indicated no change in steatitis lesions (Nichols *et al*., 1986).

Pansteatitis has been reported in various fish species, predominantly in an aquaculture setting (Bricknell *et al*., 1996; Goodwin, 2006; Guarda *et al*., 1997; Herman & Kircheis, 1985; Roberts *et al*., 1979; Roberts & Agius, 2008). From these studies it is evident that steatitis lesions in fish are similar to those for endothermic animals. Lesions are characterised by necrosis and an inflammatory reaction in fat tissues, the presence of multinucleated giant cells resulting from accumulated macrophages, which form the centre of epithelioid granulomas with fibrotic connective tissue infiltration (Bricknell *et al*., 1996; Herman & Kircheis, 1985).

Pansteatitis has been linked to a diet high in polyunsaturated fats, frequently of fish origin, and deficient in antioxidants, particularly vitamin E. Reduced vitamin E levels may be due to low dietary intake, or could be exhausted by oxidative pressure associated with a high intake of polyunsaturated fats (Fytianou *et al*., 2006). The disease has been induced in rabbits fed a diet enriched with herring oil and deficient in vitamin E (Jones, 1969), rats fed a diet enriched with cod-liver oil and deficient in vitamin E (Danse & Verschuren, 1978), and in cats fed cooked sardines with no vitamin E supplementation (Fytianou *et al*., 2006). Rats fed diets with high fish oil content have reduced vitamin E and antioxidant enzyme levels, and higher rates of lipid peroxidation. Lipid peroxidation was reduced and vitamin E levels were normalised by dietary supplementation with vitamin E (Cho & Choi, 1994). Ultimately the disease represents an imbalance between antioxidant defence systems and damage associated with free radicals (Halliwell, 2006). In several cases involving both endo- and ectotherms the diet implicated included high amounts of fish or fish oil (Larsen *et al*., 1983; Roberts & Agius, 2008; Niza *et al*., 2003). Fish fat, including that of *O. mossambicus*, has a high content of polyunsaturated fatty acids, particularly docosahexaenoic (22:6 (*n*-3)) and eicosapentaenoic (20:5 (*n*-3)) acids (Jabeen & Chaudhry, 2011). In fish, these fats are obtained through consumption of photosynthetic microalgae (Khozin-Goldberg *et al*., 2011). Unsaturated fatty acids in cell membranes are highly sensitive to oxidative damage and are protected by Vitamin E and enzymatic antioxidants. Vitamin E is fat-soluble and prevents the oxidative conversion of polyunsaturated fats to lipid hydroperoxides by donating electrons to free radicals. If insufficient vitamin E is present then lipid tissues undergo extensive oxidation leading to cell membrane damage and necrosis (Niza *et al*., 2003). Accumulations of ceroid pigment released during necrosis have an irritant effect which causes the inflammatory reaction typical of pansteatitis.

2.2 Aetiology in free-ranging animals

Pansteatitis has most frequently been described in captive-bred animals reared on formulated diets and has less often been reported in free-ranging animals in their natural habitat. However, accounts of this disease in free-ranging animal populations appear to be rising in frequency. In addition to the cases involving fish and crocodiles on the Olifants River, it has been reported in wild rabbits from the United Kingdom (Jones, 1969), great blue herons in Chesapeake Bay, USA (Nichols *et al*., 1986), a red-tailed hawk from Canada (Wong *et al*., 1999), common dab and long rough dab off the coast of Scotland (Begg *et al*., 2000), egrets and herons in Japan (Neagari *et al*., 2011), Mediterranean striped dolphins (Soto *et al*. 2010) and a loggerhead turtle (Orós *et al*., 2013) off the coast of Spain.

In free-ranging animals the causes are often unclear, and due to the unpredictability of acute natural mortalities, and spatio-temporal fluctuations in environmental conditions, difficult to study methodically. There is also often a lack of detailed knowledge of the diet of the animals concerned and insufficient sample sizes from which to make statistically rigorous conclusions. The fact that it occurs in animals predominantly associated with aquatic habitats (both marine and freshwater) may be indicative of deteriorating water quality or altered food webs.

2.2.1 *Fish kills and piscivory*

Fish kills may occur in polluted waters, and can be caused by a variety of factors including environmental variables and exposure to various pollutants (La & Cooke, 2010). The presence of abundant dead fish following a fish kill provides animals prepared to scavenge with a ‘cheap’ food source. The fats of dead fish quickly oxidise and become rancid. If large volumes are consumed, the rapid depletion of antioxidants such as vitamin E may result (Nichols *et al*., 1986). Although no conclusive links between fish kills and pansteatitis in free-ranging animals have been made, farmed crocodiles and alligators fed an exclusive diet of dead, unpreserved fish have developed pansteatitis (Huchzermeyer, 2003; Larsen *et al*., 1983). At least four major fish kills (one > 14 tons) were observed at Loskop Dam between 2006 and 2007 (Dabrowski, 2012) and at least one major fish kill was reported from the

Olifants River gorge at the KNP in 2009 (Huchzermeyer *et al*., 2011). Although the latter location is relatively isolated and other fish kills may not have been detected. It is conceivable that Nile crocodiles at both locations may have developed pansteatitis as a result of feeding on dead fish. This explanation may even be accommodated by the omnivorous and opportunistic feeding habits of *C. gariepinus* (Kadye & Booth, 2012). But there have never been any reports of *O. mossambicus* feeding extensively on fish in their native range, and their diet consists of detritus, phytoplankton, periphyton, macroalgae, diatoms and zooplankton (Bowen, 1979; de Moor *et al.*, 1985; Dyer *et al*., 2013; Zengeya *et al*., 2011).

Research from Australia where *O. mossambicus* is invasive presents a somewhat different picture. Studies in both laboratory and field situations showed that *O. mossambicus* consumed indigenous fish prey despite the availability of other food (Doupé *et al*., 2009), and completely digested fish in as short as one hour leaving little evidence of this food source in stomach contents (Doupé & Knott, 2011). In Sri Lanka, De Silva *et al*. (1984) reported *O. mossambicus* diets ranging from detritivory, to herbivory, to complete carnivory in different reservoirs. Within their native range *O. mossambicus* are known to exhibit trophic plasticity under different environmental conditions (Bowen & Allanson, 1982; Dyer *et al.,* 2013). But this has never been reported to include piscivorous behaviour. Assumptions regarding their diet in Loskop Dam are limited by their trophic plasticity. It is possible that *O. mossambicus* in Loskop Dam are piscivores, or may scavenge on dead fish associated with fish kills.

Animals not adapted to a diet high in polyunsaturated fat may lack effective antioxidant defence systems to control lipid peroxidation if consuming a diet of this nature. Atypical piscivorous behaviour was cited as the probable cause of pansteatitis in wild rabbits from Skokholm Island (Jones, 1969). Insufficient pasture may have caused them to forage on fish scraps from sea-birds, as indicated by the presence of polyunsatured fatty acids typical of marine organisms in their fat. However no reports of pansteatitis in free-ranging animals have made a conclusive link between the disease and piscivory. Consumption of fish (or other food items) with high quantities of polyunsaturated fats, or feeding on dead, rancid fish are two of the most straight-forward explanations of the aetiology of pansteatitis in free-ranging animals, and therefore cannot be excluded as a cause without a comprehensive dietary assessment.

2.2.2 *Algal blooms, metal bioaccumulation, and interaction effects*

Steatitis lesions were observed in a large number of farmed Sunapee trout (*Salvelinus alpines oquassa*), and were suspected of being associated with their diet. However, experiments testing the diet failed to reproduce the disease, highlighting the very broad possibility that water quality was a contributing factor (Herman & Kircheis, 1985).

One similarity between two isolated reports of pansteatitis in free-ranging animals is the presence of cyanobacteria (*Microcystis* spp.) blooms which occurred at a reservoir in Japan (Neagari *et al*., 2011), and occur during mid- to late summer at Loskop Dam (Oberholster *et al*., 2010). These blooms are considered to be an indicator of eutrophication in freshwater ecosystems. Microcystin toxins produced by cyanobacteria are known to increase lipid peroxidation and the resultant activity of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase in Nile tilapia (*Oreochromis niloticus;* Jos *et al*., 2005; Prieto *et al*., 2007). However, no microcystin toxins were detected in the water or in any of the pansteatitis-affected bird livers examined post mortem in the event in Japan (Neagari *et al*., 2011).

Apart from the production of toxins, algal blooms have associated impacts on water quality that can affect fish health. When algal blooms degrade, bacterial decomposition of the organic material can cause rapid reduction of oxygen levels resulting in fish kills (La & Cooke, 2010). Through photosynthetic absorption of carbonic acid, algal blooms in the transitional zone of Loskop Dam maintain the pH of the surface water at > 9 during the day and night (Dabrowski, 2012). This affects the speciation and bio-availability of metals such as Al (Gensemer & Playle, 1999), and increases the proportional concentration of ammonia present as un-ionised NH_3 which can also result in fish kills at high concentrations (La & Cooke, 2010). While these secondary effects of algal blooms have the capacity to kill fish, they have never directly been associated with pansteatitis. But surviving fish and crocodiles scavenging on fish kills may develop pansteatitis as described in section: 2.2.1.

Elevated concentrations of dissolved Al, Fe, Mn and Cu have been reported in the surface water of Loskop Dam (Oberholster *et al*., 2010), and Al and Fe bioaccumulation has been documented in the fat of pansteatitis-affected *O. mossambicus,* as well as filamentous algae (*Spirogyra* spp.), a potential food source (Oberholster *et al*., 2012). The presence of these metals in the fish fat may indicate a link to the oxidative stress associated with pansteatitis. Previous work found no evidence of bioaccumulation of Al or Fe in any tissues (liver, gills, brain, bone, muscle) of *O. mossambicus* from Loskop Dam (Dabrowski, 2012). Although Fe is an essential element with a number of fundamental roles in cellular biochemistry and metabolism, in excess it can initiate oxidative damage to cells through a series of redox reactions that generate free radicals including hydroxyl radicals (Bury & Grosell, 2003; Carriquiriborde *et al*., 2004). The two potential sites for the uptake of metals in fish are the intestinal (dietary borne) or branchial (water borne) epithelium. The diet of freshwater fish is their primary source of Fe (Bury & Grosell, 2003). A study investigating the effects of excessive levels of dietary Fe in *C. gariepinus* reported elevated lipid peroxidation in several tissues along with depleted vitamin E levels (Baker *et al*., 1997). The only study that has investigated the effects of elevated dietary Al was performed using *Oreochromis niloticus* (Nile tilapia) reared in tanks for one year. The study did not focus on biomarkers of oxidative stress, but reported elevated lipid content and growth of fish fed the Al-enriched diet (Santos *et al*., 2011). Incidentally, pansteatitis-affected *O. mossambicus* from Loskop Dam are among the largest specimens in South Africa according to angling records, and they have abundant mesenteric fat. The co-occurrence of elevated Al and Fe in the fish fat may result in synergistic effects on lipid peroxidation, as Fe^{2+} stimulated oxidation of phospholipid liposomes and the erythrocytes of rats is increased in the presence of Al^{3+} (Quinlan *et al*., 1988). Information on the metal content of major dietary items of *O. mossambicus*, and the physico-chemical processes leading to their absorption would be required to determine if this is a causative factor in the aetiology of pansteatitis. However, a preliminary step required is to determine the major food items that constitute their diet.

2.2.3 *Xenobiotics*

Apart from a dietary imbalance of excess polyunsaturated fats and insufficient antioxidants, oxidative stress can originate from exposure to xenobiotics. Many pollutants are known pro-oxidants in the aquatic environment including transition metals, organochlorine and organophosphate pesticides, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and dioxins (Valavanidis *et al*., 2006; van der Oost *et al*., 2003). Exposure to the oxidative effects of xenobiotics may reduce the availability of enzymatic and non-enzymatic antioxidants such as Vitamin E, rendering polyunsaturated fats in fish tissues susceptible to peroxidation (Prieto *et al*., 2007). Location specific exposure to xenobiotics has been reported in pansteatitis-affected animals. Researchers investigating a case of pansteatitis in a loggerhead turtle off the coast of Spain measured very high levels of PCBs in the fat of the animal (Oros *et al*., 2013), and elevated levels of certain insecticides were measured in the livers of pansteatitis-affected common dab off the coast of Scotland (Begg, 1994). However there has been no research to establish tissue concentrations at which induction of oxidative stress occurs, and the significance of these exposure levels to the development of pansteatitis is unknown.

The only study investigating organic pollutants in the Olifants River was conducted in the middle catchment, between Loskop and Flag Boshielo dams (Bollmohr *et al*., 2008). Out of 53 pesticides investigated, this study reported the presence of 8 PAHs, 6 phthalates, 31 organochlorine and organophosphorus pesticides, 2 carbamates, 3 pyrethroids and 3 triazines. Several pesticides were present at concentrations that posed a potential health risk to aquatic biota. Various PAHs, phthalates, organochlorine and organophosphorus pesticides, carbamates are known to disrupt thyroid function in fish (Brown *et al*., 2004; Zhai *et al*., 2014). There are potentially hundreds of man-made chemicals entering Loskop Dam from surface runoff and effluent discharged from agricultural operations, WWTWs, mining, and industrial activities in the upper catchment of the Olifants River.

2.2.3.1 *Thyroid function as a biomarker of pollutant exposure*

Apart from their ability to induce oxidative stress in aquatic biota, numerous xenobiotics can act as endocrine disrupting compounds (EDCs). The thyroid cascade is a useful biomarker of exposure to a wide range of environmental pollutants (Brown *et al*., 2004). In *O. mossambicus* thyroid hormones are involved in the control of osmoregulation (Peter *et al*., 2000), growth (Schmid *et al*., 2003), development (Reddy & Lam, 1992), and reproduction (Weber *et al*., 1992). The thyroid cascade involves two main components. Firstly, the hypothalamic-pituitary-thyroid axis controls the synthesis, secretion and metabolism of L-thyroxine (T4). The cascade begins with thyrotropin releasing hormone (TRH) from the hypothalamus in response to internal or external sensory stimuli. Along with neurological connections between the hypothalamus and pituitary gland, TRH stimulates the release of thyroid stimulating hormone (TSH) from the pituitary. Secretion of T4 is regulated by TSH. T4 is considered a prohormone, and is converted to the more metabolically active 3,5,3'-triiodo-L-thyronine (T3) by outer ring deiodination. Metabolism and receptor-mediated actions of T3 are largely under peripheral control in extra-thyroidal tissues (Eales & Brown, 1993). Thyroid function has never been measured in captive or free-ranging animals with pansteatitis, but it may be relevant in *O. mossambicus* from Loskop Dam. These fish contain abundant fat reserves which provide an extensive substrate for lipid peroxidation to occur. Lipid metabolism in fish is stimulated by increasing levels of the thyroid hormone thyroxine (T4) (Narayansingh & Eales, 1975; Sheridan, 1986), so disruption of thyroid status may interfere with lipid metabolism.

2.2.4 *Altered food webs*

The Massingir Dam wall in Mozambique was raised in 2007 which resulted in back-flooding of several rapids and pools in the Olifants River gorge (Huchzermeyer, 2012). The altered environment favoured the growth of phytoplankton, and blooms were subsequently observed in this area in 2008 (Huchzermeyer, 2012). Studies of the lipid profiles of *C. gariepinus* and *C. niloticus* from the KNP have shown that pansteatitis-affected animals have a higher n-3 to n-6 fatty acid ratio than healthy animals, indicating an increased intake of polyunsaturated fats which are prone to oxidation (Osthoff *et al*., 2010; Huchzermeyer *et al*., 2013). It has been proposed that the altered aquatic habitat presented conditions favouring phytoplankton-feeding fish, and provided an alternative prey source for both catfish and crocodiles (Huchzermeyer *et al*., 2013). The fish proposed was the invasive silver carp (*Hypophthalmichthys molitrix*). These fish are obligate plankton feeders and are known to assimilate n-3 fatty acids from their diet. Prior to 2007 they were restricted to Massingir Dam, but altered hydrodynamics of the Olifants River now permit their seasonal migration upstream into the gorge to spawn (Huchzermeyer *et al*., 2013).

The proliferation of algal blooms at Loskop Dam since 2007 represent bottom up changes in the food web. This is particularly relevant for primary consumers such as *O. mossambicus*. There are historic occurrence records of both *M. aeruginosa* and *C. hirundinella* at Loskop Dam (Butty *et al*., 1980), but at that point in time they were not as abundant, and had less of an effect on water quality. It is not clear whether the increased abundance of either species of phytoplankton is connected to pansteatitis in *O. mossambicus*. But as potentially important food sources for this fish species it is important to determine the extent to which they contribute to their diet, as well as their nutritional composition.

2.3 Local environmental drivers

Several studies reporting pansteatitis in free-ranging animals have emphasised the need to monitor and evaluate environmental conditions at these locations (Begg *et al*., 2000; Neagari *et al*., 2011; Nichols *et al*., 1986; Oros *et al*., 2013). The occurrence of pansteatitis in two isolated aquatic animal populations, at different trophic levels, on the same river system (the Olifants River), during a similar time period, presents a unique situation. This strongly suggests similarities in the environmental drivers at the two locations, and that conditions between these points differ significantly in some way. Flag Boshielo Dam is located downstream from Loskop Dam, and pansteatitis has never been reported in the fish (*O. mossambicus* and *C. gariepinus*) or crocodile populations of this dam. The crocodile population is the largest on the Olifants River outside of the KNP despite the fact that invasive silver carp implicated in food web alterations, and possibly linked to pansteatitis at the KNP, are also present in Flag Boshielo Dam (Brits, 2009). To determine why pansteatitis occurs at Loskop Dam but not at Flag Boshielo Dam it was necessary to establish the defining characteristics of each reservoir.

The nutrient dynamics, trophic status, and algal productivity at both reservoirs is particularly relevant given that intensified algal blooms appeared to coincide with the onset of pansteatitis at Loskop Dam. As fish are exposed to conditions in the entire water column and not just the surface, depth profiles within the water column of relevant water quality parameters are required, and must account for seasonal variation. Based on research showing that Al and Fe were accumulating in both algae and the fat of *O.mossambicus* at Loskop Dam (Oberholster *et al*., 2012), these parameters should include dissolved metals. Previous studies have been limited to surface water samples (Dabrowski, 2012; Oberholster *et al*., 2010), but metal concentrations can vary with depth. Soluble Fe^{2+} and Mn^{2+} in particular are known to be released from bottom sediments under reducing conditions, such as during hypolimnetic oxygen depletion associated with seasonal lake stratification (Wetzel, 1983).

The events that lead to the discovery of pansteatitis in crocodiles and fish from Loskop Dam all occurred during the mid to late 2000s, and can no longer be considered recent. It is therefore necessary to contextualise the evaluation of water quality by including the analysis of historic data collected by the Department of Water Affairs (DWA). The physico-chemical characteristics of reservoirs fluctuate climatically, seasonally, spatially, and at different depths within the water column (Wetzel, 1983). These variables must all be considered and accounted for as far as possible to increase our understanding of the environmental drivers linked to pansteatitis.

2.4 Study aims

There are numerous hypotheses surrounding the aetiology of pansteatitis in free-ranging animal populations, as outlined in this chapter. In order to explore several of these hypotheses, an in-depth understanding of the environmental conditions at the location where pansteatitis occurs is required. Therefore the first study aim was:

- To establish the major environmental drivers affecting water quality at Loskop Dam, and compare these to a suitable reference site, identified as Flag Boshielo Dam. Parameters selected for measurement were based on the findings of previous research. For instance Al, Fe and Mn have been detected previously, but mercury (Hg) and cadmium (Cd) have never been detected. In order to complete gaps in previous research, monitoring accounted for seasonal and depth variations. As the events leading to the discovery of pansteatitis at Loskop Dam are no longer recent, historic water quality monitoring data were evaluated for trends.

The most likely explanation for pansteatitis in free-ranging animal populations is a piscivorous diet or opportunistic scavenging on rancid fish during fish kills (SectionChapter 2:2.2.1). This hypothesis was tested in the second study aim which was to:

- Determine the diet of *O. mossambicus* at both Loskop and Flag Boshielo dams using a combined approach of stomach contents analysis and stable isotopes. Fish were collected on four occasions within an annual cycle to account for seasonal variation in diet availability and feeding habits. Stable isotope signatures of historic fish samples were included in the analyses to determine whether there was evidence of trophic shifts over time. If piscivory is not established in the diet of *O. mossambicus*, then identification of their dominant food sources will facilitate future research related to the nutritional and chemical composition of their diet.

The thyroid cascade is considered a sensitive biomarker of endocrine disruption by xenobiotics. Furthermore, disruption of the thyroid cascade may be linked to abundant fat deposits in *O. mossambicus*. This is an important characteristic of pansteatitis in *O. mossambicus*, because it provides an extensive substrate for the oxidation of lipids to occur. The third study aim was to:

- Establish whether there was evidence of xenobiotic interference in endocrine function using the thyroid cascade as a biomarker. Determine the thyroid status of *O. mossambicus* from Loskop and Flag Boshielo dams using plasma T3 and T4 levels, and thyroid follicle descriptors. Thyroid function is directly influenced by nutritional state, so this was determined concurrently by measuring fish plasma lipids, liver fat content, and hepatocyte descriptors.

Chapter 3: CHEMICAL CHARACTERISTICS AND LIMNOLOGY OF LOSKOP DAM ON THE OLIFANTS RIVER (SOUTH AFRICA), IN LIGHT OF RECENT FISH AND CROCODILE MORTALITIES

This chapter has been published in the journal Water SA (Appendix 1)

3.1 INTRODUCTION

Loskop Dam was constructed in 1937 to supply irrigation water to downstream agricultural areas (Van Vuuren, 2008). The irrigation canal system, measuring 480 km, was completed just over 10 years later, in 1948. The system provides water to the Loskop Irrigation Board, which is the second largest irrigation area in South Africa, supplying 700 properties and covering an area of 16 117 ha (Oberholster & Botha, 2011). The dam is located about 32 km south of the town of Groblersdal in Mpumalanga Province, and is surrounded by a nature reserve. The main inflow to Loskop Dam is the Olifants River, which has the Wilge and Klein Olifants Rivers as its main tributaries. From Loskop Dam, the Olifants River flows north-east through the Drakensberg mountain range, the KNP, and into Massingir Dam in Mozambique, before continuing to the Indian Ocean.

Land uses in the upper Olifants River catchment that impact freshwater ecosystems include coal mining, power generation, industry, agriculture, and WWTWs associated with urban areas. Coal mining is a dominant activity and the Witbank-Highveld coalfield produces 81% of the coal in South Africa, thereby contributing significantly to the South African economy (DMR, 2009). One of the adverse impacts of coal mining is the production AMD, which occurs when pyrite associated with coal-bearing units is oxidised by exposure to air and moisture, forming sulphuric acid (H_2SO_4) (Pinetown *et al*., 2007). Acid mine water decanting from abandoned mines in the upper Olifants River catchment is characterised by low pH (< 3), high sulphate (SO_4^{2-}) concentrations (> 2000 mg/ℓ) and elevated Al, Fe and Mn concentrations (Bell *et al*., 2001; Bell *et al*., 2002; Vermeulen & Usher, 2006). The increased metal concentrations may be attenuated by the receiving water body through a number of processes, including absorption and adsorption by aquatic flora and fauna, thus entering the food web. Research has shown that algae growing in the AMD-impacted Blesbokspruit (tributary of the Klipspruit) have elevated levels of Al, Fe, Zn, Cu, Ni, Mn and Pb (Bell *et al*., 2002), and several trophic levels have shown evidence of Al and Fe bioaccumulation in Loskop Dam (Oberholster *et al*., 2012).

Previous studies have reported concentrations of Al and Cu in the water in Loskop Dam that exceeded the target water quality ranges (TWQR) for ecosystem health (Oberholster *et al*., 2010) set by the Department of Water Affairs (DWAF, 1996a). Along with dissolved metals, another impact associated with extensive coal mining in the catchment is the ongoing increase in SO_4^{2-} concentrations, which have risen more than 7-fold in Loskop Dam since the 1970's (De Villiers & Mkwelo, 2009). Nutrient enrichment in the form of inorganic nitrogen (mainly nitrate and nitrite) and phosphate (orthophosphate) has increased, resulting in eutrophication of the dam and subsequent algal blooms (Oberholster *et al*., 2010). While agricultural sources have not been eliminated, a major source of nutrients is thought to be the WWTWs in the catchment that, according to the latest Green Drop Report (DWA, 2011), are largely dysfunctional (to varying degrees), releasing partially treated or untreated sewage into water resources.

Serious concerns about water quality in Loskop Dam were raised in the mid-2000s when a number of environmental problems were reported. These events occurred at several trophic levels indicating wide-scale changes in the structure and function of the ecosystem. At the level of primary production, the cyanobacterium *M. aeruginosa* and dinoflagellate *C. hirundinella,* which have been present in the dam for many years (Butty *et al*., 1980), began reaching bloom proportions around 2007 (J. Coetzee, Mpumalanga Tourism and Parks Agency, pers. comm. 2011). *Oreochromis mossambicus* were the next trophic level affected, with seasonal (usually spring) die-offs of predominantly large (> 30 cm) males occurring in the transitional zone of the dam. Early work conducted by veterinarians from the Faculty of Veterinary Science at the University of Pretoria confirmed that the fish had pansteatitis, a disease characterised by obesity and lipid peroxidation. Apart from these chronic fish die-offs, several large-scale fish kills also occurred with up to 14 t of fish, predominantly rednose mudfish (*Labeo rosae*), dying in an event that lasted around 30 days in

August 2007. This event was accompanied by a strong smell of H_2SO_4 from the water and bubbling at the water surface (J. Coetzee, Mpumalanga Tourism and Parks Agency, pers. comm. 2011). The top predator in the aquatic ecosystem was also affected. In 2005 the first Nile crocodile (*Crocodylus niloticus*) found in a poor condition was taken to the Faculty of Veterinary Science where it was diagnosed with pansteatitis. The problem has been ongoing since then, with a documented decline in the crocodile population at Loskop Dam to 6 individuals (Botha *et al*., 2011). During fieldwork for the current study, another crocodile was found weak and dying near the Kranspoortspruit inflow (pers. obs.) in April 2011 and pansteatitis was confirmed during post mortem examination at the Faculty of Veterinary Science section of Pathology, University of Pretoria.

The only other incidence of pansteatitis in wild animal populations in South Africa was reported further downstream, in the Olifants River gorge in the KNP, where over 180 crocodiles died and were diagnosed with the condition (Huchzermeyer *et al.,* 2011). Since then the population has been in decline (Ferreira & Pienaar, 2011). The condition has also been reported in *C. gariepinus* at the same location (Huchzermeyer *et al.*, 2011). The occurrence of the disease in two geographically distinct fish and crocodile populations on the same river is peculiar, especially as there have been no reports of the disease occurring between these locations. This suggests that there is something unique about the combined environmental impacts present at both locations, that are not present elsewhere on the Olifants River.

Water quality impacts and their effects in Loskop Dam need to be well described and understood so that appropriate management strategies can be planned and prioritised. The last limnological surveys of Loskop Dam were completed several decades ago by Gieskes (1960) and Butty *et al.* (1980). Current information on limnology and water chemistry of the epilimnion and hypolimnion, including seasonal variations in Loskop Dam, was therefore required as a starting point to determine the physical and chemical processes that may be influencing water quality from an ecosystem health perspective.

The aetiology of pansteatitis in fish and crocodiles from Loskop Dam is as yet unknown, although it is recurrently described elsewhere as being related to a diet high in polyunsaturated fats and deficient in antioxidants, in captive-bred animals (Fytianou *et al*., 2006; Goodwin, 2006; Larsen, 1983; Roberts *et al*., 1979; Roberts & Agius, 2008). The diet of *Oreochromis mossambicus* in Loskop Dam was investigated during the present study, and these results are reported in Chapter 5. A thorough assessment of current water quality provided a backdrop to the dietary study, and was essential in understanding possible causes of fish kills, and pansteatitis in fish and crocodiles. The aims of this study were to assess: (i) the current water chemistry and limnology of Loskop Dam over an annual cycle; (ii) the trophic state of Loskop Dam; (iii) time-series trends of physico-chemical data collected by the DWA using a seasonal Mann-Kendall trend test; (iv) water quality according to the DWAF guidelines for ecosystem health (DWAF, 1996a) where applicable.

3.2 METHODS

3.2.1 *Study site*

Loskop Dam (25º 26’ 57.05” S, 29º 19’ 44.36” E) is in the Olifants Water Management Area. Vegetation in the upper Olifants River catchment consists of Highveld grassland in the upper reaches of the catchment and mixed bushveld and thornveld in the lower reaches around Loskop Dam (Mucina & Rutherford, 2006). Established study sites at 5 locations across the dam were sampled (Figure 3.1). The sites were selected to reflect a spatial gradient across the dam from the riverine (LK1), through the transitional (LK2, LK3), to the lacustrine zone (LK4, LK5). The DWA water quality monitoring site (Station B3R002) is located near the dam wall and close to LK5 in the lacustrine zone (Figure 3.1).

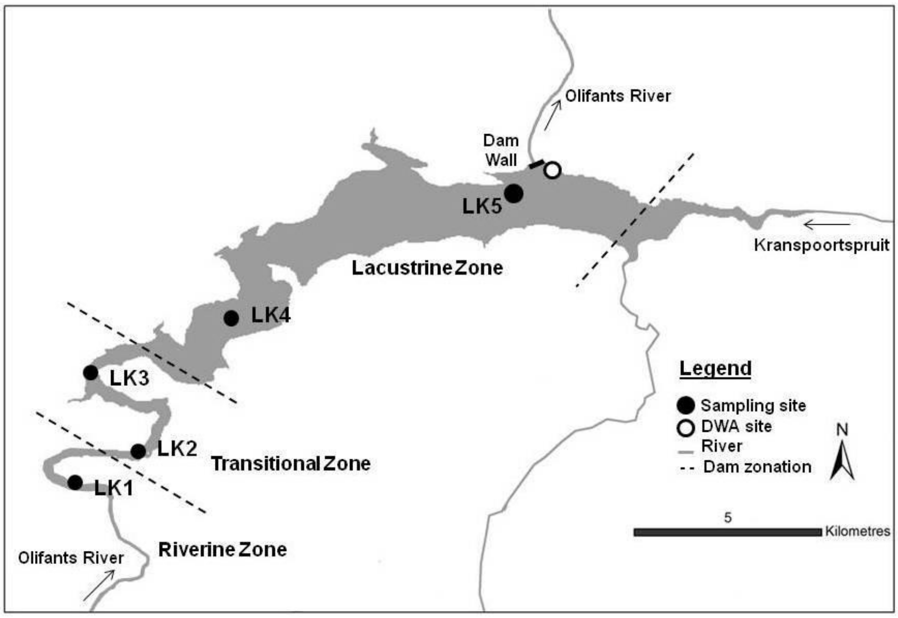

Figure 3.1 Map of Loskop Dam showing the location of five sampling sites and the Department of Water Affairs (DWA) site in various zones across the dam.

3.2.2 *Reservoir physiography*

Several reservoir attributes were revised as part of this study, as previous calculations of mean depth and mean retention time were completed in 1977 and 1978, prior to the dam wall being raised by 9 m in 1979 (Butty *et al*., 1980; Midgely *et al*., 1994). Mean annual runoff was calculated from the sum of annual flows recorded at DWA gauging stations B1HO15 (Klein Olifants River), B1H004 (Klipspruit), B1H010 (Olifants River), B1H002 (Spookspruit), and B2H015 (Wilge River). Only complete annual records (12 months) monitored simultaneously at all stations were included in the calculation. This limited the dataset to 14 years between 1994 and 2010. The mean retention time was calculated by dividing the mean annual runoff by the reservoir volume (Dodds & Whiles, 2010), and the mean depth was calculated by dividing lake volume by lake area (Timms, 2010). Shoreline length was obtained from the DWA Directorate of Spatial and Land Information Management. All other values in Table 1 were obtained from the DWA station catalogue (DWA, 2013). Shoreline development (D_L) is an index that quantifies the irregularity of the shore. A perfect circle would have a D_L value of 1, with larger values indicating that the shoreline is more convoluted. The index was calculated as follows:

$$D_L = \frac{L}{2\sqrt{\pi A_O}}$$

Where L is shoreline length (km), and A_O is reservoir surface area (km^2). Lakes with high shoreline development are often naturally more productive than those with low shoreline development (Dodds & Whiles, 2010).

3.2.3 *Surface water chemistry*

Loskop Dam was sampled on 5 occasions in 2011, during February, April, June, October and December, to cover seasonal variation in an annual cycle. One-litre integrated (2 m) water samples were collected using a plastic hose (diameter 5 cm) lowered vertically into the water and then emptied into a plastic bucket and mixed prior to collecting the sample with a plastic container rinsed in water from the site. Samples were refrigerated at 4°C in the field and then frozen until they were delivered to the accredited CSIR Analytical Laboratory in Stellenbosch. Samples were filtered through 0.45 µm pore size Whatman GF filters prior to being analysed for dissolved nutrients, metals and major ions using standard methods (APHA, 1998). Concentrations of Al, Fe, Mn, Se, V, Zn and Cu were determined using inductively coupled plasma mass spectrometry (ICP-MS). All major ions except chloride (Cl) were analysed using inductively coupled plasma optical emission spectrometry (ICP-OES). Chloride and dissolved nitrogen and phosphorus were measured using a flow injection analyser (FIA). A 1-ℓ sub-sample of water was collected from the bucket and stored at 4°C covered in foil for analysis of chlorophyll-*a* concentrations. Samples were filtered using Whatman GF filters and then lyophilised. Chlorophyll-*a* was extracted using N,N-dimethylformamide for 2 h at room temperature, then measured spectrophotometrically at 647 nm and 664 nm (Porra *et al*., 1989).

Field measurements of pH, dissolved oxygen, conductivity, and temperature of the surface water were taken along with water transparency using a Secchi disc (25 cm) at each site. Measurements were made using a Hach HQ40D multiparameter meter using a pH gel intellical probe, luminescent dissolved oxygen intellical probe and a standard 4-pole graphite type electrical conductivity intellical probe (USEPA compliant).

3.2.4 *Near-bottom water chemistry and reservoir profiles*

Near-bottom water sampling was carried out at each site in Loskop Dam once during low flow (July 2011, winter) and again during high flow (December 2011, summer). A Van Dorn sampler (6 litres) was lowered to the last metre from the dam bottom at each site and a sample of water was brought to the surface. Samples were analysed for concentrations of Al, Fe, Mn, Si, nutrients, and SO_4^{2-} using the methods described above. Because routine surface water sampling was not carried out in July, a surface water sample was collected simultaneously and analysed for the same constituents to serve as a comparison to the near-bottom samples. Vertical profiles of pH, dissolved oxygen, conductivity and temperature were measured to a maximum depth of 30 m using the same meter at each site and each sampling period.

3.2.5 *Statistical analyses*

Water chemistry results were compared to the TWQR, chronic effect values (CEV) and acute effect values (AEV), where applicable, as set out in the water quality guidelines for ecosystem health in South Africa (DWAF, 1996a). Molar ratios of SO_4^{2-}/Cl were calculated as an indicator of sulphate inputs of mining or industrial origin.

3.2.6 *Trophic indicators*

The trophic status of Loskop Dam was determined using data collected between 1985 and 2011 from the DWA monitoring station. Trophic state index (TSI) values were determined using the Carlson (1977) method as follows:

$$TSI(TP) = 14.42(\ln TP) + 4.15$$
$$TSI(SD) = 60 - 14.41(\ln SD)$$
$$TSI(CHLA) = 9.81(\ln CHLA) + 30.6$$

where TP is total phosphorus (μg/ℓ), SD is Secchi-depth (m), CHLA is chlorophyll-*a* concentrations (μg/ℓ), and ln is the natural logarithm of the variable.

The annual median values for total phosphorus, Secchi-disk depth and chlorophyll-*a* were used to calculate TSI values. Censored values were replaced at 0.5 times the detection limit and included in the calculation of annual medians. The DWA records occasionally included samples collected at various depths, but only surface and integrated (5 m) sample values were used in the calculations.

The TSI was developed in order to determine the state of eutrophication of a water body, with phosphorus limitation as an underlying assumption. All three variables are meant to be normalised by the indices, allowing any of the three to describe the trophic state of a water body. A reservoir is considered oligotrophic (nutrient-poor) up to a TSI of 30, mesotrophic (moderate nutrients) up to a TSI of 50 and eutrophic (nutrient-enriched) above 50.

3.2.7 *Trend analysis*

Data compiled from the DWA monitoring station at Loskop Dam were analysed for increasing or decreasing trends using a nonparametric seasonal Mann-Kendall test (Hirsch *et al*., 1982) performed in XLSTAT version 2012.6.08 © Addinsoft. The test removes seasonal effects that can obscure trends, does not require data to be normally distributed, and permits the use of censored values. The dataset was modified to include only surface and 5 m integrated samples. Sampling frequency was predominantly monthly, but varied randomly, so data were aggregated to a single value for 4 seasons each year using the median of the available data. The seasonal Mann-Kendall test was computed on each season individually and results were then combined to form an overall trend result. Multiple detection limits were common so censored data were re-censored to the highest detection limit. Trends for each parameter were only calculated if less than 50% of the data were censored and more than 5 years of data were available (Stevens, 2003). This meant that trends could not be analysed for several parameters, including dissolved oxygen, temperature, Secchi depth, orthophosphate and dissolved metals. The sodium (Na) adsorption ratio (SAR) was also included in the analysis due to the importance of Loskop Dam for irrigation. High SAR values indicate that water may not be suitable for irrigation as Na replaces calcium (Ca) and magnesium (Mg) in the soil (DWAF, 1996b).

3.3 RESULTS

3.3.1 *Reservoir physiography*

The revised attributes of Loskop Dam are presented inTable 3.1. The revised mean retention time was 12.2 months, and the mean depth was 15.43 m. The revised mean annual runoff was calculated as 323.31 Mm^3 which was substantially lower than 10 780 Mm^3 (Midgely *et al*., 1994), but similar to 304.5 Mm^3 calculated by Butty *et al*. (1980). The shoreline development value of 3.96 was greater than 3, which indicated that Loskop Dam had a highly irregular shoreline that could be described as dendritic or tree-shaped (Timms, 2010).

Table 3.1 Attributes of Loskop Dam at full supply level, and the upper catchment of the Olifants River.

Catchment attributes	
Mean annual runoff (Mm^3)	323.31
Catchment area (km^2)	12,262
Reservoir attributes	
Maximum depth (m)	36
Mean depth (m)	15.43
Surface area (km^2)	24.27
Volume (Mm^3)	374.3
Mean retention time (months)	12.2
Elevation (m)	1001
Shoreline length* (km)	69.3
Shoreline development (D_L)	3.96

* Scale 1:2000, shoreline contour 1001.61 m

3.3.2 *Surface water chemistry*

A summary of the major water quality constituents measured in the upper 2 m at all 5 sites is presented in Table 3.2, and selected parameters are presented in boxplots

(Figure 3.2). For the duration of the study concentrations of Se, Zn, V and Cu were below analytical detection limits and were, therefore, not reported in detail.

Origins of patterns in water quality across the dam could be broadly categorised as those influenced by cycles within the dam, and those influenced by the inflow of the Olifants River. Examples of parameters influenced by internal cycles were chlorophyll-*a*, dissolved oxygen, orthophosphate, and pH, all of which were linked to algal blooms, and peak in the transitional zone before decreasing across the dam to Site LK5 (Figure 3.2).

Peak algal growth was reflected by chlorophyll-*a* concentrations which were highest in the transitional zone, especially at Site LK2, which peaked at 26.79 µg/ℓ in April (Table 3.2) during a bloom of *M. aeruginosa.* Secchi depths in this area were consistently <100 cm and were always lower than at Site LK1, indicating that turbidity was of biological as opposed to mineral origins. In contrast, Secchi depth values ranged widely from 103 cm to 575 cm at LK5, with the highest values corresponding to non-detectable chlorophyll-*a* concentrations in a distinct clear water phase during October (Table 3.2). The pH at the inflow site (LK1) was generally in the neutral range of 7 to 8, compared to the other sites where the pH was frequently above 9 (Figure 3.2e). Dissolved oxygen levels also ranged widely in the transitional zone with values at Site LK2 peaking at 14.5 mg/ℓ in April and dropping to 2.1 mg/ℓ at the surface in December (Table 3.2). This coincided with the lowest observed concentration of chlorophyll-*a* (0.08 µg/ℓ) and pH (7.35) at the site, indicating that oxygen depletion was probably due to the collapse of an algal bloom. Furthermore, the chemical oxygen demand was consistently highest at Sites LK2 and LK3 in the dam (Table 3.2).

Orthophosphate and inorganic nitrogen (nitrate plus nitrite) showed contrasting patterns across the sampling sites. Orthophosphate concentrations were highest in the transitional zone, measuring 185 µg/ℓ at Site LK2 in February, and indicated an internal source of phosphorus within the dam, fluctuating with the growth and collapse of algal blooms (Figure 3.2c). Concentrations recorded in February, April and June at Site LK2 fell within the eutrophic range of 25–250 µg/ℓ (DWAF, 1996a). Inorganic nitrogen concentrations were highest at the inflow site LK1 and decreased across the sites to the dam wall, indicating that the main source was the Olifants River (Figure 3.2d). The highest concentration at Site LK1 was 2.7 mg/ℓ, measured in June, which fell within the eutrophic category of >2.5 mg/ℓ, while the remaining values fell within the mesotrophic range between 0.5 and 2.5 mg/ℓ (Table 3.2; DWAF, 1996a).

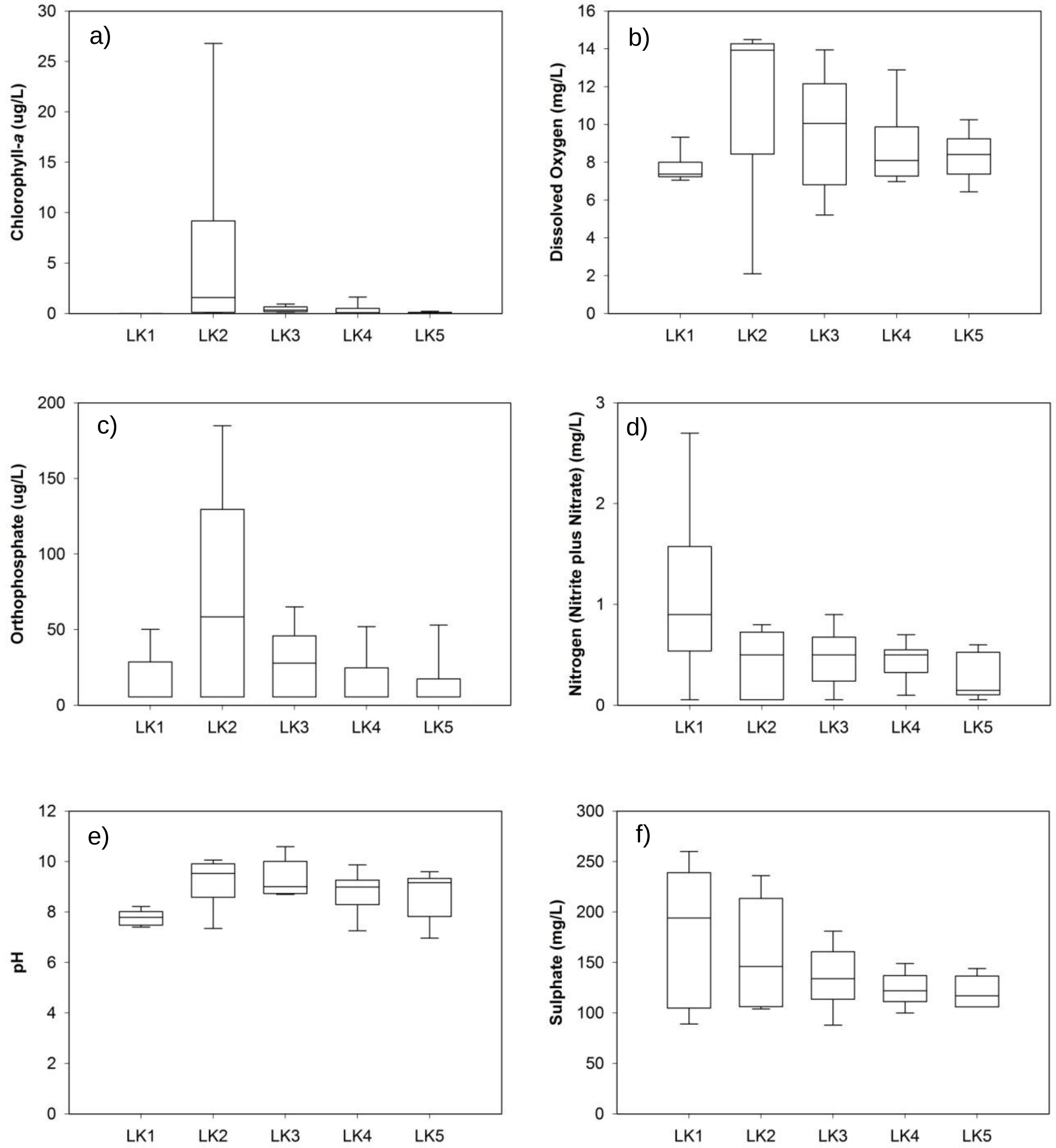

Figure 3.2 Boxplots of selected water quality constituents measured in 2 m integrated surface water samples (*n* = 5) from five sites in Loskop Dam during 2011. Plots are of the median (centre line), 10th, 25th (box), 75th, and 90th (whiskers) percentiles.

The inflow was also the main source of SO_4^{2-} which was highest at Site LK1 measuring 260 mg/ℓ in October with a SO_4^{2-}/Cl ratio of 10.1, and decreasing across the dam to LK5 where the highest measurement was 144 mg/ℓ in December. Dissolved Al concentrations exceeded the TWQR of 10 µg/ℓ (for pH >6.5) during October at LK3, and during December at Sites LK1, LK2, LK3 and LK4. The highest

Al concentration exceeded the CEV of 20 µg/ℓ (for pH >6.5) at LK3 in April. Dissolved Mn only exceeded the TWQR of 180 µg/ℓ at Site LK1 in June (Table 3.2).

A noteworthy event occurred in June at the inflow site LK1 when the water was a milky-blue colour, which coincided with elevated conductivity (503 µS/cm), high SO_4^{2-} concentrations (194 mg/ℓ) and the highest observed Mn concentrations (220 µg/ℓ) at this site (Table 3.2).

Table 3.2 Summary of constituents measured in 2 m integrated surface water samples from five sites in Loskop Dam during 2011.

Site	Date	pH	Conductivity (µS/cm)	Dissolved oxygen (mg/ℓ)	Orthophosphate (µg/ℓ)	Ammonia (mg/ℓ)	Nitrate plus Nitrite (mg/ℓ)	Chemical Oxygen Demand (mg/ℓ)	Total Alkalinity (CaCO$_3$, (mg/ℓ)	Sulphate (mg/ℓ)	Sulphate : chloride ratio	Aluminium (µg/ℓ)	Iron (µg/ℓ)	Manganese (µg/ℓ)	Chlorophyll-*a* (µg/ℓ)	Secchi depth (cm)
LK1	Feb	7.95	372	7.30	2.1	0.1	0.7	5.7	53	110	6.6	-	-	-	0.006	49
	Apr	7.79	329	7.56	50	0.2	0.9	51	46	89	5.8	-	-	103.4	0.01	42
	Jun	7.51	503	9.33	-	0.2	2.7	10	25	194	9.6	-	-	**220**	0.002	279
	Oct	7.40	643	7.37	-	-	1.2	20	33	260	10.1	2.9	2.4	2.0	-	169
	Dec	8.22	654	7.05	-	-	-	33	53	232	7.7	**17.5**	-	4.2	-	95
LK2	Feb	9.86	364	10.55	185	-	-	69	60	104	6.3	-	-	0.02	26.8	0
	Apr	10.06	400	14.50	59	-	0.8	71	27	107	7.2	-	-	60	3.31	21
	Jun	9.00	409	14.20	111	0.2	0.7	93	49	146	7.7	-	-	30	1.58	51
	Oct	9.53	583	13.93	-	-	0.5	64	30	236	9.5	9.3	4.5	15.3	0.12	85
	Dec	7.35	653	2.10	-	0.2	-	55	54	206	8.5	**19**	17.4	1	0.08	69
LK3	Feb	10.59	364	10.05	39	-	-	23	51	88	6.2	-	-	--	0.933	0
	Apr	8.70	397	7.34	28	0.1	0.9	47	37	122	7.6	**<u>20</u>**	20	10	0.33	115
	Jun	9.01	398	11.56	65	-	0.6	43	50	134	7.1	-	-	10	0.2	122
	Oct	9.81	464	13.95	-	-	0.5	104	27	154	6.9	**10.8**	13	3.3	0.55	83
	Dec	8.74	505	5.21	-	-	0.3	35	49	181	7.4	**16.2**	5.8	0.7	0.13	121
LK4	Feb	9.87	363	12.89	-	-	-	34	48	100	6.4	-	-	-	1.63	81
	Apr	9.06	385	8.09	16	-	0.4	75	45	115	7.3	-	-	-	0.14	170
	Jun	7.26	400	6.98	52	-	0.7	23	52	133	7	-	-	-	0.07	284
	Oct	8.64	426	8.87	-	-	0.5	16	42	122	6	3.9	3	4.3	0.02	405
	Dec	8.99	456	7.37	-	-	0.5	22	41	149	6.9	**11.4**	3.9	0.4	-	374
LK5	Feb	9.60	363	10.25	-	-	0.12	22	58	106	6.6	-	-	-	0.07	103
	Apr	9.16	369	8.91	-	-	0.15	51	40	117	7.5	-	17	-	0.23	164
	Jun	6.97	404	6.44	53	0.1	0.6	27	53	134	7.1	-	-	40	0.02	268
	Oct	8.11	423	8.41	-	-	-	20	45	106	5.3	-	2.5	3.5	-	575
	Dec	9.24	451	7.69	-	-	0.5	15	44	144	6.6	9.9	-	0.3	-	350

-, values below detection limit; bold print, value exceeds TWQR; underline, value exceeds CEV

3.3.3 *Near-bottom water chemistry and reservoir profiles*

Concentrations of inorganic and total nitrogen and phosphorus in near-bottom water were very similar to those measured at the surface at all sites, and therefore the results were not presented. Concentrations of dissolved Al, Fe and Mn in the surface and near-bottom water at all sites are presented in Figure 3.3. Profiles of temperature, dissolved oxygen, pH and conductivity are presented for Site LK2 (Figure 3.4) and LK3 (Figure 3.5) in the transitional zone, and Site LK5 (Figure 3.6) in the lacustrine zone. Site LK1 was omitted because it was well mixed without stratification, and LK4 was omitted because the conditions were very similar to LK5.

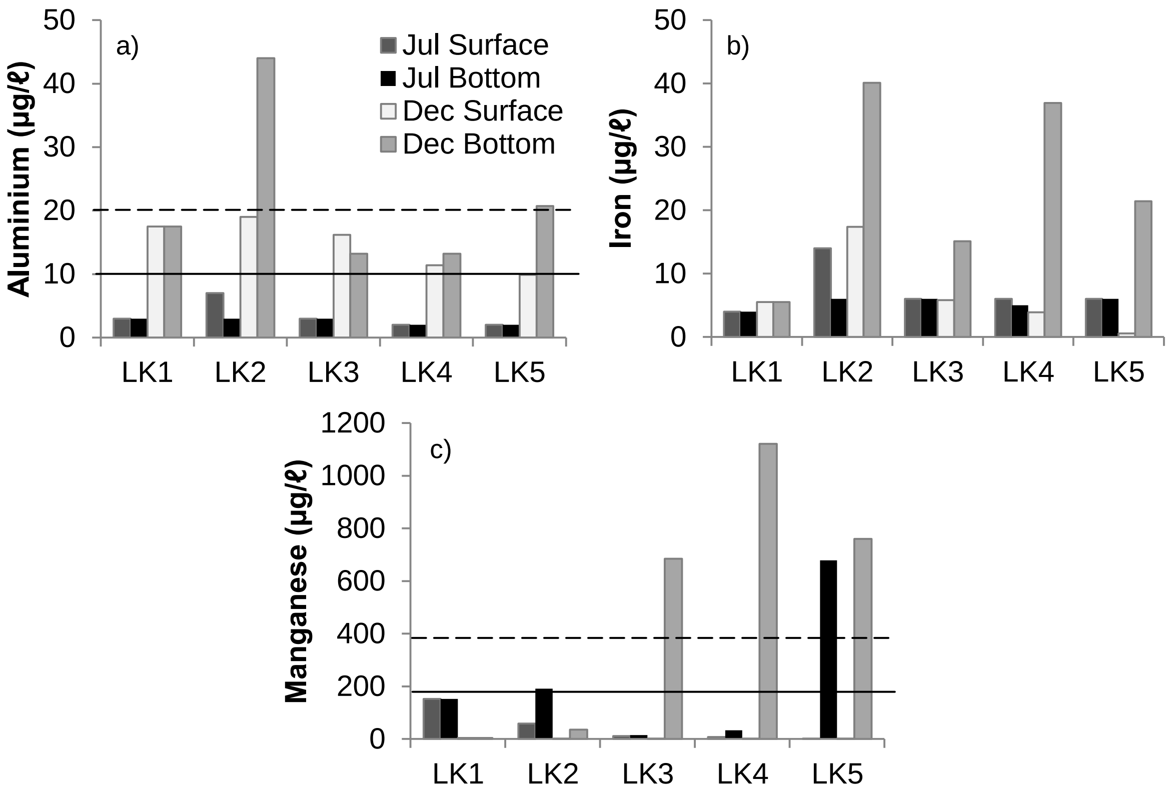

Figure 3.3 Surface and near-bottom concentrations of dissolved Al (a), Fe (b) and Mn (c) at five sites in Loskop Dam sampled during July and December 2011. Solid lines indicate the target water quality range (TWQR) and dashed lines indicate the chronic effect value (CEV) (DWAF, 1996).

Dissolved Al, Fe and Mn measured at the inflow of the Olifants River (LK1) confirm the riverine conditions at this site, because they were approximately uniform from the surface to the bottom at a depth of 4 m in July, and 3 m in December 2011 (Figure

3.3). No marked stratification of pH, temperature, dissolved oxygen, or conductivity was observed at LK1; this was consistent with the well-mixed conditions associated with higher river flows and relatively shallow depth.

Dissolved Al, Fe and Mn differed between surface and near-bottom depths on several occasions at the other sites. There was a clear seasonal difference in Al concentrations between July and December (Figure 3.3a). Aluminium exceeded the TWQR of 10 µg/ℓ at Sites LK1, LK2, LK3 and LK4 in the surface and near-bottom water during December. At Sites LK2 and LK5, Al exceeded the CEV of 20 µg/ℓ in the near-bottom water in December, which coincided with low oxygen levels at Site LK2 (1.38 mg/ℓ; Figure 3.4b) and anoxic conditions > 10 m at LK5 (Figure 3.6b). In July all recorded Al concentrations were within the TWQR.

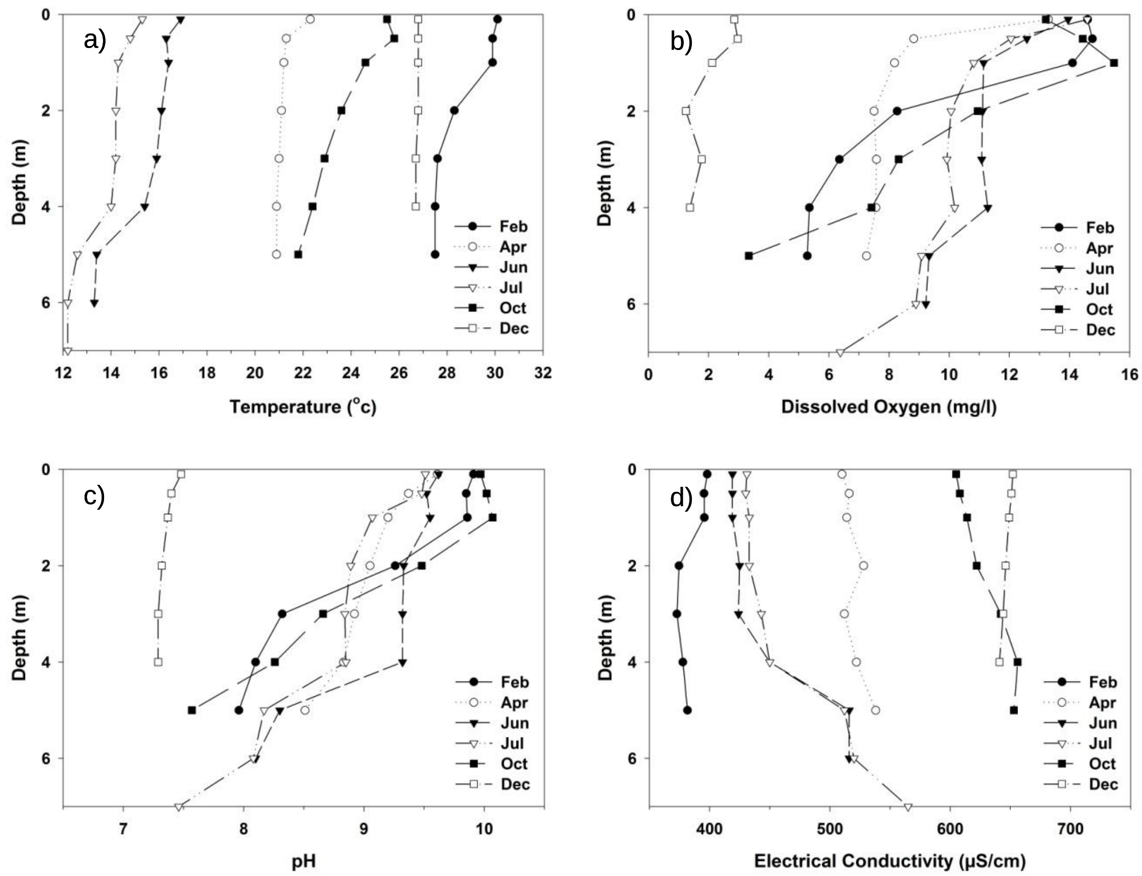

Figure 3.4 Field measurement profiles of various parameters at site LK2 in the transitional zone of Loskop Dam.

Surface water concentrations of Mn were consistently within the TWQR at all sites except as already mentioned at Site LK1 in June. However, concentrations exceeded the CEV of 370 µg/ℓ in the near-bottom water at Sites LK3, LK4 and LK5 during December (Figure 3.3c). Conditions in the hypolimnion during December at these sites were anoxic (Figure 3.4, Figure 3.5, Figure 3.6), with a near-neutral pH and increased conductivity in the last 2 m of water above the dam bottom (Figure 3.6c and d). In July the near-bottom concentrations exceeded the TWQR of 180 µg/ℓ at Site LK2, and the CEV at LK5, despite the water column being oxygenated at this time.

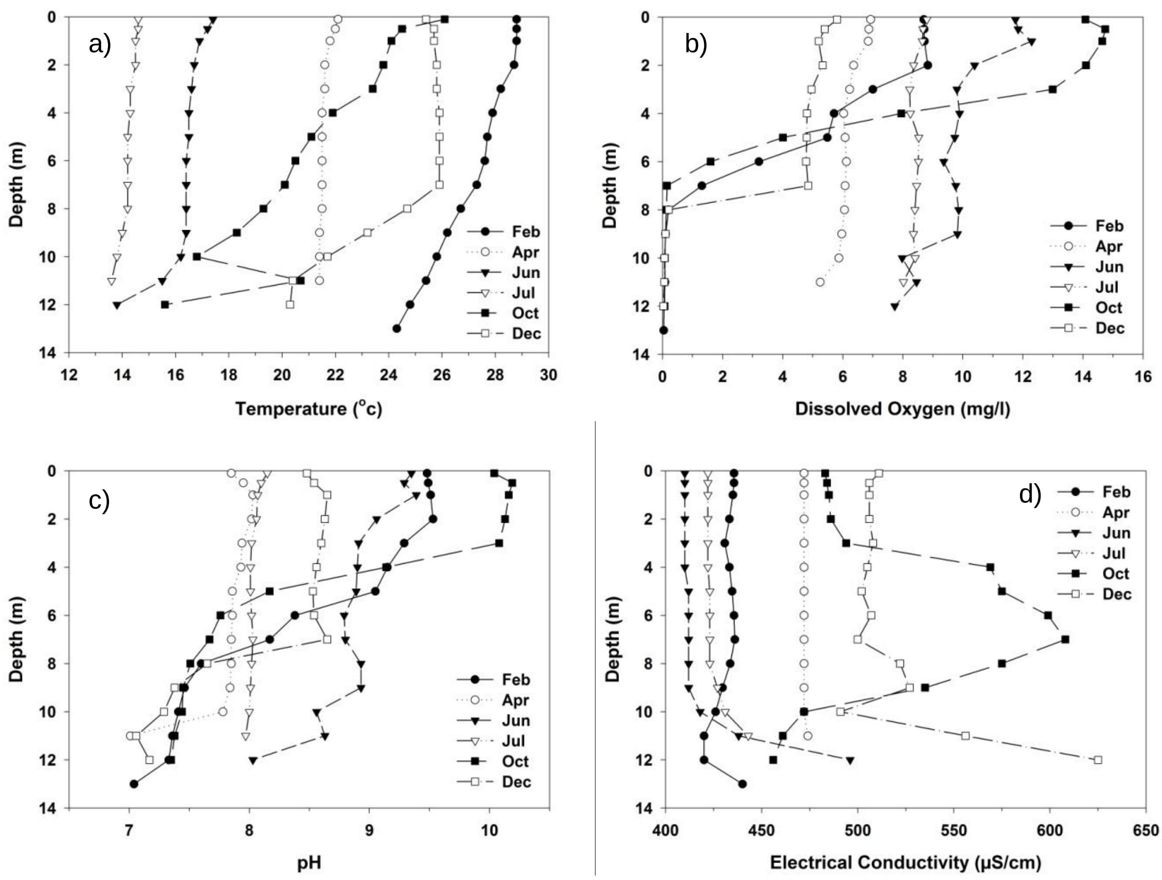

Figure 3.5 Field measurement profiles at site LK3 in the transitional zone of Loskop Dam.

The TWQR for Fe stipulates that concentrations should not vary by more than 10% of the background concentrations, which is a tentative guideline (DWAF, 1996a). The lack of historic knowledge of Fe concentrations under unimpacted conditions in Loskop Dam complicates interpretation of the results. Iron followed a similar pattern to Mn, with elevated concentrations observed in the near-bottom waters in

December at all sites except the inflow at Site LK1, where there was little seasonal change or difference between surface and near-bottom concentrations (Figure 3.3b). This indicates that fluctuating Fe and Mn concentrations are related to internal cycles within the dam.

Loskop Dam showed a monomictic pattern of thermal stratification in summer (October to April) and holomictic winter circulation (June to July). It was evident that although turnover had not yet occurred during monitoring in April, the water column was cooling towards winter isothermy as the thermocline deepened to 23 m (Figure 3.6a). Temperatures declined rapidly with the water column cooling from 21.5ºC in April to 16.5ºC in June. Thermal stratification was already well developed in October, indicating that surface warming must have occurred earlier in August or September. Surface temperatures in summer were high, peaking in February at > 30ºC. Temperature profiles showed pronounced seasonal variability, with an annual thermal range of > 15ºC in the upper 2 m of the water column (Figure 3.4a, Figure 3.5a, Figure 3.6a).

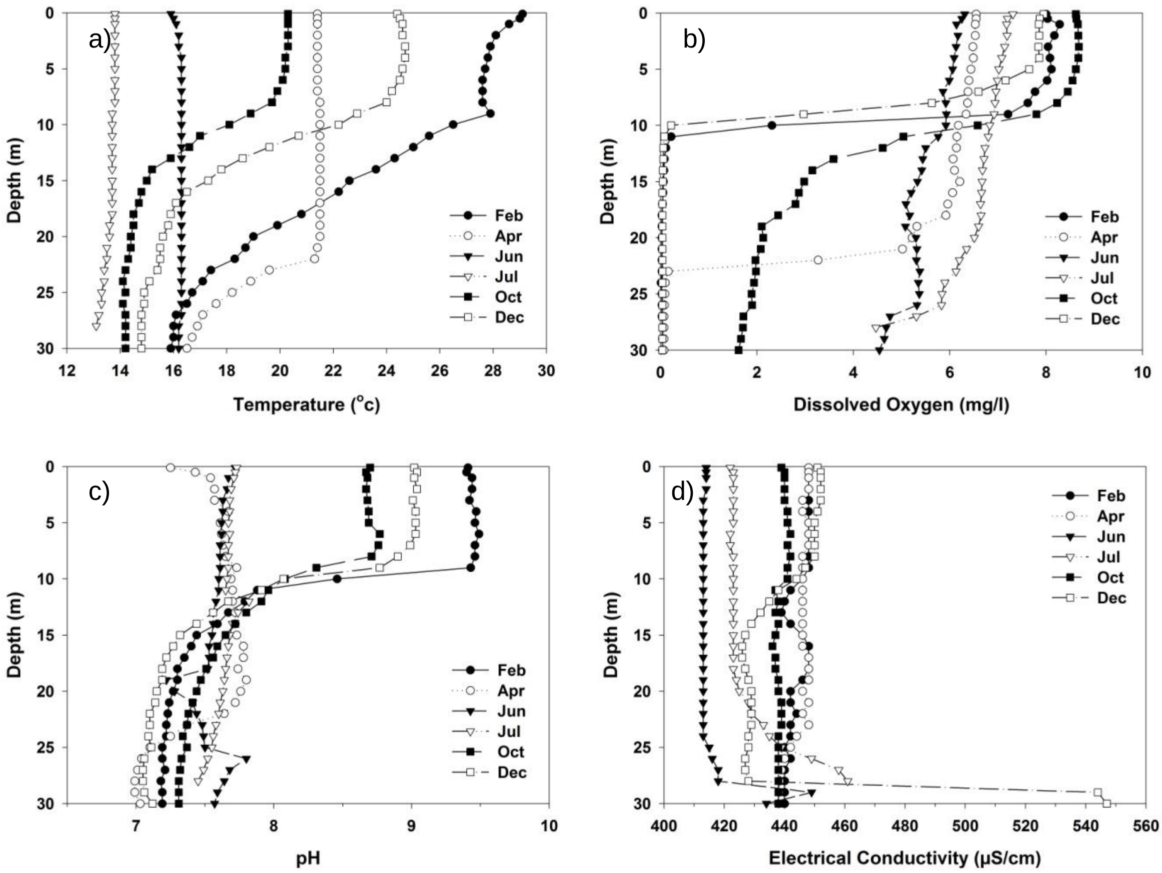

Figure 3.6 Field measurement profiles at site LK5 near the dam wall in the lacustrine zone of Loskop Dam.

Sites LK3 and LK5 demonstrated anoxic conditions in the hypolimnion with distinct oxyclines during December, while LK2 had very low dissolved oxygen ranging from 2.86 mg/ℓ at the surface to 1.38 mg/ℓ at the bottom. Anoxic conditions persisted at Sites LK3 and LK5 in October and February, and at LK5 in April prior to lake overturn. Dissolved oxygen in the epilimnion at LK2 was frequently > 13 mg/ℓ and at LK3 was >10 mg/ℓ in June and October which reflected the algal blooms at these sites (Figure 3.4b). The pH followed a similar pattern to DO at LK2, with values > 9 recorded in the upper 2–3 m every month except December (Figure 3.4c).

Differences in the water chemistry profile of LK2 in July may have been driven by temperature. The water temperature was distinctly stratified, measuring 15.3°C at the surface and 12.2°C at 7 m, a difference of 3.1°C with a thermocline between 4 m and 5 m (Figure 3.4a). The sub-surface water was similar in temperature to the inflow site LK1, at 11.7°C, which implies that the cooler and denser river water submerged beneath the warmer surface waters of the dam before mixing. The conductivity at the surface was 431 µS/cm, compared to 565 µS/cm at 7 m depth (Figure 3.4c), a difference of 134 µS/cm and more similar to the inflow measurement of 528 µS/cm (Table 3.2). Furthermore, near-bottom SO_4^{2-} concentrations reflected riverine conditions at 185 mg/ℓ compared to 135 mg/ℓ in the surface water at LK2.

3.3.4 *Long-term trophic indicators and phytoplankton assemblage*

Secchi disk measurements were only collected by DWA from 1990 to 1996; therefore the dataset was supplemented with values collected at LK5 as part of ongoing research conducted by the CSIR between 2008 and 2011. Most of the TSI values were in the mesotrophic range but phosphorus showed an upward trend into the eutrophic category from 2005 onwards, with a peak in 2008 (Figure 3.7). This trend was not reflected in Secchi depth and chlorophyll-*a* values which remained within the mesotrophic range throughout.

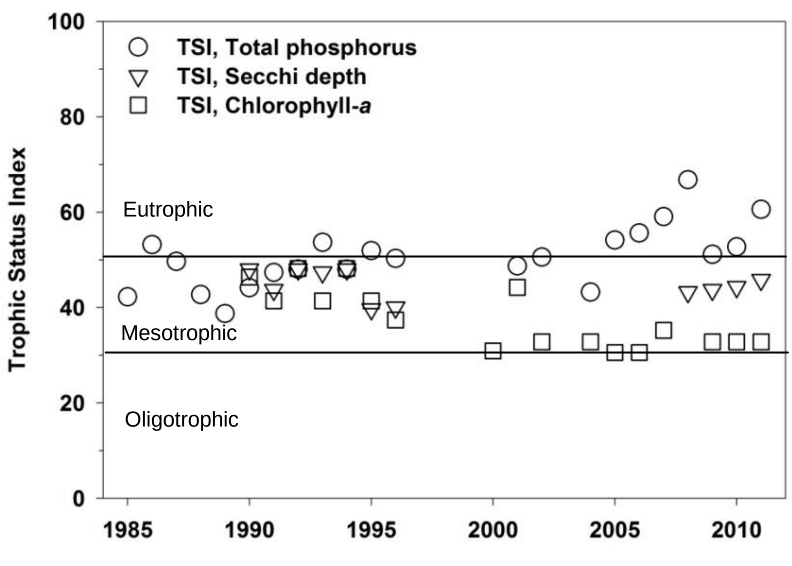

Figure 3.7 Trophic status index (TSI) calculated using the Carlson method (1977) for total phosphorus, secchi depth and chlorophyll-a using annual median values at Loskop Dam.

The phytoplankton assemblage at Loskop Dam was monitored by DWA from 1990 onwards. Unfortunately the record was inconsistent with breaks of several years at a time when monitoring was not conducted. However, what was clear, was an increasing trend in the dominance of *C. hirundinella*. Prior to 2005 it never contributed more than 16%. But from 2006 onwards it became the dominant species, contributing between 50% and 90% to the phytoplankton assemblage.

3.3.5 *Trend analysis*

Results of the Seasonal Mann-Kendall trend analyses are presented in Table 3.3. Scatterplots of selected parameters were presented with a LOWESS (Locally Weighted Scatterplot Smooth) trace in order to integrate results visually and clarify the data pattern. The time-series data for pH was non-monotonic, demonstrating downward and upward trends within the data range (Figure 3.8a). From 1968 until the early 1980s pH values steadily decreased, then began to increase until around 1996 at which time they approximately levelled off. Overall the pH showed a significant increasing trend (Tau = 0.375; Table 3.3).

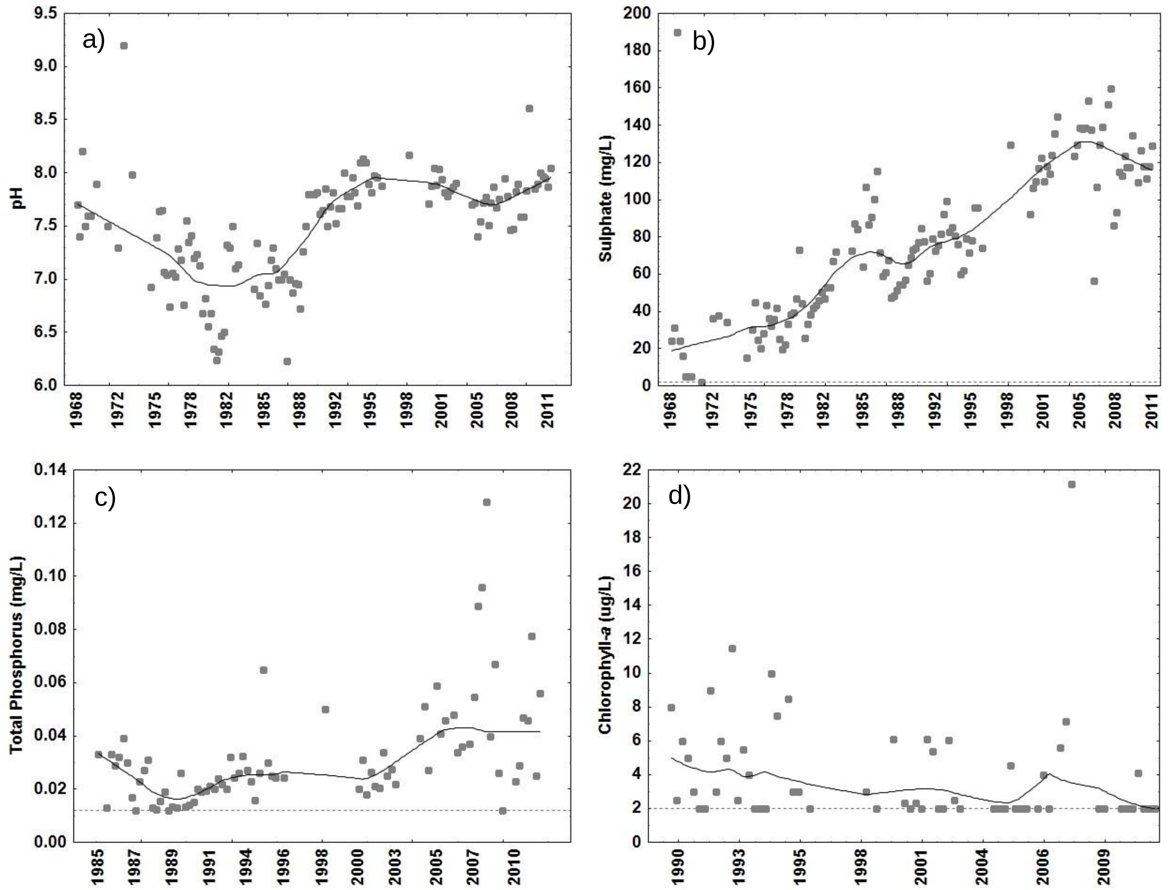

Figure 3.8 Seasonal Mann-Kendall trend test results with LOWESS smooth curves plotted for selected constituents. Dotted line indicates detection limits.

Electrical conductivity and the major ions all showed significant increases over time (Table 3.3). The SO_4^{2-} data had a generally monotonic distribution with a significant increasing trend (Tau = 0.694) that peaked in 2007 at around 160 mg/ℓ, after which there was a slight decrease (Figure 3.8b). While there was no clear trend in any direction in total nitrogen (Table 3.3), total phosphorus (Figure 3.8c), showed a significant increasing trend (Tau = 0.422), with the highest levels observed in 2007 and especially 2008 at 0.128 mg/ℓ. Given the concern about algal blooms and the increasing trend in total phosphorus in Loskop Dam, the decreasing trend (Tau = -0.298) observed in chlorophyll-*a* concentrations was unexpected (Table 3.3; Figure 3.8d). However a very high proportion of the values were censored (46.7%) and their inclusion in the calculation of quarterly medians for the trend analysis meant that occasional peaks were lost. The year 2008 also had the highest recorded value for chlorophyll-*a* which was noteworthy because total phosphorus peaked at a similar time.

Table 3.3 Summary of Seasonal Mann-Kendall trend analyses.

Parameter	**Date range (*n*)**	**% Censored data**	**Trend**	**Kendall's Tau**
pH (standard units)	1968 – 2011 (129)	0	+	0.375 ***
EC (µS/cm)	1968 – 2011 (130)	0	+	0.734 ***
Calcium (mg/ℓ)	1968 – 2011 (129)	0	+	0.696 ***
Magnesium (mg/ℓ)	1968 - 2011 (129)	0	+	0.708 ***
Potassium (mg/ℓ)	1972 – 2011 (119)	0	+	0.498 ***
Sodium (mg/ℓ)	1968 – 2011 (128)	0.8	+	0.415 ***
Sodium Adsorption Ratio	1968 – 2011 (128)	0	+	0.351 ***
Chloride (mg/ℓ)	1968 – 2011 (124)	0	+	0.284 ***
Fluoride (mg/ℓ)	1972 – 2011 (119)	0.8	+	0.159 *
Sulphate (mg/ℓ)	1968 – 2011 (128)	0	+	0.694 ***
Ryznar Index	1976 – 2011 (110)	0	-	-0.665 ***
Total alkalinity as CaCO3 (mg/ℓ)	1968 – 2011 (129)	0	+	0.264 ***
Total Phosphorus (mg/ℓ)	1985 – 2011 (80)	3.8	+	0.422 ***
Total Nitrogen (mg/ℓ)	1985 – 2011 (78)	6.4	0	0.038
Inorganic Nitrogren (mg/ℓ)	1976 – 2011 (114)	44.7	0	-0.065
Silica (mg/ℓ)	1976 – 2011 (114)	2.9	-	-0.209 *
Chlorophyll-*a* (µg/ℓ)	1990 – 2011 (60)	46.7	-	-0.298 *

(+), upward trend; (-), downward trend; (0), no significant trend
Significance: ***, <0.0001; **, <0.001; *, <0.05

The increasing trend in the sodium adsorption ratio is of concern as the primary use of the dam is for irrigation. However, the highest value recorded in the data range was 1.22 in 2011 which was still well within the TWQR of < 2 according to the water quality guidelines for irrigation (DWAF, 1996b).

Trends could not be computed on all of the metals because of the high proportion of censored data and limited time period of data collection. Specifically, Al, Fe and Mn were only monitored sporadically from around 1991 with gaps as long as 10 years between samples. From around 2005 onwards, sampling occurred on a more regular basis, but for all three metals the number of censored values exceeded 50% and therefore trends were not computed.

The relationship between total phosphorus and dam level for the 10-year period between 2000 and 2010 is shown in Figure 3.9. The dam level reduced drastically between 2001 and 2004, reaching a critical minimum of 12 m (24% capacity) in January 2004 before beginning to increase from 2005 onwards.

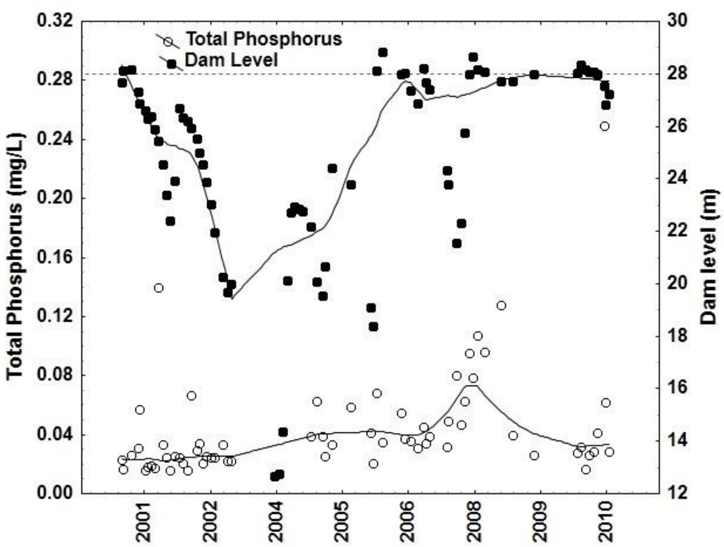

Figure 3.9 LOWESS smoothed scatterplot of total phosphorus concentrations and dam levels between 2000 and 2010. The dotted horizontal line indicates the gauge plate level at 28m (full supply capacity).

This event represented a 16 m drawdown which exposed significant areas of the littoral zone (Figure 3.10). Corresponding total phosphorus concentrations appeared relatively stable before 2004, but increased steadily to a peak in 2008, before decreasing to similar levels observed in 2005 and 2006.

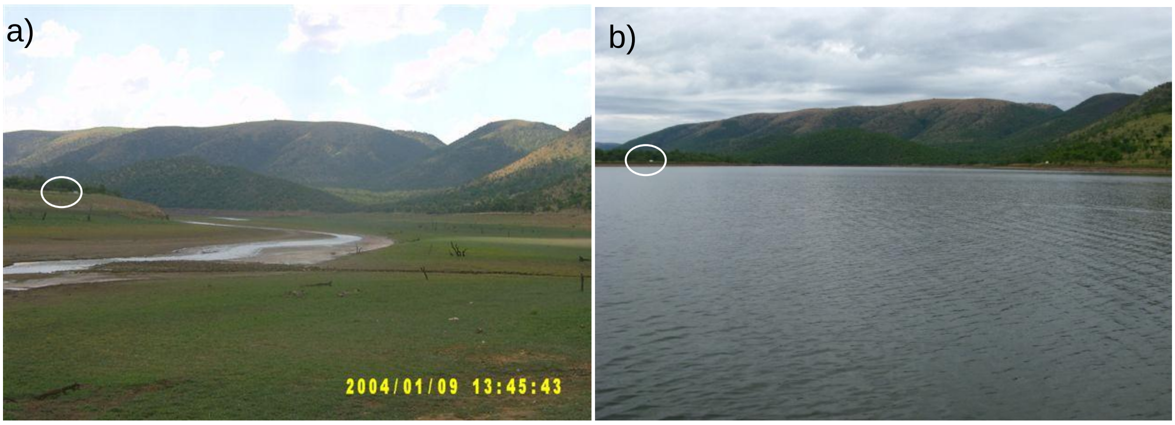

Figure 3.10 Picture taken near sampling site LK3 (notice board circled) when Loskop Dam was 24% full in 2004 (a), and at 100% full in 2011 (b) for comparison of water levels. (Fig. 10a courtesy Jannie Coetzee)

3.4 DISCUSSION

There is a marked difference in water quality across Loskop Dam, largely influenced by the algal blooms that currently characterise the transitional zone. In this area, the system changes from one that supports organisms suited to lotic environments to one that supports those adapted to lentic environments, such as *M. aeruginosa* and *C. hirundinella*. During algal blooms, photosynthetic activity in daylight results in elevated pH (> 9) through carbonic acid absorption, and increased dissolved oxygen levels (> 10 mg/ℓ) as a result of oxygen release. As blooms degrade, oxygen levels can plummet, as observed in December 2011 when dissolved oxygen dropped to 2.1 mg/ℓ in the surface water. The elevated pH levels can cause an increase in the concentration of the toxic un-ionised form of ammonia (NH_3) relative to the less toxic ammonium ion (NH_4^+) (DWAF, 1996[a]). The solubility and bioavailability of Al is also increased at pH values > 8 (Gensemer & Playle, 1999). All of these factors may negatively impact the health of aquatic biota, especially fish.

Through the use of algal assays, phosphate was reported as the limiting nutrient in Loskop Dam (Toerien *et al*., 1975), so the increasing trend in total phosphorus is a cause for concern. The revised mean retention time of 12.2 months is double the frequently referenced time of 6 months (Butty *et al*., 1980). The longer retention time and high shoreline development value increase the sensitivity of Loskop Dam to nutrient enrichment. However, the increased mean depth of 15.43 m offsets this sensitivity to a degree due to decreased mixing (Dodds & Whiles, 2010; Oberholster *et al.,* 2013). The results of the TSI indicated that Loskop Dam was in the meso- to eutrophic category. However, the lack of agreement between all three indices suggests there may be other factors at play such as seasonal grazing of phytoplankton by zooplankton which would lower the Secchi depth and chlorophyll-*a* TSI's. Another factor affecting the results is that a high proportion of the chlorophyll-*a* data were censored, in which case the annual median value was often a censored value which excluded seasonal peaks associated with algal blooms.

What was clear from the data collected during 2011 was that the dam was heterogeneous with regard to nutrient concentrations, algal biomass and dissolved metals. Fluctuating concentrations of orthophosphate in the transitional zone, independent of the inflow, indicate internal phosphorus loading that may continue to stimulate algal blooms despite nutrient controls in the catchment. The shallow depth of this area (4–7 m) may also play a role in the re-suspension of sediments with phosphorus due to wind mixing (Jones & Welch, 1990). Different zones in the dam display different eutrophication characteristics, and, for this reason, monitoring water quality at the dam wall alone may fail to detect changes at the onset of eutrophication. While internal phosphorus cycling from sediments has been shown to limit the recovery rates of phosphorus-enriched lakes (Van der Does *et al.,* 1992), the borderline trophic classification of Loskop Dam suggests that a regime shift is incomplete and the dam may respond positively to decreased phosphorus loads in the catchment.

The convergent peaks of total phosphorus and chlorophyll-*a* in 2008 were intriguing and suggest an event of common origin. One explanation is linked to the dam level and rainfall patterns preceding 2010. The dam level dropped substantially to 24% in January 2004 after below average annual rainfall (407.8 mℓ in 2002 and 351.5 mℓ in 2003) in the catchment. This period was followed by increasing rainfalls which culminated in a very high annual rainfall season in 2007 (631.8 mℓ) and 2008 (751.6 mℓ). Chlorophyll-*a* measured 38.24 µg/ℓ in February 2008, and the annual median total phosphorus approximately doubled from 0.048 mg/ℓ in 2007 to 0.089 mg/ℓ in 2008, at which point the dam level was back to 103%. The flushing rates of Loskop Dam are low, with a substantial hydraulic retention time of 12.2 months, which increases its susceptibility to enhanced nutrient loadings (Vollenweider & Kerekes, 1980) and favours the establishment of inherently slow-growing species like *C. hirundinella* and *M. aeruginosa* (Reynolds *et al*., 2012). The large volume of rainfall from a nutrient-enriched catchment may have resulted in an extensive first flush which increased nutrient loads in the dam and stimulated the growth of algae. The first flush effect may have been enhanced by the reduced dilution capacity associated with very low dam levels at the time which resulted in high concentrations of nutrients. An alternative explanation may be linked to internal nutrient cycles. Lake drawdown is frequently used as an effective remediation technique for the control of macrophytes, but it has been known to result in algal blooms upon refilling as a result of nutrient release from re-wetted, organically enriched sediments (James *et al*., 2001).

Loskop Dam is a monomictic reservoir characterised by anoxic conditions in the hypolimnion during a large portion of the year, until the autumn overturn which had begun to occur in April. The depth, intensity, and duration of hypolimnetic oxygen depletion has increased since profiles were recorded in previous studies in 1960 (Gieskes, 1960) and 1978 (Butty *et al*., 1980). This was probably due to the increased frequency and intensity of algal blooms that provide a significant source of organic matter. Bacterial decomposition of algal blooms exacerbates oxygen depletion, extending the depth and duration over which anoxic conditions occur. This is particularly significant at the sediment-water interface where oxygen consumption is most intense (Wetzel, 1983). Increasing inputs of organic matter and availability of SO_4^{2-} strongly influence the decomposition of organic matter through sulphate reduction in anaerobic environments (Holmer & Storkholm, 2001). This process increases the production of H_2SO_4 which can be toxic to fish.

In addition, bottom water anoxia causes reducing conditions that release soluble Fe^{2+} and Mn^{2+} from bottom sediments (Wetzel, 1983), thus accounting for the elevated concentrations of dissolved Fe and Mn observed in the near-bottom water during 2011. Furthermore, the onset of anoxic conditions can cause the release of phosphorus from bottom sediments, further stimulating algal blooms by positive feedback (Nurnberg, 1984). In contrast, Al speciation is not redox sensitive (Gensemer & Playle, 1999) and therefore the elevated concentrations of dissolved Al observed at the near-bottom of LK2 and LK5 in December are unlikely to be attributed to anoxic conditions at these sites. The seasonal increase in Al above the TWQR in December may be linked to mine-water released through the controlled release scheme in the upper Olifants River catchment, which occurs during high flows. This is supported by the fact that the highest SO_4^{2-} concentrations were also recorded at LK1 in October and December 2011. On a positive note, the recent decrease in sulphate concentrations observed in the trend analysis indicates that desalination efforts in the upper catchment may be having an impact (Mey & Van Niekerk, 2009). It is tempting to attribute the increasing trend in pH values observed in the early 1980s to treatment of acid mine drainage in the catchment, however the Brugspruit Mine Water Treatment plant near the town of Witbank was only constructed in 1995 (Mey & Van Niekerk, 2009) and therefore cannot account for this. The dam wall was raised by 9 m in 1980, which corresponds to the increase in pH and provides a possible explanation. The increased volume of the reservoir may have had a dilution effect on water significantly impacted by AMD.

The elevated concentrations of dissolved Al, Fe and Mn, above guideline levels, in the near-bottom water of Loskop Dam indicate a chemocline that warrants further monitoring. Potentially toxic dissolved metal concentrations may be restricted to the hypolimnion while the water column is stratified, but mixing associated with autumn turnover may temporarily increase their distribution in the water column. Therefore, profiles need to be taken more than twice in the year and at more frequent depth intervals to clarify the distribution of dissolved metals. Additional parameters should be measured such as redox, so that metal speciation can be modelled, and the processes influencing water chemistry in the hypolimnion can be delineated.

The occurrence of milky-blue coloured water, observed at Site LK1 in June, has also been observed further upstream on the Olifants River, below the confluence with the AMD-impacted Klipspruit (pers. obs.). The colour could be due to gypsum precipitation, which is a common by-product of lime neutralisation, and may originate from the Brugspruit Mine Water Treatment plant in the Klipspruit catchment. The blue colour may also be linked to Al precipitation as a result of neutralisation reactions in pH-neutral waters (McCarthy & Pretorius, 2009). The fact that the plume of discoloured water reached the inflow of Loskop Dam is a strong indicator that the Klipspruit may significantly influence water quality. Dilution by the Olifants River and downstream Wilge River may at times be insufficient during low flows to mitigate the impact of the Klipspruit.

The lack of chemical equilibrium in the transitional zone of the reservoir presents fish with many physiological challenges as they may periodically be exposed to elevated levels of H_2SO_4, ammonia, and dissolved metals, as well as low dissolved oxygen. While these factors may result in acute fish kills and physiological stress, they have never been reported to cause symptoms associated with pansteatitis in *O. mossambicus* on their own. However, the combined effects of these factors on fish health have never been researched. An important piece in the pansteatitis puzzle is the increased frequency and extent of algal blooms, particularly in the transitional zone. From a fish health perspective, this represents a modified food web which may have important links to the development of pansteatitis, particularly as the disease is so often attributed to dietary causes (Fytianou *et al*., 2006). Generally *O. mossambicus* feed on a combination of detritus, phytoplankton, filamentous algae, diatoms and plant material (De Moor *et al*., 1985), but there are ontogenetic and site-specific differences which have not been researched in Loskop Dam. This is especially pertinent as previous research at Loskop Dam has reported elevated levels of Al and Fe in the stomach contents and body fat of *O. mossambicus* (Oberholster *et al*., 2011).

Chapter 4: WATER QUALITY OF FLAG BOSHIELO DAM, OLIFANTS RIVER, SOUTH AFRICA: HISTORICAL TRENDS AND THE IMPACT OF DROUGHT

This chapter has been published in the journal Water SA (Appendix 2)

4.1 INTRODUCTION

Flag Boshielo Dam was built in 1987 for irrigation of agriculture downstream of the dam, to supply municipal water to the town of Polokwane, and to ensure dry-season water storage for mines in the area (van Koppen, 2008). It is located at the confluence of the Olifants and Elands rivers, approximately 30 km from the town of Marble Hall in the Limpopo Province. In March 2006, the dam wall was raised by 5 m in order to secure water for mining development, and to improve supply to rural communities through the Olifants River Water Resource Development Programme (van Koppen, 2008). The raising of the dam wall coincided with the end of a drought that lasted approximately three years, which provided additional motivation to increase the reservoir capacity. The drought ended in January 2006 with the first significant flows into the reservoir occurring two months prior to completion of construction on the dam wall.

Wide-scale irrigated agriculture along the Olifants River is the predominant catchment land use downstream of Loskop Dam, while dryland and irrigated agriculture occur in the Elands River catchment. Upstream of Flag Boshielo Dam, Loskop Dam supplies water to the Loskop Irrigation Board, the second largest in South Africa. The irrigation board supplies 700 properties which cover an area of 16 117 ha within the Flag Boshielo Dam catchment (Oberholster & Botha, 2011). Major crops include cotton, wheat, citrus and grapes, a significant proportion of which are grown for export. In contrast to Loskop Dam, there is very little mining and industry in the catchment although its location downstream from Loskop Dam means that Flag Boshielo Dam is susceptible to the same impacts affecting water quality in the upper catchment. Subsistence farming in the catchment has lead to land degradation with extensive areas of soil erosion, and subsequent elevated suspended sediment loads in waterways. Inadequate water supply, sanitation and waste disposal systems place additional pressure on the water resources in the area (Moolman *et al*., 1999; Magagula *et al*., 2006). Not all WWTW in the catchment function optimally, which can result in high inputs of phosphorus-enriched effluent discharged into receiving water bodies. In particular, the Marble Hall WWTW, which discharges into the Elands River, was categorised as being in a critical state at the time of this study, scoring only 23.4% in the 2011 Green Drop report (DWA, 2011).

Recent water quality concerns upstream in Loskop Dam include eutrophication, *M. aeruginosa* blooms, AMD-related impacts, and the as yet unexplained occurrence of the disease, pansteatitis, in Nile crocodiles and *O. mossambicus* (Ashton, 2010; Oberholster *et al.,* 2010). To date, none of these issues have been reported in Flag Boshielo Dam. Pansteatitis has also been reported further downstream in the Olifants River in the KNP, where over 180 pansteatitis-affected crocodiles died (Huchzermeyer *et al*., 2011), and the population is in decline (Ferreira & Pienaar, 2011). The condition has also been described in *C. gariepinus* at this location (Huchzermeyer *et al.,* 2011). With around 135 individuals, Flag Boshielo Dam has the highest concentration of Nile crocodiles in the Olifants River system, outside of the KNP. Their number has reportedly decreased by 27% since 2005, before the dam wall was raised. However this decrease was attributed to habitat loss, as opposed to water quality concerns or pansteatitis (Botha, 2010).

The occurrence of pansteatitis in two geographically distinct aquatic animal populations on the same river is remarkable. Flag Boshielo Dam is conspicuous by the absence of pansteatitis in fish and crocodiles, despite being located downstream of Loskop Dam. This makes it a suitable negative control site for research into the aetiology of the disease. During a study of the health of *O. mossambicus* from Loskop Dam, historic trends and selected water quality parameters were evaluated in an effort to determine the environmental drivers that may be involved in the aetiology of pansteatitis (Chapter 3). The study focussed on parameters influenced by dominant catchment land uses such as the trophic state and metal concentrations. The primary objective of this study was to provide comparative information on the same parameters, and the limnology and water chemistry of Flag

Boshielo Dam. While the two reservoirs are certain to have inherent differences related to factors such as their physiography, differences in physico-chemical dynamics related to various anthropogenic impacts may provide insights into the causes of pansteatitis. In addition, long-term monitoring data collected by the DWA were analysed for trends to provide a more comprehensive assessment of water quality. This assessment included a multivariate analysis of water quality characteristics associated with the drought between 2002 and 2006.

4.2 METHODS

4.2.1 *Study Site*

Flag Boshielo Dam (24º 46’ 50” S; 29º 25’ 32” E) is located near the town of Marble Hall in Limpopo Province, South Africa. Four water quality monitoring sites were selected for this study. At the inflow of the Olifants River (FB0), the confluence with the Elands River in the dam (FB1), after the confluence with the Elands River (FB2), and in the main basin near the dam wall (FB3). The DWA water quality monitoring station (B5R002) is also located near the dam wall (Figure 4.1). When FSL is exceeded water flows over a spillway. There is a continuous outflow of water via a pipeline for drinking water purposes, and downstream flow in the Olifants River is augmented as required by water released for irrigation, mining and domestic use. Vegetation in the catchment consists predominantly of mixed Thornveld and Bushveld, much of which has been degraded, with a small proportion of Highveld Grassland in the upper catchment of the Elands River (Mucina & Rutherford, 2006). The catchment is underlain by intrusive igneous rocks (granite and gneiss) of the Lebowa Granite Suite (LGS), which are part of the Bushveld Igneous Complex (Johnson *et al.,* 2006).

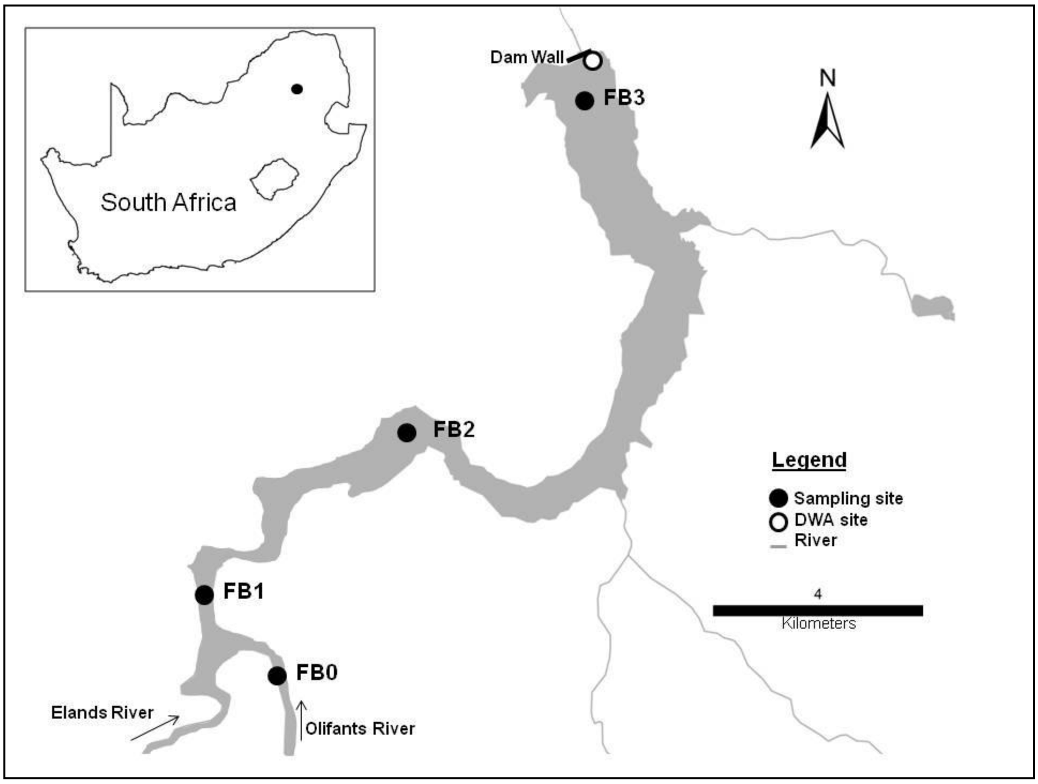

Figure 4.1 Map of Flag Boshielo Dam showing the location of four sampling sites monitored during 2011, and the Department of Water Affairs (DWA) monitoring site.

4.2.2 *Reservoir Physiography*

Mean annual runoff was calculated from the sum of annual flows recorded at DWA gauging stations B3H001 (Loskop North) and B3H021 (Scherp Arabie). Only complete annual records (12 months) monitored simultaneously at both stations were included in the calculation. This limited the dataset to 17 of the years between 1990 and 2009. The mean retention time was estimated by dividing the mean annual runoff by the reservoir volume (Dodds & Whiles, 2010), and the mean depth was calculated by dividing reservoir volume by reservoir area (Timms, 2010). Shoreline length was obtained from the DWA Directorate of Spatial and Land Information Management. All other values in Table 4.1 were obtained from the DWA station catalogue (DWA, 2013). Shoreline development (D_L) is an index that quantifies the irregularity of the shore. A perfect circle would have a D_L value of 1, with larger values indicating that the shoreline is more convoluted. The index was calculated as follows:

$$D_L = \frac{L}{2\sqrt{\pi A_0}}$$

Where L is shoreline length (km), and A_0 is reservoir surface area (km^2). Reservoirs with high shoreline development are often naturally more productive than those with low shoreline development (Dodds & Whiles, 2010).

4.2.3 *Physico-chemical characteristics*

Each of the sites in Flag Boshielo Dam was sampled on five occasions during February, April, June, October and December 2011 to cover seasonal variation in an annual cycle. One-litre integrated (2 m) water samples were collected using a plastic hose (diameter 5 cm) lowered vertically into the water and then emptied into a plastic bucket and mixed prior to collecting the sample with a plastic container rinsed in water from the sampling site. Samples were refrigerated at 4ºC in the field and then frozen until they were delivered to the accredited CSIR Analytical Laboratory in Stellenbosch. Samples were filtered through 0.45 µm pore size Whatman GF filters prior to being analysed for dissolved nutrients, metals and major ions using standard methods (APHA, 1998). Acid soluble concentrations of Al, Fe, Mn, Se, V, Zn and Cu were determined using ICP-MS. Acid soluble concentrations of all major ions except Cl and fluoride (F) were analysed using ICP-OES. Chloride, and dissolved reactive nitrogen (N) and phosphorus (P) were measured using a FIA. Fluoride was measured using ion-selective electrode analysis. A one-litre sub-sample of water was collected from the bucket and stored at 4˚C covered in foil, for analysis of chlorophyll-*a* concentrations. Samples were filtered using Whatman GF filters and then lyophilised. Chlorophyll-*a* was extracted using N,N-dimethylformamide for two hours at room temperature, then measured spectrophotometrically at 647 nm and 664 nm (Porra *et al*., 1989).

Field measurements of pH, dissolved oxygen, conductivity, and temperature of the surface water were taken along with water transparency using a Secchi disc (25 cm) at each site. Measurements were made using a Hach HQ40D multiparameter meter using a pH gel intellical probe, luminescent dissolved oxygen intellical probe and a standard four-pole graphite type electrical conductivity intellical probe (USEPA compliant). Vertical profiles of pH, dissolved oxygen and temperature were measured to a maximum depth of 30 m using the same meter at Site FB3 during each sampling period.

Censored values were replaced at 0.55 of the detection limit. All values were compared to guidelines for aquatic ecosystem health where provided (DWAF, 1996[a]). The guidelines provide a management objective known as the TWQR. This is the concentration at which no adverse effects on the health of aquatic ecosystems are expected. The SO_4^{2-} to Cl^- ratio was calculated as an indicator of mining and industrial contributions to changes in water quality. It is calculated from the ionic activity as SO_4^{2-} / Cl^-. A value greater than 5.0 indicates that these land uses are having an adverse impact on water quality (Ashton & Dabrowski, 2011).

4.2.4 *Drought Characterisation*

The Standardised Precipitation Index (SPI) developed by McKee *et al.* (1993) is widely recommended as the preferred index to characterise meteorological droughts (Svoboda *et al.,* 2012). The SPI fits data to a gamma probability distribution, which is then transformed to a normal distribution so that the mean SPI for a location and time period is zero (Edwards & MKee, 1997). The index can be used to monitor both wet and dry events. A drought event occurs when the SPI first falls below zero, and ends when the SPI becomes positive (Svoboda *et al.,* 2012). Drought intensity is defined by the following categories: 0 to -0.99 = mild drought; -1.00 to -1.49 = moderate drought; 1.50 to -1.99 = severe drought; $\leq$ -2.00 = extreme drought (McKee *et al.,* 1993). The 12-month SPI was calculated as it represents a long-term timescale and is usually tied to streamflows and reservoir levels (Svoboda *et al.,* 2012). Monthly rainfall (mm) records from 1952 until 2011 (59 years) were obtained for three stations in the catchment of Flag Boshielo Dam (Loskop Irrigation Board; Aquaville; Marble Hall), from the South African Weather Service (SAWS). The SPI was calculated using an open source SPI program (SPI_SL_6.exe) recommended by the World Meteorological Organisation, and downloaded from http://drought.unl.edu/MonitoringTools/DownloadableSPIProgram.aspx.

4.2.5 *Department of Water Affairs Monitoring Data*

4.2.5.1 *Data reliability*

The data record collected during routine water quality monitoring by DWA had several shortcomings which were addressed as follows, prior to analysis using standard methods (APHA, 1998; Ashton & Dabrowski, 2011). Where duplicate samples were recorded on several dates, only surface water samples were retained; the first complete set of analyses for a particular date was selected, and the rest of the data were discarded. The ionic activity balance was calculated for each sample using available cations Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, and ammonium-N (NH_4^{+}), and anions Cl^{-}, SO_4^{2-}, carbonate (CO_3^{2-}, calculated from total alkalinity), nitrate (NO_3^{-}) and F^{-}. Where the difference between the cation charge and anion charge was greater than 5% of the ionic charge, samples were excluded from the analyses (APHA, 1998). A total of 37 samples (12.3%) were unreliable and subsequently rejected.

Between 2009 and 2011 F was not measured in several samples. However, samples that balanced were retained, despite the exclusion of F, as it is not considered to be a major constituent of total dissolved salts (Wetzel, 1983). Sampling frequency varied from monthly to weekly, so data were aggregated to single monthly values using the median of the available data. Multiple detection limits were common so censored data were recensored to the highest detection limit in the data range (Helsel & Hirsch, 2002).

4.2.5.2 *Trophic status*

The trophic status and eutrophication potential of Flag Boshielo Dam was calculated using chlorophyll-*a* and total P concentrations measured between 2005 and 2011. Classification was determined according to the National Eutrophication Monitoring Programme (DWAF, 2002). The Carlson (1977) method was not used (as per Chapter 3) because secchi depth measurements were not made prior to 2012. Phytoplankton samples collected by DWA during the same time period were evaluated in order to compare the dominant species in Flag Boshielo Dam to those upstream in Loskop Dam.

4.2.5.3 *Trend Analyses*

Data were tested for trends using a nonparametric procedure performed in XLSTAT version 2012.6.08 Addinsoft. The Seasonal-Kendall test was used to detect monotonic trends in the data, which are continuous changes in a variable over time (Hirsch *et al*., 1982). The null hypothesis of this test is that a random variable is independent of time. The Seasonal-Kendall test accounted for seasonal variation by computing statistics for each season (each month in this study), summing the test statistics, and evaluating the overall summed statistic (Hirsch *et al.,* 1982).

Dam level (m) had a considerable influence on several parameters, and was therefore included in the Seasonal-Kendall test as an exogenous variable. This was accomplished by modelling the relationship between dam level and each *Y* variable using the LOWESS smoothing technique with a smoothing parameter (span) of 0.5, which does not assume linearity or normality of residuals (Helsel & Hirsch, 2002). The residuals were then analysed, thereby removing the effect of dam level and allowing any trends to be more clearly observed. Trends for each parameter were only calculated if less than 50% of the data were censored and more than five years of data were available (Stevens, 2003).

4.2.5.4 *Principal Component Analysis*

With no *a priori* presumptions of data relationships, a principal components analysis (PCA) was used to detect groupings in the water samples collected by the DWA. Twelve water quality parameters were analysed including pH, major ions, dissolved nutrients and Si concentrations. Samples were grouped according to normal flows or drought periods as determined by the SPI and examining inflow data from the Olifants River and Elands River, along with water levels in Flag Boshielo Dam. The analysis was completed in Statistica Version 11 (StatSoft. Inc, Tulsa, USA).

4.3 RESULTS

4.3.1 *Reservoir physiography*

The mean annual flow from the Olifants River was almost eight times greater than that from the Elands River (Table 4.1). An extensive area (9.03 km^2) surrounding Flag Boshielo Dam was flooded when the dam wall was raised in March 2006, and vegetation was purposely not cleared from this area beforehand in order to provide habitat in the form of dead trees for waterfowl. As a result, submerged dead trees are scattered throughout most of the littoral zone. After the dam wall was raised, the mean depth increased, the volume and surface area almost doubled, and the mean retention time increased to 4.9 months (Table 4.1). The shoreline development value of 4.94 is greater than 3, which means that Flag Boshielo Dam has a highly irregular, dendritic or tree-shaped shoreline (Timms, 2010).

Table 4.1 Attributes of Flag Boshielo Dam at full supply level before and after the dam wall was raised by 5 m in March 2006, including relevant catchment attributes.

Catchment Attributes	**Pre March 2006**	**Post March 2006**
Elands River mean annual flow (x 10^6 m^3)	NA	28.51
Olifants River mean annual flow (x 10^6 m^3)	NA	218.45
Mean annual runoff (x 10^6 m^3)	NA	382.89
Catchment area (km^2)	NA	23,555
Reservoir Attributes		
Dam wall height (m)	35	40
Maximum Depth (m)	28	33
Mean Depth (m)	7.69	8.6
Volume (x 10^6 m^3)	98.99	188.85
Surface area (km^2)	12.87	21.9
Mean retention time (months)	3.9	4.9
Elevation above sea level (m)	817	822.09
Shoreline length* (km, scale 1:5000)	ND	82.1
Shoreline development (D_L)	ND	4.94

NA, Not Applicable; ND, Not Determined; * scale 1:5000, shoreline contour 823 m

4.3.2 *Physico-chemical characteristics*

4.3.2.1 *Surface water chemistry*

A summary of selected water quality constituents measured during 2011 is presented in Table 4.2. Water in Flag Boshielo Dam was alkaline, with pH ranging from 7.38 to 9.42, and relatively high total alkalinity (as $CaCO_3$) ranging from 40 mg/ℓ to 87 mg/ℓ. Dissolved oxygen was also consistently high with a median of 10.02 mg/ℓ (Table 4.2). While some spatial patterns in water quality constituents across the dam were evident, these were not very distinct, and seasonal patterns at sites were more apparent.

Sulphate concentrations were relatively constant across the dam, with an overall median of 92.5 mg/ℓ (Table 4.2), and while concentrations showed limited seasonal variation, the lowest values were observed in October (Figure 4.2a). The SO_4^{2-} / Cl^- ratio ranged widely from 2 to 7 with a median value of 4, and all sites showed similar seasonal patterns. Values were higher in February, April and June, than in October and December (Figure 4.2b) which was as a result of elevated Cl concentrations in the latter months (Figure 4.2c). Both Na and Cl showed distinct and similar seasonal variation, with the lowest medians observed in February steadily increasing throughout the year to peak in December (Figure 4.2c & d).

Concentrations of dissolved Cu, Se, V and Zn were never above instrument detection limits and were thus excluded from Table 4.2. Manganese concentrations were consistently within the TWQR throughout the study. The TWQR guideline for Fe stipulates that values should not vary by more than 10% from the background concentrations. Several values were censored during monitoring, making it impossible to determine a background concentration. However, when values were detected, they were relatively low and did not show substantial variation (Table 4.2). Dissolved Al concentrations exceeded the TWQR of 10 µg/ℓ in October and December at Sites FB0 and FB1, and at Site FB3 in December. The highest concentration of 14.9 µg/ℓ was still well below the CEV of 20 µg/ℓ. In February, April and June, the detection limit was 20 µg/ℓ, and no Al was detected above this limit.

Table 4.2 Summary of constituents measured in surface water samples from four sites in Flag Boshielo Dam during 2011.

Site	Date	pH	Electrical Conductivity (µS/cm)	Dissolved oxygen (mg/ℓ)	Inorganic phosphorus (DIP) (mg/ℓ)	Dissolved inorganic nitrogen (mg/ℓ)	DIN:DIP ratio	Total Alkalinity ($CaCO_3$) (mg/ℓ)	Sulphate (mg/ℓ)	SO_4 : Cl ratio	Aluminium (Al) (µg/ℓ)	Iron (Fe) (µg/ℓ)	Manganese (Mn) (µg/ℓ)	Chlorophyll-*a* (µg/ℓ)	Dissolved Organic Carbon (mg/ℓ)	Silica (mg/ℓ)	Secchi depth (cm)	* Total suspended solids (mg/ℓ)	* Turbidity (NTU)
FBO	Feb	7.93	355	7.49	0.005	0.40	72.7	65	90	5	11.0	27.5	5.5	0.009	5.9	6.4	66	ND	ND
	Apr	7.38	381	7.04	0.005	0.15	18.1	40	78	7	11.0	20.0	5.5	ND	4.0	1.4	85	ND	ND
	Jun	8.91	439	13.62	0.062	0.35	4.84	73	111	4	11.0	5.5	10.0	0.128	8.8	3.4	46	ND	ND
	Oct	9.08	531	7.60	0.050	0.10	ND	75	78	2	**14.9**	12.4	3.9	0.110	7.0	5.5	64	ND	ND
	Dec	9.39	576	14.23	0.050	0.10	ND	77	121	2	**14.3**	18.2	0.6	0.320	8.0	5.7	49	ND	ND
FB1	Feb	8.69	177	10.44	0.010	0.35	36.7	70	87	4	11.0	27.5	5.5	0.015	5.8	4.9	75	ND	ND
	Apr	8.28	396	8.39	0.065	0.50	3.1	59	98	5	11.0	20.0	5.5	0.050	8.0	2.8	75	ND	ND
	Jun	8.98	401	12.05	0.084	0.15	0.7	58	106	4	11.0	5.5	10.0	0.062	9.0	3.1	41	ND	ND
	Oct	9.07	490	7.19	0.050	0.10	ND	75	73	2	**11.9**	10.2	3.1	0.080	8.0	5.3	56	ND	ND
	Dec	9.42	507	12.97	0.050	0.10	ND	69	113	3	**10.5**	2.4	0.5	0.600	9.0	5.1	45	ND	ND
FB2	Feb	9.19	371	10.41	0.014	0.10	7.9	73	81	4	11.0	27.5	5.5	0.036	6.9	6.2	75	ND	ND
	Apr	9.07	405	9.78	0.036	0.10	3.0	87	127	4	11.0	5.5	5.5	0.150	9.0	10.1	69	ND	ND
	Jun	8.76	398	10.90	0.056	0.10	1.9	59	102	4	11.0	5.5	5.5	0.224	9.2	3.1	49	ND	ND
	Oct	9.11	473	7.66	0.050	0.10	ND	76	73	2	6.8	7.3	3.6	0.080	8.0	5.2	59	ND	ND
	Dec	9.4	473	12.58	0.050	0.10	ND	58	95	3	8.6	9.4	0.3	0.110	8.0	4.7	62	ND	ND
FB3	Feb	9.17	369	10.26	0.013	0.10	8.2	70	81	4	11.0	27.5	5.5	0.025	7.7	6.0	ND	7.0	0.50
	Apr	8.92	386	8.66	0.023	0.10	4.8	61	89	4	11.0	5.5	5.5	0.030	7.0	6.7	72	7.4	0.50
	Jun	8.37	400	10.28	0.064	0.10	1.7	67	102	4	11.0	5.5	10.0	0.196	9.1	3.1	55	**21.2**	1.73
	Oct	8.84	451	7.85	0.050	0.10	ND	75	73	2	4.4	9.2	4.1	0.070	7.0	5.2	65	5.8	1.23
	Dec	8.94	465	8.14	0.050	0.10	ND	71	105	3	**11.4**	0.5	0.2	0.550	8.0	5.0	59	7.7	1.49
Median		8.96	403	10.02	0.050	0.10	ND	70	93	4	11.0	9.3	5.5	0.080	8.0	5.2	62	7.4	1.23
Min.		7.38	177	7.04	0.005	0.10	0.7	40	73	2	4.4	0.5	0.2	0.009	4.0	1.4	41	5.8	0.50
Max.		9.42	576	14.23	0.084	0.50	72.7	87	127	7	14.9	27.5	10.0	0.600	9.2	10.1	85	21.2	1.73

Underlined, censored values replaced at 0.55 of detection limit; ND, value not determined; bold print, value exceeds TWQR; ***** values collected by DWA during routine monitoring

Seasonal patterns in inorganic N showed a generally inverse relationship to chlorophyll-*a* concentrations (Figure 4.2e & g). The highest values were observed in February with a median value of 0.23 mg/ℓ, which decreased in April and June, and was below detection limits at all sites in October and December. No inorganic N was detected at Sites FB2 and FB3 at any time during the study period, and detected levels at Sites FB0 and FB1 never exceeded 0.5 mg/ℓ (Table 4.2), which categorises Flag Boshielo Dam as oligotrophic (DWAF, 1996[a]). The inorganic P concentrations were also frequently censored, particularly during October and December when no inorganic P was detected at any sites (Table 4.2). During February, April and June, the values ranged from 0.01 to 0.084 mg/ℓ (Figure 4.2f) which fall into the meso- to eutrophic category (DWAF, 1996[a]).

Inorganic N and P concentrations were frequently below the detection limit, which prevented the calculation of ratios for most sampling periods. Ratios were calculated when N and P were above detection limits, but not when both values were censored. The resulting ratios fluctuated widely, ranging from < 1 up to 72 (Table 4.2).

The reservoir was relatively turbid throughout monitoring. Secchi disk transparency was consistently low, with a median of 0.62 m and very limited spatial variation across the dam (Figure 4.2h), although there was an apparent inverse relationship between Secchi depths and chlorophyll-*a* concentrations (Figure 4.2g & h). Chlorophyll-*a* concentrations were generally low with a median of 0.08 μg/ℓ and a maximum of 0.6 μg/ℓ which was measured in December (Table 4.2). No algal blooms were observed during the study period.

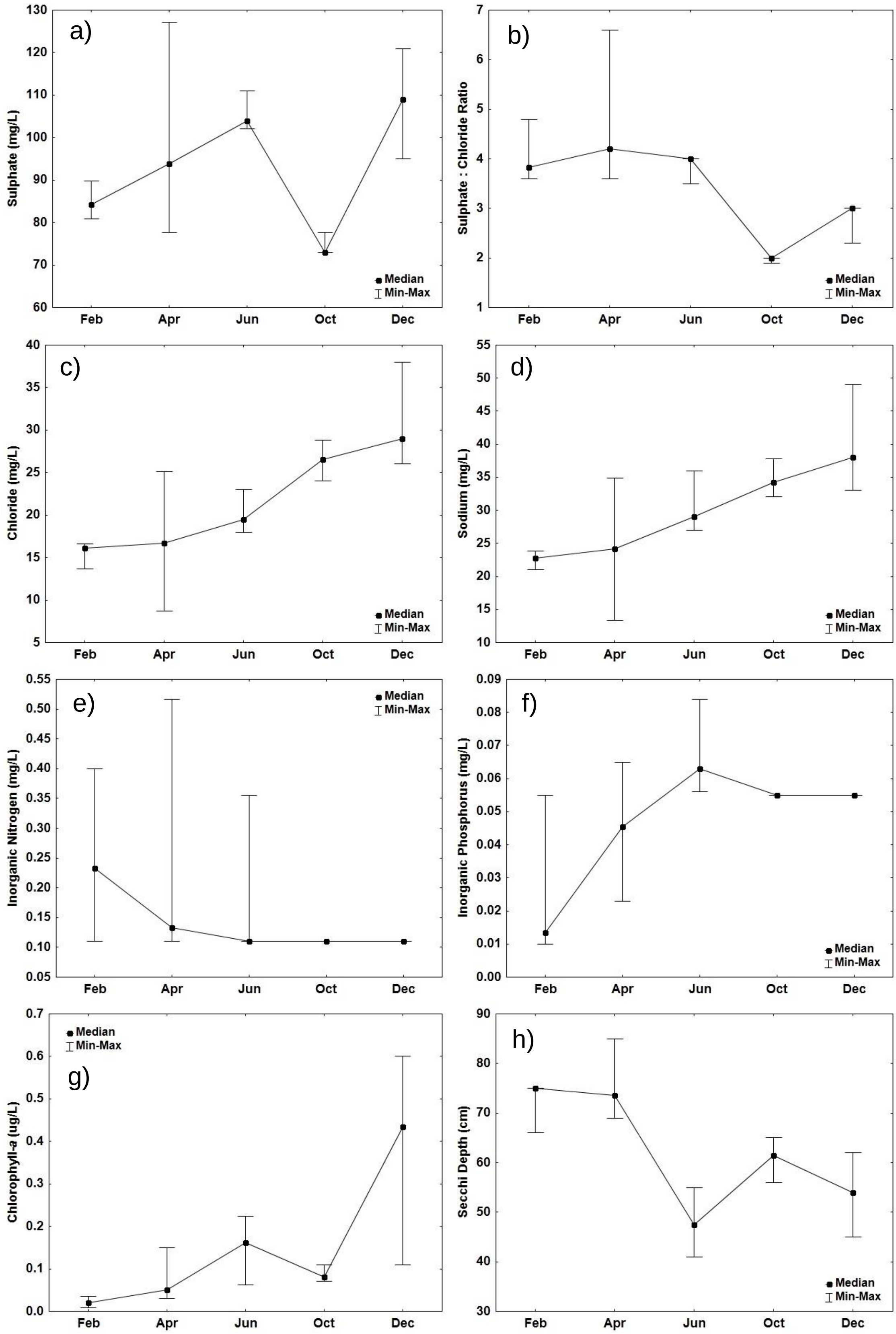

Figure 4.2 Seasonal variation in the median and range of sulphate (a), sulphate : chloride ratio (b), chloride (c), sodium (d), inorganic nitrogen (e), inorganic phosphorus (f), chlorophyll-a (g) and secchi depth (h) measured in Flag Boshielo Dam during 2011.

Total suspended solids (TSS) and turbidity were not monitored as part of this study; however, DWA measured these parameters monthly at their site during 2011. To better explain the origins of turbidity, these results were included in Table 4.2 under Site FB3 as it is located near the DWA site. The background value for TSS was calculated as 5.68 mg/ℓ, which was the median of the DWA dataset ranging from 2006 until 2011 (n=40). In June the TSS measured 21.2 mg/ℓ, which was > 10% of the background value and therefore exceeded the TWQR. However, the background value is well within range of the guideline for all aquatic ecosystems of < 100 mg/ℓ (DWAF, 1996[a]). Turbidity values were below detection limits in February and April, and were below 2 NTU in June, October and December. The median concentration of dissolved organic carbon was 8 mg/ℓ which was fairly consistent in different seasons and across sites in the dam. Silica concentrations ranged widely from 1.4 mg/ℓ to 10.1 mg/ℓ, with no clear seasonal or spatial trends across the dam (Table 4.2).

4.3.2.2 *Reservoir Profiles*

Flag Boshielo Dam demonstrated a typical monomictic mixing regime. Turnover occurred between April and June, which was the only period when approximately isothermal conditions were observed at around 16°C (Figure 4.3a). Surface heating commenced from October onwards, resulting in well defined thermal stratification from October until February. Surface water temperatures varied substantially from 21°C in June to 28°C in February. During periods of stratification, dissolved oxygen (Figure 4.3b) and pH values (Figure 4.3c) were elevated at the surface. Distinct oxyclines around 10 m depth were evident, where values decreased from between 6 and 10 mg/ℓ at the surface, to anoxic conditions in the hypolimnion in all months except June (Figure 4.3b). The pH followed a similar trend to dissolved oxygen, with values of approximately 9 in the upper 5 m, decreasing to between 7 and 8.8 in the metalimnion and hypolimnion (Figure 4.3c).

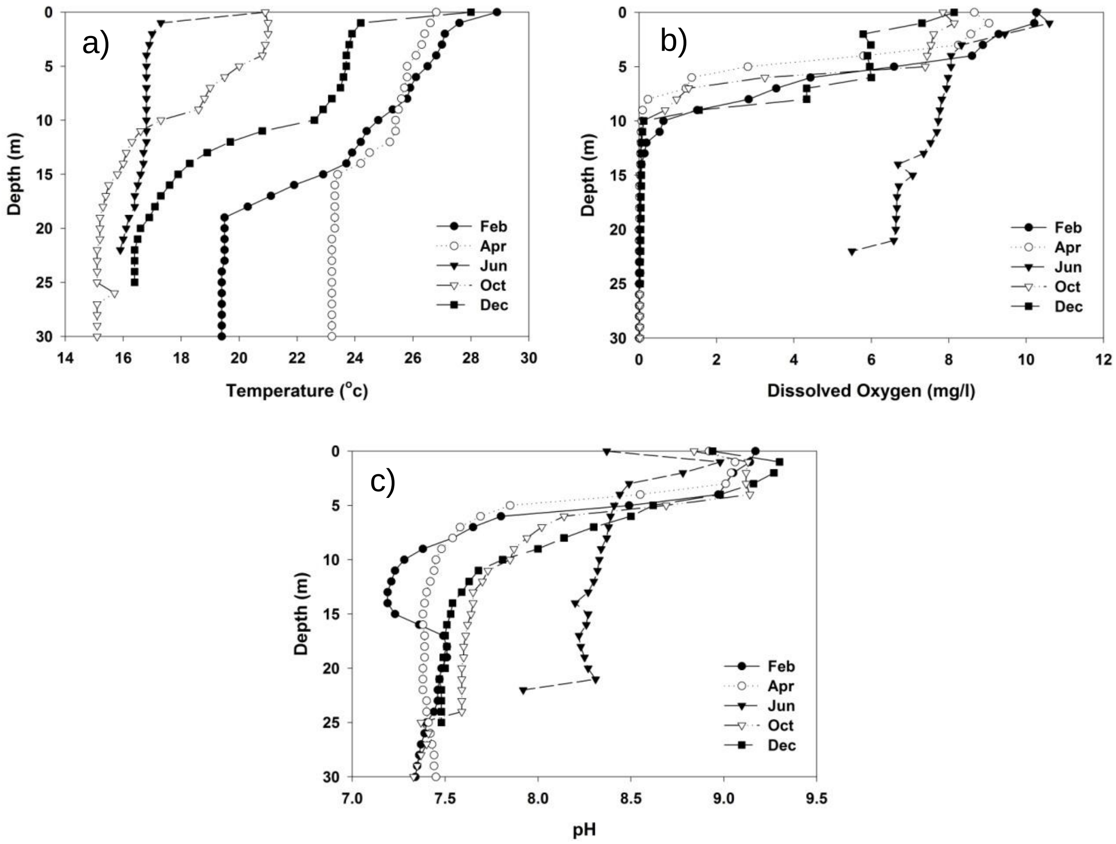

Figure 4.3 Field measurement profiles of temperature (a), dissolved oxygen (b) and pH (c), collected during 2011 at site FB3 near the dam wall of Flag Boshielo Dam.

4.3.3 *Drought Characterisation*

The 12-month SPI results from all three rainfall stations were positively correlated with dam level (%), but values from the Loskop Irrigation Board showed the strongest correlation (Spearman's r_s = 0.61; p < 0.001) and are presented in Figure 4.4. The rainfall station is located downstream of Loskop Dam, and adjacent to the Olifants River (- 25.4; 29.367). Although data from 1952 to 2011 were used to determine the 12-month SPI, only the time period from 1998 to 2011 was presented (Figure 4.4) as it corresponded to the DWA monitoring period. There was an extended drought between November 2002 and December 2005. During this period the SPI dropped below zero and was frequently around -3.00, which was representative of extreme drought conditions. This event was typified by the lowest SPI values in the long-term data from 1952 to 2011 at this station.

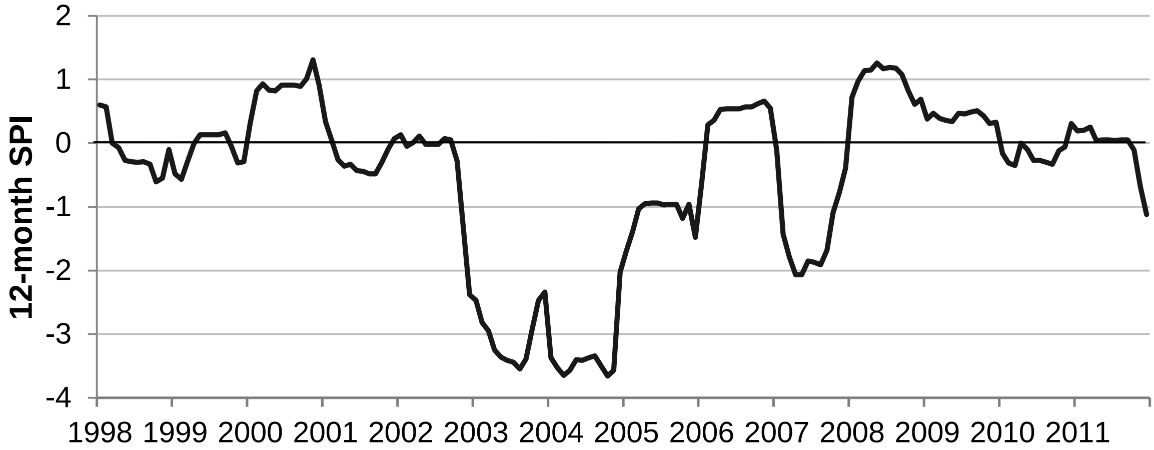

Figure 4.4 Selected time series from January 1998 to December 2011 of 12-month SPI values for the Loskop Irrigation Board rainfall station. Calculations were based on long-term records from 1952 until 2011.

During the drought, prolonged low flows from the Olifants and Elands rivers resulted in very low water levels in Flag Boshielo Dam (Figure 4.5). The lowest water level was recorded at 7.7 m (23.7% of FSL) in February 2004. At this point the area exposed was equivalent to 8.14 km^2, or 63% of the surface area of the reservoir at FSL (1997 sediment survey, DWA Spatial and Land Information Management). The water level rose briefly in early 2004, but was not sustained due to consistently low inflow volumes. Renewed high flows in February 2006 coincided closely with raising the dam wall by 5 m in March 2006, although the two events were unrelated. Construction work to raise the dam wall did not require any release of water to enable work to proceed, and low water levels prior to completion of construction were purely related to the drought.

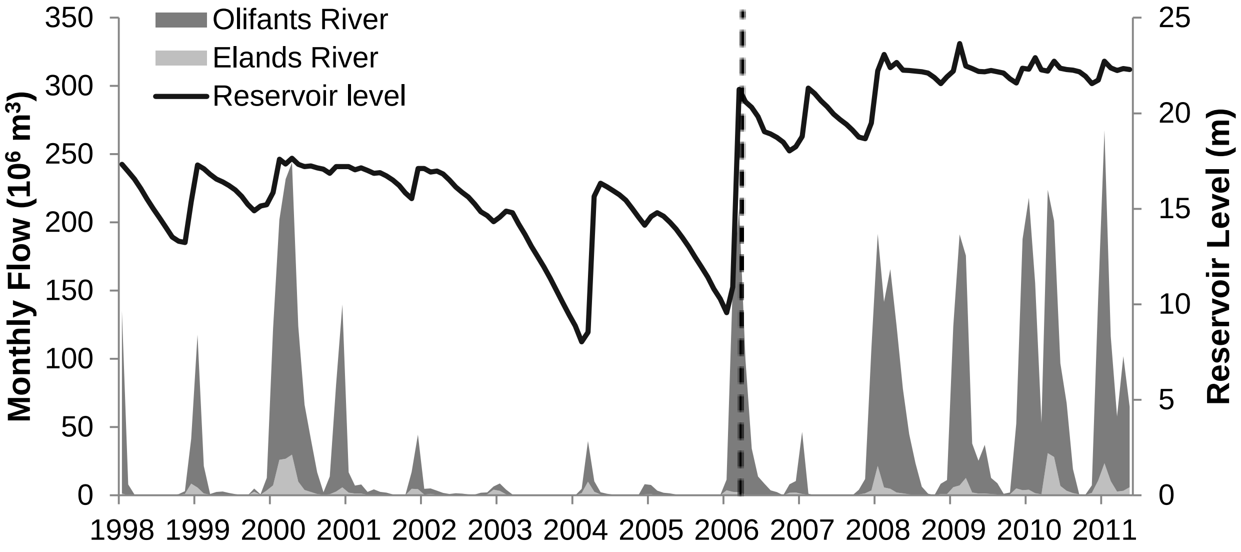

Figure 4.5 Monthly water levels in Flag Boshielo Dam plotted with monthly inflow values from the Olifants River (DWA site B3H001) and Elands River (DWA site B3H021) between 1998 and 2011. The dashed vertical line represents the end of a drought that coincided with the completion of the raised dam wall in March 2006.

4.3.4 *Department of Water Affairs Monitoring Data*

The only parameters with a relatively high proportion of censored data were dissolved inorganic P and N, total suspended solids, and chlorophyll-*a*. However, the proportions were less than 50% allowing trends to be analysed. All parameters were monitored regularly from 1998 except total P, total N, chlorophyll-*a*, and TSS, which were only monitored from around 2005 onwards. Trends could not be calculated for Secchi depth as no measurements before 2012 were available.

Mean annual chlorophyll-*a* concentrations classified the reservoir as oligotrophic with a moderate nuisance value for algal bloom productivity (Table 4.3). Mean annual total P concentrations represented a moderate potential for algal or plant productivity (Table 4.3). Both moderate classifications are fairly low because there are two categories (significant and serious) above this level.

Table 4.3 Trophic status and eutrophication potential of Flag Boshielo Dam calculated using DWA monitoring data collected between 2005 and 2011 (DWAF, 2002).

Statistic	**Unit**	**Value**	**Classification**
Mean annual chlorophyll-*a*	µg/ℓ	7.41	Trophic status: **Oligotrophic**
% time chlorophyll-*a* > 30 µg/ℓ	%	2.81	Current nuisance value of algal bloom productivity: **Moderate**
Mean annual total phosphorus	mg/ℓ	0.045	Potential for algal or plant productivity: **Moderate**

4.3.4.1 *Seasonal-Kendall trend analysis*

Results of the Spearman's rank correlation (r_s) between dam level (m) and each parameter for the full duration of the data range, are presented in Table 4.4. All major ions except SO_4^{2-} showed significant negative correlations with dam level (i.e., as dam level (X) increases, variable (Y) concentration decreases). The only parameter that was positively correlated with dam level was the SO_4^{2-} / Cl- ratio (r_s 0.75). Parameters that were not significantly correlated with dam level were SO_4^{2-}, total P, total N, dissolved inorganic N, TSS and chlorophyll-*a*.

Table 4.4 Summary of the seasonal Mann-Kendall trend analysis performed on LOWESS residuals adjusted for dam level (m) for the entire range of the dataset. Results of the Spearmans rank correlation (r_S) between dam level (m) and each parameter are included, along with median values of each parameter after the dam wall was raised and high flows resumed in March 2006.

Parameter	**Data range (*n*)**	**% Censored**	**Spearmans r_s**	***p***	**Median post March 2006**	**Trend**	**Kendall's Tau**	***p***
pH (standard units)	1998-2011 (156)	0	-0.53	***	7.9	-	-0.273	***
Electrical Conductivity (µS/cm)	1998-2011 (154)	0	-0.76	***	444.75	+	0.384	***
Calcium Ca^{2+} (mg/ℓ)	1998-2011 (155)	0	-0.47	***	29.2	0	0.023	NS
Magnesium Mg^{2+} (mg/ℓ)	1998-2011 (151)	0	-0.37	***	18.1	+	0.154	*
Potassium K^{+} (mg/ℓ)	1998-2011 (138)	0	-0.60	***	4.9	0	0.066	NS
Sodium Na^{+} (mg/ℓ)	1998-2011 (149)	0	-0.81	***	27.03	-	-0.179	*
Sodium Adsorption Ratio	1998-2011 (153)	0	-0.81	***	1	-	-0.197	*
Chloride Cl^{-} (mg/ℓ)	1998-2011 (149)	0	-0.75	***	23	+	0.336	***
Fluoride F^{-} (mg/ℓ)	1998-2011 (138)	0	-0.68	***	0.51	0	0.023	NS
Sulphate SO_4^{2-} (mg/ℓ)	1998-2011 (146)	0	-0.03	NS	102.3	+	0.179	*
Total Alkalinity as CaCO3 (mg/ℓ)	1998-2011 (148)	0	-0.76	***	79.3	0	-0.096	NS
Total Phosphorus (mg/ℓ)	2005-2011 (47)	2	0.15	NS	0.035	0	0.056	NS
Total Nitrogen (mg/ℓ)	2005-2011 (46)	0	-0.03	NS	0.801	-	-0.500	*
Dissolved Inorganic Phosphorus (mg/ℓ)	1998-2011 (149)	43	-0.16	*	0.014	0	-0.093	NS
Dissolved inorganic Nitrogen (mg/ ℓ)	1998-2011 (146)	28	-0.04	NS	0.123	-	-0.294	***
Inorganic N : P ratio	1998-2011 (141)	7	0.04	NS	5.41	-	-0.225	*
Silica Si (mg/ℓ)	1998-2011 (149)	0	-0.50	***	5.14	-	-0.144	*
Sulphate : Chloride Ratio	1998-2011 (152)	0	0.75	***	3.21	+	0.129	*
Total suspended solids (mg/ℓ)	2006-2011 (40)	38	-0.24	NS	6.2	0	0.164	NS
Chlorophyll-*a* (µg/ℓ)	2005-2011 (46)	34	-0.11	NS	6.85	0	-0.167	NS

(+), upward trend; (-), downward trend; (0), no significant trend; (NS), Not Significant; (ND) Not Determined. Significance: ***, <0.0001; **, <0.001; *, <0.05

Due to the drought and raising of the dam wall that influenced dam levels during the evaluation period (Figure 4.4 & Figure 4.5), the Seasonal-Kendall trend analysis was performed on LOWESS residuals adjusted for dam level (m) in order to evaluate long-term trends in parameters excluding variation related to this factor. No significant trends were observed for calcium, potassium, fluoride, total alkalinity, total suspended solids or chlorophyll-*a*.

A significant decreasing trend in pH values was observed (Tau = -0.273) and the median value after March 2006 was 7.9 (Table 4.4). However, the trend was very gradual and pH values were consistently alkaline with low variation (

Figure 4.6a). Electrical conductivity showed a significant increasing trend (Tau = 0.384; Table 4.4) despite a substantial decrease in concentrations after the the dam wall was raised and normal flows resumed (
Figure 4.6b).

Sodium concentrations illustrated clear seasonal patterns that were evident for most parameters as diagonal lines of increasing concentrations over periods of time (

Figure 4.6c). These seasonal patterns spanned various time periods from less than 12 months to almost 24 months. A Spearman's rank correlation showed that Na and Cl concentrations were significantly positively correlated (r_s = 0.95; $p < 0.001$), and patterns in the Cl data were almost identical to those for Na (

Figure 4.6c). Both Na and Cl showed the most significant negative correlations with dam level, at r_s = -0.81 and r_s = -0.75 repectively (Table 4.4), and concentrations of both ions dropped substantially after March 2006 (
Figure 4.6c). While Na showed a weakly significant negative trend over time (Tau = -0.179), Cl increased significantly over time (Tau = 0.336; Table 4.4). During the drought, the sodium adsorption ratio continuously exceeded the TWQR for irrigation water which is < 2 (
Figure 4.6d). However, post March 2006, the median value reduced to 1 and there was a significant decreasing trend over time (Tau = - 0.197; Table 4.4).

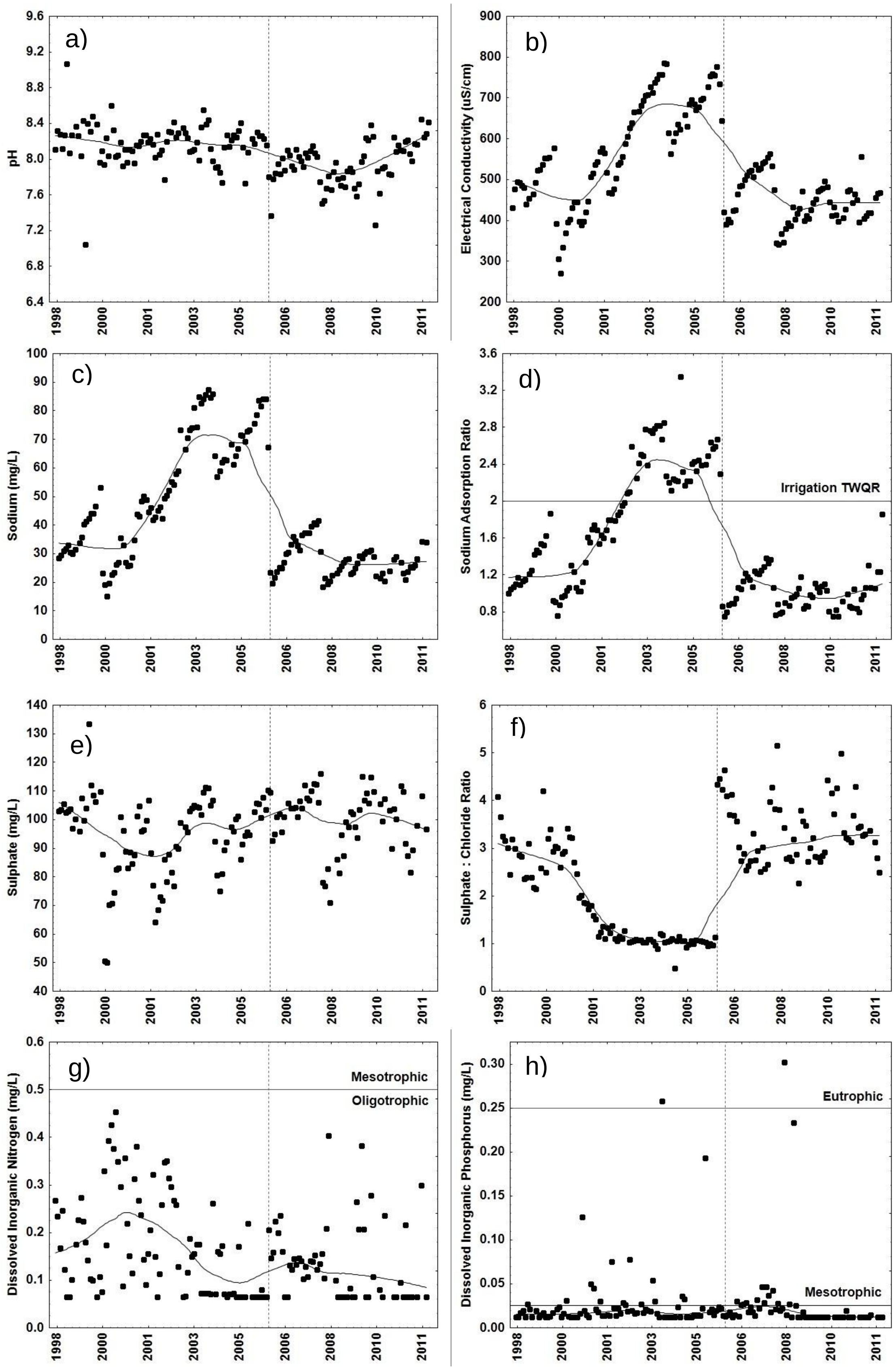

Figure 4.6 Scatterplots of various water quality constituents with LOWESS smooth curves. The dotted vertical line indicates March 2006 when the dam wall was raised.

Sulphate concentrations showed a significant increase over time (Tau = 0.179; Table 4.4), although this was a very gradual trend, and concentrations appeared more or less stable (
Figure 4.6e). The drought period did not appear to influence SO_4^{2-} concentrations to a large degree, which was supported by the fact that SO_4^{2-} was the only ion that was not significantly correlated with dam level (Table 4.4). While sulphate concentrations remained relatively constant, the decrease in Cl concentrations after the dam wall was raised, resulted in a significant increasing trend in the SO_4^{2-} / Cl^- ratio (Tau = 0.129) and the median value was 3.21 after March 2006 (Table 4.4).

The LOWESS trend-line for both inorganic N and P showed a small increase after the dam wall was raised from 2006 to 2007, followed by a decrease from 2008 onwards (
Figure 4.6g & h). Inorganic N showed a significant decreasing trend over time (Tau = -0.294; Table 4.4), and concentrations never exceeded 0.5 mg/ℓ, which classified Flag Boshielo Dam as oligotrophic (DWAF, 1996[a]). Total N also showed a significant decreasing trend (Tau = - 0.5), but there was no trend in total P or inorganic P over time. The median inorganic P concentration after raising the dam wall was 0.014 mg/ℓ which also classified the reservoir as oligotrophic (DWAF, 1996[a]). The inorganic N : P ratio showed a significant decreasing trend (Tau = - 0.225), the median ratio of 5.41 after construction of the dam wall was indicative of N limitation (Table 4.4). Between 2006 and 2011 there was no significant monotonic trend in the chlorophyll-*a* concentrations, and the median value was 6.85 µg/ℓ (Table 4.4).

The phytoplankton assemblage was generally dominated by nitrogen-fixing species, especially *Cylindrospermopsis* sp., between 2005 and 2008. No *Microcystis* spp. were detected throughout the monitoring period. *Ceratium* sp. was never dominant, and not frequently detected in samples (Figure 4.7)

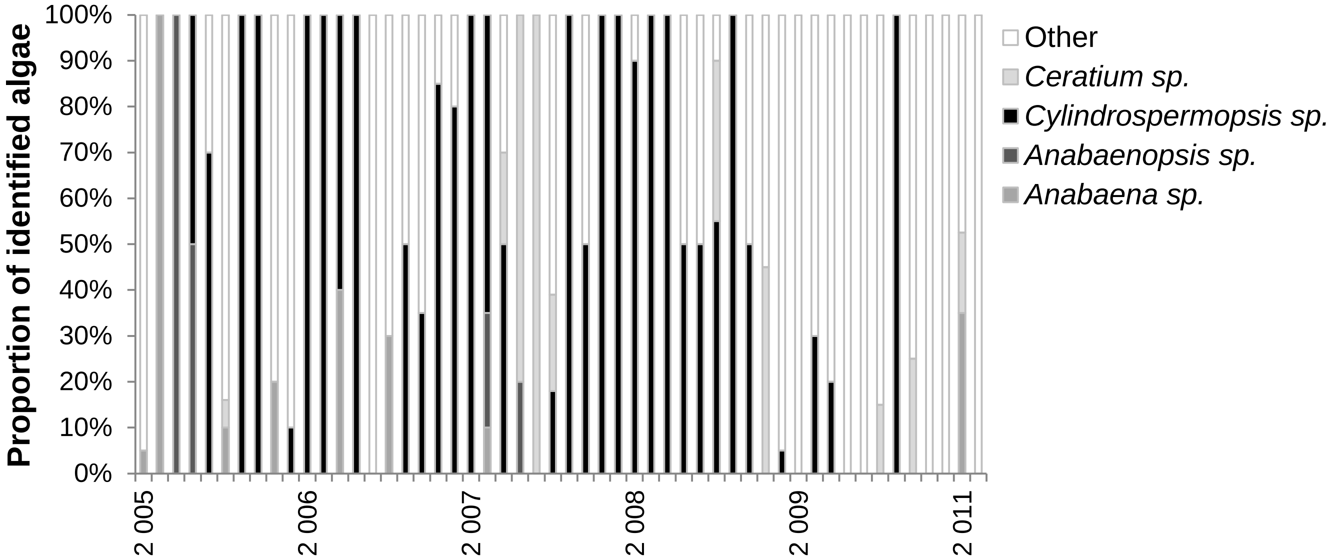

Figure 4.7 Proportional contribution of nitrogen-fixing species (C*ylindrospermopsis* sp.*; Anabaenopsis* sp.*, Anabaena* sp.) and *Ceratium* sp. identified in monthly phytoplankton samples collected by DWA between 2005 and 2011 in Flag Boshielo Dam.

4.3.4.2 *Principal component analysis*

Results of the PCA of water samples collected during the drought from November 2002 until December 2005, and normal flows before and after this period, are presented in Figure 4.8, with related factor loadings in Table 4.5. Only parameters that were measured from 1998 onwards were included in the analysis.

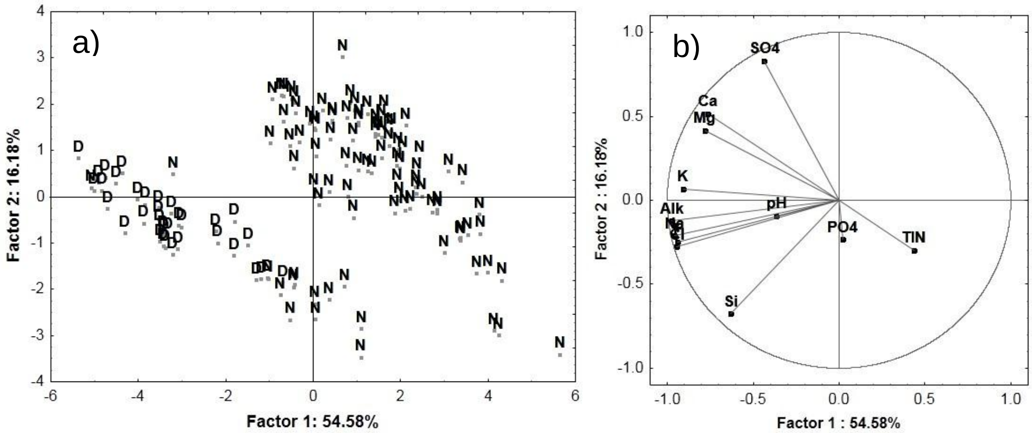

Figure 4.8 Principal components analysis (PCA) showing monthly samples (a), and variables (b) projected on the factor plane. Monthly samples were composed of median values for 11 water quality variables measured by the DWA at Flag Boshielo Dam between 1998 and 2011. Samples were grouped according to collection date as either drought (D), between November 2002 to December 2005, or normal (N), before and after this period.

There was a very clear distinction between water samples collected during both periods. The first and second factors accounted for 54.58% and 16.18% of the variation in the data, respectively. Drought water samples were characterised by high levels of dissolved salts, especially K, Na, Cl, F and total alkalinity, all of which had factor loadings > 0.9 in the first factor (Table 4.5). Sulphate had the strongest influence in the second factor (0.83) which separated samples collected during both normal flows and the drought period.

Table 4.5 Factor loadings of each water quality parameter in factor one and factor two from the PCA shown in Figure 6. 8

Variable	Factor 1	Factor 2
pH	-0.36	-0.09
Calcium Ca^{2+}	-0.76	0.51
Magnesium Mg^{2+}	-0.78	0.41
Potassium K^{+}	-0.91	0.06
Sodium Na^{+}	-0.95	-0.21
Chloride Cl^{-}	-0.94	-0.27
Fluoride F^{-}	-0.93	-0.24
Sulphate SO_4^{2-}	-0.43	0.83
Total Alkalinity ($CaCO_3$)	-0.97	-0.12
Dissolved Inorganic N (DIN)	0.43	-0.29
Dissolved Inorganic P (DIP)	0.02	-0.23
Silica Si	-0.62	-0.67

4.4 DISCUSSION

Results of short-term and long-term monitoring following the drought indicated that water quality in Flag Boshielo Dam was of a good standard from an ecosystem health perspective. On the basis of inorganic N and P measured during 2011, and long-term measurements of chlorophyll-*a* and total P, Flag Boshielo Dam can be considered oligotrophic to mesotrophic, with N-limited primary production. In this respect it does not fit conventional assumptions that P is the yield-limiting nutrient for most South African impoundments (Toerien, 1975; Walmsley & Butty, 1980), and does not follow the trend of increasing eutrophication observed in many of our reservoirs (van Ginkel, 2011), including Loskop Dam (Chapter 3; Oberholster *et al*., 2013). Given that irrigated and dryland agriculture dominate catchment land uses, providing a potential source of nutrients, this finding is both positive and remarkable.

While regular blooms of *M. aeruginosa* and *C. hirundinella* occur at Loskop Dam, no algal blooms occurred at Flag Boshielo Dam, and the phytoplankton assemblage was dominated by nitrogen-fixing species. Although *C. hirundinella* occurs at Flag Boshielo Dam, it was never dominant, and *Microcystis* spp. were never recorded between 2005 and 2011. Dissolved metal concentrations at Flag Boshielo Dam were consistently either below detection limits, or very low, while Al, Mn and Fe periodically exceeded various thresholds of the water quality guidelines at Loskop Dam (Oberholster *et al*., 2010; Chapter 3). Furthermore, elevated concentrations of Al and Fe have been measured in the fat of *O. mossambicus* as well as in a potential food source, *Spirogyra* spp., from Loskop Dam (Oberholster *et al*., 2012). Of the metals measured at Flag Boshielo Dam during 2011, only Al was ever detected at levels that periodically exceeded the TWQR. However, given the pH range at which these concentrations were measured (pH 8.94 – 9.39), the chemical speciation would predominantly have been in the form of relatively non-toxic Al hydroxides $Al(OH)_3$ and $Al(OH)_4^-$, as opposed to the highly toxic Al^{3+} which predominates below a pH of 5 (Gensemer & Playle, 1999).

The relatively shallow mean depth and high shoreline development value are factors that naturally increase the susceptibility of Flag Boshielo Dam to eutrophication (Dodds & Whiles, 2010). These factors are offset to an extent by the relatively short retention time of 4.9 months which increases flushing rates (Vollenweider, 1968). This is more than half the retention time of 12.2 months at Loskop Dam (Chapter 3). The reservoir showed a typical monomictic pattern of stratification, with prolonged anoxic conditions in the hypolimnion below 10 m during all sampling periods except June, when the water column was approximately holomictic. There was no indication of a deepening thermocline or oxycline related to lake turnover during sampling in April. This event occurred between late April and June. Elevated pH levels in the surface waters followed roughly the same pattern of stratification as dissolved oxygen up to around 10 m. Through photosynthesis and the absorption of carbonic acid, phytoplankton growth is known to influence both of these variables (Wetzel, 1983).

Flag Boshielo Dam was moderately turbid throughout 2011, with Secchi depth values ranging from 0.62 – 0.85 m. Turbidity was not obviously mineral in character because total suspended solids and turbidity values were not very high during 2011. However, the consistently low chlorophyll-*a* concentrations indicated that algal productivity was also unlikely to influence turbidity to a great extent. The median dissolved organic carbon concentration of 8 mg/ℓ in 2011 could be considered moderate when compared to highly coloured rivers such as those in the Western Cape, where values range from 10 mg/ℓ to 21 mg/ℓ (Nkambule *et al.,* 2012).

Water levels approximately doubled from 9.7 m in January 2006 to around 21 m in March 2006 following the drought, after which there was a clear pulse of inorganic N and P between 2006 and 2008. Although the water level in March 2006 only represented 90% of the new FSL, it was still around 4 m higher than the previous FSL. Newly inundated areas of the littoral zone including vegetation, and soils rich in organic matter and leaf litter, would have stimulated high levels of microbial decomposition, leading to an increase in nitrification and partially explaining the pulse of N and P. Additional nutrients may have been released upon re-wetting of the sediments after the drought, a process caused by drying-induced microbial-cell lysis (Qiu & McComb, 1995; Baldwin & Mitchell, 2000). Although inorganic N was not significantly correlated with dam level, the LOWESS curve showed a distinct decrease in concentrations during the drought which contributed to the overall decreasing trend in inorganic N. The reasons for lower inorganic N concentrations were not clear, but may include reduced inputs from the Olifants and Elands Rivers during the drought period. Alternatively, internal N loss associated with anoxic conditions during extended seasonal stratification is one of the main causes of N limitation in lakes at lower latitudes (Lewis, 2002).

Despite the flush of nutrients after re-wetting, inorganic N levels were still considered representative of oligotrophic conditions (DWAF, 1996[a]). Nutrient concentrations in lakes are known to show system-specific responses during extended drought periods, and after re-wetting. These include reductions, increases, and no change (Lake, 2011). These responses are governed by a variety of factors, such as the intensity and duration of drought, the extent of drying and oxidation levels of reservoir sediments, trophic status of the waterbody, and microbial activity associated with nutrient cycling (Lake, 2011).

Increasing salinity, turbidity and alkalinity during periods of drought in lakes and rivers as a result of decreased dilution, increased evaporation, and increased residence time, are well documented (Lake, 2011). Elevated salinity observed in the PCA during the drought in Flag Boshielo Dam was probably due to a combination of these factors, along with inputs of saline seepage associated with sub-surface agricultural return flows from the catchment (Loehr, 1984). The PCA showed that elevated salinity during the drought was characterised by high concentrations of Na, Cl, K, F and total alkalinity. Concentrations of these ions reduced substantially when normal flows resumed in March 2006. The sodium adsorption ratio permanently measured > 2 during the drought period, which exceeded the TWQR for irrigation purposes increasing the risk of Na uptake by sensitive crops (DWAF[b], 1996). However, this value reduced to a median of 1 from March 2006 onwards, which was well within guideline levels. The strong correlation between reservoir water levels and Na and Cl concentrations is the most likely driver of patterns of increasing Na and Cl over various time periods. Seasonal reductions in water level would lead to increased Na and Cl concentrations due to evaporation, decreased dilution and increased residence time.

Sulphate concentrations showed distinct seasonal variation, but were largely independent of water levels, as shown by the PCA and the Spearman's rank correlation. Fluctuations in the SO_4^{2-}/Cl^- ratio were driven by low-flow mediated changes in Cl values, while SO_4^{2-} was relatively stable. Values ranged from around 1 during the drought, to a median of 3.21 following resumed normal flows in March 2006. This value, along with the median sulphate concentration of 102.3 mg/ℓ suggested that the reservoir was influenced by treated and untreated acid mine drainage originating in the upper Olifants River catchment. However, the increasing trend in SO_4^{2-} concentrations observed upstream in Loskop Dam (De Villiers and Mkwelo, 2009; Chapter 3), was not reflected in Flag Boshielo Dam. The very gradual increasing trend in SO_4^{2-} concentrations and very low levels of dissolved metals suggest that the effects of mining and industry on the water chemistry of Flag Boshielo Dam are currently limited, and are not yet a major cause for concern.

The results of this study emphasise the need to acknowledge and account for water levels (flow or reservoir level) when examining trends in long-term data sets. A case in point is the analysis of Na concentrations. Had the trend test been conducted prior to March 2006, not accounting for water levels, the Na concentrations would have shown an alarmingly steep increase that may have been attributed to other sources. The significant decreasing trend in Na concentrations after adjustment by LOWESS shows what occurs when water levels are held constant. However, aquatic biota experience the effect of actual concentrations and therefore both scenarios must be considered. Developing a hydrological model of Flag Boshielo Dam would be the next step in understanding the response of the system to changes in streamflow and water quality.

Chapter 5: PISCIVORY DOES NOT CAUSE PANSTEATITIS (YELLOW FAT DISEASE) IN *OREOCHROMIS MOSSAMBICUS* FROM AN AFRICAN SUB-TROPICAL RESERVOIR*

* The term ‘reservoir’ was used instead of ‘dam’ in this chapter due to international journal requirements.

This chapter is published in the journal Freshwater Biology (Appendix 3)

5.1 INTRODUCTION

Oreochromis mossambicus is one of the most widely distributed exotic fish species globally (Costa-Pierce, 2003) mainly due to their high value as an aquaculture species. The species is endemic to south-eastern Africa where it is found in a range of habitats including lakes, rivers, and estuaries from the lower Zambezi River in Mozambique, to the Bushmans River in South Africa (Skelton, 2001). Their distribution includes the Olifants River, one of the main river systems in South Africa, which is beset by increasing demands for water, discharge of effluents from coal-mining, urban, industrial and agricultural activities, and variable rainfall (Ashton & Dabrowski, 2011).

The adverse effects of deteriorating water quality culminated in the deaths of large numbers of Nile crocodiles at two distinct locations along the river between the years 2005 and 2009 (Ashton, 2010). Mortalities were attributed to pansteatitis (also known as yellow fat disease), and occurred in Loskop Reservoir (LR) in the upper catchment (Oberholster *et al*., 2010), and approximately 520 km downstream in the Olifants River gorge in the KNP (Osthoff *et al.,* 2010; Ferreira & Pienaar, 2011). The Nile crocodile population at LR has since declined to ca. 6 individuals (Botha *et al*., 2011), while at KNP, South Africa’s premier conservation area, the population is in decline after ca. 200 mortalities (Ashton, 2010; Ferreira & Pienaar, 2011). Pansteatitis was also described in *C. gariepinus* in the Olifants River gorge at KNP (Huchzermeyer *et al*., 2011), and associated symptoms were observed in the majority of *O. mossambicus* examined from LR during this study. The simultaneous occurrence of the disease at two distinct locations on the same river system, at various consumer trophic levels and in different fish species is remarkable, and represents the first documented case of this type.

Pansteatitis has infrequently been reported in other free-ranging animals associated with aquatic environments. These include several species of egrets and herons in Japan (Neagari *et al*., 2011), Mediterranean striped dolphins, and a loggerhead turtle off the coast of Spain (Soto *et al*., 2010; Orós *et al.,* 2013), common dab and long rough dab off the coast of Scotland (Begg *et al*., 2000), and great blue herons in Chesapeake Bay in the USA (Nichols *et al*., 1986). However, it is more commonly reported in various captive-bred or farmed animals including several species of marine and freshwater fish (Roberts *et al*., 1979; Herman & Kircheis, 1985; Bricknell *et al*., 1996; Guarda *et al.,* 1997; Goodwin, 2006*;* Roberts & Agius, 2008), alligators (Larsen *et al*., 1983), and crocodiles (Huchzermeyer, 2003). The disease is characterised by an inflammatory reaction to necrotic fat cells, and is a nutritionally mediated condition. In captive-bred animals it is recurrently attributed to consumption of a diet high in rancid or unsaturated fats, frequently of fish origin, and deficient in antioxidants such as vitamin E (Roberts *et al*., 1979; Fytianou *et al.,* 2006; Roberts & Agius, 2008). Consumption of a diet of this nature can further deplete reserves of vitamin E and other antioxidants, compounding the effects of oxidative stress, which include an accumulation of reactive peroxides in fat tissues (Fytianou *et al*., 2006).

The specific aetiology of pansteatitis in *O. mossambicus* from LR, and in most other cases involving free-ranging animals, is unknown. Several theories have been suggested and are mainly linked to known causes of oxidative stress. These include exposure to the hepatotoxin, microcystin, produced by *Microcystis* spp. blooms (Rattner & McGowan, 2007). However, no evidence of intake or exposure to microcystin was detected when more than 70 pansteatitis-affected egrets and herons died concurrent to a bloom of *M. aeruginosa* (Neagari *et al*., 2011). Inorganic pollutants have not been measured in LR, but warrant further investigation because high concentrations of polychlorinated biphenyls (PCBs) have been found in the fat of a pansteatitis-affected loggerhead sea turtle (Orós *et al.,* 2013). In LR, elevated levels of aluminium and iron have been detected in the water (Oberholster *et al*., 2010; Dabrowski *et al*., 2013), the fat of *O. mossambicus*, and a potential food source, *Spirogyra* spp. (Oberholster *et al*., 2012). High levels of ingested iron have been linked to lipid peroxidation in fish (Baker *et al*., 1997; Elbaraasi *et al*., 2004), suggesting a possible connection between bioaccumulation of metals and oxidative stress associated with pansteatitis. Recent work in the KNP has implicated altered trophic relationships resulting from back-flooding in the Olifants River gorge, when the Massingir Reservoir dam wall was raised (Woodborne *et al*., 2012). These include the proliferation of invasive silver carp as a possible food source rich in polyunsaturated fat for pansteatitis-affected *C. gariepinus* (Huchzermeyer *et al*., 2013). Studies of the lipid profiles of *C. gariepinus* and crocodiles from the KNP have shown that pansteatitis-affected animals have a higher n-3 to n-6 fatty acid ratio than healthy animals, indicating an increased intake of polyunsaturated fats which is prone to oxidation (Osthoff *et al*., 2010; Huchzermeyer *et al*., 2013). Given the dietary links established in captive-bred animals, the results of this research highlight the need to determine the diet of free-ranging animals with pansteatitis.

Crocodile mortalities in LR occurred concurrent to several fish kills of various species (including *O. mossambicus*), the culmination of an extended dry period (Chapter 3), and escalating blooms of the cyanobacterium *M. aeruginosa,* and the dinoflagellate *C. hirundinella* due to eutrophication (Oberholster *et al.,* 2010). Although *O. mossambicus* examined during fish kills were diagnosed with pansteatitis post-mortem, the cause of death may have been related to fluctuating environmental conditions that affected several fish species. The algal blooms are indicative of bottom-up changes in the food web, and have significantly altered physico-chemical parameters such as pH and dissolved oxygen levels in the reservoir (Dabrowski *et al*., 2013). While the consumption of rancid (rotting) fish following fish kills may explain the occurrence of pansteatitis in crocodiles, persistent symptoms of the disease observed in mature *O. mossambicus* in LR are not easily explained. In their native range their diet usually consists of detritus, phytoplankton, periphyton, macroalgae, diatoms and zooplankton (Bowen, 1979; de Moor *et al.*, 1985; Dyer *et al*., 2013; Zengeya *et al*., 2011). However, *O. mossambicus* are known to exhibit trophic plasticity under different environmental conditions (Bowen & Allanson, 1982; Dyer *et al.,* 2013). In Australia, they have been shown to prey on juvenile indigenous fish in both laboratory and field situations (Doupé *et al*., 2009), and to completely digest fish prey in as short as one hour leaving little evidence of this food source in stomach contents (Doupé & Knott, 2011). In Sri Lanka, De Silva *et al*. (1984) reported *O. mossambicus* diets ranging from detritivory, to herbivory, to complete carnivory in different reservoirs.

Assumptions about their diet are thus limited by their omnivorous feeding habits. It is possible that *O. mossambicus* from LR are piscivores, or may scavenge on dead fish associated with fish kills, causing them to develop pansteatitis. This shift in trophic level may be apparent in their $\delta^{15}N$ values. If there is no evidence of piscivory, then it is important to establish the specific and dominant constituents of their diet to facilitate further research into their chemical and nutritional composition. The aims of this study were to determine whether there is any evidence of piscivory in pansteatitis-affected fish from LR, and how their diet compares to healthy fish in the same river system. We used the combined approach of stomach contents analysis and stable isotopes incorporating seasonal variation to assess the fish diet. This was applied to the *O. mossambicus* population in LR as well as a reference population in Flag Boshielo Reservoir (FBR). The latter reservoir is located approximately 100 km downstream from LR, and was selected because pansteatitis has never been reported at this location. The reservoir has a large population of *O. mossambicus* and the highest concentration of crocodiles in the Olifants River system outside of the KNP (Botha, 2010). Muscle samples of *O. mossambicus* collected from LR during large-scale fish kills in 2007, and a fish health study undertaken in 2010, were analysed to determine whether there was any evidence of historic variation in isotopic signatures relative to the situation in 2011.

5.2 METHODS

5.2.1 *Reservoir characteristics*

The Olifants River is the main inflow for both LR and FBR, which are located in the Mpumalanga and Limpopo provinces of South Africa respectively (Figure 5.1). An extensive assessment of the current state and historic trends in water chemistry, physiography and limnology was undertaken concurrent to fish sampling at both LR (Chapter 3) and FBR (Chapter 4). Regular algal blooms at LR resulted in higher maximum chlorophyll-*a* values than FBR, where no algal blooms were observed during the study period. The trophic state of LR was meso- to eutrophic (Oberholster *et al*., 2013), while FBR was oligotrophic. Dominant vegetation types differ markedly between the catchments of both reservoirs. The catchment of LR is dominated by Highveld grassland in the upper reaches, and mixed Thornveld and Bushveld (semi-arid savanna dominated by grassland and *Acacia* spp. trees and various shrubs) in the lower reaches around the reservoir. The catchment of FBR is dominated by mixed Thornveld and Bushveld, much of which has been degraded (Mucina & Rutherford, 2006).

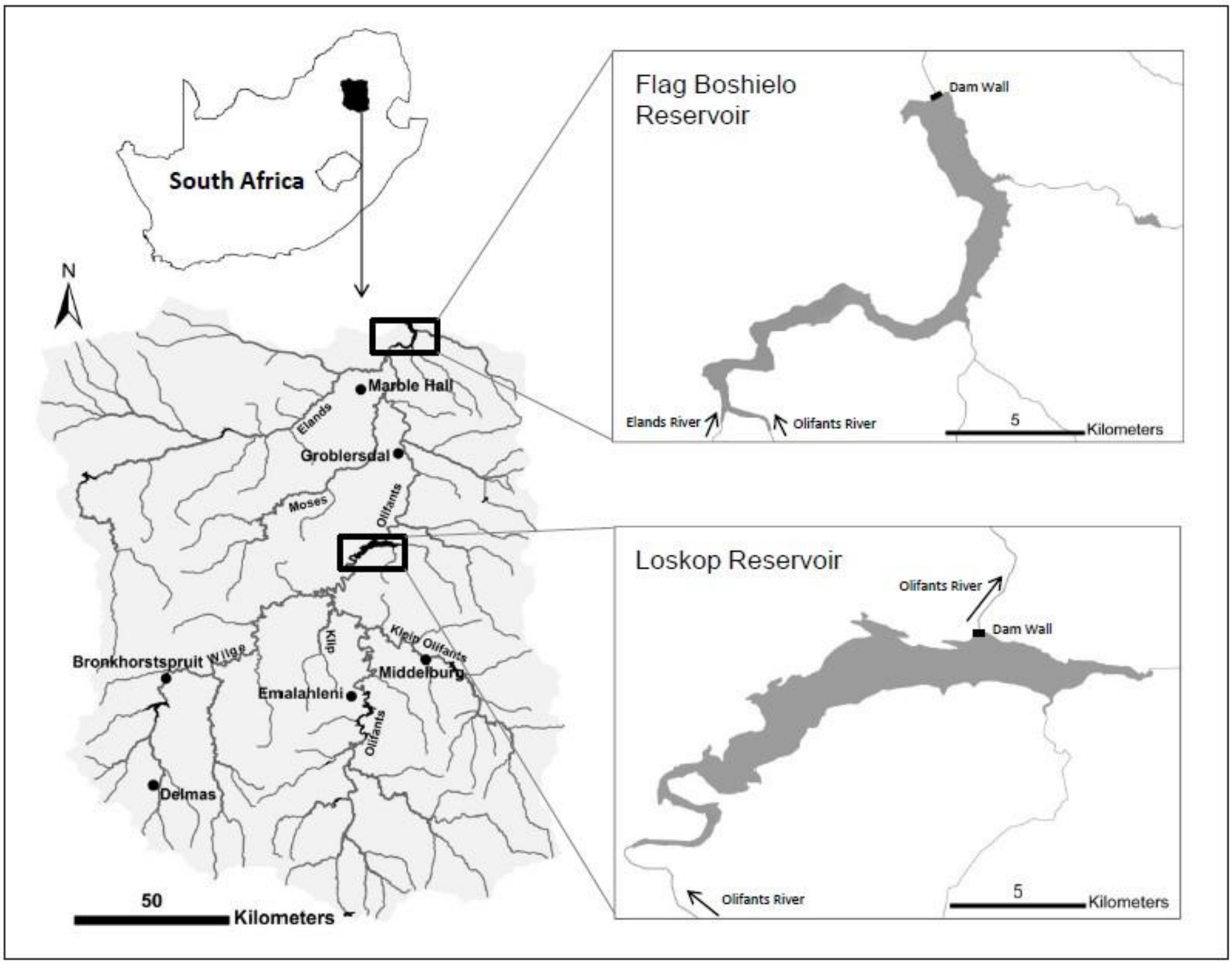

Figure 5.1 Location of Loskop (LR) and Flag Boshielo Reservoirs (FBR) in the Olifants River catchment, South Africa.

5.2.2 *Sample collection*

In order to study the dietary composition of *O. mossambicus* from both reservoirs, samples of approximately 20 fish per season were collected during April (Autumn), June (Winter), October (Spring) and December (Summer) in 2011 to represent an annual cycle. To reduce variation associated with ontogenetic dietary shifts, only fish > 200 mm total length (TL) were collected (Table 5.1). Gill nets were set during daylight hours using three 25 m panels with 70, 90 and 130 mm multi-filament stretched-mesh nets. After collection, fish were weighed and their TL was recorded. A section of approximately 10 g of muscle tissue was dissected from the left flank of each fish for analysis of $\delta^{15}N$ and $\delta^{13}C$ isotopes. Tissue samples were frozen for subsequent isotopic analysis.

Potential food sources for *O. mossambicus* fluctuated seasonally and were sampled when abundant throughout 2011 at both reservoirs. Filamentous green algae (Chlorophyceae) were handpicked from various substrates including rocks and submerged trees. Sediment organic matter (SOM) samples were collected at all sites using an Ekman grab lowered to the reservoir bottom at various depths. The top 5 cm of sediment was collected from the grab sample. Near-shore detrital material was collected by hand in polycarbonate containers from above the sediment in areas where it accumulated, and was distinct from the fine material in SOM. All samples were stored on ice in the field and subsequently frozen prior to laboratory processing. Plankton were sampled from both reservoirs by using a plankton net (30 µm mesh, 25 cm diameter) lowered to the reservoir bottom at each study site and brought to the surface in order to obtain an integrated sample of the water column. An amount of 10 ml was sub-sampled and preserved in 10% buffered formalin for identification and sorting of zooplankton. Zooplankton replicates consisted of > 50 whole individuals, predominantly *Daphnia* spp. and copepods. Samples of *C. hirundinella*, *M. aeruginosa* and the pelagic diatom *Fragilaria crotonensis* were collected by water filtration using 3µm filters in LR. Dominance of these taxa was confirmed by inspection of samples with a compound microscope at 1250 x magnification.

5.2.3 *Historic Oreochromis mossambicus samples*

Isotopic signatures were determined for samples of muscle tissue from *O. mossambicus* that were collected and frozen on several occasions prior to this study. In 2007, around the time when several large fish kills occurred, three pansteatitis-affected fish were collected by a veterinary pathologist. During an unpublished fish health study in 2010, 24 fish were collected using gill nets (Dabrowski, 2012). Concurrent to that study, samples were collected from isolated mortalities of large *O. mossambicus* displaying severe symptoms of pansteatitis. All fish measured greater than 200 mm TL.

5.2.4 *Dietary composition: stomach contents analysis*

Fish stomachs from *O. mossambicus* were removed and preserved in 10% buffered formalin. Dietary composition was assessed by frequency of occurrence (Hyslop, 1980) as follows:

$\%F_i = (N_i / N) \times 100$

Where $\%F_i$ is the percentage frequency of occurrence of prey item *i*, *Ni* is the number of fish with prey *i* in their stomachs, and *N* is the total number of fish stomachs containing food examined. Stomach contents were divided into the fore-, mid- and hindgut and three subsamples of each region were spread on a Sedgwick rafter counting cell to determine the proportional contribution (% number) of each dietary item to the gut contents of each fish (Hyslop, 1980). Items in the stomach contents were identified to the lowest taxonomic level possible, and then assigned to the following categories: diatoms; *C. hirundinella*; green algae; *M. aeruginosa*; zooplankton; sediment; detritus. The proportion of fish with empty stomachs was recorded.

5.2.5 *Dietary composition: stable isotope analysis*

All samples were analysed by the environmental isotope facility at the Council for Scientific and Industrial Research (Pretoria, South Africa). Lipids were extracted from fish muscle tissue using a 2:1 chloroform:ethanol mixture following the method of Logan *et al.* (2008). All other samples were treated with 1% HCl to remove possible biogenic carbonates, and then repeatedly rinsed with distilled water. Samples were oven-dried at 70ºC overnight before being homogenised. Aliquots of each sample were weighed (0.8 – 1 mg) into tin capsules and combusted at 1020ºC in an elemental analyser (Flash EA, 1112 series, Thermo Fisher Scientific, Bremen, Germany). A continuous flow isotope ratio mass spectrometer (CF-IRMS, Delta V Plus, Thermo Finnigan, Bremen, Germany) coupled to the EA via a Conflo IV interface, was used to measure the $\delta^{13}C$ and $\delta^{15}N$ isotopic composition of the samples. Isotope ratios were expressed as parts per thousand (‰) relative to the reference standards of air for ^{15}N, and Vienna Pee Dee belemnite for ^{13}C (Coplen, 1994). Isotope ratios were expressed relative to standards as follows:

$X = [(R_{sample} / R_{standard})-1] \times 1000$

where X = $\delta^{13}C$ or $\delta^{15}N$ (‰) and R=$^{13}C/^{12}C$ or $^{15}N/^{14}N$ respectively.

An in-house laboratory standard (Merck gelatine) and a blank were run after every 12 samples. To ensure reproducibility of the results, approximately every 11th sample was measured in duplicate. The variance from replicates was 0.09‰ for $\delta^{13}C$ and 0.14‰ for $\delta^{15}N$ which was very close to the instrumental reproducibility achieved from the standards. Fish muscle $\delta^{13}C$ was not adjusted for lipid content because of recent uncertainties about lipid-normalisation methods (Fagan *et al*., 2011). Zooplankton values were adjusted because preservation in formalin has been shown to marginally affect isotopic signatures of freshwater zooplankton, with $\delta^{15}N$ increasing by 0.25‰ and $\delta^{13}C$ decreasing by 0.2‰ on average (Rennie *et al*., 2012).

5.2.6 *Trophic Position*

The trophic position of *O. mossambicus* was calculated using the formula outlined by Post (2002):

$$\text{Trophic position} = \lambda + (\delta^{15}N_{consumer} - \delta^{15}N_{base}) / \Delta_n$$

Where λ is the trophic level of the base, $\delta^{15}N_{consumer}$ is the nitrogen isotope signature of *O. mossambicus*, $\delta^{15}N_{base}$ is that of the organism used to estimate the baseline, and Δ_n is the average trophic enrichment of nitrogen (3.4‰). The species used to estimate $\delta^{15}N_{base}$ should share the same habitat as the target species and be relatively long-lived so as to minimise short-term variation in isotopic signatures of the food web (Post, 2002). Redbreast tilapia (*Tilapia rendalli*) is abundant at both reservoirs, shares a similar habitat to *O. mossambicus*, and was used to estimate $\delta^{15}N_{base}$. As a primary consumer that also feeds on invertebrates and crustaceans, they were assigned the trophic level of 2.5. The previously described gill nets, and a seine net (5 m length, 1 mm mesh) were used to capture *T. rendalli* during summer which limited the calculation to this season only.

5.2.7 *Statistical Analyses*

A one-way analysis of similarity (ANOSIM) was used to detect differences in the stomach contents (% number) of *O. mossambicus* between reservoirs (Primer-E, ver.5.2.9, Primer-E Ltd., Plymouth, UK). This test produces the statistic R which measures the effect size, where $R = 1$ indicates that samples within groups are more similar than between groups, and $R = 0$ indicates that within-group similarity is equal to between group similarity. A non-metric multidimensional scaling (NMDS) plot of the data from both reservoirs, accounting for four seasons, was constructed from a similarity matrix calculated using the Bray-Curtis similarity coefficient. In a two-dimensional ordination plot, samples that are grouped more closely together represent more similar assemblages than samples spread further apart.

Isotopic composition and trophic position of *O. mossambicus* was evaluated using Analysis of Covariance (ANCOVA), with $\delta^{15}N$, $\delta^{13}C$ and trophic position as dependent variables, reservoir, season and sex as independent variables, and fish length as the covariate. The assumption of homogeneity of slopes was tested and there were significant interactions between several independent variables and fish length. Therefore a separate slopes model was used. A one-way ANOVA was used to detect differences in the isotopic signatures of *O. mossambicus* collected from LR in 2007, 2010 and 2011. Data were square root transformed where necessary to ensure the assumption of normality was met for all parametric tests. Statistical analyses were completed in Statistica Version 11 (StatSoft. Inc, Tulsa, USA).

The proportional contribution of potential food items to the diet of *O. mossambicus* in both reservoirs was assessed with an isotopic mixing model in Stable Isotope Analysis in R (SIAR) version 4.2 according to Parnell *et al*. (2010). Given the isotopic ratios in a range of possible food sources and consumers, the model uses Bayesian inference to provide probability distributions of dietary proportions. In contrast to the stomach analyses which are based on presence/absence of prey ingested at a single point in time, the stable isotope mixing model results reflect the proportional mass contributions of various prey to consumer tissues after assimilation, and integrated over a longer period of time (Phillips, 2012). Trophic enrichment factors (mean ± SD) of 3.4‰ (± 1.0) for $\delta^{15}N$ and 0.4 (± 1.3) for $\delta^{13}C$ were applied to food sources in order to account for isotopic shifts between consumer and diet (Post, 2002), and uninformative priors were used. Comparisons between food sources were made using the 95% Bayesian credibility intervals which were considered significantly different when they did not overlap.

5.3 RESULTS

5.3.1 *Oreochromis mossambicus catch summary*

Oreochromis mossambicus from LR were characterised by abundant mesenteric fat containing distinct yellow, orange and brown spots (ceroid pigment) which varied in intensity and hardness, and are typical lesions of pansteatitis. In contrast, fish from FBR had little to no mesenteric fat, which when present, was off-white with no discolouration (Figure 5.2).

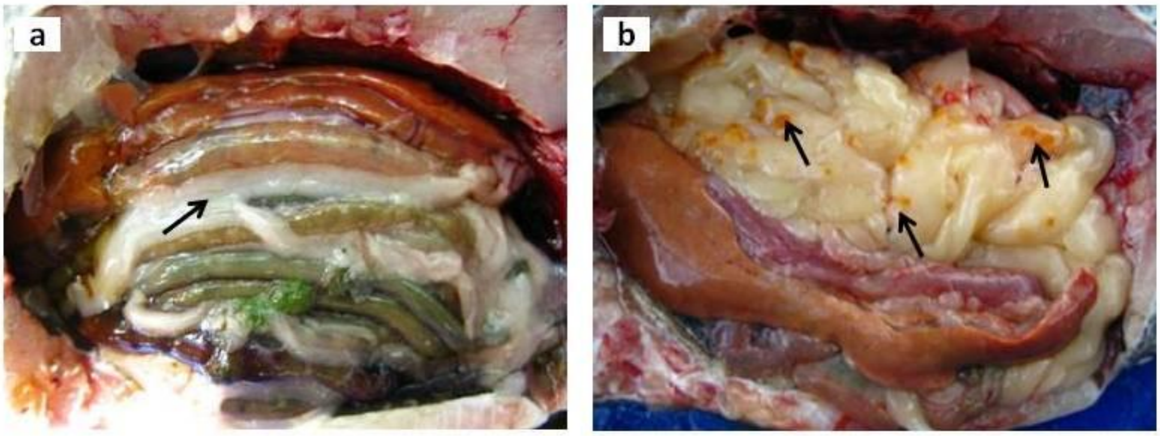

Figure 5.2 Macroscopic comparison of mesenteric fat typical of healthy *Oreochromis mossambicus* from Flag Boshielo Reservoir (a), and pansteatitis-affected fish from Loskop Reservoir (b). Arrows indicate fat tissue and highlight concentrations of yellow-brown ceroid pigment in pansteatitis-affected fish.

The mean TL in fish from LR (398 mm) was significantly longer than fish from FBR (325 mm; T-test, $p < 0.0001$). The fish from LR were also significantly heavier (T-test, $p < 0.0001$), with a mean weight of 1458 g which was almost double the mean weight of fish from FBR (Table 5.1).

Table 5.1 Summarised catch statistics for *Oreochromis mossambicus* sampled from Loskop (LR) and Flag Boshielo (FBR) Reservoirs in 2011 including the sex ratio (M : F), total length (TL) and weight (Mean ± SD).

Site	Season	*n*	M : F	Empty stomachs (%)	TL (mm)	Weight (g)
LR	Autumn	31	17 : 14	0	372 ± 6.5	1207 ± 597
	Winter	20	7 : 13	50	421 ± 3.9	1725 ± 468
	Spring	20	11 : 9	25	420 ± 3.4	1683 ± 337
	Summer	20	6 : 14	30	395 ± 4.8	1353 ± 448
	Total	**91**	**41 : 50**	**23**	**398 ± 5.4**	**1458 ± 532**
FBR	Autumn	21	16 : 5	19	365 ± 6.3	876 ± 404
	Winter	20	15 : 5	20	353 ± 4.8	906 ± 312
	Spring	20	7 : 13	20	321 ± 5.2	662 ± 252
	Summer	20	4 : 16	45	283 ± 3.8	474 ± 223
	Total	**81**	**42 : 39**	**26**	**325 ± 5.7**	**730 ± 348**

5.3.2 *Dietary composition: stomach contents analysis*

The highest proportion of empty fish stomachs at LR was during winter, while at FBR it was during summer (Table 5.1). Stomach contents analysis revealed a more diverse range of prey items in fish from LR with seven categories compared to five in FBR (

Figure 5.3). At LR, *C. hirundinella* was the most frequently consumed prey, occurring in more than 80% of the fish stomachs (Table 5.2), with a substantial contribution to stomach contents during all seasons (

Figure 5.3). In autumn *M. aeruginosa* occurred in 87.1% of the fish stomachs, and contributed 26% of the % number in stomach contents of fish from LR, but was not abundant during other sampling periods. Despite occurring in more than 60% of the fish stomachs from LR, zooplankton contributed a low biomass, and consisted predominantly of *Daphnia* spp. and copepods. Diatoms also occurred at a high frequency but made a relatively low proportional contribution, and were dominated by the pelagic species *F. crotonensis*. An increase in the occurrence and % number of sediment was evident in winter, while detritus increased in spring.

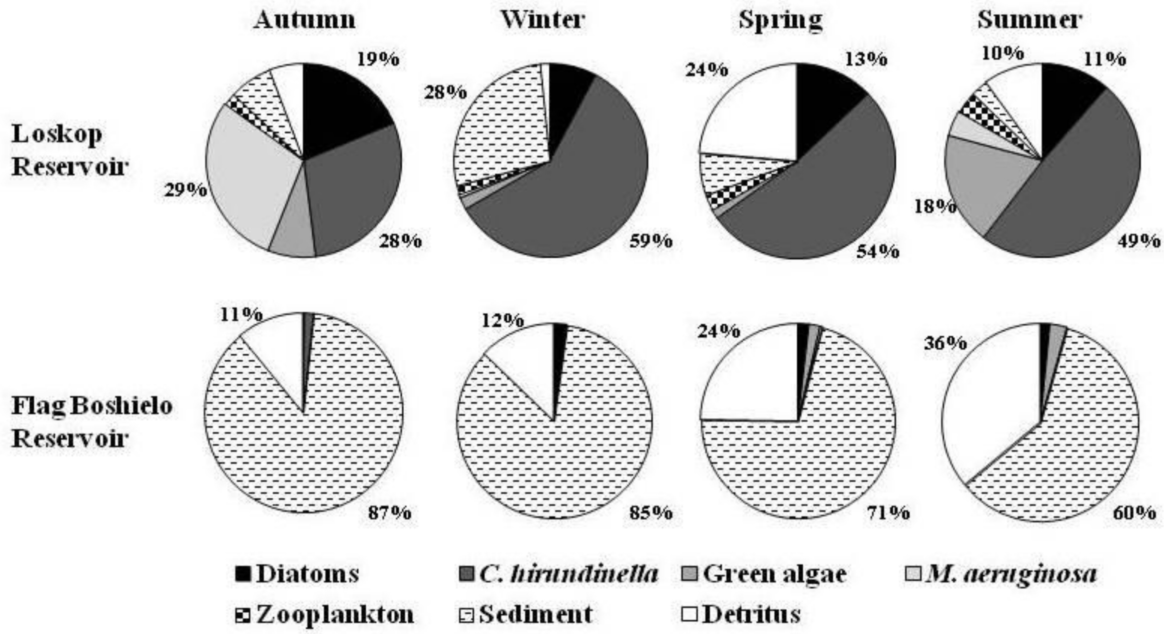

Figure 5.3 Proportional contributions of major dietary items (shown for items ≥ 10%) in the stomach contents of *Oreochromis mossambicus* collected over four seasons from Loskop and Flag Boshielo Reservoirs, South Africa

Seasonal variation in the stomach contents of fish from FBR was low with the highest contribution from sediment in 100% of the fish stomachs examined in all seasons (Table 2). Secondary dominance by detritus was evident and contributions increased over the duration of the sampling period. Diatoms, predominantly the benthic species *Fragilaria ulna,* made a low proportional contribution, and ranged in occurrence from 5.88% in autumn to 50% in spring. Despite a high occurrence in spring, green algae never contributed more than 10% to stomach contents. In contrast to LR, *C. hirundinella* was never detected at high frequencies or in large quantities from the fish stomachs. Zooplankton and *M. aeruginosa* were never recorded from the fish stomach contents in FBR.

Table 5.2 Dietary composition by frequency of occcurrence (*% F*) for *Oreochromis mossambicus* from Loskop and Flag Boshielo Reservoirs, South Africa.

	Loskop Reservoir				**Flag Boshielo Reservoir**			
	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer
Diatoms	100	100	86.67	100	5.88	25	50	45.45
C. hirundinella	100	100	86.67	100	29.41	-	-	9.09
Green algae	87.1	90	40	100	-	6.25	87.5	18.18
M. aeruginosa	87.1	10	-	7.14	-	-	-	-
Zooplankton	64.52	70	60	71.43	-	-	-	-
Sediment	61.29	100	46.67	50	100	100	100	100
Detritus	74.19	20	100	64.29	94.12	56.25	100	100

The NMDS showed clear separation in the stomach contents of fish from both reservoirs, which was supported by a low stress value of 0.09 (Figure 5.4). This was reinforced by the results of the ANOSIM which indicated a significant difference in dietary composition between reservoirs (R = 0.929). The NMDS showed a high degree of seasonal overlap within each reservoir, except in LR in autumn when *M. aeruginosa* (29%) and diatoms (19%) increased in dominance (

Figure 5.3) resulting in a level of separation between these fish in the NMDS (Figure 5.4).

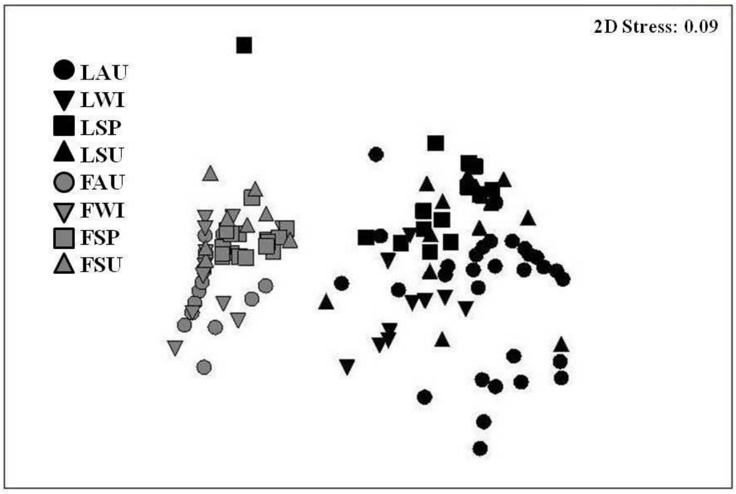

Figure 5.4 Non-metric multidimensional scaling (NMDS) plot of stomach contents (% number) from *O. mossambicus* sampled in two reservoirs. L: Loskop Reservoir; F: Flag Boshielo Reservoir; AU: autumn; WI: winter; SP: spring; SU: summer.

5.3.3 *Variation in isotopic signatures of Oreochromis mossambicus*

The ANCOVA showed that isotope signatures of *O. mossambicus* differed significantly between both reservoirs. The $\delta^{15}N$ values of fish from LR were significantly higher ($F_{1,149}$ = 61.3, p < 0.0001), and $\delta^{13}C$ values were significantly less depleted ($F_{1,149}$ = 365, p < 0.0001), compared to fish from FBR (Table 5.3). At LR the $\delta^{15}N$ values were significantly higher in winter (ANCOVA, $F_{3,78}$ = 5.7, p = 0.001) with an average of 15.6‰ compared to other sampling periods, which ranged from 15.2 to 15.4‰. At FBR the $\delta^{15}N$ values were significantly lower in autumn with a mean of 13.6 compared to other seasons which ranged from 13.8 to 14.1‰. (ANCOVA, $F_{3,67}$ = 8.1, p < 0.001). Although statistically significant, the seasonal differences in means were very low and unlikely to be of great biological significance. There was no effect of season on $\delta^{13}C$ values at either reservoir, and sex had no influence on $\delta^{15}N$ or $\delta^{13}C$ signatures. There was a positive relationship between $\delta^{15}N$ and TL in fish from FBR (ANCOVA, $F_{16,149}$ = 3.4, p < 0.001), but not fish from LR. The $\delta^{13}C$ values were significantly more depleted as fish increased in length at both LR and FBR (ANCOVA, $F_{16,149}$ = 2.1, p < 0.001).

All food items identified in the stomach contents of fish from LR were collected and analysed, however, several comparable food sources could not be collected from FBR. *Microcystis aeruginosa* was never detected in the stomach contents or water column during this study, and the dinoflagellate *C. hirundinella*, diatoms and zooplankton were so scarce that they could not be sampled in quantities sufficient for analysis. The only zooplankton detected in water samples from FBR were rotifers and there were less than 10 individuals collected from all samples combined. All fish species and food sources from LR were significantly enriched with $\delta^{15}N$ and less depleted in $\delta^{13}C$ when compared to the same taxonomic group in FBR (Table 5.3).

Table 5.3 Carbon and nitrogen stable isotope values (Mean ‰ ± SD) and sample sizes for *Oreochromis mossambicus* dietary sources and *Tilapia rendalli* (baseline organism) collected from Loskop Reservoir and Flag Boshielo Reservoir on the Olifants River, South Africa.

Species / group	**Loskop Reservoir**			**Flag Boshielo Reservoir**		
	$\delta^{15}N$	**$\delta^{13}C$**	***n***	**$\delta^{15}N$**	**$\delta^{13}C$**	***n***
Ceratium hirundinella	7.3 ± 0.1	-11.1 ± 1.3	2	-	-	-
Detritus	7.4 ± 0.9*	-15.4 ± 1.9*	4	3.2 ± 1.1*	-27.0 ± 2.0*	4
Diatoms[1]	18.2 ± 1.3	-26.3 ± 2.7	2	-	-	-
Green algae	12.6 ± 2.8*	-13.7 ± 3.6*	4	7.4 ± 0.5*	-21.5 ± 4.1*	4
Microcystis aeruginosa	16.2 ± 0.1	-18.1 ± 0.4	3	-	-	-
Oreochromis mossambicus†	15.4 ± 0.4	-13.8 ± 0.6	91	13.9 ± 0.6	-26.3 ± 0.7	81
Oreochromis mossambicus‡	15.2 ± 0.5	-13.7 ± 0.5	24	-	-	-
Oreochromis mossambicus◊	15.7 ± 0.5	-13.3 ± 0.4	3	-	-	-
Oreochromis mossambicus∆	14.6 ± 0.4	-14.3 ± 0.5	3	-	-	-
Sediment organic matter	8.9 ± 1.4*	-19.80 ± 1.4*	9	6.1 ± 1.1*	-22.9 ± 0.9*	7
Tilapia rendalli	18.7 ± 0.5*	-14.3 ± 0.3*	7	12.8 ± 1.1*	-22.3 ± 2.8*	13
Zooplankton[2]	21.4 ± 0.7	-12.5 ± 0.1	2	-	-	-

[1] Diatoms consisted primarily of the pelagic species *Fragilaria crotonensis*

[2] Zooplankton consisted primarily of *Daphnia* spp. and copepods.

* Indicates significant difference ($p < 0.05$) in isotopic signatures between reservoirs using a *t*-test.

Oreochromis mossambicus symbols: †collected in 2011; ‡ collected in 2010; ◊mortalities in 2010; ∆collected in 2007.

5.3.4 *Trophic Position*

The mean trophic position of *O. mossambicus* was significantly lower at LR (mean 1.5 ± 0.1) than FBR (mean 2.8 ± 0.1; ANCOVA, $F_{1,33} = 266$, $p < 0.0001$) which was over a full trophic position higher. Trophic level was not significantly related to fish length in either reservoir or in males and females.

5.3.5 *Variation in historic isotopic signatures of <u>Oreochromis mossambicus</u>*

The one-way ANOVA showed that $\delta^{15}N$ and $\delta^{13}C$ values of fish collected in 2007 differed significantly to various other time periods (Figure 5.5). The mean $\delta^{15}N$ value in 2007 was significantly lower (approximately 1‰) than mortalities collected in 2010 (p = 0.02) and samples collected in 2011 (p = 0.02; Table 5.2). The mean $\delta^{13}C$ value in 2007 was more depleted than mortalities (p = 0.04) and samples collected in 2010 (p = 0.03), but only differed by 0.5‰ compared to samples from 2011 (Table 5.2).

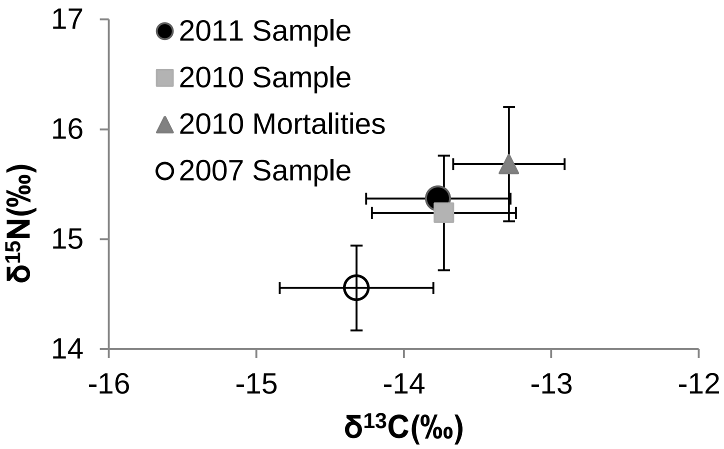

Figure 5.5 Stable isotope biplot showing the mean ‰ (± SD) $\delta^{15}N$ and $\delta^{13}C$ values for *Oreochromis mossambicus* collected during various time periods from Loskop Reservoir.

5.3.6 *Dietary composition: stable isotopes*

No seasonal variation was evident in the range of solutions provided by the mixing model, so results were pooled for each reservoir. In LR the SIAR model indicated that *C. hirundinella* constituted the majority of the diet (29-40%; 95% credibility interval) which was in agreement with the stomach contents analysis (Figure 5.6a). This was followed by zooplankton despite the low frequency of this food item in the fish stomachs. Lower contributions were indicated from *M. aeruginosa* which was similar to detritus. Diatom $\delta^{13}C$ values were approximately 13‰ more depleted than *O. mossambicus* (Table 3), and although they frequently contributed greater than 10% to stomach contents, the SIAR model indicated that they were the least important food source. The contributions of *M. aeruginosa* and zooplankton were negatively correlated in the mixing model (-0.88) meaning that proportional increases in the contribution of one would be at the expense of the other.

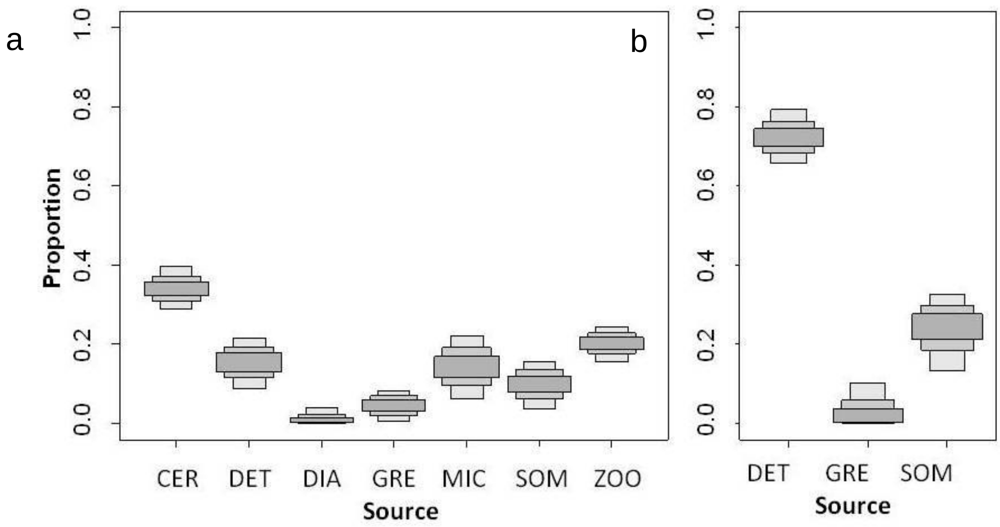

Figure 5.6 Proportional contributions of dietary sources for *Oreochromis mossambicus* from Loskop Reservoir (a) and Flag Boshielo Reservoir (b) pooled across four seasons. Dietary sources are *Ceratium hirundinella* (CER); detritus (DET), diatoms (DIA), green algae (GRE), *Microcystis aeruginosa* (MIC), sediment organic matter (SOM) and zooplankton (ZOO). The boxes indicate 50, 75 and 95% Bayesian credibility intervals based on the Stable Isotope Analysis in R mixing model.

The SIAR model indicated that detritus was the dominant food source for fish in FBR (Figure 5.6b), followed by sediment organic matter and green algae. However, the mean $\delta^{15}N$ for *O. mossambicus* (13.9%) was enriched by between 6.5‰ (compared to green algae) and 10.7‰ (compared to detritus; Table 5.3). The trophic enrichment factor incorporated into the SIAR model for $\delta^{15}N$ was 3.4‰ (±1) which means that the $\delta^{15}N$ values were highly enriched relative to these items. No combination of these three food sources explains the isotopic values for *O. mossambicus* in FBR, and the system remained undetermined despite the distribution of solutions provided by the mixing model.

5.4 DISCUSSION

The results of this study showed no evidence of current or historic piscivory in *O. mossambicus* from LR that may explain pansteatitis. The 2011 $\delta^{15}N$ values for *O. mossambicus* were very similar when compared to samples collected in 2010, and were slightly higher than samples collected in 2007. Their trophic level indicated their position as between a primary and secondary consumer, which was consistent with the dietary analysis. Their calculated trophic level also confirmed that although their $\delta^{15}N$ values were significantly enriched compared to fish from FBR, this was purely due to differences in the trophic baseline at each reservoir. Their diet was herbivorous / detritivorous and the dominant food items identified indicated that they mostly fed in the pelagic zone. Based on the stomach contents and mixing model results, their diet in LR is very much what would be expected according to previous studies in their native range (Bowen, 1979; de Moor *et al.*, 1985; Dyer *et al*., 2013; Zengeya *et al*., 2011). Both analytical methods showed that *C. hirundinella* was the dominant food source. Secondary dominance by zooplankton in the mixing model was contrary to the stomach contents where this food group accounted for less than 10%. However, the relatively large body size of cladocerans and cyclopods relative to phytoplankton species clearly makes a significant nutritional contribution. The stomach contents reflected seasonal variation in the abundance of different food sources such as *M. aeruginosa* which made a higher contribution during late summer to autumn. However, the stable isotope analysis indicated little to no seasonal variation, which is probably due to the longer integration time of muscle isotopic values compared to stomach contents.

Although our results gave no indication that *O. mossambicus* from LR were regularly feeding at a higher trophic level, we cannot exclude the possibility of a brief period of opportunistic piscivory associated with a fish kill that went undetected due to the confounding effect of tissue turnover rates. Muscle tissue half-lives of adult fish can range from 116 to173 days (Weidel *et al.,* 2011) and no major fish kills were observed at LR during 2010 or 2011. There were however, at least three large-scale fish kills in 2007 at LR. If *O. mossambicus* had been opportunistically feeding on fish during these events it seems unlikely that this would not have reflected in the $\delta^{15}N$ values of fish we analysed from that time. These fish were sampled in September of that year, and displayed severe symptoms of pansteatitis, but their $\delta^{15}N$ values were actually slightly lower than fish collected in subsequent years. It is also not known whether the lesions typical of pansteatitis in fish from LR are indicative of ongoing oxidative stress, or are the remnants of a historic event related to feeding or other causes. Observations of farmed crocodiles have shown that while pansteatitis may cause acute mortalities, some animals survive, continue growing, and the lesions are only discovered at slaughter (Huchzermeyer, 2003).

There were vast differences in the stomach contents and isotopic signature of pansteatitis-affected *O. mossambicus* from LR compared to unaffected fish from FBR. Compared to LR, the isotopic signatures of *O. mossambicus* from FBR are located very differently, being approximately less enriched on the δ^{15}N axis and utilising a distinctly depleted δ^{13}C source. The stomach contents of fish from FBR were dominated by sediment and detritus with very little seasonal variation, and the mixing model indicated that detritus was the most important food source. However their high levels of δ^{15}N enrichment relative to the food sources we measured showed that their diet was still undetermined to an extent. One explanation is that we only measured sediment organic matter and detritus from the benthic zone, and did not include suspended particulate matter which may have been a significant food source. The presence of the benthic diatom *F. ulna* in the stomach contents of fish suggests that they were feeding in the benthic zone at least periodically. In addition to a missing food source, it is speculated that the fish in FBR may be experiencing a degree of nutritional stress. A study of carp (*Cyprinus carpio*) has shown that when feeding levels were experimentally manipulated, δ^{15}N values increased as feeding level decreased, introducing an error of up to 1‰ for both δ^{13}C and δ^{15}N values (Gaye-Siessegger *et al*., 2004). This provides one possible explanation for their elevated trophic position compared to LR. In addition, the positive relationship between fish length and δ^{15}N values for fish from FBR was opposite to that predicted from previous research which demonstrated ontogenetic dietary shifts of *O. mossambicus* from zooplankton to phytoplankton, detritus and algae as they mature (de Moor *et al.,* 1985; Zengeya *et al.,* 2011). This could indicate increasing nutritional stress as fish mature, which has been observed in a previous study which showed how habitat segregation between juvenile and adult *O. mossambicus* resulted in variable protein content of detrital food resources, with the consequence that the adult population was stunted and malnourished (Bowen, 1979).

Eutrophication in LR has resulted in blooms of *C. hirundinella* and *M. aeruginosa* (Oberholster *et al*., 2010), providing a year-round abundant food source for consumers such as *O. mossmabicus*. The larger range of dietary items in LR compared to FBR indicated more trophic levels and trophic diversity. The baseline enrichment with δ^{15}N at LR is probably influenced by effluent originating from wastewater treatment works in the catchment of LR. In contrast, the oligotrophic, nitrogen-limited status of FBR provides limited food sources for *O. mossambicus* as shown by the low diversity of their stomach contents, low chlorophyll-*a* concentrations, and the scarcity of phyto- and zooplankton in the water column.

The $\delta^{13}C$ values of the food web at FBR were significantly more depleted than at LR. These differences are probably a reflection of vegetation types in the respective catchments and within the reservoirs themselves. The catchment of LR is predominantly grassland, consisting of C_4 grasses while the catchment of FBR is predominantly bushveld consisting of C_3 pathway shrubs and trees. In addition, the dam wall of FBR was raised by 5 m in 2006 and vegetation was purposely not cleared in order to provide habitat for waterfowl. As a result, submerged dead trees are scattered throughout the littoral zone providing a large source of C_3 carbon to the food web. In contrast, the littoral zone of LR is largely sand or rocky substrate with little to no vegetation.

Previous research has demonstrated that the aetiology of pansteatitis is exclusively dietary (it is not a contagious disease) and is linked to a diet rich in unsaturated or rancid fats frequently of fish origin (Fytianou *et al.,* 2006; Roberts *et al*., 1979; Roberts & Agius, 2008). The dietary reconstruction for *O. mossambicus* from LR, where pansteatitis is ubiquitous, indicated that fish made no observable contribution to their diet. This is a distinctive feature of pansteatitis in LR, as elevated levels of piscivory have been associated with the disease in *C. gariepinus* in the KNP (Woodborne *et al*., 2012). If piscivory is not associated with the aetiology of pansteatitis in LR, then one distinguishing feature of the dietary comparison between LR and FBR is the presence of abundant planktonic food sources dominated by *C. hirundinella*. While the food sources identified are not unique to LR, the numerous pollutants originating from various land uses in the catchment may affect their nutritional quality in some way. Given the well-established dietary aetiology of pansteatitis, an investigation of the fatty acid composition of dominant food sources for *O. mossambicus* at LR would make a significant contribution to further unravelling the complex aetiology of this disease.

Chapter 6: THYROID AND NUTRITIONAL STATUS OF *OREOCHROMIS MOSSAMBICUS* FROM TWO SUB-TROPICAL AFRICAN RESERVOIRS

* The term ‘reservoir’ was used instead of ‘dam’ in this chapter due to international journal requirements.

This chapter is currently being prepared for submission to the Journal of Fish Biology

6.1 INTRODUCTION

Due to its popularity as an aquaculture species, *O. mossambicus* is one of the most widely distributed fish globally. Their native distribution extends from the Bushmans River in South Africa to the lower Zambezi River in Mozambique (Skelton, 2001), including the Olifants River system. Water quality in the Olifants River has declined as a result of impacts associated with coal mining, power generation, industry, agriculture and wastewater treatment works (Ashton & Dabrowski, 2011). In the mid to late 2000s these impacts culminated in the deaths of large numbers of fish (several species including *O. mossambicus*) and Nile crocodiles at LR. At approximately the same time, crocodile mortalities (> 170 individuals) were reported downstream at the Olifants River gorge in the KNP, South Africa’s premier conservation area (Ashton, 2010; Osthoff *et al*., 2010; Ferreira & Pienaar, 2011). Although factors such as pollution and low dissolved oxygen were implicated in several of these events, mortalities of crocodiles at both locations and *O. mossambicus* at LR were linked to pansteatitis (yellow fat disease), a nutritionally mediated condition characterised by an inflammatory reaction to necrotic fat cells. In captive-bred animals it is recurrently linked to consumption of a diet high in rancid or unsaturated fats, frequently of fish origin, and deficient in antioxidants such as vitamin E (Roberts *et al*., 1979; Fytianou *et al.,* 2006; Roberts & Agius, 2008). Pansteatitis has been reported in other free-ranging species associated with aquatic environments, including African sharptooth catfish (Huchzermeyer *et al.*, 2011), egrets and herons (Neagari *et al*., 2011), Mediterranean striped dolphins (Soto *et al*., 2010), a loggerhead turtle (Orós *et al.,* 2013), long rough dab and common dab (Begg *et al*., 2000), and great blue herons (Nichols *et al*., 1986). In all reports of pansteatitis in free-ranging species the aetiology of the disease has not been conclusively determined.

Oreochromis mossambicus from LR are particularly large specimens. Many South African angling records have been set at this location, and the current International Game Fish Association (IGFA) world record fish weighing 3.11 kg was caught at LR in 2003. Pansteatitis persists in the population of *O. mossambicus* from LR, and the Nile crocodile population has declined to ca. 6 individuals as a result of the disease (Botha *et al*., 2011). A distinctive characteristic of these fish is their abundant mesenteric fat resulting in an abnormally distended abdomen. This is an important predisposing factor in the pathogenesis of pansteatitis because fish predominantly store polyunsaturated fat which is susceptible to lipid peroxidation, and in the presence of pro-oxidants, provides an extensive substrate for oxidative damage to occur (Lall, 2010).

Loskop Reservoir has become increasingly eutrophic with frequent algal blooms which significantly alter physico-chemical conditions such as pH and dissolved oxygen of the water (Chapter 3; Oberholster *et al.,* 2010). The cyanobacterium *M. aeruginosa* is dominant in summer and the dinoflagellate *C. hirundinella* is dominant in winter, ensuring a year-round food supply for primary consumers (Dabrowski *et al*., 2013). Piscivorous feeding habits or opportunistic scavenging during fish kills may have explained the condition in *O. mossambicus*, but this was regarded as highly unlikely in a recent study of their diet which was described as herbivorous / detritivorous (Chapter 5) and was consistent with previous dietary studies in their native range (Bowen, 1979; de Moor *et al.*, Dyer *et al*., 2013; 1985; Zengeya *et al*., 2011). With no direct evidence of piscivory, conventional theories about the aetiology of pansteatitis are challenged, and the role of chemical contaminants must be considered. It is conceivable that *O. mossambicus* from LR are fat because they consume large quantities of food. Alternatively, metabolic processes regulated by the endocrine system, and in particular the thyroid cascade, may be under the influence of environmental contaminants.

In *O. mossambicus* thyroid hormones are involved in the control of osmoregulation (Peter *et al*., 2000), growth (Schmid *et al*., 2003), development (Reddy & Lam, 1992), and reproduction (Weber *et al*., 1992). The thyroid cascade involves two main components. Firstly, the brain-pituitary-thyroid axis controls the synthesis, secretion and metabolism of L-thyroxine (T4). Secretion of T4 is regulated by thyroid stimulating hormone (TSH) produced by the pituitary gland. Secondly, T4 is converted to the more metabolically active 3,5,3’-triiodo-L-thyronine (T3) by outer ring deiodination.

Metabolism and receptor-mediated actions of T3 are largely under peripheral control in extra-thyroidal tissues (Eales & Brown, 1993). Thyroid follicles are the functional unit of the thyroid system and occur in the subpharyngeal region of *O. mossambicus* (Geven *et al*., 2007).

Thyroid status is known to vary with age, gender, reproductive stage, nutritional status, physiological condition and season (Rolland, 2000). These factors can complicate the assessment of thyroid status in free-ranging fish populations, and field studies have been limited to various locations in North America (Brar *et al*., 2010; Leatherland, 1993; Zhou *et al.,* 2000) and Europe (Schnitzler *et al.,* 2012). Dietary regulation of thyroid function in fish has been well established, with several authors reporting changes in thyroid function in response to food deprivation or restriction (Power *et al*., 2000; Toguyeni *et al*., 1996; Wunderink *et al*., 2012). While the actions of thyroid hormones are known to affect basal metabolic rates in endotherms, this calorigenic action has not been shown convincingly in teleosts (Eales & Brown, 1993). However, experimental studies have shown that lipid metabolism is stimulated by increasing levels of T4 (Narayansingh & Eales, 1975; Sheridan, 1986), but remains unaltered with increased levels of T3 (Woo *et al*., 1991).

Contaminant effects on thyroid function have been studied in over 40 fish species and have been extensively reviewed (Brown *et al*., 2004; Carr & Patiño, 2011; Rolland, 2000). Around 116 environmentally relevant chemicals have been identified (Howdeshell, 2002), which include polycyclic aromatic hydrocarbons, polychlorinated hydrocarbons, chlorinated paraffins, metals, organophosphorous, carbamate and organochlorine pesticides, phenols, ammonia, acid loads, sex steroids, cyanide compounds, methyl bromide and pharmaceuticals (Brown *et al*., 2004). The two most significant impacts on water quality in LR are AMD originating from numerous operating and especially abandoned coal mines, and partially treated effluent from poorly functioning WWTWs in the catchment (Dabrowski & de Klerk, 2013). Effluent from these two sources alone may contain numerous chemicals that are known to exert acute or chronic effects on the thyroid cascade (Brown *et al*., 2004). Studies of the environmental conditions in LR have reported elevated concentrations of dissolved Al, Fe, Mn and Cu in the water column (Oberholster *et al*., 2010; Chapter 3), and evidence of bioaccumulation of Al and Fe at several trophic levels including the fat tissues of *O.*

mossambicus (Oberholster *et al*., 2012). Apart from these known antagonists, it is likely that a complex mix of chemical contaminants with the potential to modify fish thyroid function is present in LR.

The primary objective of this study was to evaluate the thyroid status of pansteatitis-affected *O. mossambicus* from LR to establish whether there is any evidence of disruption by environmental contaminants. A combined approach of plasma hormone analysis and histological measurements of thyroid follicles was used. Results are compared to a reference population of fish unaffected by pansteatitis in the same river system. The reference site selected was FBR, which is located less than 100 km downstream from LR. This reservoir has the largest population of crocodiles outside of the KNP on the Olifants River (Botha, 2010), and *O. mossambicus* are an important part of the indigenous fish community. As yet, pansteatitis has never been reported in any species from FBR. The thyroid system is highly sensitive to acute and chronic alterations in dietary quality and quantity, making consideration of the nutritional status of fish in this study essential (Eales, 1988). This is especially relevant because fish from LR have access to relatively large quantities of a diverse diet compared to fish from FBR that predominantly feed on sediment and detritus (Chapter 5), which in itself may influence thyroid function (MacKenzie *et al*., 1998). This is the first study to report thyroid status in a free-ranging population of *O. mossambicus* in their native range, as well as in animals affected by pansteatitis.

6.2 METHODS

6.2.1 *Study site*

An extensive assessment of the water chemistry, physiography and limnology of both reservoirs was undertaken concurrent to fish sampling using five sites in LR and four sites in FBR (Figure 6.1). In addition, historic trends in water quality were analysed at both reservoirs and these results are available in Chapter 3 and Chapter 4. Loskop Reservoir was constructed in 1937 to supply irrigation water to downstream agricultural areas via a network of irrigation canals. The main inflow is the Olifants River which drains a catchment of 12,262 km^2. The reservoir has a mean depth of 15.4 m and a surface area of 24.27 km^2. The winter minimum temperature was 15.4ºC and the summer maximum was 32.2ºC. Catchment land uses affecting water quality include power generation, industry, agriculture and sewage treatment works associated with human settlements. Coal mining is also prevalent and the Witbank-Highveld coalfield produces 81% of South Africa's coal (DMR, 2009). Flag Boshielo Reservoir was constructed in 1987 to supply water for irrigation, mining and municipal use. The main inflows are the Olifants and Elands Rivers which drain a combined catchment area of 23,555 km^2. The mean depth is 8.6 m and the surface area is 21.9 km^2. The minimum temperature measured in winter was 19ºC and the summer maximum was 31.8ºC. Catchment land uses are dominated by dryland and irrigated agriculture.

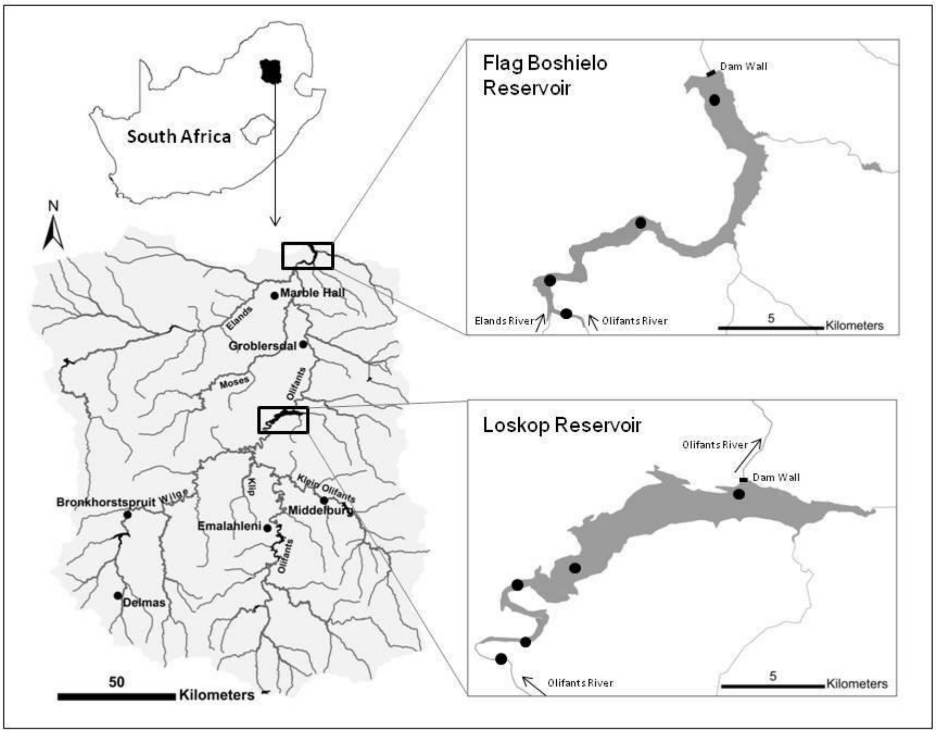

Figure 6.1 Location of Loskop (LR) and Flag Boshielo Reservoirs (FBR) in the Olifants River catchment. Solid circles indicate the location of water quality sampling sites assessed in Chapters 3 and 4.

6.2.2 *Fish collection*

Approximately 20 adult fish were collected using gill nets during April (autumn), June (winter), October (spring) and December (summer) in 2011 to incorporate variation related to feeding and reproductive cycles. Nets were set during daylight hours using three 25 m panels with 70, 90 and 130 mm multi-filament stretched-mesh. An approximately even sex ratio and consistent size class (> 200 mm total length) was collected from each reservoir. Fish were weighed (g) and measured (TL, cm). Condition factor (*K*) was calculated as; $K = 100\ W\ L^{-3}$ where *W* is body weight (g) and *L* is length (TL, cm). Fish were transported to a field laboratory in a 260ℓ tank filled with water from the site and aerated with a mobile pump. Blood samples were taken within two hours of capture. After collection of blood samples, fish were sacrificed by severing the cervical spinal cord. Collection methods and animal handling followed approved university guidelines and relevant permits were obtained.

6.2.3 *Plasma lipids and thyroid hormones*

While fish were alive a sample of blood was collected from the caudal vein. After centrifugation of whole blood, 0.5 ml of plasma was collected and frozen for analysis of total cholesterol and triglyceride concentrations (Wahlefeld, 1974). Analyses were conducted on the Cobas Integra 400 chemistry analyzer by Roche Diagnostics. Cholesterol concentrations were compared to reference intervals for *Oreochromis* hybrids in low-density production systems (Hrubec *et al*., 2000). A further sample of 0.5 ml of serum was collected and frozen at -80ºC for analysis of total T3 and total T4 concentrations using Siemens Coat-A-Count® commercial kits by radioimmunoassay (RIA) according to manufacturer's guidelines. In order to measure total T3 and T4, the assay takes place in the presence of blocking agents which liberate bound thyroid hormones from carrier proteins. Sensitivity of the assay was defined by standards which ranged from 0.31 to 9.22 nmol/L for T3, and 6.4 to 193 nmol/L for T4. All samples were run in duplicate for each hormone in a single assay and the intra-assay coefficient of variation (% CV) was 3.26% for the T3 assay and 13.89% for the T4 assay. Thyroid hormone levels were expressed as nmol/L. As an indicator of peripheral conversion of T4 to T3 (5'-deiodination), the ratios of T3/T4 were calculated (Brar *et al*., 2010).

6.2.4 *Histomorphology of thyroid follicles and liver*

The ventral pharyngeal tissue of a sub-sample of 30 fish from each reservoir was dissected and fixed in 10% buffered (pH 6.5-6.8, $CaCO_3$) formaldehyde solution for histological and morphological analysis of thyroid follicles. Approximately even sex ratios were evaluated with 14 females and 16 males from LR, and 17 females and 13 males from FBR. Thyroid tissue was not collected during winter; therefore this season was excluded from the analysis. Liver tissue was collected and fixed from the previously mentioned fish, as well as an additional 10 fish sampled during winter from both reservoirs, allowing a complete seasonal comparison. All fish included in the analysis had corresponding values for plasma T3, T4, cholesterol and triglycerides. Tissue sections were processed and stained with haematoxylin and eosin (H & E) following standard methods (Bancroft & Gamble, 2002).

Tissue sections were analysed with an Olympus BX43 light microscope fitted with an AxioCam ICc 3 Zeiss microscope camera, and all parameters were measured using AxioVision 4 software. The same tissue section for each fish was used to make all histological observations. Tissue sections were selected from those available on the slide depending on the most numerous intact follicles. As the size of follicles varied, the ten largest intact thyroid follicles were selected per fish. The perimeter (µm) and area (μm^2) of each follicle was measured. Stimulation of the thyroid gland with thyroid stimulating hormone (TSH) produces predictable dose-dependent increases in epithelial cell height, decrease in colloid density and number of endocytotic colloid droplets at apical borders of epithelial cells (Eales & Brown, 1993). These parameters are a reliable indirect measurement of TSH and are frequently used to measure thyroid gland function (Carr & Patiño, 2011). For each follicle, the number of colloid droplets along the inner perimeter was counted (colloid droplets / µm), and the epithelial cell height of three thyrocytes was measured. The appearance of colloid within each follicle was subjectively scored numerically along a gradient with lower values indicating depleted levels, and higher values indicating more concentrated storage: 0 = empty; 1 = granular appearance; 2 = light eosinophilic stain; 3 = intense eosinophilic stain. The mean of these values for ten follicles per fish was presented as the average colloid density.

One of the methods commonly used to assess the nutritional status of fish is to measure the size (circumference, diameter or area) of hepatocytes (Power *et al.,* 2000; Lu *et al*., 2013). The extent of individual hepatocytes was difficult to delineate as they were often unclear, and an alternative method was devised. Hepatocyte density was measured by counting all the visible nuclei in a rectangular frame measuring 180µm x 130µm placed in five random, non-overlapping areas of the liver. Each frame was examined at 40 x magnification. The size (area in μm^2) of 10 randomly selected nuclei per frame was also measured as this has been shown to be a good indicator of nutritional status (Strüssmann & Takashima, 1989; Power *et al*., 2000).

6.2.5 *Liver lipid and moisture content determination*

Total lipids from liver samples were quantitatively extracted according to the method of Folch *et al*. (1957), using chloroform and methanol in a ratio of 2:1. Solvents were removed from the dry fat extracts by evaporation, and the extracts were further dried overnight in a vacuum oven at 50°C, using phosphorus pentoxide as moisture adsorbent. Total extractable fat was determined gravimetrically and expressed as % fat (w/w) per 100 g tissue. The moisture content was determined by weight difference before and after drying tissue to a constant weight.

6.2.6 *Statistical analysis*

Thyroid hormone data violated assumptions of normality and homogeneity of variance, despite data transformation efforts, and were therefore analysed using a non-parametric Kruskal-Wallis analysis of variance (ANOVA) by ranks, with site, season and gender as grouping variables. Thyroid follicle histomorphology, plasma cholesterol and triglycerides, and liver measurements were analysed using a multi-factor analysis of variance (MANOVA) with site, season, and gender as factors. Results from ten thyroid follicles per fish were used to calculate mean values that were used in the analysis, and variables were transformed to meet assumptions where necessary. To determine whether fish size influenced the measured endpoints, the relationship between fish TL and all parameters was assessed using a Spearman's rank correlation. No significant correlations were found and therefore fish length was not used as a covariate in any statistical analyses. Relationships between thyroid hormones and all other endpoints were evaluated using Spearman's rank correlation analysis. All statistical analyses were completed using Statistica Version 12 (StatSoft, Inc. Tulsa OK) and significance level (α) was set at 0.05.

6.3 RESULTS

6.3.1 *Fish collection*

Fish from LR had abundant mesenteric fat which contained clearly visible lesions typical of pansteatitis, characterised by yellow, orange and brown (ceroid pigment) clearly demarcated consolidated masses ranging from multifocal 1 mm pin point to large coalescing irregularly shaped foci. Fish from FBR had little to no fat, which when present, was off-white in colour. The mean body weight of fish from LR (1458 g) was significantly heavier (T-test, $p < 0.0001$), and approximately double the mean weight of fish from FBR (730 g; Table 6.1). The mean TL of fish from LR was also significantly longer than fish from FBR (T-test, $p < 0.0001$). Fish from LR had a significantly higher condition factor than fish from FBR (T-test, $p < 0.0001$). Although this metric is based on the basic premise that fatter fish are healthier fish, this is not necessarily the case in fish from LR, where fat fish are affected by pansteatitis.

Table 6.1 Summarised catch statistics for *Oreochromis mossambicus* sampled from Loskop (LR) and Flag Boshielo (FBR) Reservoirs in 2011 including the sex ratio (M : F), weight, total length, and condition factor (*K*) (TL; Mean ± SD).

Site	Season	*n*	M : F	Weight (g)	TL (mm)	*K*
LR	Autumn	31	17 : 14	1207 ± 597	372 ± 6.5	2.2 ± 0.2
	Winter	20	7 : 13	1725 ± 468	421 ± 3.9	2.2 ± 0.2
	Spring	20	11 : 9	1683 ± 337	420 ± 3.4	2.8 ± 0.2
	Summer	20	6 : 14	1353 ± 448	395 ± 4.8	2.1 ± 0.2
	Total	**91**	**41 : 50**	**1458 ± 532**	**398 ± 5.4**	**2.2** ± 0.2
FBR	Autumn	21	16 : 5	876 ± 404	365 ± 6.3	2.0 ± 0.2
	Winter	20	15 : 5	906 ± 312	353 ± 4.8	2.0 ± 0.2
	Spring	20	7 : 13	662 ± 252	321 ± 5.2	1.9 ± 0.2
	Summer	20	4 : 16	474 ± 223	283 ± 3.8	1.9 ± 0.1
	Total	**81**	**42 : 39**	**730 ± 348**	**325 ± 5.7**	**1.99** ± 0.2

6.3.2 *Plasma thyroid hormones*

Kruskal-Wallis tests revealed significantly higher T3 levels in fish from LR than FBR ($H = 85$, $df = 1$, $p < 0.0001$) while T4 levels did not differ significantly between reservoirs (Figure 6.2a & b). At LR, seasonal variation significantly affected both T3 ($H = 28$, $df = 3$, $p < 0.0001$) and T4 ($H = 43$, $df = 3$, $p < 0.0001$) levels, but only affected T4 values at FBR ($H = 16$, $df = 3$, $p < 0.001$). Seasonal variations in T4 followed a similar pattern at both reservoirs with an increasing trend over the study period. A similar trend was observed in T3 levels in fish from LR, where a *post-hoc* Mann-Whitney test showed that T3 values were higher in summer than autumn, winter and spring ($p < 0.05$) and higher in spring than in autumn (p < 0.01). Fish gender had a significant effect, with higher concentrations of both T3 ($H = 4$, $df = 1$, $p < 0.05$) and T4 ($H = 9$, $df = 1$, $p < 0.01$) in females than males from both reservoirs. The T3/T4 ratio was significantly higher in fish from LR ($H = 40$, $df = 1$, $p < 0.0001$; Figure 6.2c). The ratio was not affected by fish gender and results were therefore pooled according to reservoir and season in Figure 6.2c. The T3/T4 ratio showed a decreasing trend over the duration of the study period at both reservoirs ($H = 13$, $df = 3$, $p < 0.01$). At LR, the ratio in autumn was significantly higher than summer (p < 0.001), and at FBR the ratio in autumn was significantly higher than spring ($p < 0.01$) and summer ($p < 0.001$).

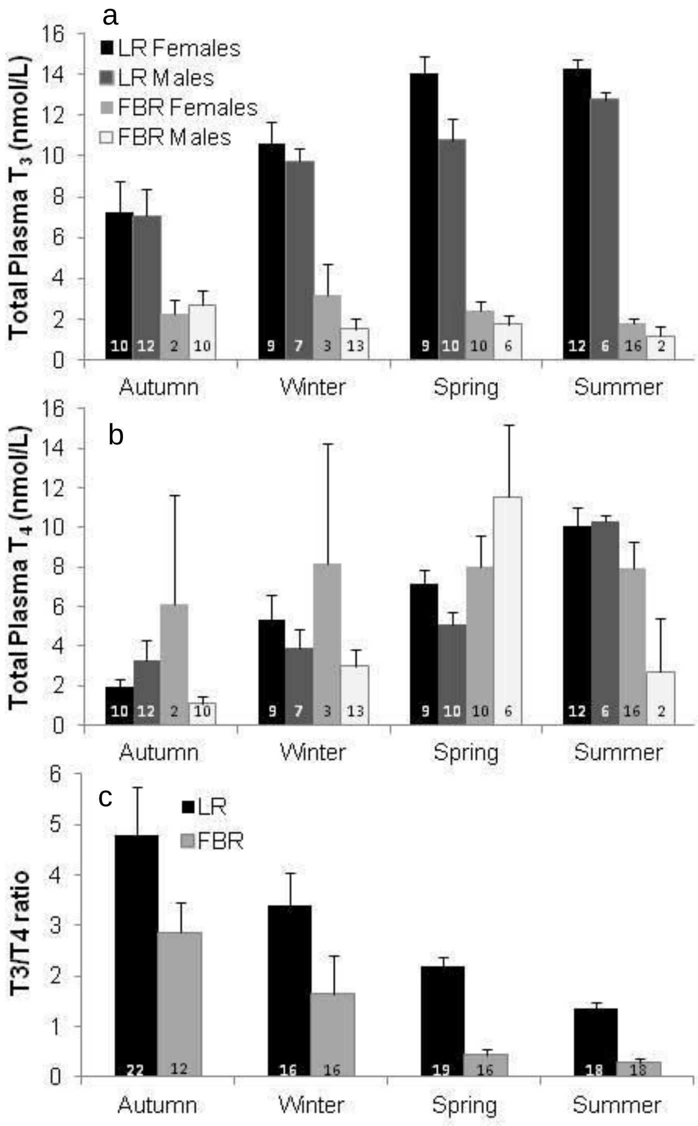

Figure 6.2 Plasma T3 (a) and T4 (b) concentrations, and T3/T4 ratio (c) measured in *Oreochromis mossambicus* sampled from Loskop Reservoir (LR) and Flag Boshielo Reservoir (FBR). Bars represent mean values ± S.E., with the number of individuals per mean shown within bars.

6.3.3 *Thyroid follicle histomorphology*

In fish from LR, thyroid follicles were frequently enlarged with hypertrophy of the follicular epithelium and increased numbers of colloid droplets along the epithelial-colloid interface. Follicles in fish from FBR showed a large degree of variation in size and colloid staining intensity (Figure 6.3).

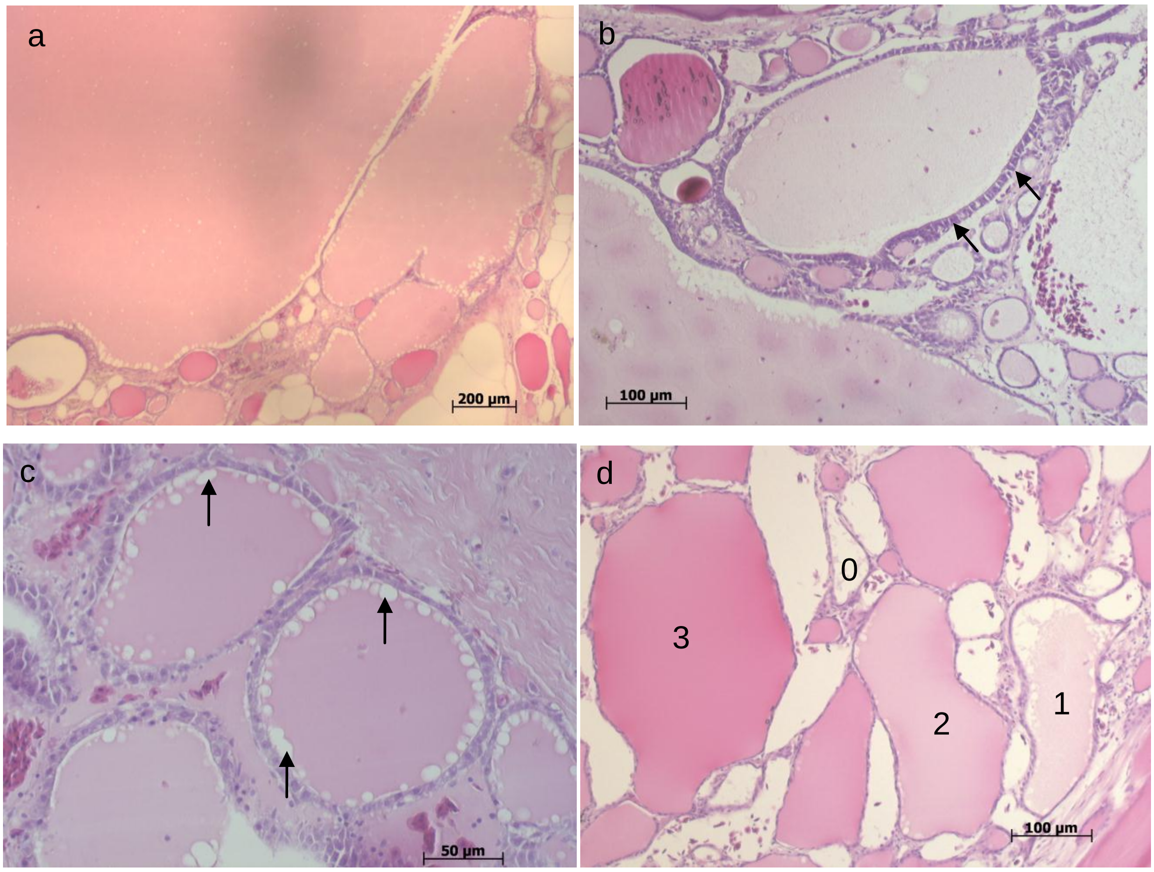

Figure 6.3 Photomicrograph examples (H&E) of thyroid follicle sections collected from *Oreochromis mossambicus*. a. follicles in fish from Loskop Reservoir were significantly enlarged with greater surface area (x 40 magnification); b. hypertrophy of the follicular epithelium (indicated by arrows) in fish from LR (x 100 magnification); c. increased number of epithelial-colloid interface follicular droplets (indicated by arrows) in fish from LR (x 200 magnification); d. typical variation in size, colloid staining intensity (indicated by numbers 0-3), resting epithelium and low vacuolar activity of follicles in fish from FBR (x 100 magnification).

Results of the MANOVA showed that all four follicle descriptors were significantly affected by reservoir as a factor. Neither sex nor season had a significant effect on any of the descriptors so the results were grouped by reservoir (Table II). In fish from LR, thyroid follicles were larger ($F_{1,48} = 7$, $p < 0.05$), with a greater epithelial cell height ($F_{1,48} = 17$, $p < 0.0001$), and more colloid droplets along the follicle epithelium ($F_{1,48} = 16$, $p < 0.001$). Average colloid storage was lower in fish from LR ($F_{1,48} = 10$, $p < 0.01$).

Table 6.2 Mean (±SE) histological and morphometric descriptor values for thyroid follicles (10 per fish) evaluated in *Oreochromis mossambicus* from Loskop Reservoir (*N*=30) and Flag Boshielo Reservoir (*N*=30).

Thyroid follicle descriptors	Loskop Reservoir	Flag Boshielo Reservoir
Area (μm^2) ^{L}	64,005 ± 9562 *	25,639 ± 4118 *
Epithelial Cell Height (µm)	4.5 ± 0.09 ***	3.5 ± 0.06 ***
Colloid droplets / µm ^{S}	0.04 ± 0.00***	0.02 ± 0.00***
Average colloid storage	2.02 ± 0.07**	2.3 ± 0.05**

*** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$. Statistics based on MANOVA

^{L} Log transformed; ^{S} Square root transformed for statistical analysis.

6.3.4 *Plasma cholesterol and triglycerides*

There was a significant effect of reservoir on both cholesterol ($F_{1,130} = 120$, $p < 0.0001$) and triglyceride ($F_{1,130} = 9$, $p < 0.01$) concentrations, with higher concentrations of both plasma lipids in fish from LR (Figure 6.4). While cholesterol and triglyceride levels were not affected by season, the interaction between sex and season was significant with higher concentrations in female fish in spring and summer at both reservoirs ($F_{3,130} = 6$, $p < 0.001$). Female fish from LR had the highest cholesterol and triglyceride levels of all fish sampled and exceeded the published reference interval for tilapia hybrids. Although female fish from FBR showed similar seasonal variation, their cholesterol levels were still within the reference range (Figure 6.4a).

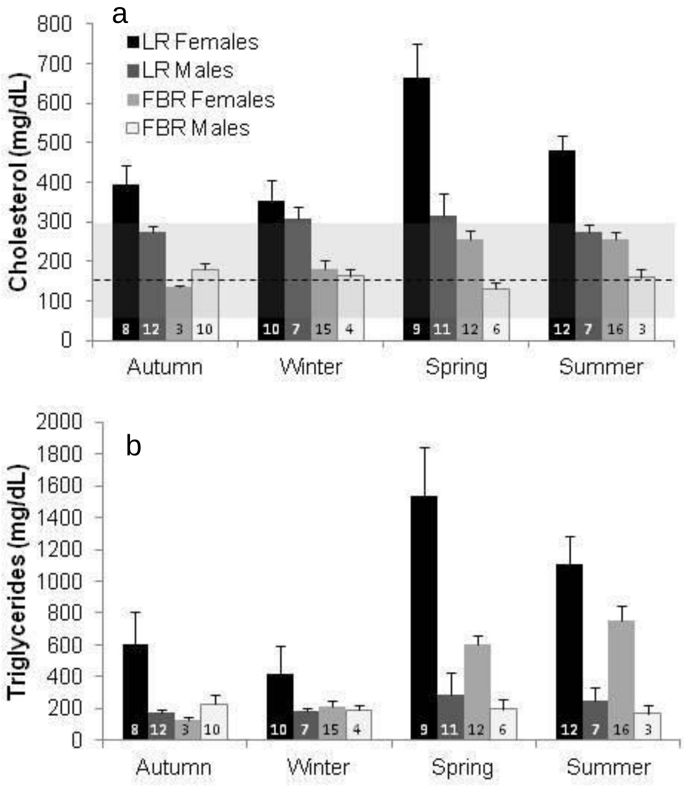

Figure 6.4 Concentration of (a) cholesterol and (b) triglycerides (Mean ± S.E.), measured in *Oreochromis mossambicus* plasma sampled from Loskop Reservoir (LR) and Flag Boshielo Reservoir (FBR). Dashed horizontal line and shaded area for cholesterol indicate the respective range and median reference intervals for *Oreochromis* hybrids (Hrubec *et al.,* 2000). Bars represent mean values ± S.E., with the number of individuals per mean shown within bars.

6.3.5 *Hepatocyte histomorphology*

Extensive lipid vacuolisation was generally evident in hepatocytes of fish from LR, even during winter months (Figure 6.5a). Hepatocytes of fish from FBR were frequently atrophied, depleted of lipid reserves, with accentuated sinusoids and accumulations of lipofuscin (Figure 6.5b). Hepatocyte density was significantly lower in fish from LR ($F_{1,64}$ = 25, p < 0.0001), which is equivalent to increased cell area by comparison to fish from FBR. This parameter was not significantly affected by season or sex. The nuclear area of hepatocytes was also significantly larger in fish from LR ($F_{1,64}$ = 9, p < 0.01). The interaction between reservoir and sex was significant, with larger nuclei in females from LR than females from FBR.

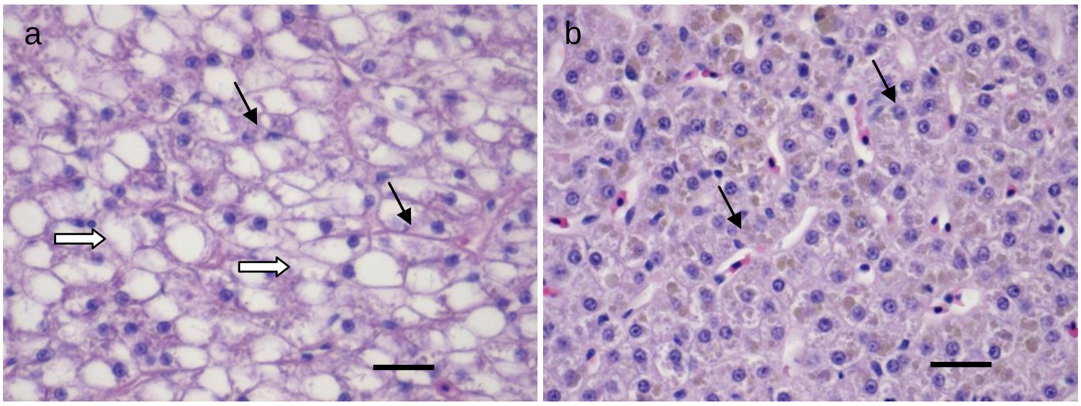

Figure 6.5 Photomicrographs (H&E) of *Oreochromis mossambicus* liver sections collected during winter from (a) Loskop Reservoir, where extensive lipid vacuolisation was frequently evident (white arrows) resulting in enlarged hepatocytes, and sinusoids appear squeezed among hepatocyte tubules (black arrows); (b) Flag Boshielo Reservoir, where cell area was reduced, sinusoids were enhanced (black arrows), and vacuolisation was minimal. Scale bar = 20 µm

6.3.6 *Liver lipid and moisture content*

The mean liver lipid content at LR ranged from 18.8% in autumn to 31.3% in summer (Figure 6.6a), and was significantly higher in fish from LR ($F_{1,61} = 28$, $p < 0.0001$). At FBR the mean lipid content ranged from 9.6% in spring to 15.4% in winter (Figure 6.6b). Liver moisture content was lower in fish from LR than fish from FBR ($F_{1,61} = 29$, $p < 0.0001$). Both lipid and moisture content were not affected by season or sex, and there were no significant interaction effects.

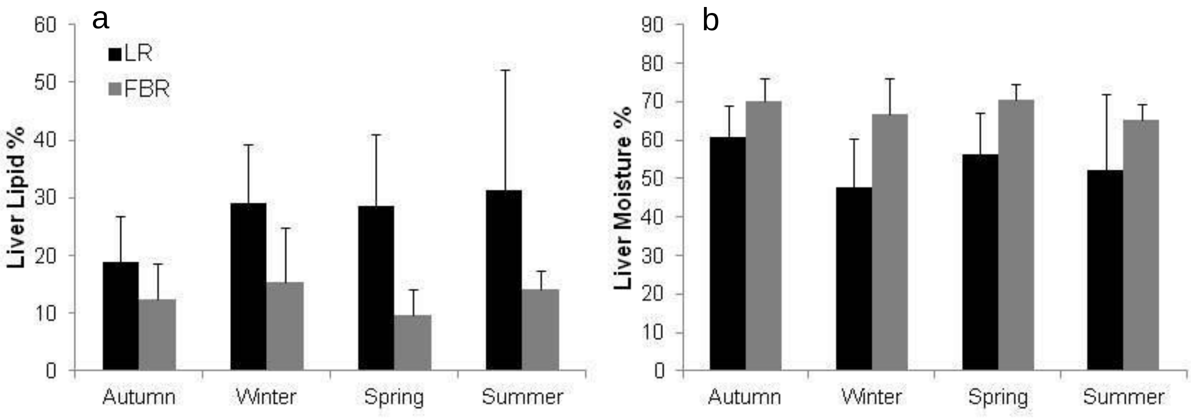

Figure 6.6 Liver lipid and moisture levels (%) measured in *O. mossambicus* from Loskop Reservoir (LR) and Flag Boshielo Reservoir (FBR).

6.3.7 *Relationships between thyroid and nutritional status*

There was a weak but very similar positive correlation between condition factor and T3/T4 ratios in fish from both reservoirs (Table 6.3). Thyrocyte height was positively correlated with T3 levels and negatively correlated with T4 levels in fish from FBR, but showed no relationship to hormone levels in fish from LR. Colloid droplets were positively correlated with T4 in fish from LR. There was a significant positive correlation between triglycerides and cholesterol and both T3 and T4 in fish from LR. The relationship was similar, but correlation coefficients were much weaker in fish from FBR. Hepatocyte density was negatively correlated with T3 in fish from FBR, or in other words, smaller hepatocytes were correlated with lower T3 values.

Table 6.3 Spearmans rank correlation coefficients (*R*) and *p*-values of relationships between nutritional and thyroid parameters measured in *Oreochromis mossambicus* from Loskop Reservoir and Flag Boshielo Reservoir. Bold font indicates that the correlation coefficient is significant ($p < 0.05$).

Loskop Reservoir						Parameters	Flag Boshielo Reservoir					
T3		T4		T3/T4			T3		T4		T3/T4	
R	*p*	*R*	*p*	*R*	*p*		*R*	*p*	*R*	*p*	*R*	*p*
-	-	**0.50**	***	0.05	NS	T3	-	-	0.20	NS	**0.41**	**
0.50	***	-	-	**-0.76**	***	T4	0.20	NS	-	-	**-0.77**	***
0.05	NS	**-0.76**	***	-	-	T3/T4	**0.41**	**	**-0.77**	***	-	-
0.05	NS	-0.15	NS	**0.29**	*	Condition factor	**0.30**	*	-0.10	NS	**0.27**	*
-0.25	NS	-0.14	NS	0.07	NS	Thyroid follicle area	0.12	NS	0.24	NS	-0.08	NS
-0.18	NS	-0.17	NS	0.01	NS	Thyrocyte height	**0.39**	*	**-0.41**	*	**0.62**	**
0.32	NS	**0.40**	*	-0.12	NS	Colloid droplets	0.16	NS	0.03	NS	-0.09	NS
-0.09	NS	-0.07	NS	-0.00	NS	Colloid storage	0.14	NS	0.15	NS	-0.08	NS
0.66	***	**0.26**	*	0.06	NS	Cholesterol	**0.31**	*	0.23	NS	-0.06	NS
0.59	***	**0.36**	**	-0.07	NS	Triglycerides	**0.34**	*	**0.27**	*	-0.12	NS
-0.25	NS	-0.02	NS	-0.16	NS	Hepatocyte density	**-0.31**	*	0.22	NS	**-0.41**	*
0.01	NS	**0.32**	*	-0.25	NS	Nuclear area	-0.00	NS	0.04	NS	0.00	NS
0.28	NS	0.23	NS	-0.03	NS	Liver lipid %	0.19	NS	-0.10	NS	0.16	NS
-0.14	NS	-0.09	NS	-0.03	NS	Liver moisture %	-0.28	NS	-0.02	NS	-0.08	NS

*** $P \leq 0.0001$; ** $P \leq 0.001$; * $P \leq 0.05$

6.4 DISCUSSION

Every aspect of the thyroid cascade examined in *O. mossambicus* from LR indicated very high levels of activity in comparison to fish from FBR. Elevated epithelial cell height, decreased colloid staining, and a greater number of colloid droplets within thyroid follicles are all indirect but reliable indicators that TSH was being actively released by the pituitary gland (Eales & Brown, 1993). These results also indicated that T4 was being actively synthesized and secreted, and no disruption was detected at this level of the thyroid cascade. This was further confirmed by the similar T4 concentrations observed in fish from both reservoirs, which emphasised the homeostatic capacity of the thyroid system at the central level and has been previously observed (Eales *et al*., 1999; Van der Geyten *et al.*, 2005). In hyperthyroidism, colloid stains less intensively, eipithelial cell height increases and follicles are smaller (Eales, 1988). The latter is not consistent with our observations in fish from LR, as their follicles were significantly larger than those from FBR. Elevated T3 levels, reduced colloid staining, and hypertrophy of follicle epithelial cells can be attributed to iodine deficiency (Lavado-Autric *et al*., 2013). However, prolonged exposure to iodine deficiency usually results in decreased T4 levels which were not observed in fish from LR. The T3 and T4 levels of *O. mossambicus* from FBR, and T4 levels in fish from LR were comparable to control fish used in experimental studies, but T3 levels in LR fish were higher than published values (Peter, 2009; Peter & Peter, 2009). We did not directly measure deiodination in peripheral tissues, but there was no evidence of inhibition of extrathyroidal conversion of T4 to T3 because elevated T3 levels and the T3/T4 ratio indicated high rates of deiodination in fish from LR (Brar *et al*., 2010).

Elevated T3 levels were positively correlated with plasma cholesterol and triglycerides in fish from LR, which showed a similar but weaker relationship in fish from FBR. This result indicates a link between lipid metabolism and the thyroid system, and is the first field study to support similar findings observed in experimental studies (Narayansingh & Eales, 1975; Sheridan, 1986). Major differences in the nutritional state of fish from both reservoirs were observed. The larger hepatocytes, nuclei and higher liver lipid concentrations, along with elevated plasma cholesterol and triglycerides in fish from LR were all indicative of their abundant nutritional intake and energy reserves. This was also reflected in the significantly higher condition factor in fish from LR. These results are not surprising given the greater diversity and abundance of food items in the diet of fish from LR compared to those from FBR (Chapter 4). Dietary quantity and quality plays an important role in regulating thyroid hormones (Eales, 1988; MacKenzie *et al.,* 1998). Higher T3 levels have been reported when fish were fed a high-protein and high-calorie diet compared to equal amounts of a low-protein and low-calorie diet (Higgs & Eales, 1979). Increasing feeding levels in Nile tilapia (*Oreochromis niloticus*) have resulted in significantly higher plasma T3 levels, while T4 levels remained unaffected (Toguyeni *et al.,* 1996). This effect has been reported in several other fish species (Eales & Shostak, 1985; McCormick & Saunders, 1990) and is directly comparable to fish from LR, suggesting that their elevated T3 levels may be in response to higher nutritional inputs. The negative correlation between T3 and hepatocyte density (size) observed in fish from FBR further confirmed the influence of nutritional state on T3 levels. In *O. mossambicus*, the expression of hepatic insulin-like growth factor (IGF-I), a mitogenic factor that induces growth and differentiation, is stimulated by increasing T3 levels (Schmid *et al.,* 2003). Elevated T3 levels associated with high nutritional inputs may explain the large body size of fish from LR. Despite the abundant and relatively diverse diet of fish from LR, there is the possibility that their elevated plasma and liver lipids are the result of exposure to metabolic pathway disrupting obesogenic compounds (Meador *et al*., 2011). Future studies will be required to establish all the factors that underlie the homeostatic shift that leads to elevated plasma T3 in LR fish.

Seasonal increases in plasma T3 and T4 in spring and summer were observed in fish from both reservoirs. In addition to variation in feeding rates, this pattern may be related to reproductive processes as thyroid hormones are known to be elevated during gonadal development (Arcand-Hoy & Benson, 1998) which would occur at this time of year. The active mobilisation of reserved lipids can occur during distinct periods in the biological cycle of fish, ie. Fasting periods during winter months, and the gonad development phase (Corraze, 2001). Elevated cholesterol and triglyceride levels in female fish from both reservoirs in spring and summer may be related to increased feeding rates, or reproductive cycles. Cholesterol in particular is converted to various classes of gonadal steroids by steroidogenic enzymes (Young *et al.,* 2005) and in females large amounts of fat are mobilised during the formation of ovaries and their constituent eggs (Tocher, 2012). During winter, 50% of the fish stomachs from LR, and only 20% from FBR were empty (Chapter 5). This indicates that fish were still actively feeding despite low temperatures (< 16ºC at LR; 19ºC at FBR) and probably explains why plasma lipids remained stable in winter relative to the other seasons.

Thyroid activity and nutritional status were reduced in fish from FBR compared to fish from LR. Fish from this reservoir have access to a restricted diet dominated by detritus and sediment, although their diet was undetermined to an extent and other items may make minor contributions (Chapter 5). In contrast to fish from LR, their T3 values were consistently lower than T4 which has been reported for control values of *O. mossambicus* in previous laboratory based studies (Peter & Peter, 2009; van Anholt *et al.,* 2003; Weber *et al*., 1992), and is the general pattern reported for most fish species (Power *et al.*, 2001). Their liver lipid values, although significantly lower than fish from

LR, were indicative that energy stores were present and available for mobilisation. Their plasma cholesterol was within the reference intervals for *Oreochromis* hybrids (Hrubec *et al.,* 2000), and triglycerides were comparable to those of male fish from LR. The T4 levels of food restricted fish have mostly been shown to decrease (Eales *et al.,* 1988; Power *et al*., 2000), but increased levels have also been reported (Toguyeni *et al*., 1996). The fact that T4 levels were similar in fish from both reservoirs indicates that food restriction was not affecting fish from FBR. We conclude that although fish from FBR had lower nutritional inputs and energy reserves, these were not indicative of nutritional stress.

The results of this study indicated that highly active thyroid function in fish from LR is partly associated with their nutritional state. Abundant fat reserves are therefore likely to be due to high nutritional inputs, as opposed to disruption of the thyroid cascade. As a sensitive biomarker of exposure to pollutants, the lack of evident disruption of the thyroid axis at the levels measured strongly suggested that pollutant exposure was not influencing fish health at the time of this study. This does not rule out exposure to a pollutant at some historic point in time. Pansteatitis can cause acute mortalities, but some animals recover, survive and continue growing, and pansteatitis lesions are only discovered at slaughter (Huchzermeyer, 2003). Observed variation related to seasons and gender may be associated with reproductive state which was not determined in this study, but should be considered in future. Our results emphasised the need to consider the nutritional status of fish when evaluating thyroid function in free-ranging populations. This is particularly relevant when fish are likely to have access to diets that differ in quality and quantity in their respective habitats.

Chapter 7: GENERAL DISCUSSION AND CONCLUSIONS

7.1 Introduction

An in-depth understanding of the environmental conditions at Loskop Dam was required to further explore several hypotheses surrounding the aetiology of pansteatitis. The first aim of this study was to improve the understanding of environmental drivers affecting water quality at Loskop Dam, and compare these to a suitable reference site identified as Flag Boshielo Dam. The main finding was that eutrophication has occurred to a large extent at Loskop Dam, and is a significant driver of physico-chemical variation and bottom-up changes in the food web. Flag Boshielo Dam was found to be oligotrophic with reduced impacts associated with mining and industry.

Pansteatitis in captive-bred animals has frequently been associated with the consumption of diets high in polyunsatured or rancid fats, frequently of fish origin. The second study aim was therefore to examine the diet of *O. mossambicus* at both reservoirs, and determine whether there was evidence of piscivorous feeding behaviour. The results showed no evidence of piscivory and their diet was described as detritivorous / herbivorous. The dominant food items were identified enabling future research into their nutritional and elemental composition.

The thyroid cascade is considered a sensitive biomarker of endocrine disruption through exposure to xenobiotiecs, and thyroid disruption may be linked to abundant fat deposits in fish. The third study aim was to determine whether there was evidence of xenobiotic disruption of endocrine function using the thyroid cascade as a biomarker. There was no evidence of disruption at any level of the thyroid cascade measured in this study, which is a good sign that xenobiotics were not influencing endocrine function at this time. The thyroid was highly active in fish from Loskop Dam and was positively correlated with several indicators of nutritional state. These indicators confirmed that fish from Loskop Dam had large energy reserves resulting from an abundant and diverse food supply.

During fieldwork every fish sampled from Loskop Dam displayed steatitis lesions to varying extents, while no lesions were evident in fish from Flag Boshielo Dam. No large-scale fish kills or die-offs of *O. mossambicus* were observed during monitoring at either dam, which took place throughout 2011. This indicates that steatitis lesions observed in *O. mossambicus* from Loskop Dam were possibly due to historic oxidative stress, and the stressor responsible had subsided, or is intermittent. This is supported by the lack of intermediary steatitis lesions observed in the mesenteric fat of fish from Loskop Dam (Dr. J. Steyl, Faculty of Veterinary Science, University of Pretoria, pers. comm. 2014). One of the few remaining crocodiles at Loskop Dam died as a result of pansteatitis in April 2011, which may indicate a species-specific aetiology of the disease. Alternatively, the lack of observed fish mortalities despite the high incidence of pansteatitis in the *O. mossambicus* population may reflect better survival rates of fish than crocodiles. This pattern was also observed in KNP where pansteatitis-affected catfish were increasingly prevalent with no major die-offs, despite reduced, but ongoing crocodile mortalities (Huchzermeyer, 2012).

7.2 Dominant environmental drivers

In comparison to Loskop Dam, water quality is greatly improved at Flag Boshielo Dam, which had reduced impacts associated with coal mining and wastewater treatment works. For instance, while sulphate concentrations showed a significant increasing trend at Loskop Dam, they remained approximately stable at Flag Boshielo Dam. This confirmed that Loskop Dam is acting as a sink for pollutants originating in the upper Olifants River catchment. Concentrations of dissolved Al, Fe and Mn were periodically elevated in surface and near-bottom water at Loskop Dam, while Al exceeded the TWQR only once at Flag Boshielo Dam. A distinct difference between both reservoirs was their respective trophic states. Eutrophication at Loskop Dam resulted in frequent blooms of *M. aeruginosa* and *C. hirundinella* that altered the physico-chemical conditions in the water column, particularly in the transitional zone. In contrast, algal blooms were never observed at oligotrophic Flag Boshielo Dam, and nitrogen-fixing species dominated the phytoplankton community. One common environmental factor identified at Loskop Dam and the Olifants River gorge in the KNP is the presence of high inputs of phosphorous leading to eutrophication. At the KNP, these predominantly originate from a phosphate mine near Phalaborwa, and algal blooms were observed in the gorge in 2008 (Huchzermeyer, 2012). However the species' present and population dynamics of phytoplankton at this location have not been reported. Fluctuating variables associated with algal blooms such as pH and dissolved oxygen can directly and indirectly lead to numerous reactions that cause fish kills, but are not known to cause pansteatitis. While consumption of rancid fish associated with fish kills may explain the occurrence of pansteatitis in crocodiles, the results of this study showed that it does not explain the disease in *O. mossambicus* from Loskop Dam.

Both reservoirs are monomictic with prolonged anoxia in the hypolimnion during periods of stratification. Unfortunately comparable metal concentrations were not measured in near-bottom water samples from Flag Boshielo Dam due to cost constraints. The elevated concentrations of dissolved Al, Fe and Mn in near bottom water from Loskop Dam during stratification warrants further investigation. Depth profiles within the water column showing the distribution and concentrations of these metals throughout the year, coupled with measurements such as redox potential will be imperative to determine their chemical speciation and bioavailability. Concurrent measurements of the elemental concentrations of dominant food sources for *O. mossambicus* would help to explain the high concentrations of Al and Fe previously reported in the fat of *O. mossambicus* and *Spirogyra* spp. (Oberholster *et al*., 2012).

7.2.1 *The consequences of drought*

The synchronous nature of the occurrence of pansteatitis at two distinct locations on the same river system strongly suggests that a wide-spread environmental factor affecting the entire catchment was somehow involved. The severe drought that lasted from late 2002 to late 2005 caused water levels to drop significantly, and extensive areas of lake sediments were exposed and oxidised at both Loskop and Flag Boshielo dams. After water levels rose in early 2006, total P and chlorophyll-*a* concentrations increased at Loskop Dam and inorganic N and P increased slightly for several months at Flag Boshielo Dam. The latter concentrations were still low enough to be categorised within the oligotrophic to mesotrophic category respectively, and did not result in increased algal growth (measured as chlorophyll-*a*). During monitoring for this study at Loskop Dam, the highest concentrations of inorganic P were recorded beyond the inflow in the transitional zone, suggesting that internal cycling of sediment-bound P played an important role in nutrient dynamics. This may continue to stimulate algal blooms in spite of any management interventions to reduce P inputs in the catchment. Research conducted throughout 2009, reported pH levels ranging from 5.9 to 6.8, Al concentrations as high as 1610 µg/ℓ, total N as high as 17000 µg/ℓ, and total P reaching 711 µg/ℓ at the inflow to Loskop Dam (Oberholster *et al*., 2010). The water re-filling the reservoir post-drought was therefore of very poor quality, and likely to result in numerous environmental perturbations. The results of this study show that water quality at the inflow has improved since then.

Reservoir sediments act as a sink for a variety of organic and inorganic pollutants as reduced flow allows them to settle out of suspension. It is well known that fluctuations in water chemistry parameters such as dissolved nutrients (especially P) and metals can be linked to release from reservoir sediments in different states of oxygen reduction. This was directly observed in Loskop Dam where dissolved Al, Fe and Mn concentrations were periodically elevated in near bottom water samples. What is not known is how chemical interactions between the sediment and overlying water column alter after extended periods of drying (drought) and re-wetting (post-drought). Evidence from international literature has shown that re-wetting can result in acidification, eutrophication and disrupt nutrient cycling and microbial processes (Lake, 2011). Changes in water quality related to re-wetting post-drought are likely to be system specific and related to pollutant inputs from the catchment. Pansteatitis in fish and crocodiles, numerous fish kills and increasing algal blooms at Loskop Dam occurred during and post-drought when water levels had risen to FSL. This highlights the sensitivity of Loskop Dam to drought conditions, and emphasises the need to investigate the effects of drying and re-wetting reservoir sediments on water quality, in order to better anticipate the consequences of drought. Such as study may also reveal a range of organic and inorganic pollutants that are mobilised from the sediment under different environmental conditions thus providing a historic perspective of water quality following the drought.

7.3 Diet as a cause of pansteatitis

The simplest explanation for pansteatitis in free-ranging animals associated with aquatic habitats is a diet consisting of fish with a high proportion of polyunsaturated fats, or feeding on rancid fish associated with fish kills. Using the combined approach of stable isotopes and stomach contents, the diet of *O. mossambicus* from Loskop Dam was determined as detritivorous / herbivorous, with no evidence of piscivorous behaviour during any of the time periods evaluated.

In order of importance, the dominant food items in the diet of *O. mossambicus* from Loskop Dam were identified as *C. hirundinella*, zooplankton (*Daphnia* spp. and copepods), detritus and *M. aeruginosa*. High nutrient inputs at Loskop Dam have resulted in an abundant food supply for *O. mossambicus* that is available year-round, as *C. hirundinella* blooms throughout winter, and fish were still observed to be feeding at this time. Given the dietary links to pansteatitis, this bottom-up change in the food web at Loskop Dam still provides the most likely link to the disease aetiology. The lack of piscivory in their feeding behaviour does not mean that diet can be excluded as a possible cause of pansteatitis, rather that future research should investigate aspects such as the fatty acid profile and elemental concentrations of the dominant food sources identified.

Pansteatitis-affected *C. gariepinus* from the KNP were found to have elevated n-3 to n-6 fatty acid ratios, which were indicative of large quantities of polyunsaturated fats in their diet (Huchzermeyer *et al*., 2013). Certain n-3 and n-6 fatty acids such as α-linolenic and linoleic acid cannot be synthesized by fish, and must be obtained through dietary sources (Tocher, 2003). The subsequent ratios of n-3 to n-6 fatty acids in fish are reflective of their diet. Freshwater phytoplankton are known to contain large quantities of polyunsaturated fats including α-linolenic acid (Tocher, 2003). Their fatty acid composition fluctuates as a function of environmental factors such as pH, nutrient concentrations, salinity, light and temperature (Guschina & Harwood, 2009). Freshwater zooplankton such as *Daphnia* spp. and copepods predominantly accumulate a high proportion of n-3 fatty acids, especially eicosapentaenoic and docosahexaenoic acids, relative to their diet. The fatty acid composition of *Daphnia* spp. in particular is strongly influenced by their diet (Brett *et al*., 2009). A high polyunsaturated fat intake can lead to increased consumption of enzymatic and non-enzymatic antioxidants, as these fats are highly susceptible to autoxidaiton. Fluctuating environmental conditions may have influenced the fatty acid composition of algae at Loskop Dam, increasing the ratio of n-3 to n-6 in a highly abundant food source. This is a topic for further research.

Bioaccumulation of Al and Fe reported in the fat of *O. mossambicus* and at other trophic levels in Loskop Dam may not be unique to this location. High accumulations of Fe were reported in various tissues of pansteatitis-affected *C. gariepinus* from the KNP. It could not be determined whether these deposits represented iron storage as haemosiderin due to excessive haemolysis, or indicated abnormal uptake of Fe from the environment (Huchzermeyer *et al.,* 2011). Oxidative stress is known to be associated with the ingestion of excessive quantities of Fe (Baker *et al.,* 1997), and is enhanced in the presence of Al (Quinlan *et al*., 1998), which emphasises the importance of determining the elemental composition of dominant food sources for pansteatitis-affected fish.

Fish from Flag Boshielo Dam were on the opposite side of the food availability spectrum. Their diet was mainly restricted to detritus and sediment with limited contributions from phytoplankton, and a very low contribution from zooplankton. However, their diet remained somewhat undetermined, due to the scarcity of food items available for sampling in the pelagic zone and an important food source may have been overlooked during sampling. The oligotrophic, nitrogen-limited status of the reservoir lends itself to the assumption that algal productivity is nutrient limited. While this may be partially true, the presence of a large population of invasive silver carp may exert a significant top-down effect on phytoplankton and zooplankton as they are specialist filter feeders (Rosemberg *et al*., 2010) capable of consuming 20 – 40 % of their own body mass per day (Kolar *et al*., 2007), and can have a considerable impact on lake plankton communities (Lu *et al.,* 2002).

7.4 Thyroid function and nutritional state

The presence of inorganic pollutants related to mining and industry has been confirmed in Loskop Dam (Oberholster *et al*., 2010), and given the large-scale impacts associated with the discharge of effluent from WWTWs in the catchment (Ashton & Dabrowski, 2011; Dabrowski & De Klerk, 2013), the presence of organic pollutants is highly likely. There was no evidence that exposure to any of these pollutants was impacting negatively on the thyroid axis at the time of this study, which is considered a good biomarker of exposure to xenobiotics. Although thyroid function was elevated in fish from Loskop Dam, this was considered to be partially related to the nutritional state of the fish, and was not considered pathological. Additional variation was likely to be related to the reproductive state of the fish which was not addressed in this study, but should be considered in future research. The lack of evidence for any xenobiotic affect on thyroid function may be interpreted in two ways. Firstly, that if endocrine dysfunction was in any way involved in the aetiology of pansteatitis, then the pollutant involved was not present at the time of this study, and the steatitis lesions observed in *O. mossambicus* were historic in nature. Secondly, that endocrine dysfunction was not involved in the aetiology of panteatitis.

Differences in food diversity and abundance between Loskop Dam and Flag Boshielo Dam were reflected in the nutritional state of *O. mossambicus*. Fish from Loskop Dam had higher condition factors, larger body size, higher energy stores (measured as liver fat content), larger hepatocytes and hepatocyte nuclei, and higher plasma cholesterol and triglyceride levels. Apart from the plasma lipids which showed an increasing trend over the study period and were probably related to reproductive state, the other variables showed no seasonal trends. All of these factors are considered good indicators of the nutritional state of an animal, and indicated that fish from Loskop Dam had access to abundant food supplies and were actively feeding throughout the year. This pattern was also apparent in the results of the dietary study (Chapter 5).

The finding that abundant fat reserves are likely due to high nutritional inputs as opposed to disruption at measured levels of the thyroid axis is significant. Abundant fat provides an extensive substrate for lipid peroxidation associated with pansteatitis to occur. Furthermore, this puts strain on the availability of enzymatic and non-enzymatic antioxidants as polyunsaturated fats typically stored by fish are prone to lipid peroxidation. This may be the reason for drastically reduced Cu concentrations in the livers of fish from Loskop Dam that were observed during research conducted in 2010 (Dabrowski, 2012). Copper is an important co-factor in the antioxidant enzyme Cu-Zn-superoxide dismutase which is present in the majority of cells. Damage to fat cell membranes in steatitis-affected fat tissues can result in reduced lipolysis, which despite abundant fat reserves decreases the availability of fat stores to the animal (Danse & Verschuren, 1978). This reportedly occurred in pansteatitis-affected *C. gariepinus* (Huchzermeyer, 2012), and may also partially explain the abundant fat reserves evident in fish from Loskop Dam.

One of the reasons suggested for elevated $\delta^{15}N$ signature in *O. mossambicus* from Flag Boshielo Dam relative to measured food items was that they could have been under a degree of nutritional stress. Their liver lipid content would suggest that this was not the case. Although lower than that of fish from Loskop Dam, the consistent presence of > 9% lipid in their livers indicated that energy stores were available for mobilisation. This indicates that elevated $\delta^{15}N$ values were probably due to consumption of an unsampled food source, as opposed to nutritional stress.

7.5 Conclusions and the way forward

The purpose of this study was to test certain hypotheses about the causes of pansteatitis in *O. mossambicus* from Loskop Dam, while providing an informative insight into the environmental conditions where the disease occurs. Through a process of elimination of likely factors involved in the disease aetiology, it is hoped that the combination of factors involved will eventually be elucidated. Therefore the results of this study represent an important foundation upon which future hypotheses can be built and tested.

Feeding on fish associated with fish kills or as a regular part of the diet has been eliminated as a possible cause of pansteatitis. The next hypotheses to be tested revolve around the fatty acid and elemental composition of the major dietary items identified. These are predominantly *C. hirundinella*, zooplankton (*Daphnia* spp. and copepods) detritus and *M. aeruginosa*. Food sources with an elevated n-3 to n-6 ratio, and / or enriched with Fe and Al could possibly be linked to the disease through an elevated demand for antioxidants and their oxidative effects respectively.

Given that research has already confirmed bioaccumulation of Al and Fe in the fat of *O. mossambicus*, as well as *Spirogyra* spp. (Oberholster *et al*., 2012), the mechanism of this accumulation must be determined. This requires an understanding of the chemical speciation and bioavailability of dissolved metals. Future studies will need to establish more complete depth profiles of dissolved metals with concurrent measurements of reduction potential in order to determine the chemical speciation, which can be accomplished using models such as PHREEQC.

The influence of extended drying and oxidation of reservoir sediments associated with extended droughts, and subsequent re-wetting on water quality is unknown and has not been investigated in Loskop Dam, or elsewhere in South Africa. The environmental perturbations that occurred when Loskop Dam re-filled emphasise the role of drought in affecting both water quantity, and water quality. Given our dependence on stored water resources, a study investigating reservoir specific responses of sediment to drying and re-wetting would inform the management framework of reservoirs in general, and provide insights into the environmental changes observed at Loskop Dam. This study should address both inorganic and organic pollutants released from the sediment, and combine the use of toxicity bio-assays and appropriate endocrine endpoints to assess the impacts on invertebrates and fish.

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APPENDIX: RESEARCH PUBLICATIONS RELATED TO THIS STUDY

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