Health assessment of tilapia (*Oreochromis* spp.) aquaculture systems in the northern provinces of South Africa

By

Gillian Denise Taylor

A dissertation in fulfilment of the requirements of the degree Master of Science in the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria

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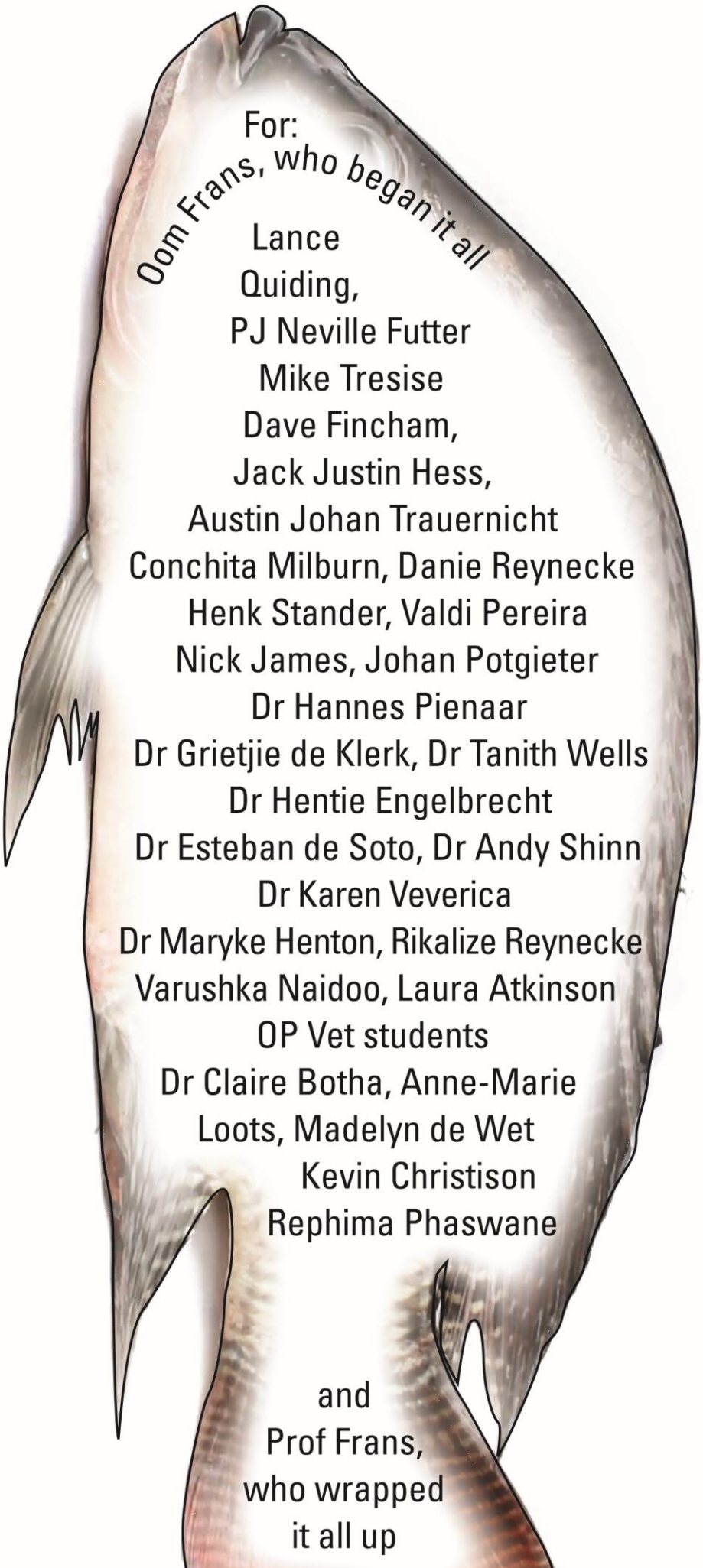
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SUMMARY

Health assessment of tilapia (*Oreochromis* spp.*)* aquaculture systems in the northern provinces of South Africa

By

Gillian Denise Taylor

Supervisor: Dr J.C.A. Steyl

Co-supervisor: Dr D. Scarfe

Department: Paraclinical Sciences

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This study evaluated eighteen commercial farms within Gauteng, Northwest and Limpopo provinces, where a representative sample of grow-out fish from each farm, was humanely euthanased, weighed, measured, and each fish's overall health assessed through microscopic examination of skin and gills. A full necropsy and histo-pathological evaluation of all key organs followed.

Farm production parameters were assessed by means of a questionnaire with a detailed history and a comprehensive water analysis that included water temperature, dissolved oxygen, CO_2, ammonia, nitrite, nitrate, pH, hardness and alkalinity. These production parameters, together

with stocking density and underlying nutrition, were compared with the macro- and microscopic findings for positive and negative correlations / relationships.

Significantly high burdens of ecto-parasites and very poor water quality, with compromisingly low dissolved oxygen and temperatures, and high carbon dioxide and nitrite, were found in association with severe gill pathology. This was compounded by inadequate filtration for the density of fish stocked, with resultant toxic nitrogenous waste accumulation. Other key abnormalities observed were chronic-active hepatic lipid oxidation, low hepatocellular lipid, evidence of secondary opportunistic infectious disease, and extremely poor growth. Poor farm management practises were prevalent, with evidence of uneconomical fish sex ratios, and poor implementation of biosecurity and disease management.

The study serves to highlight the factors that currently dominate as critical issues affecting overall health and growth of aqua-cultured tilapia in the South African context.

CHAPTER 1 - INTRODUCTION

With global food security under increasing pressure, focus has turned to previously underdeveloped water- and land-rich areas, to farm fish, as a cost-effective protein source. The South African freshwater tilapia aquaculture industry, despite a slower start behind the rest of Africa, and globally, has shown steady growth in the last few years, with particular effort and investment being placed in the development of inland recirculating aquaculture systems.

Recirculating aquaculture systems (RAS) have been widely promoted and adopted in South Africa over the last 4 to 5 years within the developing tilapia aquaculture sector. This is largely because of the benefits of water-reuse in a water-constrained country, easier management of local challenges with cold winter temperatures, the low land surface-area system requirements, reduced environmental impact, and good economy of scale with highest production per unit area and labourer.

It is well understood that compromised fish health and poor growth, whether from underlying infectious causes, sub-optimal nutrition, or environmental stressors, exert significant economic impact on production levels and profit in the aquaculture sector, with losses believed to reach up to US 9.58 billion dollars per year (Shinn *et al.,* 2015).

With awareness of the obvious negative impact that disease or chronic suboptimal health potentially could leverage against success of this new aquaculture industry, the South African tilapia farming sector recognized the value of proactive assessment of fish population health, with the aim to identify key problem areas, address them, and thereby optimize productivity and success of the sector.

This project was initiated with assistance from and on their behalf, to assess current fish and farm health levels, using routinely performed veterinary clinical assessment methods. The chief objective of the study was to evaluate overall fish and farm health by assessing key parameters like growth, parasite burdens and macroscopic or microscopic pathology, in correlation with husbandry practices, nutrition, and key water quality stressors.

Declaration of freedom of disease was not an objective, and could not be inferred from this once-off study.

As with most industries where animals are produced for food, assessing overall levels of health and disease management, within the aquaculture sector, required a herd-health approach rather than a fish-specific approach. Collated sample data could be used to develop an impression of overall health of each unit, and unit data together provided information about the health status of the industry. It is important to acknowledge, however, the complexity involved of the assessment of numerous variables and their effects upon each other in a survey like this. This study, attempted to determine patterns of variable associations, with the aim to improve production using gained knowledge to manipulate correlating parameters.

No similar assessment has previously been carried out on the farmed tilapia (*Oreochromis* spp.) populations within South Africa.

CHAPTER 2 - LITERATURE REVIEW

2.1 AQUACULTURE SYSTEMS

Aquaculture systems can be classified as open, semi-open or closed with various categories within each group, but with a general trend of increasing intensification, human intervention and costs as one moves towards an increasingly closed structure design (Tidwell, 2012).

Through-flow systems fall within the semi-closed group, where the management of key water quality parameters like oxygen, temperature and nitrogenous organic waste are largely controlled by continuous high-level input of clean water from source and output of waste water out of the system. There is no recirculation of water and little need for filtration. But the challenge of this type of system on fish health is the resultant poor level of control that exists on maintaining good and consistent water quality, high volumes of water required, and limits to stocking densities achievable (Noga, 2010.i).

Recirculating aquaculture systems (RAS), on the other hand, fall within the closed group and are classified as highly intensive (Timmons and Ebeling, 2013.e). Because they rely on repeated reuse of water, desired water quality suitable for fish health, has to be maintained with a combination of filtration mechanisms, whereby solid waste is removed mechanically, and toxic nitrogenous compounds detoxified through high density bacterial bio-filtration. They require additional aeration to maintain adequate oxygen levels for the high concentration of fish in the system as well as supporting the biofilter (Tidwell, 2012). Although these systems allow for good control of water parameters and high stocking densities, the temptation often exists to

push the boundaries of system stocking capacity beyond the fine line of balance, with resultant dramatic effect on water quality, stress on fish, and disease.

An aquaponic system is an integrated-type of RAS system (Timmons and Ebeling, 2013.f), where cultivation of plants within the system produces a second marketable product, as well as replacing part of the function of the biofilter, in its utilization of nitrogenous by-products. These integrated systems are becoming increasingly popular as a dual crop form of aquaculture, carrying the same benefits and challenges of a conventional RAS system, but with the added benefit of a secondary harvest. However, in these systems, effective disease management remains a challenge, in the event of a disease outbreak in either fish or plants.

Within these systems, exists a dynamic finely balanced equilibrium within the host fish, its watery environment, and potential disease challenges, with significantly greater variability than terrestrial habitats, and having the contribution of additional man-made stressors as well (Wedemeyer *et al.,* 1976.a): the greater the intensification of the system, the greater the risk of imbalance and the greater the need for vigilant monitoring and surveillance.

2.2 STRESSORS AND IMMUNITY

Stress can be defined as an inability to maintain normal physiologic state because of negatively impacting chemical or physical factors (Rottmann *et al.*, 1992). These stressors serve to divert resources from non-essential physiological processes like growth and reproduction, to essentials like energy production, in order to survive (Pankhurst and Van Der Kraak, 2007). This compromised physiological state, together with physical injuries, are considered the chief contributing factors to fish disease and mortalities in the aquaculture industry (Rottmann *et al.*,

1992). To attempt to assess the level of stressful impact upon a fish population and potential impact upon health, one needs to start with a broad outlook of all aspects with potential to do harm. This includes chemical stressors like water pH, hardness, alkalinity, excess nitrogenous waste, diet, and pollutants; physical stressors like water temperature, light and sound intensity and duration, levels of dissolved gases in the water body; biological stressors like species variation, stocking density and both presence and virulence of pathogens; and finally, management or husbandry stress factors like handling, disease management and biosecurity implementation (Rottmann *et al.*, 1992). Snieszko, 1976, described this interaction through the equation $H(A+S^2) =D$, where H= the species of host, A=aetiological agent, S=environmental stressors, and D= disease. The importance of environmental effect is seen through the squaring of S, showing how effect of this group of parameters increase geometrically as fish approach the limit of their adaptive capacity or when cumulative environmental stressors come into play (Plumb, 1994).

Fish health becomes compromised by effect upon the immune barriers of epithelial mucus layers, scale and skin integrity, inflammatory response, antibody production, and effects upon growth hormone, plasma testosterone, oestradiol, gonadotrophin and cortisol levels (Rottmann *et al.*, 1992; Sumpter, 1997). As examples: a study by Chen *et al.,* 2002, demonstrated the dramatic effect of cold stress on *Oreochromis aureus,* in terms of an increase in stress hormones (adrenalin, noradrenalin, and cortisol), as well as a reduction in leukocyte phagocytic activity and immunoglobulin production. Temperature stress, especially cold temperatures, has been shown to halve the activity of killer cells in the immune system, and sharp decreases are known to reduce antibody production (Rottmann *et al.*, 1992). Another study by Barcellos *et al.*,

1999, showed elevated plasma cortisol levels in response to both chronic high stocking density stress, as well as acute stress, with resultant lower growth rates. Physiological increase in fish thrombocytes have also been demonstrated under stressful conditions, with potential physiological impacts (Wedemeyer *et al.,* 1976.b).

2.3 ASSESSING FISH AND SYSTEM HEALTH

Sub-optimal fish health is a nebulous state of compromised physiologic functioning, presenting with measurable parameters like increased morbidity or mortality levels, as well as less noticeable or subclinical states like slow growth, or poor reproductive performance (Macmillan, 1991). Because of the challenge of assessing these subtle and often unnoticed effects there is very little data available on their realistic economic impact (Macmillan, 1991). As a species, tilapia is believed to be more resistant to many common environmental and pathogenic influences, yet are not exempt from the effects of disease, water and husbandry related stress (Boyd, 2004). In addition, most fish pathogens have quick and direct effect upon their fish host and population because of the rapid transmission of disease through the water body. A large number of pathogens also have the ability to exist in an asymptomatic carrier state, making diagnosis and evaluation of their impact upon health very difficult (Huchzermeyer, 2015).

2.3.1 Euthanasia for disease diagnostics

Euthanasing fin-fish in a humane manner without compromising accurate disease diagnosis is challenging. Although chemical euthanasia is a humane and effective option, there are currently no drugs approved for finfish euthanasia (Leary *et al.,* 2013) and challenges exist with their use for disease diagnostic purposes. Noga, 2010.f, describes the dramatic effect of

parasite loss with use of chemical immobilization, and advocates rather “pithing” or cervical severance as alternatives to avoid a compromised parasite assessment for meaningful disease diagnostics. This is corroborated by other authors (Alvarez-Pellitero, 2008; Callahan and Noga, 2002; Lewbart, 1998.b). Rapid chilling with an ice slurry, alone, is considered an acceptable method of euthanasia for tropical and sub-tropical (warm water) fish and small bodied (below 3.8cm in length) finfish that cannot survive at 4 degrees Celsius or below (Leary *et al.*, 2016). Being poikilothermic, a reduction in water temperature not only rapidly reduces metabolism but also allows for easy handling and culling (Robb, 2008). Some authors also suggest that a reduction in body temperature may serve as an anaesthetic as physiological processes are slowed down (Wedemeyer *et al.,* 1976.e). Cervical transection or decapitation, as a mechanical alternative method, would usually be followed by pithing (destroying the brain tissue) in a two-step slaughter process to ensure death (Leary *et al.*, 2013; Leary *et al.,* 2016), but will have a negative impact on results if brain tissue is to be examined.

In all situations, minimal handling is important to reduce additional stress to the fish (Leary *et al.*, 2013). Useful indicators of death include: cessation of movement, loss of reaction to external stimuli, loss of muscular tone, no opercular movement or loss of eyeroll reflex (Leary *et al.,* 2013).

2.3.2 Fish diagnostics (Noga, 2010.f)

Assessing health of a fish requires a systematic assessment and can include a number of different tools, each offering a deeper layer of information. These can include an external physical examination, skin and gill biopsies, blood examination, faecal examination, necropsy,

histopathology, cultures, and molecular diagnostics, amongst others, and should be tailored to meet the end goal.

A thorough physical assessment should include a visual inspection for abnormalities like skin lesions, external macroscopically visible parasites, fraying of fins, exophthalmia, excess mucus on skin or gills, colour changes, abdominal swelling, morphometrics: length and weight, and overall condition of body.

Wet mount diagnostics are considered one of the most informative diagnostic tools in assessing fish health, with the skin scrape/ biopsy technique regarded as one of the most valuable and, in fact, together with a water analysis, can be diagnostic in many cases. Valuable information on ecto-parasite presence and burden, water mould infections, some bacterial infections, and even gas-supersaturation can be gleaned.

Visualization of non-septate filamentous hyphae on wet mount or H&E stained histopathology sections offers a presumptive diagnosis of *Saprolegnia* (Noga, 2010.e). Demonstration of asexual sporangia, characteristic of *Oomycetes* (Noga, 2010.e), and absence of an associated inflammatory response would further substantiate presumptive findings. Confirmation of this pathogen requires fungal culture in specialized low nutrient culture media.

There are many pros and cons to the various diagnostic protocols for bacterial and viral diseases, with conventional PCR techniques proving time-consuming, and some lacking sensitivity. qRT-PCR (real-time PCR) techniques offer increased range of detection, higher sensitivity and specificity, and reduced turnaround time, but are expensive and need real time PCR analysers. In addition, they are able to quantify the pathogen which is a useful feature in

sub-clinical carriers. Despite this, for most RNA-viral diseases, RT-PCR seems to be an approved molecular diagnostic tool in terms of the value it offers in quick and "sensitive-enough" diagnosis. However, in many instances the presence of antigen i.e. a positive RT-PCR reaction, does not always imply causality of disease nor does it differentiate it from carrier states or incidental presence (Bootland and Leong, 1999), which is a problem. qRT-PCR, despite its advantages, is yet to be validated by official global organizations. Viral isolation and serological identification are still considered the gold-standard protocol in terms of proof of viral presence and infectivity for viral disease. However, it is also expensive, labour-intensive, requires skill, and is time-consuming. LAMP (Loop-mediated isothermal amplification) is a useful tool to consider for rapid field detection of fish viruses, and is considered sensitive and specific (Biswas and Sakai, 2014).

In general, conventional culture and biochemical identification are still regarded as gold-standard testing for bacterial pathogens, despite the time constraints, labour and cost. qRT-PCR with sequencing of the 16S rRNA gene sequence (Keeling *et al.,* 2012*)* is another alternative. However, there are some exceptions. Although bacterial culture on TSA, 5% sheep blood or McConkey agar with replating and biochemical identification was the traditional method of identification for *Edwardsiella* spp. (Soto *et al.,* 2012), now a repetitive sequence mediated PCR is recommended for *Edwardsiella* spp. differentiation (Griffin *et al.,* 2016). A multiplex PCR test for *Aeromonas* spp*., Yersinia ruckeri , Flavobacterium columnaris* and *Renibacterium salmoninarum,* has also been developed, which is considered sensitive and specific enough for all pathogens (Altinok *et al.,* 2008). LAMP technology is increasingly being advocated as a practical, field-applicable, quick, sensitive and specific test (Saleh *et al., 2*008).

Gold nano-particle based assays are emerging as future technologies to be considered (Kumar *et al.,* 2015).

The gold standard test for *Streptococcus* spp. is still bacterial culture of brain or anterior kidney on blood agar, TSA, BHIA, or enriched Colombia agar and remains confirmative for this pathogen (Soto, 2015). Culture shows good sensitivity for subclinical infections. It requires follow up with biochemical identification or PCR techniques to differentiate to species level (M. Henton, personal communication, 17th August, 2016). Protocols for PCR are available in pre-cited articles. However, the techniques need to be validated and more specifically, specificity and sensitivity values are needed.

Lactococcus spp. are conventionally diagnosed still with bacterial culture and biochemical identification or fluorescent antibody techniques (Salati, 2011), but real time qPCR based on the 16S rRNA gene sequence is replacing this technique as the preferred gold standard test due to its rapid, sensitive and specific benefits. Definitive differentiation of the *Lactococcus* genus, is known to be extremely difficult, with culture and biochemical differentiation with the Clindamycin phenotypic test, time consuming and difficult. Conventional PCR can duplicate this quickly, within 5 hours, and detect carrier state, but lacks specificity and produces false positives (Zlotkin *et al.,* 1998). As another alternative, there is a multiplex PCR that has been developed for *Streptococcus agalactiae, S. iniae,* and *Lactococcus garviae* that can be used (Itsaro *et al.,* 2012). A non-lethal monoclonal antibody-based IFAT test also exists (Klesius *et al.,* 2006).

Diagnosis of *Flavobacterium columnare* bacterial infection is routinely performed with microscopy wet mounts of gills, demonstrating the typical "haystack" appearance of bacteria (Noga, 2010.e), or on H&E stained histopathology sections. Culture is considered difficult and requires a special low nutrient media (M. Henton, personal communication, 17th August, 2016; Huchzermeyer, 2015), with following biochemical tests or agglutination (Declercq *et al.,* 2013). PCR and sequencing of the 16s rRNA gene is a useful technology to provide a quick definitive confirmation within hours. Serological tools like ELISA and fluorescent antibody also offer rapid diagnosis (Panangala *et al.,* 2006; Speare e*t al.,* 1995)*,* with fluorescent antibody offering simultaneous detection of *Edwardsiella ictaluri* .

Although molecular diagnostic tests have been developed, including a multiplex PCR (Altinok *et al.,* 2008) for *Aeromonas hydrophila* infections, traditional bacterial culture on TSA or BHIA agar with biochemical identification is regarded as acceptable.

Presumptive diagnosis of *Francisella noatunensis* subsp*. orientalis* can be made on histopathology, with demonstration of granulomas in H&E stained tissue and the presence of gram-negative intracellular bacteria (Soto *et al.,* 2013). The gold standard test is still bacterial culture on specialised culture medium (cysteine heart agar with antibiotics: polymyxin B, and a source of iron) (Soto, 2015), but is costly and requires technical skill (M. Henton, personal communication, 13th September, 2016).

Visualization of the characteristic sporulating hyphae on H&E or Gomori-methenamine stained histopathology sections causing a deep branchial infection, often associated with base of the primary lamellae, are pathognomonic for *Branchiomyces* (Noga, 2010.e).

Definitive diagnosis of *Aphanomyces invadens* (Epizootic Ulcerative Syndrome) can be made either through identification of characteristic broad aseptate hyphae on histopathology sections of deep ulcers, with associated inflammatory response or through PCR techniques . Culture is challenging.

2.3.3 The influence of water quality

Poor water quality is widely considered the most common cause of fish mortalities and predisposing factor to secondary disease issues (Shelton, 2010).

Sub-optimal water parameters are directly implicated in fish mortalities, but more often act as a chronic stressor upon fish and disruptor of the fine balance between host, environment and potential pathogens (Boyd, 2017). In addition, the propensity of water parameters to fluctuate dramatically and rapidly, particularly in intensive fish culture, further contribute to stress, compromising immunity, and elevating the effect of pathogens and toxins in the water body (El-Sayed, 2006.b). Ultimately, the health and productivity of a population suffers.

2.3.3.1 Dissolved oxygen

Dissolved Oxygen (DO) is arguably the most important water parameter affecting immunity, health and growth of a fish (Boyd 2000.b; Noga, 2010.d; Swann, 1997). It is difficult and probably inaccurate to specify exact DO concentrations that are species-specific or system-specific, because of the significant number of factors involved in the physiological process of respiration and oxygen metabolism (Timmons and Ebeling, 2013.c). However, generally, fish grow well and maintain good health at DO levels exceeding 5mg/L (ppm) or 100% saturation (Timmons and Ebeling, 2013.c) and levels below will always carry negative impact on growth,

reproduction and physiology (Wedemeyer *et al.,* 1976.d). Tilapia have the capacity to withstand extremely low DO levels (Swann, 1997), even as low as 0.1-0.5mg/L for varying short periods (Abdel Magid and Babiker, 1975). However, in poorly managed systems, where DO is dramatically reduced by high water temperature (Becker and Fishelson, 1986; Franklin *et al.*, 1995), overstocking of fish, poor handling protocol (Ross and Ross, 1983), high phytoplankton levels, high organic load or inadequate supplementary aeration, the coping capacity of the fish, as well as the bacterial nitrification processes within the biofilter are often exceeded (Speare, 2008), with resulting direct impact upon fish health as well as water quality. In addition, borehole water may also naturally be low in dissolved oxygen (Petrie-Hanson *et al.*, 2004.a). Hypoxia has been known to induce acidotic conditions in fish, thought to be induced by release of stress-triggered catecholamines like noradrenalin (Fievet *et al.,* 1988) triggering lactate production (Wedemeyer *et al.,* 1976.b). This acidosis, in turn, reduces the oxygen- carrying capacity of the haemoglobin molecule, and further exacerbates the situation. In addition, low carbon dioxide (CO_2) assists in unloading oxygen off the haemoglobin molecule into tissues through the Bohr effect. Thus, if CO_2 concentration increases in the water, acidosis will be exacerbated. Hypoxic conditions also result in a physiological increase of the gill respiratory surface area, which in turn causes an increase in osmoregulatory load which poses further stress upon the fish (Wedemeyer *et al.,* 1976.b).

In addition, although fish may still actively feed when DO levels are suboptimal, nutrient absorption from the feed is compromised (Huchzermeyer, 2015), because the total metabolic rate and thus oxygen demand increases dramatically in the process of digesting feed: known as the specific dynamic action (SDA) of fish (Wedemeyer *et al.,* 1976.b).

Another aspect of dissolved gases to consider is the partial pressure of dissolved gases, which, according to Huchzermeyer, 2015, may possibly be one of the most neglected aspects of water quality affecting fish health. It can be an underlying predisposing factor in many secondary disease situations in cultured fish. Low grade on-going exposure to gas super-saturation is difficult to diagnose as there are so few clinical signs, but it is more likely to predispose to gas-bubble disease with visible macroscopic lesions and gas emboli developing in small blood vessels, than short periods of super-saturation (Boyd, 2000.b). Although total gas super-saturation, often from high water turbulence, is composed of nitrogen, oxygen, and to a lesser degree, argon and CO_2, nitrogen carries the greatest pathological impact upon fish (Speece, 2007). Oxygen super-saturation up to 200% is tolerated by most species, as excess oxygen is absorbed by haemoglobin in the blood (Huchzermeyer, 2015), so is less of a factor. Common underlying aetiologies in closed RAS systems include the pumping of water from deep boreholes, leaking pipes that result in a sucking of air, cavitating pumps, long lengths of piping, and venturis (Noga, 2010.e), as well as rapid heating of water and excessive algal or plant photosynthesis or respiration processes (Loh and Landos, 2011.e).

Table 2-1 Summarized effect of dissolved oxygen on tilapia health

21mg/L at 12^{o}C	Top threshold (Speece, 2007)
5mg/L-7mg/L or 100% saturation	Ideal for commercial production (De Long *et al.,* 2009; Noga, 2010.d; Swann, 1997)
1.5<5mg/L	Poor growth. Reduced feeding efficiency (Hollerman and Boyd, 1980). Slower growth (Andrews *et al.,* 1973). Increased stress and predisposition to secondary infection (Boyd, 2000.b; Scott and Rogers, 1980). Limited effect if maintained over 3mg/L (Boyd, 2004)
0.3<1.5mg/L	Significant effect on growth and disease resistance. Exposure over a few hours potentially lethal (Boyd, 2000.b)
0<0.3mg/L	High mortalities. Only small fish able to survive short exposure (Boyd, 2000.b)

Perhaps of more concern in cultured fish, are not so much situations of acute hypoxia, but more often the sustained chronic suboptimal levels, with their often inapparent effect on growth and health (Noga, 2010.d). It is important, also, to bear in mind the diurnal and nocturnal fluctuation of DO levels as photosynthetic and respiratory processes are respectively adding and removing oxygen from the system. It is of value to measure levels at least twice daily.

2.3.3.2 Carbon Dioxide

CO_2 is an underestimated water parameter, and tolerance of tilapia to CO_2 levels has not been well studied. It is an often ignored, poorly-monitored water parameter, because of lack of understanding about it and the challenges in measuring it (Southgate, 2005).

Most finfish grow optimally with CO_2 levels below 10mg/L (Southgate, 2005) and an upper limit of 15-20mg/L (Timmons and Ebeling, 2013). Warm water species are thought to be more tolerant, and tilapia are thought to be fairly resistant to high CO_2 levels, coping with levels up to as high as 72.6mg/L (Fish, 1956). According to Aquatic Network, 2012, CO_2 should be maintained under 20mg/L, yet, a study by Kaya *et al.*, 2016, showed that CO_2 levels as low as 14mg/L still had an impact on haematological parameters of fish, although not affecting mortality. Wedemeyer *et al.*, 1976.d, recommends levels under 3mg/L, and describes negative effects upon fish at 20mg/L. Noga, 2010.g, classifies CO_2 levels over 12mg/L as a "Hypercarbia". Loh and Landos, 2011.e, recommend keeping CO_2 levels below 6mg/L, with levels as low as 15mg/L being implicated in fish kills. However, they maintain that fish can generally tolerate levels up to 60mg/L. It is thus clear that the literature differs considerably on this matter and further studies are required to reach consensus.

CO_2 levels in aquaculture facilities are composed of and affected by exposure to the atmosphere, fish respiration, significantly by organic material (fish waste and uneaten feed) decomposition, as well as the level of zoo and phytoplankton in the water body. In addition, borehole water can have levels as high as 100mg/L (Timmons and Ebeling, 2013.c; Noga, 2010.g), so introduction of such water without use of holding tanks could undoubtedly significantly raise the water CO_2 levels within a system. It is also often seen in systems where liquid oxygen is used to aerate to support extremely high stocking densities (Noga, 2010.g). It is considered a dynamic parameter because of the on-going effect of photosynthesis and respiration activities. Although CO_2 has the ability to exercise significant physiological effect upon fish, Boyd, 2000.e, explains that generally environmental CO_2 levels are not high enough

to have the narcotic effect upon fish as seen with euthanasia methods, yet exercise dramatic effect upon the gills and respiration processes. Loh and Landos, 2011.e and Wedemeyer *et al.,* 1976.d describe anaesthesia occurring at CO_2 levels of 100mg/L and higher. High water CO_2 levels have a direct physiological effect upon the fish by reducing CO_2 loss by the fish, reducing oxygen-carrying capacity by the haemoglobin molecule, and causing a state of hypercapnia and acidosis to develop. Fish become less tolerant of low DO as they steadily develop more of an acidotic state, with associated lower affinity and ability to carry oxygen through the Bohr and Root effects respectively (Noga, 2010.g). Thus, a combination of low DO and high CO_2 is potentially more lethal (Plumb, 1994). Perry and Wood, 1989, however, propose that CO_2 alone has the capacity to affect ventilatory effort and volume, independently of water DO levels. The long-term effects of high CO_2 on fish are still poorly understood. An interesting association between high CO_2 and "nephrocalcinosis" has been shown in salmonids, where high CO_2 levels are thought to result in mineral (phosphorus and calcium) deposition in the excretory kidneys as a compensatory mechanism (Southgate, 2005). High CO_2 is also an additional stressor upon the fish with all the potential secondary immunosuppressive and disease repercussions (Southgate, 2005). Fish are even known to actively avoid areas of high CO_2 in the water body (Boyd, 2000.e; HACH®, 1999.c).

On site measuring with a titration method is recommended, although delayed testing can be carried out within 24 hours, providing sample containers are filled completely, not agitated and cooled to 4^{o}C (HACH®, 1999.h). Noga, 2010.g, advocates testing levels within 2 hours of collection as long as the sample is maintained below the temperature at which the water was

collected. Laboratory testing is considered complex and time delays potentially impact the readings (Southgate, 2005).

2.3.3.3 Nitrogenous compounds: ammonia, nitrite, nitrate

Elevated ammonia and nitrite levels are a common problem in intensively cultured tilapia in RAS systems, where the high stocking density required to maintain profitability, requires highly efficient filtration systems to effectively remove organic waste and facilitate conversion of toxic ammonia and nitrite, to the less toxic nitrate (Atwood *et al.*, 2001). Low DO levels within the system, further compound the problem by affecting the functioning and health of the aerobic *Nitrosomonas*, *Nitrobacter* and other bacterial species involved in the nitrification process within the biofilters. In addition, low DO levels result in suboptimal digestion and absorption of feed by the fish, with resultant higher organic waste levels with nitrogenous by-products (Wedemeyer *et al.,* 1976.d). Thus, low DO in a system often leads to secondary elevated ammonia or nitrite levels.

Ammonia

Fish respiration and organic matter decomposition are the primary sources of ammonia within an aquaculture system (Loh and Landos, 2011.e). Ammonia exists in a dynamic equilibrium between an ionized form (NH_4^+) which is non-toxic for fish, and an un-ionized form (NH_3/ NH_3-UIA), which is highly toxic for fish (Heath, 1995.b) because of its increased ability to permeate cell membranes (Timmons and Ebeling, 2013.c). There are many variables that affect the chemical state of the ammonia molecule, among others, the variable parameters of temperature and pH, with higher temperatures and pH facilitating the conversion of NH_4^+ to

NH_3 (Heath, 1995.b; Wedemeyer *et al.,* 1976.d). This results in daily fluctuation of total ammonia nitrogen (TAN), NH_4^+, and NH_3 levels (Boyd, 2000.c) and toxicity to fish. pH exerts the greatest influence on conversion between NH_4^+ and NH_3 (Loh and Landos, 2011.e), with a higher pH predisposing to higher NH_3 levels.

Great controversy exists in the literature regarding tolerance levels.

J.W. Meade (1985) quoted: "A truly safe maximum acceptable concentration of un-ionized or of total ammonia for fish culture systems is not known", yet many fish health professionals advocate that only a zero level can be considered acceptable (Shelton, 2010).

Taking into account the large species tolerance variance that seems to exist, and the good adaptive response of fish to higher NH_3 (Speare, 2008), Timmons advocates working on a TAN level of 1mg/L for chronic exposure, and aiming for a maximum NH_3 level of 0.025mg/L. Noga, 2010.d, proposes that NH_3-UIA levels over 0.05mg/L offer sub-lethal exposure with effect on growth and overall immune function and health, and mortalities start to appear from levels as low as 0.2mg/L, with dramatic effect at levels greater than 1mg/L. A study by Hargreaves and Kucak, 2001, showed that brief sub-lethal peaks in NH_3 had little effect on tilapia performance, yet another study by El-Shafey, in 1998, demonstrated that sub-lethal NH_3 levels had the capacity to reduce the oxygen saturation level in arterial blood and induce a state of alkalosis, with potential detrimental health impact. High water ammonia levels have been shown to cause a hyperammonaemia within the fish tissues and fluids, raise the blood pH, increase water uptake, and disrupt metabolic function, all with resultant increased oxygen requirements, and pathological impact on tissues, particularly targeted towards the gills, suppressed immune

function and increased risk of disease (Boyd, 2000.c). Renal failure can result from stress upon kidneys and the high tissue ammonia levels can induce cytopathic or neuropathic effect (Loh and Landos, 2011.e). Loh and Landos, 2011.e, suggest that levels as low as 0.2mg/L can lead to gill injury and impaired gaseous exchange, with high mortalities having been recorded at such readings.

High NH_3 levels, in addition, inhibit bacterial nitrification of nitrite to nitrate, within the biofilter (Loh and Landos, 2011.e)

Typical clinical signs associated with high NH_3 levels would include gill hyperaemia, "piping" behaviour at the water surface or around inlet points, increased opercular movements, and non-specific signs of stressed fish like increased skin mucus and darkening of skin (Loh and Landos, 2011.e).

Thus, for production purposes, it is suggested to keep NH_3-UIA below 0.05mg/L.

Nitrite (NO_2^-)

Nitrite is an extremely important parameter to assess especially with recirculating systems, where improper nitrification within the bio-filter, results in a rapid accumulation of nitrite in the system. Nitrite is considered highly toxic to fish, with high levels affecting their physiology, inducing a state of functional hypoxia in the form of methemoglobinemia and impaired growth (Boyd, 2000.c). Toxicity is exacerbated with low temperature, low DO and pH beyond range tolerance, and is also affected by chloride and calcium levels in the water (Boyd, 2000.c). Nitrification slows down at temperatures below 25^oC and pH levels out of the 7-8 range (Boyd, 2000.c). Tolerance levels also vary according to fish size, species, previous exposure, and overall

health and immune status (Shelton, 2010). Because of this, again, it is difficult to specify exact levels of toxicity for fish.

Some references maintain that nitrite should ideally be maintained below 0.3mg/L (Aquatic Network, 2012), or 0.2mg/L (Loh and Landos, 2011.e). Symptoms of lethargy and anorexia may already be apparent around 0.5mg/L, yet levels as low as 0.1mg/L can induce methemoglobinemia (Huchzermeyer, 2015). An article by Wedemeyer and Yasutake, 1978, describes methemoglobinemia in sunfish at levels as low as 0.015-0.060mg/L. Levels over 1.6mg/L are believed to cause death (Loh and Landos, 2011.e), yet the author has seen Koi carp exhibiting normal swimming and feeding behaviour at levels greater than 1mg/L. Many aquaculturists advocate aiming for zero nitrite levels. In addition, high nitrite may also exercise a toxic effect upon leukocytes, with resultant immunosuppression and susceptibility to secondary infections (Speare, 2008). High nitrite levels can also be associated with biofilter bacterial die-off (*Nitrobacter -, Nitrococcus* - and *Nitrospira* spp.) from sudden water temperature changes, or low prevalent DO levels (Huchzermeyer, 2015) dropping below 2mg/L (Loh and Landos, 2011.d). The toxic impact of nitrite upon fish also increases with size and becomes of increasing significance as fish are grown out (Atwood *et al.*, 2001).

Nitrate (NO_3^-)

High NO_3^- levels in the water body are generally considered to be of less concern than NH_3-UIA or nitrite, and held to be less toxic. However, there is suspicion that the combined chronic exposure of high nitrate levels, coupled with concomitant increased algal growth feeding on the nitrate, and reduced buffering capacity can act as a chronic stressor upon fish (Shelton, 2010)

and predispose to compromised immune function and disease resistance, as well as slower growth (Loh and Landos, 2011.e). Slow accumulation in older systems, is a common phenomenon and high nitrate readings are often an indication of poor underlying husbandry with inefficient waste removal from the system (Loh and Landos, 2011.e).

Tilapia are considered to be more tolerant of high nitrate levels than many other fish species, and a study by Broders *et al.*, 2005, demonstrated ability to survive and grow well in water with nitrate levels up to 27mg/L. Loh and Landos, 2011.e, suggest an optimum range of 20-60mg/L for freshwater fish, with an upper limit of 100mg/L.

2.3.3.4 Temperature

As with all poikilothermic animals, temperature plays a dramatic role in regulating metabolic processes and affects feed intake and growth (Loh and Landos, 2011.e), as well as many physiological responses, including immune function (Bly and Clem, 1992).Temperature is considered one of most important key factors affecting the balance between host and environment, and resulting metabolic, reproductive and growth potential of tilapia (Ellis *et al.*, 1978 ; El-Sayed, 2006.b). Although aquatic temperature fluctuations are less dramatic than in terrestrial habitats, when it does occur, fish are limited in their ability to escape from such challenges (Wedemeyer *et al.,* 1976.a). One of its most significant impacts lies in its inverse relationship with dissolved oxygen availability in the water body. In addition, potential impact of toxins and ammonia in the water body is enhanced under the influence of lowered temperature, with added challenge upon the fish population (Loh and Landos, 2011.e).

Various temperature ranges are suggested for *Oreochromis* spp. to support rapid growth and maintain health, but tolerance varies dramatically according to species, the individual adaptive response, life stage, breeding status, genetic adaptation to previous geographical locations (Smitherman, 1987) and other factors. *O. mossambicus* are generally more cold-tolerant than *O. niloticus* (James, 2014). Aquatic network, 2012, recommends maintaining tilapia in a range between 26.7 to 37.8^{o}C with optimum growth occurring between 27.8 and 29.5^{o}C. Watanabe *et al.*, 1993, demonstrated peak performance at 27^{o}C, while Boyd, 2004, suggests an optimal range of 28-32^{o}C. A study conducted on *O. niloticus* fry (El-Sayed and Kawanna, 2008) demonstrated a doubled growth rate at 28^{o}C, compared to 24 or 32^{o}C, clearly highlighting the dramatic impact of temperature on fish growth, as well as the value of optimizing, to the exact degree, the water temperature, and not just maintaining within a broader range (Wedemeyer *et al.,* 1976. d). Tilapia, as a species, are considered more heat tolerant than cold tolerant (El-Sayed, 2006.b). However physiological tissue injury is more likely to occur close to the upper lethal temperature limit, with higher metabolic tissue demand, and lower oxygen saturation levels, rather than lower, where an anaesthetic-type effect develops, and metabolic processes slow down (Wedemeyer *et al.*, 1976.b). Increased bacterial activity and lowered immune function with high temperatures, further compound risk of disease (Wedemeyer, *et al.*, 1976.b). A study by Amoudi *et al.*, 1996, demonstrated a better tolerance capacity of *Oreochromis* spp. to higher temperature shock, than lower temperature shock, which has more bearing and relevance to local conditions in South Africa, where winter temperatures often plummet rapidly. Low temperatures are known to significantly suppress antibody production

and the immune response (Ellis, 1978; Wedemeyer *et al.,* 1976.b) but the threshold of immune response failure is variable among species.

In addition, sudden reduction in temperatures also has effect upon the metabolic rate of the bacteria within the biofilter, with reduced nitrification efficacy (Speare, 2008).

Findings on the effect of low temperature on fish health can be summarized as follows, clearly demonstrating the progressive loss in appetite, growth, reproductive performance and secondary disease with decreasing temperatures:

Table 2-2 Summarized effect of temperature on tilapia health

Below 23.9^{o}C	Appetite suppression (Aquatic Network, 2012)
Below 20^{o}C	No breeding and significant reduction in feeding (El-Sayed, 2006.b; Towers, 2005)
Below 17^{o}C	Lethargy (Aquatic Network, 2012). No feeding (Boyd, 2004). No growth (Towers, 2005). High disease risk
9-12^{o}C	Extremely high disease risk. Death (Aquatic Network, 2012; Boyd, 2004; James, 2014)

Hatchery management did not form part of this study, but it is worth mentioning that water temperature has also been shown to be a factor affecting sex ratios (Baroiller *et al.*, 1995).

Higher water temperatures can also be problematic in terms of increasing the toxicity of certain pollutants, like heavy metals (Munro, 1978) as well compounding the effect of low DO, suppressing immunity and stimulating pathogen proliferation (Wedemeyer, *et al.,* 1976.e; Whittington and Chisholm, 2008). To avoid the impact of excessively high water temperatures,

husbandry practices like maintaining water depth at 2-3 meters or ventilating tunnels, then become of great importance in offering a measure of control or providing a refuge from extreme temperature (Atwood *et al.*, 2001). Chronic hypo or hyperthermia will trigger the stress, immunosuppression, and poor health cascade (Shelton, 2010).

2.3.3.5 pH

pH is defined as the negative logarithm of the hydrogen ion concentration (Boyd, 2000.e). Although any extreme pH levels can severely stress fish health, its greatest impact lies in its interactions with other variables, most importantly the conversions of ammonia and nitrite between ionized and non-ionized states, as well the buffering capacity of the water, rather than the pH itself (Wedemeyer *et al.,* 1976.d).

Most aquatic animals are believed to tolerate pH levels between 6.5 and 9 (Timmons and Ebeling, 2013.c), and most freshwater aquatic species are believed to achieve optimal health and growth within this range (Boyd and Tucker, 1998). Aquatic Network, 2012, suggests 7-7.5 as an optimum. pH has a diurnal curve, peaking in late afternoon because of photosynthetic consumption of CO_2. It is lowered by microbial decomposition processes in the water. One of the biggest risks concerning pH in aquaculture, comes not so much from the ambient pH, but more often dramatic pH swings by more than ½ unit/day in a poorly buffered system, or transport shock from transferal between two systems of significantly differing pH (Loh and Landos, 2011.e). Low pH is believed to stimulate mucus production in gills with resultant respiratory and osmoregulatory dysfunction, as well as potentiate toxic effect of heavy metals

(Boyd, 2000.e), while high pH's are linked to gill pathology like goblet cell hypertrophy, gill epithelial separation, and ocular pathology (Boyd, 2000.e).

2.3.3.6 Total Alkalinity (TA)

We define this as the buffering capacity of the water, and it is essentially, a measure of the total levels of titratable bases like carbonates, bicarbonates, phosphates, sulphates and to a small degree the hydroxides and borates (Swann, 1997), with carbonates and bicarbonates being the largest component and most important (Timmons and Ebeling, 2013.c). Calcitic limestone and dolomitic limestone serve as the primary source of alkalinity in water, and dissolution in the water body depends largely on a threshold level of dissolved CO_2 being present (Boyd, 2000.e). Where the alkalinity is low, pH can fluctuate dramatically because of the effect of respiration overnight, producing CO_2, and photosynthesis in the day, consuming CO_2 and carbonic acid.

Alkalinity levels below 20mg/L (Loh and Landos, 2011.e, propose levels as high as 55mg/L) can cause dramatic pH swings through the 24-hour cycle, with fluctuations seen between pH's of 6 and 10 (Wurts, 2002). Although most aquatic species are tolerant of varying alkalinity levels, this degree of fluctuating pH acts as a significant stressor. Although a TA of 50mg/L is considered acceptable, values over 75mg/L are preferred (Wurts and Durborow, 1992). Loh and Landos, 2011.e, recommend an ideal range of 60-80mg/L for freshwater fish species. Boyd has found lower productivity in waters with alkalinity below 50mg/L and above 200mg/L (Boyd, 2000.e; Petrie-Hanson and Hanson, 2004.b). Levels over 800mg/L are considered lethal (Loh and Landos, 2011.e).Wedemeyer *et al.,* 1976.d, recommend a lower limit of 20mg/L for chronic exposure. In addition, total alkalinity fulfils an additional role as an important energy source for

the biofilter nitrifying bacteria (Loh and Landos, 2011.e), in their elimination of ammonia and nitrite, and low levels in water often reflect chronic removal through nitrification processes in the biofilter (Loh and Landos, 2011.e).

2.3.3.7 Complete Hardness (CH)

We define complete hardness as the total value of divalent salts, with calcium and magnesium forming the largest component thereof, and trace amounts of iron, copper, zinc and lead contributing (Wedemeyer *et al.,* 1976.d). Composition is largely related to area geology (Loh and Landos, 2011.e). Levels of calcium are of particular importance in terms of impact upon fish osmoregulation (Wurts, 2002). Soft water is generally categorized with a hardness of 0-60mg/L, moderately soft with 60-120mg/L, hard as 120-300mg/L, and very hard as over 300mg/L (Boyd, 2000.d; Munro, 1978), but variants exist. CH levels over 30mg/L are desirable for freshwater systems, with hard water (100-150mg/L) being a preferred range (Shelton, 2010). Loh and Landos, 2011.e, suggest levels closer to 150mg/L are preferred. Low CH levels have been related to "Hole-in-the Head" disease in Cichlids, while high levels show association with zinc-deficiency cataracts and nephrocalcinosis (Loh and Landos, 2011.e). Low CH levels would also potentiate the effect of toxins like heavy metals, through low competition for calcium sites as well as reduced complex formation between carbonates and the heavy metals (Olsson, 1998).

2.3.3.8 Hardness to Alkalinity ratio

De Holanda Cavalcante *et al.,* 2014, emphasizes the importance of not only assessing hardness or alkalinity individually, but looking at the CH to TA ratio as well. Even in the presence of an appropriate CH or TA, one can have an improper ratio between them. In their study, ratios

below 1 were shown to result in high afternoon pH spikes, often exceeding 9, with potential dramatic stress effect upon fish, while ratios over 1, but particularly over 5, were shown to cause osmotic stress to the fish. De Holanda Cavalcante *et al,* 2014, advocates an ideal ratio of 1:1. Both ratios below 1 or over 5 predispose to elevated TAN levels in the water body, with its suppressive effect on fish appetite and feeding. This in turn, further contributes to increased organic load and worsens the nitrogenous load in the water body.

A higher CH:TA ratio reflects a high amount of sulphate and chloride in the water body, in comparison to the carbonate and bicarbonate amount, while a low CH:TA ratio points to a large amount of sodium and potassium compared to calcium and magnesium (Boyd, 2000.d). Harder waters, with resulting higher ratios, have been shown to have greater impact on grow-out fish feed conversion rate (FCR), while higher alkalinity seems to exert its greatest impact on larval stage fish (De Holanda Cavalcante *et al.,* 2014).

2.3.3.9 Therapeutic chemicals

Use of chemicals and antibiotics is rare in tilapia aquaculture (Boyd, 2004).

2.3.3.10 Hydrogen Sulphide (H_2S)

Formation of hydrogen-sulphide in aquaculture units, is indicative of anaerobic pockets in the water, where sulphates become reduced to sulphides. It is often associated with build-up of organic matter on the pond/tank base, with associated increased rate of decomposition seen often in warmer water temperatures. It is highly toxic to fish and appears to interfere with respiration and cause a functional hypoxia (Noga, 2010.g). Levels can be measured in the water body, but more often can simply be detected by the characteristic rotten-egg sulphurous smell.

2.3.4 The influence of nutrition

Sub-optimal nutrition often presents with little evidence other than signs of inappetence or poor body condition or growth (Noga, 2010.g), and diagnosis can often be complicated with secondary debilitating disease, like high parasite burdens, associated with general immunosuppression and poor health. On necropsy, such fish normally have low visceral fat, and distended gall bladders.

Tilapia should be fed multiple times in the day because by nature they are continuous feeders, and also have limited stomach size (Boyd, 2004). However, this needs to be tempered by water temperature, DO levels and metabolic activity of fish, to avoid feed not being consumed and contributing to organic waste build-up (Noga, 2010.g). Knowing the tank biomass is extremely important in order to calculate correct feed volumes, and avoid overfeeding or underfeeding. Fish also avoid pockets of water where CO_2 is high or DO low and feed may accumulate there (Huchzermeyer, 2015).

It is extremely important to ensure that feed quality is of a high standard. Excessively high carbohydrates are known to suppress feeding (possibly from a suppressive effect on fish appetite or increased toughness of pellets), increase visceral fat (Noga, 2010.g), and reduce growth (Hawkins *et al.*, 2002). High protein quality is important to stimulate appetite and encourage good growth (Huchzermeyer, 2015). On the other hand, feed excessively high in protein can lead to other sequelae like renal mineralization (Wedemeyer *et al.,* 1976.d). The correct ratio of carbohydrate and protein preferentially directs protein usage into growth rather than for energy purposes (Wedemeyer *et al.,* 1976.b) but prevents excess glycogen

deposition in livers and fatty degeneration. Known protein requirements for different tilapia species are still inconclusive and depend on age of fish, type of protein used as well as the carbohydrate to protein ratio (El-Sayed, 2006.c).

Fish generally have a high amino acid dietary requirement (Huchzermeyer, 1993) and deficiencies are common, due to poor feed composition or oxidation (Huchzermeyer, 1993).

Lipid in feed is important for grow out fish, as an energy source and a source of essential fatty acids (Huchzermeyer, 2015). Low levels may inhibit growth (El-Sayed, 2006.c). Although fatty acid dietary requirements of tilapia are still uncertain, research with diets low in omega-3 have been shown to significantly slow growth and reproductive performance (El-Sayed, 2006.c). Deficiencies of essential fatty acids in the diet have been associated with fatty infiltration of the liver, and reduced capacity to cope with other stressors (Wedemeyer *et al.,* 1976 d). Chronic essential fatty acid deficiency results in reduced weight gain (Gatlin, 2008; Huchzermeyer, 1993). Higher demand for linolenic acid (omega-3 fatty acid) is associated with colder water fish, and may be a requirement of tilapia farmed in cooler areas (Huchzermeyer, 2015), as well as clear water systems. Waters higher in zoo and phytoplankton provide some of the omega-3 requirements.

Oxidation of unsaturated fats in the feed is a common sequel to improper storage where feed is exposed to heat or moisture, resulting in increased anti-oxidant demand, often manifesting as hepatic lipofuscinosis (Evenson, 2006).

Many mineral imbalances in the feed are known to reduce growth and FCR (Hawkins *et al.*, 2002; Noga, 2010.g). Vitamin levels in feed are also of paramount importance with deficiencies

of many vitamins adversely affecting growth, health and fish FCR in various ways. Of particular importance are the inclusion of antioxidants like Vitamin E in the feed to reduce oxidation of lipids and facilitate erythrocyte maturation, and the addition of Vitamin C, with its multiple beneficial properties boosting growth, reproduction, wound healing, reduced stress response and disease resistance. Deficiencies of this vitamin are also known to result in cartilaginous deformities (Huchzermeyer, 2015). Low levels of Vitamins E and C, Selenium, and incorrect amino acid ratios, together with feed high in oxidized lipid, all predispose to an inefficient antioxidant system, with secondary lipofuscinosis, anaemia, poor growth (Evenson, 2006; Speare, 2008) and often permanent hepatic injury (Gatlin, 2008; Hawkins *et al.,* 2002). Deficiencies of vitamin D have been associated with poor growth and production and impaired calcium homeostasis (Loh and Landos, 2011.f). Excessive levels of calcium and magnesium have also been linked to renal tubule mineralization (Loh and Landos, 2011.e).

2.3.5 The influence of stocking density

This is defined as the mass of fish that can be accommodated per unit volume of a tank or system (Timmons and Ebeling, 2013.d). It is considered the most important determining factor in intensive aquaculture systems (El-Sayed, 2006.a; Wedemeyer *et al.,* 1976.e). Because of the extensive number of variables that come into play when determining the ideal stocking rate of any system, which include design, water quality, species of fish, management practices, additional disease stressors, available quantity and quality of nutrition, age of fish and more, there is no “ideal” stocking rate, and research to date has yielded confusing results (El-Sayed, 2006.a). Stocking density is broadly determined by oxygen requirements of the population,

efficiency of waste removal from system, filtration capacity and also the level of aggression between fish (Huchzermeyer, 2015).

Poor management of stocking density is often reflected in systems where there is no breeding management, with resultant mixed male and female populations. This results in a rapidly expanding population with resultant stress, competition for feed, and a harvest of small fish (Boyd, 2004). Cannibalism is another common sequel to high fish densities (Huchzermeyer, 2015). Competition for feed can also result in physical trauma to fins and eyes, predisposing to secondary infections (Huchzermeyer, 2015), however, an interesting phenomenon was observed in Salmon, where a lower stocking density predisposed to increased territorial behaviour and aggression (Speare, 2008), thus social behaviour patterns of fish also need to be considered.

Approaches have been designed whereby stocking density can be calculated based upon average fish length (Tidwell, 2012; Timmons & Ebeling, 2013.d), with Timmons and Ebeling calculating mass of fish stocked per unit volume as: average fish body length divided by a factor of 0.24 (tilapia spp.).

Although RAS systems have the capacity to stock fish at extremely high stocking rates, this comes with the cost of smaller fish, often differing sizes, increased strain upon filtration systems to maintain water quality, and increased stress and risk of disease in fish (El-Sayed, 2006.a).

2.3.6 Ecto-parasites: both indicator and pathogen

Although the interplay between host, parasite and environment cannot be overemphasized, parasites as a factor, particularly the protozoals, play a significant contributory role in many disease outbreaks and should not be underestimated (Macmillan, 1991). In fact, parasites account for annual global production losses in the aquaculture industries, estimated to amount to between 62 to 175 million US dollars, excluding mortalities from parasite-induced secondary infections (Shinn *et al.*, 2015). Not only do they reflect a compromised immune barrier and health, but contribute to further health deterioration and challenge upon the host fish.

Overcrowded conditions, commonly associated with intensive systems, with associated stress and trauma to fish, predispose to a proliferation of parasites and disease (Reed *et al.*, 2009). High numbers of parasites, in turn, exert their own virulent effect upon the host fish, largely through their modes of attachment and feeding behaviour (Macmillan, 1991). As a result of the lack of keratinization in fish skin, it is an organ that is easily injured and once the immune barrier is compromised, is further targeted by secondary infectious pathogens esp. fungal and bacterial. Heavy parasite infestation of gills, with associated pathology, potentially compromise vital physiological processes like respiration, osmoregulation, acid-base balance, and excretion of ammonia (Loh and Landos, 2011.c). Tolerance to suboptimal water quality conditions, especially low DO, is also adversely affected.

2.3.6.1 The Ciliophorans

Ambiphrya and *Epistylus* are sessile peritrichs, are associated with organic-polluted water, and colonize skin and gills where the barrier is already compromised (Colorni, 2008). Concurrent

bacterial disease may often be implicated (Esch *et al.*, 1976; Miller and Chapman, 1976). Although primarily using the fish as attachment substrate from which to feed from the surrounding water body, high numbers irritate the fish and further damage the skin, as well as interfering with osmoregulatory, excretory and respiratory function. They are extremely significant parasites within the intensive environment, due to their rapid reproductive rates, modes of direct transmission and lack of species specificity and can overwhelm an entire fish population literally within days (Colorni, 2008). In addition, they may predispose fish to secondary bacterial or fungal infections (Colorni, 2008).

Trichodinids are common commensals of fish and, in low numbers, cause little pathological damage or gill interference (Colorni, 2008). Stress, compromised immunity and ill health, stimulate their proliferation, and the high numbers cause severe irritation to the fish and epithelial injury through their adhesive attachment as well as direct feeding on epithelial cells (Paperna, 1996.a).This, in turn, results in increased mucus production, hyperplastic epithelial responses, and haemorrhage (Colorni, 2008), predisposing to secondary bacterial infection (Lio-Po and Lim, 2002). They are known to commonly occur with monogeneans (Barker *et al.*, 2002; Colorni and Diamant, 2005) with proposed synergistic effect and are also believed to be often associated with suboptimal water quality (Huchzermeyer, 2015), stress, overcrowding and poor nutrition (Loh and Landos, 2011.h).

According to Basson and Van As, 2006, any ecto-parasitic ciliophoran outbreak, points to an imbalance in the host-parasite-environment equilibrium, with resulting compromised fish immunity and health, and always warrants a deeper investigation into all involved factors.

2.3.6.2 Flagellates

Ichthyobodo necator complex is a flagellated obligate fish parasite, with a free-swimming stage and feeding stage which attaches to fish skin and gills. It is often associated with significant tissue injury, low-grade continual mortalities, with resulting high economic impact (Alvarez-Pellitero, 2008; Macmillan, 1991; Shinn *et al.*, 2015). The feeding stage is attached by a proboscis-like appendage, that deeply enters the host cell. Thus, even low infections cause severe irritation to the fish, increased mucus production, epidermal sloughing, hyperaemia and ulceration of the skin (Loh and Landos, 2011.h). Associated gill changes include hyperplasia, secondary lamellae fusion, and even necrosis (Lom and Dykova, 1992). It has a predilection for fry to juvenile stage (Shinn, 2016). Infestations are often associated with poor water quality, with high suspended solids and overcrowding (Shinn, 2016). *Ichthyobodo necator* complex can only be introduced into a closed system via fish introductions or from natural waters (Huchzermeyer, 2015).

2.3.6.3 Monogeneans

Although monogeneans are rarely implicated in wild fish disease and occur naturally in low numbers in healthy populations (Whittingdon and Chisholm, 2008), they are potential harmful parasites within the warm-water intensive culture system because of their rapid direct life cycles. High reproductive rates can occur in systems where high fish stocking densities and provision of artificial substrates for eggs further facilitate their proliferation and transfer between hosts (Whittingdon and Chisholm, 2008). Under these high-density intensive systems, multiple other stressors increase host susceptibility to parasitic infestation. Under such

circumstances where numbers proliferate, they are thought to damage host epithelium through their highly sclerotized haptor attachment structures, their mode and level of attachment, and feeding behaviour on host epithelial cells (Whittington and Chisholm, 2008). Secretions from posterior glands and proteolytic pharyngeal or gut digestive enzymes are further believed to stimulate a local host immune response (Whittington and Chisholm, 2008). They are known to stimulate epithelial hyperplasia and mucus production. These actions can cause host debilitation, with predisposition to secondary pathogen invasion (Lio-Po and Lim, 2002). Their possible role as vectors for bacterial or viral pathogens has been suggested, but requires further investigation (Cone, 1995).

The two monogenean orders Gyrodactylidea and Dactylogyridae can be differentiated based on the presence or absence of eyespots, presence of embryos or eggs within the adults, haptor attachment structural differences, and preferred areas of attachment and feeding on fish (Reed *et al.*, 2009). Gyrodactylidea shows a predilection for skin and fins, an absence of eyespots, a pair of large anchors on the opisthohaptor, and adults often contains a mature embryo. Dactylogyridae, on the other hand, have two pairs of eyespots, one to two pairs of attaching anchor hooklets, adults often carry eggs, and they show a predilection for gills, except for juvenile stages which can often be found on skin as well. The most common Dactylogyridae group associated with tilapia are the *Cichlidogyrus* spp. Monogeneans in a system would have had to be introduced from infested fish or from natural waters (Huchzermeyer, 2015).

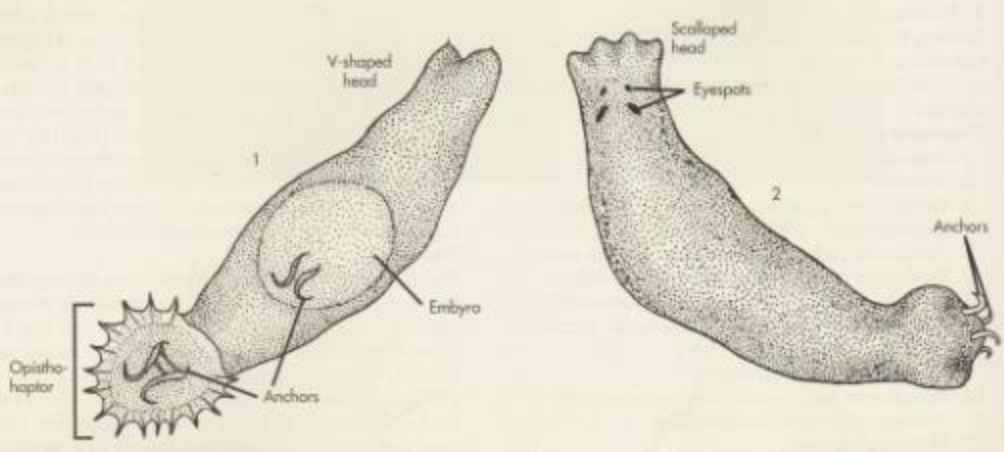


Fig. 20-1: Comparison of key diagnostic features differentiating gyrodactylid (left) and dactylogyrid (right) monogeneans (Noga, 2010.e)

Parasitic crustaceans like *Lernaea* spp. and *Argulus* spp. are important aquaculture pathogens but their impact in warm-water systems is still largely unknown. *Lernaea* are thought to cause gross epithelial injury at sites of attachment and predispose to secondary invaders. They are believed to either feed on host blood or cells. *Argulus* spp. attach and feed on the mucus, blood (Loh and Landos, 2011.h), and epithelial layer of the fish, possibly secreting toxins, damaging deep skin layers, and gills and also predispose to secondary invasion. Population mortalities can be high (Lio-Po and Lim, 2002). *Argulus* spp. has been shown to act as a viral vector of "Spring Viraemia of Carp" and "Carp Pox" (Lio-Po and Lim, 2002; Loh and Landos, 2011.h). They are easily visually demonstrated macroscopically or on wet mounts.

2.3.7 Other disease conditions

Digenean trematodes, cestodes and nematodes are uncommon in cultured fish (Noga, 2010.c; Shinn, 2016). Their impact in aquaculture is largely unknown still and there is no link with large scale mortalities (Lio-Po and Lim, 2002).

Tilapia, especially *O. niloticus* are considered resistant to Epizootic Ulcerative syndrome (EUS) (Huchzermeyer, 2015; Lilley *et al.,* 1998). EUS syndrome was surmised to have multiple pathogens as causes, including *Aeromonas hydrophila, Aphanomyces invadens* and a rhabdovirus (Lio-Po and Lim, 2002), but *Aphanomyces* is now believed to be the sole aetiology, and just often associated with secondary pathogens. Outbreaks are associated with lower water temperatures. Affected fish are thought to always present with clinical signs and carrier status is unlikely (OIE, 2018).

Aeromonas hydrophila is considered a ubiquitous facultative bacterium and is probably the most common bacterial disease of freshwater fish. It is associated with stress, most commonly hypoxia, overcrowding, high organic load and high levels of suspended solids (Noga, 2010.b). It can manifest as acute disease with high mortalities, as well as chronic disease. Conflicting reports on the predisposing temperatures exist with Ibrahem *et al.,* 2008 and Noga, 2010.b, suggesting higher rates of infection in warmer months, and others associating the infection with cooler temperature-stress and associated immunosuppression. Pre-existing ecto-parasitic disease, with associated immune barrier compromise, predispose (Wedemeyer *et al.,* 1976.c).

Saprolegnia is ubiquitous in the environment but considered a very important pathogen and invader, secondary to primary disturbance of the skin mucus layer (Noga, 2010.e). It appears clinically as cotton-wool like tufts on fish skin and fins, erosive and ulcerated lesions, gill necrosis, and even mortality as it becomes systemic (Loh and Landos, 2011.h).

Flavobacterium columnare is considered an economically important pathogen because of the effect it has on carcass appearance in terms of ulcerative skin and fin erosions, as well as high

morbidities and mortalities. In the channel catfish industry, it is the second most prevalent bacteria, resulting in massive economic loss each year (Declercq *et al.,* 2013). It exists ubiquitously in the environment, as well as in fish in both active infection and carrier states, with heavy gill shedding post mortality. It seems to prefer harder water with high pH and organic load (Fijan, 1968) as well as higher temperatures. Higher stocking rates predispose (Wedemeyer *et al.,* 1976.c). It causes severe necrotic gill pathology as well as typical "saddleback" shaped skin erosions, which are often suggestive of the disease. Microscopically, hyperplastic changes are also often evident on gill lamellae (Declercq *et al.,* 2013).

Branchiomyces is a rare but economically devastating disease. Although, largely a disease of common carp (Paperna, 1996.b), it has been isolated in Egypt in Nile tilapia in 2014, where fish were exposed to sub-optimal water parameters including elevated ammonia, nitrite and organic matter (Khalil *et al.,* 2015).

Edwardsiella piscida and *Edwardsiella ictaluri,* are globally seen as normal water inhabitants and opportunistic pathogens (E. Soto, personal communication, 9th March, 2016).

Yersinia ruckeri has been isolated in Africa (Egypt) (Eissa and Moustafa, 2008) and trout in South Africa in 1985. Tilapia are thought to act as carriers. It is a ubiquitous bacterium, found in moribund fish, healthy carriers, biofilms, birds and invertebrates. Stress in fish is regarded as a trigger for clinical disease and spreading of the pathogen. A faecal-gill transmission is suspected, as well as vertical transmission through eggs (Kumar *et al.*, 2015).

Francisella spp. infections are associated more with colder water temperatures (Soto *et al.*, 2012) and it is a major predisposing factor, with high mortalities seen at 25°C, yet none at 30°C.

Chronic subclinical low-grade infections are very likely and are known to cause anorexia, yet have little impact on growth and feed conversion rates (Soto, 2015). It is considered a very important pathogen of tilapia (E. Soto, personal communication, 9th March, 2016). They are known to persist in the environment and possibly reside in aquatic protozoans (Soto *et al.*, 2013).

Streptococcus iniae and *Streptococcus agalactiae* are also very important pathogens in tilapia aquaculture, with higher temperatures predisposing to Streptococcal disease. Co-infection with both is very hard to manage (Soto, 2015). Although it can manifest acutely, it tends to more often present as chronic disease (Plumb, 1994).

Epitheliocystis is considered a serious emerging skin and gill disease of aqua-cultured fish (Blandford *et al.,* 2018), with commercial impact. Little is still known about it, but it is believed to be caused by intracellular bacterial pathogens with its most significant pathological effect, the formation of cysts in the gill filaments, resulting in architectural disruption, fusion of secondary lamellae and an adverse effect on gill function. Although previously believed to be caused by *Chlamydia* spp. alone, many bacterial pathogens now are postulated to be involved, often specific to the host fish. Stressors upon the host fish are believed to be a significant contributing factor to the development of the disease (Blandford *et al.,* 2018). Ciliate protozoans are advocated as possible vectors for the disease (Padua *et al.*, 2015).

2.3.8 Pathology

2.3.8.1 Gill Pathology

Causes of changes to gill structure can range from physical irritations like algae or parasites to many chemical influences including low DO and toxins in the water.

Hyperplastic responses are non-specific and have been commonly associated with poor water quality, exposure to toxicants, high parasites levels and nutritional deficiencies (Reimschussel, 2008). Water-related factors like high NH_3-UIA, high pH and green water (with its tendency to higher pH in later afternoon through the diurnal photosynthetic consumption of inorganic carbon in CO_2) have been thought to show association with gill epithelial hyperplasia (Huchzermeyer, 2015). The mechanism of effect of pH upon gills is thought to occur through the conversion of ammonia to its toxic un-ionized form NH_3. The gills become increasingly less able to secrete ammonia, causing the hyperplastic change (Shelton, 2010; Roberts, 2012.a). However, no direct link between high NH_3 and gill pathology has yet been proven, and often high levels have failed to elicit any pathological responses in gills, leaving this still as a questionable link (Speare, 2008; Daoust and Ferguson, 1984).

Immune system defence in fish gills is believed to be associated primarily with the three cell groups in the epithelial layer: rodlet cells, eosinophilic granular cells and mucus cells (Leino, 2001). Rodlet and mucus cells are thought to be activated as a first layer of defence against ectoparasites, and eosinophilic granular cells a deeper level focused more often on defence against endoparasites. The study by Leino, 2001, showed a significant increase in all these cells in freshwater *Percid* fish, in times of the year when parasites were more prolific. These cells

were thought to have a suppressive effect on parasite infestation on/in the fish, and showed a tendency to reduce in number again once parasite burdens were removed (Leino, 2001). Eosinophilic granular cells (EG cells) function in a very similar manner to mast cells of mammals, showing prevalence in chronically inflamed tissue and typical degranulation responses and release of inflammatory mediators in response to acute tissue injury (Reite and Evenson, 2006). They are believed to be mast cells of the mucosal mast cell type (Reite, 1998) and have been shown to be recruited as part of the immune reaction to parasite invasion in gills, gastro-intestinal and skin tissues. As they migrate into epithelial layers from sub-mucosae / proprial tissues, they show increased granular acidophilia and become known as "globule leukocytes". Wedemeyer *et al.*, 1976.b, also describes their osmoregulatory function, particularly those grouped at the base of the primary lamellae. They are responsible for the active inward movement of Na^+ and Cl^- ions, in exchange for ammonia and bicarbonate. Nitrite molecules are indistinguishable from Cl^- and are thus easily transported (Boyd, 2000.c) by these cells. Rodlet cells show great variation in numbers and distribution in different fish species, but seem to increase in the presence of helminths and other irritants (Reite and Evenson, 2006), particularly in gill and intestinal tissues. They are currently believed to be part of the immune response. Goblet (mucus) cell numbers are known to increase in response to the presence of any irritant in the water like ammonia and pollutants, as well as in response to parasitic invasion (Roberts and Rodger, 2012), forming part of an inflammatory response.

Chloride cells in the lamellae, are generally located at the junction of the primary and secondary lamellae and serve an osmoregulatory function, pumping Ca^{2+} and Cl^- into the fish (Genten *et al.*, 2009.a). They are rare in freshwater fishes (Endo and Oguri, 1995.a).

Telangiectasis reflects a hemodynamic change within the lamellar structure involving rupture to lamellar capillaries and pillar cells (Reimschussel, 2008). It is a non-specific pathological change and has been associated with physical or chemical trauma (Roberts and Rodger, 2012) from fish movement, parasitic irritation, bacterial or viral disease, bacterial toxins (Plumb and Hanson, 2011) as well as chemical pollutants or metabolic waste (Roberts and Rodger, 2012). It is known to be associated with physical blows to the fish head in the sacrificing process as well as with tissue removal (Endo and Oguri, 1995.a), but toxicant exposure (e.g. Mercury, chlorine) is another associated cause (Reimschussel, 2008; Noga, 2010.f). Controversial possible links with pantothenic acid deficiency have been proposed (Roberts and Rodger, 2012).

2.3.8.2 Gastritis

According to Roberts and Rodger, 2012, gastric pathology in fish is uncommon, other than gastritis, which is thought to be stress associated. Bacterial lesions in the muscularis are occasionally but rarely seen.

EG cells are commonly found in the submucosal layer of many fish species. As mentioned with respect to gill pathology, their function is still largely unknown, but they are thought to function as part of the immune defence system of the fish, with a similar function to mast cells, and are known to invade the mucosa under certain conditions (Roberts and Rodger, 2012). Apoptotic cells reflect a cell death process with non-specific cause, but high incidence is thought to be associated with exposure to infectious agents, including viral infections like Infectious Pancreatic Necrosis Virus (IPNV), as well as chemicals, among other causes (Reimschussel, 2008; Roberts and Rodger, 2012). They are an indicator of phagocytosis by adjacent functional

cells and are known to be associated with suboptimal nutrition and starvation in fish (Roberts and Rodger, 2012). Exposure to toxicants and anoxic conditions has also been correlated with increased levels of apoptosis (Nikinmaa, 2014).

2.3.8.3 Liver pathology

The ratio of liver to body weight is called the liver/somatic index. Liver size is considerably affected by hepatocyte lipid and glycogen levels and these are largely dependent on the nutritional state of the fish (Heath, 1995.a). Chronic chemical or physical stress depresses feeding, which in turn, depletes energy stores in the liver, resulting in a low somatic index.

Fish livers can be impacted by a number of factors including physicochemical parameters like DO, temperature, water pH, food quantity and quality, biotoxins from algae and fungi, parasites, infectious causes, and pollutants like heavy metals, pesticides, and more (Bruslé and Anadon, 1996). Liver lipid level is largely determined by the quality and quantity of feed. Intensive accumulation of hepatocyte lipid is more a characteristic of intensive fish production, and can be an indication of nutritional deficits in artificial feed (Strussmann and Takashima, 1995) like fatty acids (Wedemeyer *et al.,* 1976.d) as well as high lipid levels in feed (Gatlin, 2008). Microscopic hepatocyte size is largely related to their physiological state of hyper- or hypo functionality (Strussmann, 1995).

Liver lipofuscin levels vary according to factors like species, age, and health. High lipofuscin deposits, which consist of yellow-brown granules of lipoid cellular debris (Agius and Agbede, 1984; Majno and Joris, 1996.a), are formed from peroxidation of polyunsaturated fatty acids and proteins in cell membranes and organelles (Wolke *et al.,* 1985). Lipofuscin accumulation in

tissues like the liver results from the dysfunctional anti-oxidant ability of the body to neutralize free radicals and cations, or from being overwhelmed by excessive free radical challenge. They are a 'wear and tear' pigment (Majno and Joris, 1996.a) . In fish, excessive oxidative injury /stress, often result from oxidation of fats from incorrect feed storage, or presence of mycotoxins like aflatoxin. Ultimately, intake of highly oxidized feed results in reduced feed (protein) conversion, and growth performance (Huang and Huang, 2004). High degree of lipofuscin, together with shrunken lipid-poor hepatocytes, are a common feature of periods of starvation (Ellis *et al.*, 1978). Lipofuscinosis is also known to be associated with deficient anti-oxidant vitamin levels in feed, particularly vitamin E (Wedemeyer *et al.,* 1976. d), Vitamin C and Selenium (Gatlin, 2008), incorrect amino acid proportions, that often results in permanent liver damage (Hawkins *et al.*, 2002; Huchzermeyer, 1993).

Cytoplasmic laking reflects a process of smooth endoplasmic reticulum expansion and hyperplasia and is generally associated with exposure to xenobiotics (Maxie, 2015).

2.3.8.4 Other Pathology

Splenic enlargement is associated with white pulp hyperplasia, often in response to an infectious challenge. The spleen to heart ratio in crocodiles is used as a measure of splenic immunologic activity (Huchzermeyer, 2003), but does acknowledge that effect of chronic disease is likely to cause splenic atrophy making any interpretation difficult. Because part of splenic function is release of erythrocytes into the circulation, conditions of high activity and turmoil, would reduce splenic size as well, because of loss of blood reserves into the tissue (Suzuki and Yokote, 1995). On the other hand, circulatory changes like congestion may increase

the size of the spleen, hence macroscopic splenic size cannot be interpreted without histological indication of patho-mechanism/s involved.

Melanomacrophage centres (MMC's), scattered through many parenchymatous tissues, are believed to serve an immune function such as antigen scavenging (Evenson, 2006), but are speculated to possibly have other roles as well (Genten *et al.*, 2009.b). They are considered 'metabolic dump sites' where circulating macrophages deposit metabolic or microbial waste products (Roberts and Ellis, 2012).

Apoptotic changes, hyaline degeneration, necrosis, ulceration, vacuolar degeneration are all considered degenerative processes (Eiras *et al.*, 2008). Apoptotic changes in the liver are usually associated with immunological, toxin or viral causes (Evenson, 2006).

Macroscopically visible hyaline deposits in the proximal tubules of the excretory kidney can indicate protein resorption from glomerular dysfunction (Reimschussel, 2008; Oguri *et al.*, 1995). Speculative correlations with heavy metal toxicity have been proposed (Roberts, 1978). Reimschussel, 2008, acknowledges the multifactorial aetiology to this pathological feature.

"Nephro-calcinosis", defined as mineralised deposits within the excretory tissue of the kidney with associated pathology (Southgate, 2005), is considered a degenerative process (Reimschussel, 2008). It is an increasingly prevalent finding in intensively cultured fish, particularly salmonids, but has also been reported in channel catfish (*Ictaluridae)* and some marine species. It is believed to be associated with high water CO_2 levels, and dietary imbalances, particularly with respect to calcium and magnesium levels in feed (Gatlin, 2008; Roberts and Rodger, 2012). It is proposed that blood acidosis associated with high water CO_2

levels may affect calcium and phosphorus excretion and predispose to mineral deposition in the tissues (Southgate, 2005). It is also believed to be associated with chronic low water pH (Huchzermeyer, 2015), probably because of the acidifying effect of carbonic acid forming from high CO_2. A combination effect of both high CO_2 or bicarbonate hardness in the water together with excess dietary protein has also been proposed (Wedemeyer *et al.,* 1976.d). Loh and Landos, 2011.e, have also linked "nephrocalcinosis" to high total hardness levels in water. Nephrocalcinosis is characteristically an unnoticed subclinical condition unless additional stressors compromise the fish's capacity sufficiently resulting in large scale mortalities (Wedemeyer *et al.,* 1976.d). Associated links with calcified granulomas in the gastric walls, have been demonstrated (Harrison and Richards, 1979).

Cysts are defined as walled structures, either containing fluid or semi-solid material. Common causes are blocked ducts, tumours, encapsulated haemorrhages or parasites (Reimschussel, 2008).

2.3.9 Growth and productivity

Overall slow growth with less efficient feed conversion, rather than increased mortalities, are often the primary indicator of a poorly optimized system, with compromised fish health, reflecting the cumulative impact of all stressors upon fish (Plumb, 1994). Virtually every environmental factor affecting the fish, will impact on its growth (Wedemeyer *et al.,* 1976.b).

Both quantity and quality of feed will have direct impact on growth, with inadequate protein intake dramatically reducing weight gain (Gatlin, 2008). Deficiencies of thiamine and essential amino acids also negatively impact growth of fish (Wedemeyer *et al.,* 1976.d), due to

compromised protein synthesis (Gatlin, 2008), while chronic fatty acid deficiencies deplete body reserves and resultant reduced weight gain (Gatlin, 2008). The effect of feed rancidity is primarily one of lipoid degeneration (lipofuscinosis) in parenchymatous organs and adipose tissues, but also inclusive of poor growth and other changes like anaemia, and muscle necrosis (Speare, 2008). Phosphorus is a particularly important mineral not only involved in bone and scale formation, but also growth (Gatlin, 2008). Magnesium deficiencies can cause anorexia and slowed growth (Gatlin, 2008). Deficiencies of microminerals or trace elements like cobalt, chromium, copper, iodine, iron, manganese, and selenium have not been directly associated with poor growth, unless deficient for chronic periods of time (Gatlin ,2008). Their potential more serious impact relates to toxic levels in feed. Deficiencies of antioxidants in feed, like Vitamin E, C, and Selenium, *(SEE LITERATURE REVIEW: NUTRITION)*, have secondary depression on growth relating to their degenerative impact on the liver. Feed contamination by heavy metals, polychlorinated bisphenols (PCB's), and pesticides cannot be excluded as other possible nutrition-related causes of poor growth in fish (Gatlin, 2008).

Over-stocking and high competition for feed will result in inadequate feed intake and thus poor growth.

In addition, suboptimal environmental and host factors all play a role. Hypoxic conditions not only affect fish by slowing basal metabolic rate and feeding behaviour, but also suppress digestion and ability of fish to utilize available nutrients optimally (Wedemeyer *et al.,* 1976.b).

CHAPTER 3 - MATERIALS AND METHODS

3.1 EXPERIMENTAL DESIGN

The project was conducted through the northern most provinces of South Africa: Gauteng, Northwest and Limpopo, as these are, based on statistics from the Tilapia Aquaculture Association of South Africa (TAASA), and DAFF, 2016, the chief tilapia-producing provinces of South Africa.

A list of farms was obtained from the Tilapia Aquaculture Association of South Africa, as well as word of mouth. Each potentially interested farm was contacted, and informed consent obtained prior to the collection of samples. A total of eighteen farms were visited, eight in Gauteng, seven in Northwest, and three in Limpopo province.

Commercial tilapia enterprises, farming with resident *Oreochromis* spp. (*O. niloticus, O. mossambicus,* and hybrids thereof) in an aquaculture system with a capacity of more than 10 000 litres qualified for inclusion to the study.

Visitation of farms was not random and farms in outlying areas were grouped together, with sampling performed over a number of days. Sampling uniformity was achieved by keeping the sampling team constant and allowing enough time to collect specimens thoroughly. For this reason and to keep biosecurity risk to a minimum between farms, only one farm was surveyed per day. Equipment was disinfected with 10ppt (parts per thousand) chlorine solution between farms (Noga, 2010.h).

The study followed an observational targeted two-stage surveillance design (Putt, 1987), the selected tilapia farms forming the first tier of the study, and a representative sample of fish from each farm, the second tier. Early juvenile growers (100 grams-250 grams) were selected as an optimal weight group for the second stage sampling unit of interest, to optimize likelihood of presence of most diseases of concern, as well as demonstrate evidence of the effect of underlying poor husbandry practices and suboptimal water quality (A. Shinn, personal communication, 29th May, 2017). Targeted sampling was attempted, with tanks with histories of higher fish mortalities, poor growth rates or suspicious signs of disease like eye injuries or exophthalmos, being preferentially targeted within this group. Fish were sampled in a systematic manner. Ten fish were sampled per farm as an appropriate and practical number to reveal a sufficient representative picture of each farm's level of tilapia health (D. Huchzermeyer, personal communication, 29th September, 2017; G. Fosgate, personal communication, 21st September, 2017; Lom and Dykova, 1992).

3.2 AQUATIC DIAGNOSTIC TESTS

All known diagnostic laboratories within South Africa, with potential to assist with diagnostics for the project, were contacted. An overview of relevant aquatic capacity was compiled. Diagnostic modalities used in the project were restricted to what was currently available, practical and affordable for the project, as well as with consideration to what would be repeatable long-term for farmers and veterinarians in South Africa.

3.3 PARAMETERS USED TO ASSESS HEALTH OF FISH AND SYSTEM

The most important environmental factors that impact on fish health directly relate to their aquatic surroundings (Shelton, 2010; Swann, 1997; Towers, 2015)

The following water quality parameters that impact on fish health were assessed:

- DO (mg/L and % saturation level)
- CO_2
- NH_3-N/TAN and UIA-NH_3
- NO_2^-
- NO_3^-
- pH
- Temperature
- CH
- TA
- CH:TA ratio
- Other: Green water, suspicion of hydrogen sulphide

The following criteria were used to assess fish during examination:

- Behaviour in the water body
- Basic morphometrics and age
- Species
- Visual appraisal
- Gill and skin scrapes and clips

- Macroscopic evidence of lesions on necropsy
- Histo-pathological evidence of lesions in organs
- Bacterial culture of targeted fish

A questionnaire, *(APPENDIX 3)*, was compiled and vital information that may impact on fish health was recovered from farmers. The following aspects regarding the aquaculture enterprise were addressed in the questionnaire:

- System design , capacity, age of sampled fish and stocking rate
- Aquaponics vs. aquaculture
- Nutrition (Feed brand / type, quantity)
- Handling protocol of fish
- Husbandry, breeding and management practices
- Production records
- History of mortalities and morbidities
- Biosecurity measures

3.4 EXPERIMENTAL PROCEDURES AND DATA COLLECTION

3.4.1 Water quality analysis

A single water sample was analysed on each farm on the same day following the completion of the necropsies. The test sample was collected from the tank and system where fish were sampled.

As a result of diurnal fluctuation in water parameters like DO, temperature, pH and CO_2, timing of the water sample analysis for comparative statistics was important. The test sample was thus analysed at approximately the same time (around 17:00h) in the day, on each farm. Key parameters measured were: DO, CO_2, NH_3, NO_2^-, NO_3^-, pH, temperature, hardness and alkalinity.

The following equipment was used for measurement of the above parameters:

- ✓ HACH® HQd IntelliCAL Rugged field kit *(Fig. 3-2)*
- ✓ HACH® Model FF2: Fish Farming Freshwater Test Kit *(Fig. 3-1)*
- ✓ HACH® Model NI-14 Nitrate (NO_3^-) Test Kit

Fig. 3-1 HACH Model FF2: Fish farming freshwater test kit

Fig. 3-2 HACH® HQd IntelliCAL Rugged field kit

Both DO and Temperature were assessed on site (Noga, 2010.f):

3.4.1.1 DO

DO was measured on site without contact with air, because of the potential diffusive change that handling and agitation during transport, could cause (Katznelson, 2004), with a factory-calibrated luminescent probe from the HACH® HQd IntelliCAL Kit, with range 0.1- 20mg/L or 1-200% saturation. Accuracy was rated within 0.1mg/L for 0-8mg/L range, and % saturation resolution, 0.1%.

Readings were recorded in the late afternoon period on all farms, when DO readings were expected to be near their highest, as a result of photosynthetic oxygen production in the day.

The probe was suspended at an approximate depth of 200-300mm below surface level.

Following a short period of probe stabilization, a digital reading was obtained. Measurements were performed away from inflow or outflow points to get a representative assessment of tank DO levels.

Farms were assessed using the following parameter ranges:

Table 3-1 Overview of DO ranges (mg/L) used to assess farm water quality, and potential impact thereof on fish populations

>5mg/L	Ideal
>3-5mg/L	Slight impact on health and growth
>1.5-3mg/L	Moderate impact on health and growth
0.3-1.5mg/L	Significant impact on health and growth
<0.3mg/L	Mortalities

(Andrews *et al.,* 1973; Boyd, 2000.b; Boyd, 2004; DeLong *et al.,* 2009; Hollerman and Boyd, 1980; Noga, 2010.d; Scott and Rogers, 1980; Swann, 1997)

3.4.1.2 Temperature

While measuring the DO level in each tank, the tank temperature, in degrees Celsius, was concurrently recorded with the HACH® HQd IntelliCAL kit probe.

Testing temperature range of the instrument was 0-50 degrees Celsius, with a 0.1 degree Celsius resolution and 0.3 degree accuracy.

Farms were assessed using the following parameter ranges:

Table 3-2 Overview of temperature ranges (°C) used to assess farm water quality, and potential impact thereof on fish populations

> 30°C	Upper tolerance level
25-30°C	Optimal
<25°C	Reduced performance
<20°C	Significantly reduced performance
<12°C	Mortalities

(Aquatic Network, 2012; Boyd, 2004; El-Sayed, 2006.b; El-Sayed and Kawanna, 2008; James, 2014; Towers, 2005; Watanabe *et al.*, 1993)

The remaining water tests were performed within 3 hours of sampling. Water was collected in a clean, clear plastic sealed container, filled to the brim to prevent exposure to air, kept cool, and transported with minimal agitation. Analysis of these parameters needed to be performed within 6-24 hours to minimize inaccuracies (HACH®, 1999.a-g; Loh and Landos, 2011).

3.4.1.3 CO_2

The HACH® FF2 Freshwater Aquaculture Kit was used for CO_2 level assessment in the sampling tank. The CO_2 test range was 10-1000mg/L.

The protocol followed was according to the described method 8205 (HACH®,1999.g), where the acidity from the CO_2 in a 100ml water sample was titrated with Sodium Hydroxide (NaOH) to a Phenolphthalein endpoint with a light pink colour change that persisted for over 30 seconds. The reading obtained on the digital titrator was multiplied by 0.2 to determine the CO_2 reading in mg/L.

Farms were assessed using the following parameter ranges:

Table 3-3 Overview of CO_2 ranges (mg/L) used to assess farm water quality, and potential impact thereof on fish populations

<20mg/L	Ideal range
20-40mg/L	Tolerated range, potential low health impact
>40-60mg/L	Probable upper tolerance range, increasing negative impact upon health
>60mg/L	Significant health and stress impact

(Aquatic Network, 2012; Fish, 1956; Loh and Landos, 2011.e; Timmons and Ebeling, 2013.c)

To assess system organic load, and efficiency of each system biofilter, the following nitrogenous compound levels were evaluated:

- ✓ NH_3-N/TAN
- ✓ NH_3-UIA
- ✓ Nitrite
- ✓ Nitrate

3.4.1.4 Ammonia

The HACH® FF2 Freshwater Aquaculture Kit was also used for this test, using the Nessler single reagent colorimetric test (HACH®, 1999.b), where a colour comparator was used to measure total ammonia nitrogen in mg/L (TAN). In this test, Rochelle's salt solution was used to negate hardness interference.

Emerson *et al.* ,1975 Table: "Percentage un-ionized ammonia (UIA) in Aqueous solution by pH value and Temperature", together with the conversion equation: (mg/L NH_3 as N x value from table/ 100) x 1.2, were then used to calculate toxic ammonia (NH_3-UIA) levels in mg/L.

Farms were assessed using the following parameter ranges:

Table 3-4 Overview of NH_3-UIA ranges (mg/L) used to assess farm water quality, and potential impact thereof on fish populations

< 0.025mg/L	Optimal
0.025<0.05mg/L	Acceptable for production
0.05-0.2mg/L	Some effect on health and production
>0.2-1mg/L	Low level mortalities
>1mg/L	High level mortalities

(Boyd, 2000.c; Loh and Landos, 2011.e; Noga, 2010.d; Timmons and Ebeling, 2013.c).

3.4.1.5 Nitrite

Nitrite was also assessed with the HACH® FF2 Freshwater Aquaculture Kit. A very sensitive colorimetric test was used according to HACH® protocol (HACH®, 1999. e) where nitrite nitrogen (N) in mg/L is measured and converted with a 3.3 multiplication to NO_2^-(mg/L).

Farms were assessed using the following parameter ranges:

Table 3-5 Overview of NO_2^- ranges (mg/L) used to assess farm water quality, and potential impact thereof on fish populations

>0.5mg/L	Significant production stress, secondary disease
>0.3 ≤0.5mg/L	Production stress, lethargy anorexia
>0.1 ≤0.3mg/L	Acceptable
≤0.1mg/L	Ideal

(Aquatic Network, 2012; Huchzermeyer, 2015; Loh and Landos, 2011.e)

3.4.1.6 Nitrate

Nitrate levels were measured with a HACH® Colorimetric Nitrate LR test Kit (HACH, 2016), with test range of (0-1mg/L NO_3^- N). This reading was then multiplied by 4.4 to assess NO_3^- levels.

3.4.1.7 pH

A colorimetric pH test in the HACH® FF2 Freshwater Aquaculture Kit was used to assess water pH level. Reference was made to the pH scale, with range from 1-14, and 7 as neutral.

3.4.1.8 Complete Hardness (CH)

Hardness (concentration of divalent cations expressed as $CaCO_3$ equivalents), was assessed by using a buffering test in the HACH® FF2 Freshwater Aquaculture Kit, where the water sample was buffered to a pH of 10.1, coloured, and titrated with 0.800 M EDTA to a colour change endpoint (HACH®, 1999.d). Hardness was defined as the total concentration of calcium and magnesium expressed as their calcium carbonate equivalent, in mg/L.

Test range (mg/L $CaCO_3$) was 100-400mg/L.

3.4.1.9 Total Alkalinity (TA)

This assessed the level of titratable bases (bicarbonates and carbonates, expressed as $CaCO_3$ equivalents), using the HACH® FF2 Freshwater Aquaculture Kit, where the sample was titrated with Sulphuric acid (H_2SO_4) to a pH of 4.8 and corresponding colorimetric end-point (HACH®, 1999.a).

A reference sample alkalinity of approximately 150mg/L was used.

Test range (mg/L $CaCO_3$) was 100-400 mg/L.

3.4.1.10 Hardness: Alkalinity ratio (CH:TA)

The ratio between the above two parameters was assessed as Complete Hardness divided by Total Alkalinity.

3.4.1.11 Green water/ Odour

Water was subjectively assessed in terms of:

- ✓ Green water: high or low levels of Phyto/zooplankton
- ✓ Hydrogen sulphide odour

3.4.1.12 System

Visual inspection was also made of the following:

- ✓ Use of pre-system water holding tanks
- ✓ Level and design of the filtration system

3.4.2 Fish examination

3.4.2.1 Fish behaviour

Fish were assessed, pre-sampling, for evidence of abnormal behaviour symptoms like poor righting reflex, lying on the bottom, sluggish swimming, piping at the surface, or clamped fins.

3.4.2.2 Handling

In an attempt to minimise stress to the fish and prevent the occurrence of artefact/s that may have impacted on results such as autolysis or ecto-parasite detachment (Lom and Dykova, 1992), the time from tank recovery of fish to euthanasia was minimized to close to 5 minutes. For the same reasons, fish were handled gently and exposure to bright light and noise minimized as much as possible. Humane methods of fish euthanasia were employed as close to those described in the *AVMA Guidelines on Humane Slaughter and Euthanasia of Fish,* as possible (Leary *et al.,* 2013; Leary *et al.,* 2016).

A combination approach of rapid chilling, and cervical transection was used for sampled fish. Fish were collected in batches of two or three, kept in a bucket of their tank water to reduce stress (Gordon, 2004), and rapidly chilled with introduction of ice to the water. Fish were immobilized/sedated until an apparent level of deep narcosis equivalent to plane III surgical anaesthesia was achieved. This was based on the following clinical and behavioural changes:

> No voluntary swimming, total loss of equilibrium and righting reflex, significantly reduced opercula rates, and loss of reactivity to handling (Gordon, 2004; Loh & Landos, 2011.g; Noga, 2010.f).

At this point, fish were removed from the water, examined, photographed, measured and weighed as quickly as possible. Cervical transection was then performed. Complete sustained loss of rhythmic opercular activity i.e. respiratory arrest, was considered indication of death (Noga, 2010.a). Chemical sedation / euthanasia was not considered suitable for the study as chemical anaesthetic agents are known to reduce ecto-parasite motility and cause detachment of attached protozoal stages on gills and skin, which would have compromised the results of the study (Alvarez-Pellitero, 2008; Callahan and Noga, 2002; Lewbart, 1998.b; Noga, 2010.f).

3.4.2.3 Basic morphometric analysis and Age

Fig. 3-3 On-farm basic morphometric assessment A: measurement of body length, B: Weighing fish (Photographs V.Naidoo: 4th Year Veterinary student, Faculty of Veterinary Science , UP)

A measuring box and ruler was used to record total length. The weight of each fish was assessed in grams using a calibrated digital scale with a measuring accuracy of 1 gram *(Fig. 3-3).*

The age of each fish was recorded based on farm records. Because of the numerous factors potentially influencing fish growth rates (water quality ,feed quantity and quality, stocking rates, stress levels, genetics, species, underlying disease, and others), fish length and weights can only be used as an estimate of age, in optimally functioning systems, where growth rates would be more likely to match an optimal curve (Wedemeyer *et al.,* 1976.b).

3.4.2.4 Taxonomic identification

Bilateral images using an iPhone 7 of each fish were recorded, in lateral recumbency. These images were used for comparison to available taxonomic descriptions of *O. niloticus, O. mossambicus* and their hybrids *(Table 3-6, Figs. 3-4,3-5,3-6)*. The cost of genotyping was investigated but proved prohibitive for the project budget.

Numbers of dorsal and anal fin spines and soft rays could not be used to differentiate as they are almost identical in both species (Skelton, 1993).

Table 3-6 Key Taxonomic features for morphometric species identification (N. James, personal communication, 5th September, 2017; Jubb, 1961; Skelton, 1993)

Taxonomic trait	***Oreochromis niloticus***	***Oreochromis mossambicus***	***Hybrids***
Key differences in morphology of head	Straight forehead profile. Large males do not show the extended upturned snout typical of *O. mossambicus*	Concave forehead profile and upturned snout (Females have more of a straight profile). Males have a particularly prominent mouth.	Intermediate features between *O. niloticus* and *O. mossambicus*
Key features of fins	No red margins. Vertically striped caudal fins, extending into posterior parts of dorsal and anal fins. Stripes are lines of iridescent spots	Red margins to the dorsal, caudal and anal fins. Clear caudal fin, with occasional spots. Never vertical lines of spots	Varying degrees of reddening of fin margins. Frequent "ghost vertical striping" of caudal fin
Colour differences	Breeding males have a red/ plum colour over the head, lower body, dorsal and caudal fins, with blue on the snout	Breeding males have white cheeks and throat	Intermediate features between *O. niloticus* and *O. mossambicus*
Behavioural differences	More active with strong flexing when netted.	Quieter to handle. No strong flexing when netted.	No characteristic behaviour

Fig. 3-4 Adult O. mossambicus *(photograph N. James)*

Red fin margins

Concave head with large prominent mouth

Fig. 3-5 O. niloticus *(photograph N. James)*

Straight forehead with small lips

Significant barring/ stripes on dorsal, caudal and anal fins

Fig. 3-6 Oreochromis *Hybrid (photograph N James)*

"Ghost barring" of caudal fin

Intermediate features of other spp.

Any other fish species on each farm like *tilapia* spp., other *Oreochromis* spp., trout, catfish, and their association or proximity with the farmed *O. niloticus, O. mossambicus* and their hybrids, were recorded. Attempt was made to specifically record evidence of particular *Oreochromis* spp. like the introduced exotic *O. aureus,* and *O. andersonii* which was traditionally confined to the Cunene, Okavango, upper Zambezi and Kafue systems (Skelton, 1993).

3.4.2.5 External examination

Each fish was visually appraised in terms of body condition and graded subjectively as poor, average, or good *(Fig. 3-7).* These were scored from 1 to 3 respectively. An average score for each farm was determined by totalling the individual fish scores, averaging, and grading as follows:

Poor: Average score ≤ 1

Moderate: Average score >1 ≤ 2

Good: Average score >2 ≤ 3

This group of fish were characterized by their distinctive concave abdomen, and convex body shape

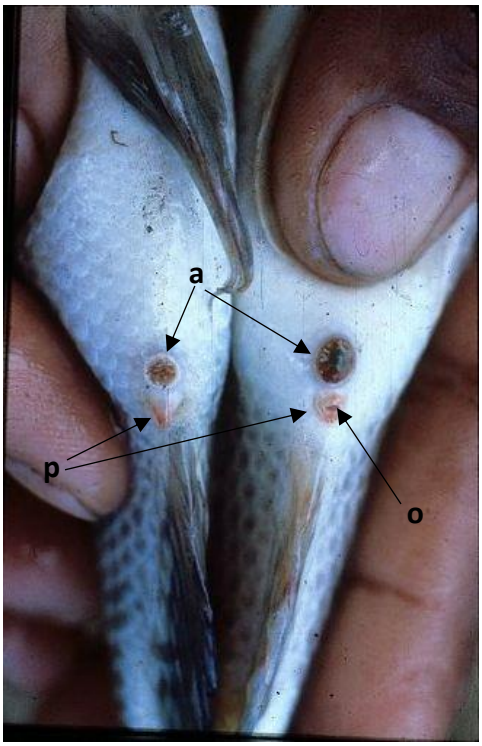
This group of fish were characterized by their straight ventral abdominal wall, and fairly elongated, linear body shape

Fish with convex ventral abdominal wall and overall rounded/ globe shape, were classified within this group

Fig. 3-7 Comparison of fish body conditions A: Poor, B: Moderate, C: Good

The skin and appendages were also assessed for lesions like ulcers, scars, missing scales, deformities and macroscopically visible crustacean parasites like *Argulus,* or the *Lernaeid* copepods, as well as *Saprolegnia* proliferative growths. In the event of visible dermal lesions, samples were collected in 10% buffered formalin for histo-pathological examination.

The gender of each fish was recorded *(Fig. 3-8).* This was confirmed later at necropsy.



Male (Left)

- Anus (a)
- Genital Papilla (p)

Female (Right)

- Anus (a)
- Genital Papilla (p) with oviduct (o) (dark stripe)

Fig. 3-8 Sexual differentiation of tilapia (Popma and Masser, 1999)
(Photograph Aquatic Network, 2013)

3.4.2.6 On Farm direct light microscopy

As soon as each fish was adequately immobilized to handle, it was positioned in right lateral recumbency for examination. In order to prevent slippage for ease of examination the fish was

placed on absorbent paper towel. A skin scrape, gill clip and gill scrape were collected from the fish's left lateral side. All slides were examined immediately, once collected, to avoid loss of parasitic viability.

An Olympus® CX21 compound microscope was used for the evaluation.

The object of this method of examination was to identify any of the many conditions that are known to target primarily the gill or skin regions, with potential adverse effect on production and fish health (Macmillan, 1991; Noga, 2010.f; Shinn, 2016).

A master list of potential key gill and skin pathogens of economic concern was compiled, that could reasonably be detected through this method (Noga, 2010.e; A. Shinn, personal communication, 29th May, 2017; K. Veverica, personal communication, 14th March, 2016).

In preparation for light microscopical examination, three drops of tank water from the sampling site were placed onto glass microscope slides as described by Noga, 2010.f and Metselaar, 2017.

Skin scrape

A glass coverslip was used to gently scrape a skin mucus sample from each fish. Sites sampled were just caudal to the left pectoral fin, left tail base, and left dorsum, just ventral to the dorsal fin (Loh and Landos, 2011.a; Reavill, 2010). When present, focal areas of injury were also targeted and included in the scrape. Scrapes were performed in a rostral to caudal direction. Care was taken not to draw blood or cause trauma. The coverslip was then laid immediately on a drop of tank water on the glass slide and examined. Parasites were identified as close as

possible to species level, and scored using a designed scoring system for each group of parasites *(Table 3-7).*

Gill clip

The left operculum was removed, and a sample of primary lamellae tips amputated with fine scissors, from the most lateral gill arch. These were targeted to potentially yield a higher parasite burden due to maximized exposure to water and the external environment. The lamellae tips were immediately placed onto a drop of tank water on the slide, covered with a coverslip, and evaluated as with the skin scrape *(Table 3-7).*

Gill scrape

The most lateral / first gill arch lamellum was gently scraped between two glass coverslips from base to tips, to remove possible parasites. The sample was immediately placed on a drop of tank water, covered with a cover slip and similarly evaluated *(Table 3-7).*

Grading of slides:

A subjective visual appraisal method was designed to grade the level of ecto-parasitic infestations encountered, in all three slide evaluations above *(Table 3-7)*. Highest and average parasite grades between all three wet mounts per fish were calculated and recorded.

Evidence of other significant findings like gas-supersaturation bubbles, bacterial disease (e.g. *Flavobacterium columnare*), water moulds (e.g. *Saprolegnia, Aphanomyces invadens*) were noted as present or absent.

Table 3-7 Description of parasite visual appraisal and grading method

Parasitic motile Ciliates **e.g. *Trichodina* spp., *Chilodonella* spp., *Ichthyophthirius multifilis***	**Grade**	**Description:**
	1	1-2 parasites/10X field
	2	3-5 parasites/10X field
	3	6-10 parasites/10X field
	4	11-15 parasites/10X field
	5	>15 parasites/10X field
Parasitic monogeneans **e.g. Dactylogyridae, Gyrodactylidea**	**Grade**	**Description**
	1	1 parasite/ 4X field
	2	2 parasites/ 4X field
	3	3-5 parasites/ 4X field
	4	6+ parasites/ 4X field
Parasitic sessile Ciliates **e.g. *Ambiphrya* spp., *Epistylus* sp., *Apiosoma* spp.**	**Grade:**	**Description:**
	1	1-2 parasites/ primary lamellum
	2	3 parasites/ primary lamellum
	3	4-6 parasites/ primary lamellum
	4	>6 parasites/ primary lamellum

<u>Parasitic flagellates</u> **e.g. *Ichthybodo necator* complex**	**Grade:**	**Description:**
	1	1-2/field*
	2	3-5/field*
	3	6-10/field*
	4	>10/field*

(* 400X magnification)

3.4.2.7 Necropsy

Following the microscopic skin and gill examinations a systematic necropsy was performed. The objective for this procedure was to identify internal pathological lesions at a macroscopic level and to collect samples for histo-pathological examination and bacterial culture.

Fig. 3-9 <u>Necropsy</u>: A: Preparation for microscopy and necropsy ; B: Ventral incision and removal of the lateral body wall to expose the viscera (Photographs T.Kersten, 4th Year Veterinary student, Faculty of Veterinary Science ,UP)

Necropsy technique and sampling (Noga, 2010.f; Noguera *et al.,* 2015; Reavill, 2010)

A ventral incision was made using dissection scissors from vent to pectoral fins. Incisions were extended dorsally as far as possible to the level of the swim bladder *(Fig. 3-9B)* and the left lateral body wall removed to expose coelomic organs. Gross organ pathology was assessed in situ *(Fig. 3-10).*

Fig. 3-10 Fish necropsy: Dissected coelomic and pericardial cavities. Key organs sampled included the testes (t)/ ovaries, stomach (s), Intestine (i), liver (l), heart (h), and anterior kidney (a). Spleen and posterior kidney not visible in this image.

Visceral fat: *(PLATE 1)*

Level of visceral fat (adipose tissue) was qualitatively assessed and graded from 0-5, to assess correlations with body condition, nutrition, and health. An average farm score was calculated from all fish scores and rounded off to the nearest whole number.

Plate 1: Necropsy: photographic comparison of visceral fat grading levels 0 to 5

A: grade 0. B: grade 1, C: grade 2, D: grade 3, E: grade 4, F: grade 5

The gender of each fish was confirmed by assessing the presence of testes or ovaries *(Fig. 3-11).*

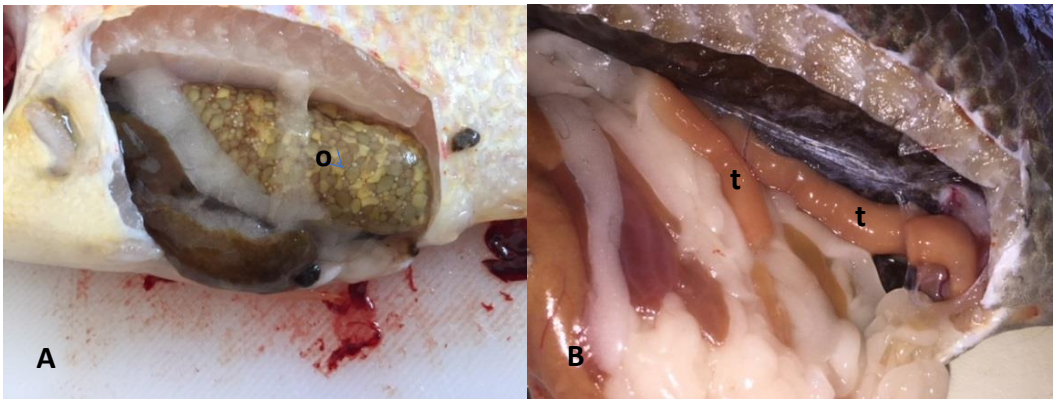


Fig. 3-11 Gender confirmation on necropsy A: female tilapia with egg-filled ovaries (o), B: male tilapia with active testes (t)

The pericardial cavity was inspected by transection and deflection of the transverse septum that separates the coelomic and pericardial cavities.

Spleen: Heart ratio

A subjective comparison was drawn between heart and spleen size with each fish *(Fig. 3-12),* to assess whether a relatively enlarged spleen could be used as an indicator of immune stimulation. The spleen to heart ratio (SHR) is used in Nile crocodiles as an indicator of splenic immunoreactivity (Huchzermeyer, 2003).

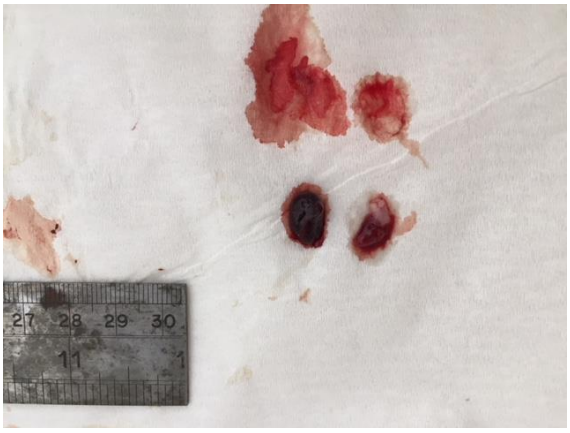


Fig. 3-12 Size comparison of dissected heart (right) and spleen (left)

Bacterial culture

The anterior kidney is considered the organ of choice for culture to detect most systemic bacterial disease in fish (M. Henton, personal communication, 13th September, 2016; Loh and Landos, 2011.b; Noga, 2010.f), and has been shown to trap more than 70% of circulating bacteria (Loh and Landos, 2011.c). A sterile Amies swab with charcoal transport medium, was used for the procedure. As a result of the decision to use histological evidence for bacterial infection prior to submission for culture, there was a delay in bacterial culture. The charcoal transport medium is preferred for samples not immediately submitted (M. Henton, personal communication, 21st October, 2016). Swabs were labelled, and stored cold at 4 to 10°C for a maximum period of 5 days.

An aerobic culture was performed to screen for common bacterial pathogens in fresh water fish. This was able to include significant pathogens like *Streptococcus* iniae and *agalactiae*, *Lactococcus garviae, Aeromonas* spp., *Yersinia ruckeri,* and *Edwardsiella* spp. (M. Henton, personal communication, 19th October, 2016). *Flavobacterium columnaris* was not included to be cultured as it required purchase of a high-cost specific culture medium, which was not warranted with the low number of cultures anticipated, and diagnosis could be confirmed with wet mount examination. *Francisella* spp. are notoriously difficult and costly to confirm with culture and biochemical identification (M. Henton, personal communication, 17th August, 2016), and can be presumptively diagnosed on histopathology (Soto *et al.,* 2013), and thus were not included.

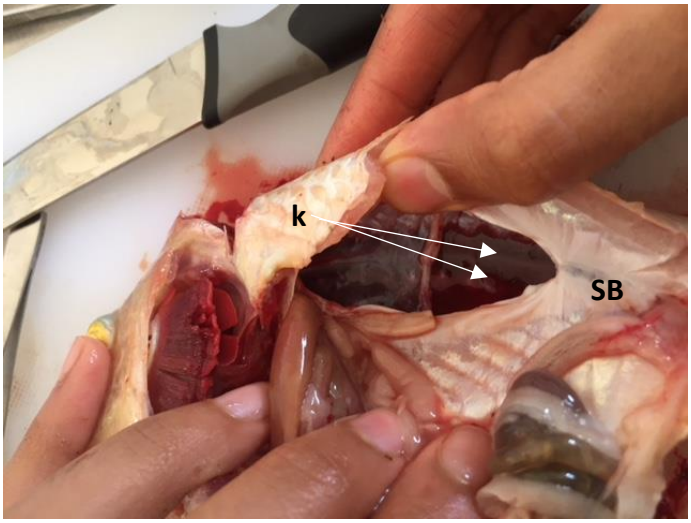


Fig. 3-13 Necropsy: exposure of the anterior kidney for bacterial culture, using a ventral approach. The base of the swim bladder (SB) is incised and deflated, to expose the dark strips of kidney (k) running ventro-laterally to the vertebral column

The right lateral operculum was removed to display gill arches and filaments.

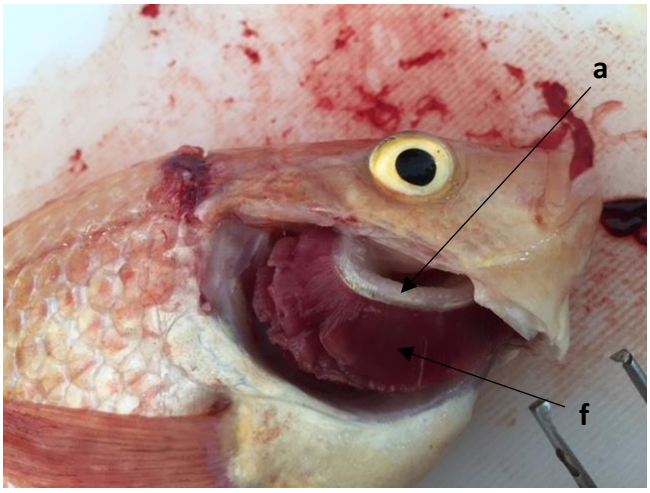


Fig. 3-14 Necropsy: branchial arch (a) and filaments (f) are exposed for assessment. The most lateral filament was removed to allow for histo-pathological sampling from a more protected inner row.

The roof of the skull was transected by means of a midline incision, to expose the cranial cavity and brain.

3.4.2.8 Microscopic Histological assessment of organ pathology

During the necropsy of the fish the following organs were sampled in 10% buffered formalin for histopathology: liver, spleen, small intestine, stomach, gonad, heart, gill, brain, anterior and posterior kidney. For consistency, attempt was made to sample the same region of the liver, stomach and intestinal loops for each fish. The heart and brain were each removed and fixed in toto. As a result of the friable nature of anterior and posterior kidney tissue, attached muscle or vertebral tissue was included to avoid tissue loss during processing. The opposite gill set to those used for parasite evaluation were sampled. The outermost row of filaments was removed, and a section taken from an inner row, with the branchial arch attached. This latter was trimmed to facilitate histological embedding.

In order to facilitate ease of processing all the samples for each fish were already trimmed to size and placed in a single perforated histology cassette at necropsy. Routine histological processing with haematoxylin and eosin (H&E) staining (Anderson and Bancroft, 2002; Gamble and Wilson, 2002; Hopwood, 2002; Horobin, 2002) was performed at the pathology laboratory, Faculty of Veterinary Science, University of Pretoria.

All tissue sections were examined using a standard compound microscope (Olympus® CX21) with optic ranges between 40 to 1000x magnification.

The following properties were evaluated in the respective organs:

Gills

The area of the gill filaments examined included the region of secondary lamellae between the primary lamellar base and its apex. The available gill filaments in the section were randomly selected, and at least 3 closely examined.

Epithelial hyperplasia:

A hyperplastic response was characterized by an increased number and/or size of epithelial cells lining the primary or secondary lamellae.

The level of hyperplasia was graded from 0-5 using the following criteria:

Table 3-8 Gill epithelial hyperplasia grading protocol

0	No epithelial hyperplasia (Normal)
1	Epithelial activation showing slight peak formation and /or nuclear expansion (euchromatism)
2	Epithelial activation with severe peak formation of the lamellae, and slight increase in epithelial cell layer at the base or on the free filament of secondary lamellae
3	Marked epithelial layer expansion at base of secondary lamellae
4	As above with expansion up to half the depth of the secondary lamellae
5	Epithelial expansion extending more than half the secondary lamellae resulting in lamellar fusion

Goblet cell hyperplasia:

This hyperplastic response was characterized as an increase in the number of goblet cells in the inter-lamellar spaces between the secondary lamellae. Five randomly selected inter-lamellar spaces in the mid primary lamellar region were selected and evaluated for goblet cells.

Hyperplasia was graded from 1-5 using the following criteria:

Table 3-9 Gill goblet cell hyperplasia grading protocol

1	Less than 5 goblet cells per 5 inter-lamellar spaces (Normal)
2	5-10 goblet cells per 5 inter-lamellar spaces
3	11-15 goblet cells per 5 inter-lamellar spaces
4	16-20 goblet cells per 5 inter-lamellar spaces
5	>20 goblet cells per 5 inter-lamellar spaces

Eosinophilic Granular Cell (EGC) infiltration:

The presence and degree of EG cell infiltration at the base of the primary lamellae was assessed. This feature was graded from 1-5 by using the following criteria:

Table 3-10 Base of primary lamellae EGC infiltration grading protocol

1	≤ 5 EG cells per field * (Normal)
2	6-10 EG cells per field *
3	11-20 EG cells per field*
4	21-30 EG cells per field*
5	> 30 EG cells per field*

* 400x magnification

Secondary lamellar fusion

Fusion of secondary lamellae was subjectively assessed and graded from 0-3 as:

Table 3-11 Grading protocol for fusion of gill secondary lamellae

0	None (Normal)
1	Mild (Focal)
2	Moderate (Multifocal)
3	Severe (Generalized)

Parasites:

Gill sections were examined for the presence of ecto-parasites, which were identified as close to species level as possible, and recorded.

Evidence of other pathological gill lesions were described.

Hepatopancreas

The following pathological changes were assessed:

Hepatocellular lipid:

As lipid content in teleost hepatocytes is closely related to overall cell size (Heath, 1995.a), a subjective ratio between the average hepatocyte size and average nuclear size was used to estimate hepatocellular lipid content, by using the following criteria:

Table 3-12 Hepatocellular lipid grading technique

1	hepatocyte size: nuclear size = < 5 : 1
2	hepatocyte size: nuclear size = 5-10 : 1
3	hepatocyte size: nuclear size = 11-15 : 1
4	hepatocyte size: nuclear size = 16-20 : 1
5	hepatocyte size : nuclear size = >20 : 1

Hepatocellular lipofuscin /ceroid concentration:

Lipofuscin deposits in the liver, which consist of damaged cellular debris (Agius and Agbede, 1984), complexes of protein and peroxides of fatty acids (Hibiya, 1982), were assessed by counting the number of hepatocytes containing the typical yellow-brown (Hibiya, 1982) pigmented oxidised lipid precipitates within a random 400X field and grading this score between 0-6 by using the following criteria :

Table 3-13 Hepatocellular lipofuscin grading protocol

0	No lipofuscin precipitates observed
1	Less than 3 hepatocytes with lipofuscin precipitates / field *
2	3-6 hepatocytes with lipofuscin precipitates / field*
3	7-9 hepatocytes with lipofuscin precipitates / field*
4	10-15 hepatocytes with lipofuscin precipitates / field*
5	16-30 hepatocytes with lipofuscin precipitates / field*
6	Greater than 30 hepatocytes with lipofuscin precipitates / field*

* 400X magnification

Hepatocellular nuclear activity

Hepatocyte nuclei were graded as active (grade 1) or inactive (grade 0) (euchromatic vs heterochromatic respectively) as a measure of hepatocellular activity. The presence of anisokaryosis as a result of variable karyomegaly was reported as a measure of possible toxin exposure (J. Steyl, personal communication, July, 2018).

Pancreatic activity:

The relative presence of zymogen granules within pancreatic acinar cells was assessed. Pancreatic tissue was graded as active or inactive. A relative absence of zymogen granules from acinar cells was interpreted a sign of inactivity (atrophy).

Portal lipofuscin (ceroid) and adipose tissue infiltration :

Hepatic portal triad lipofuscin precipitation was assessed and graded subjectively from 0-3, using the following criteria :

Table 3-14 Hepatic portal lipofuscin grading protocol

0	None
1	Low
2	Moderate
3	High

Portal fat deposits were also noted as present or absent.

Other pathological changes:

Evidence of any other pathological changes was noted.

Stomach

Inflammatory changes in the stomach sections were assessed and classified. The presence or absence of granulomatous cysts in the gastric submucosa was recorded.

Gastritis:

Gastritis was identified based of the following key changes:

a. Increase in the number of eosinophilic granular cells (EG cells) in the submucosa and lamina propria layers

b. Migration of EG cells into the gastric mucosal layer

c. Gastric mucosal epithelial vacuolation

d. Proprial and submucosal lymphocytic infiltrate

e. Mucosal epithelial apoptosis

f. Gastric mucosal erosion or ulceration

The level of gastritis was graded from 1-5 in the following way:

Table 3-15 Gastritis grading protocol

1	Low EGC cell presence in the submucosal layer (Normal)
2	A mild EGC infiltration in submucosal layer, low grade vacuolation, and/ or a few lymphocytic foci.
3	High EGC infiltration, largely in submucosal layer, and /or moderate vacuolation, and/or presence of apoptosis, and/or moderate lymphocytic infiltration
4	Severe EGC cell infiltration with significant movement into mucosal layer, and/or severe vacuolation, and/or severe apoptosis, and/ or severe lymphocytic infiltration
5	As above, with evidence of gastric mucosal erosions or ulcerations.

Spleen

The following pathological changes in the spleen were evaluated:

Lipofuscin / ceroid deposits:

The presence of lipofuscin deposits (granulomata) was recorded and subjectively graded using the following criteria:

Table 3-16 Grading protocol for splenic lipofuscin deposits

0	None
1	Low: Less than 10% / field*
2	Moderate: 10-40% / field*
3	Severe: Greater than 40% / field*

* 400X magnification

Melanomacrophage centres:

The presence of melanomacrophage centres was recorded and graded using the same criteria as with lipofuscin / ceroid deposits above.

Lymphoid (white pulp) hyperplasia :

Splenic tissue was assessed for the presence of hyperplastic lymphocytic foci.

Other splenic pathology:

Any additional splenic abnormalities were recorded and described.

Anterior kidney

The presence of lipofuscin / ceroid and melanomacrophage centres were recorded and graded using the same criteria used in the spleen.

Any additional abnormalities identified in the anterior kidney were recorded and described.

Posterior kidney

Posterior kidney tissue was assessed for the presence of interstitial mineralization. Any additional renal abnormalities were recorded and described.

Heart

Cardiac and pericardial tissues were examined for inflammatory cell infiltrates and graded as present or absent. Any additional cardiac abnormalities were recorded and described.

Brain

The brain was assessed for inflammatory changes and graded as present or absent. Any additional neural abnormalities were recorded and described.

Intestine

Intestinal sections were examined for any inflammatory infiltrates or evidence of intraluminal parasites.

3.4.2.9 Disposal

Carcass remains were made available for on-farm use or disposal.

Used glass slides and coverslips were collected in a medical waste bin and disposed by Legacy/ Envirocin®.

Fixed tissue specimens were histologically processed and are stored indefinitely at the University of Pretoria, Faculty of Veterinary Science, Histopathology laboratory. Collected,

uncultured anterior kidney swabs have been frozen to be kept indefinitely at the University of Pretoria, Faculty of Veterinary Science, Pathology laboratory.

3.4.2.10 Consistency

Water parameter testing, sampling of tissues, recording and grading of macro- and histopathological changes were performed by the author under supervision to limit interpretative bias.

3.4.2.11 Consent

Informed consent and indemnity outlining the purpose of the project, the researchers involved, procedures to be carried out and possible risks, was obtained from each visited farm as per Animal Ethics Committee Project V015-17 approval and conditions.

3.4.2.12 Biosecurity

To reduce risk of disease transfer between farming enterprises, the following principles and procedures were applied:

- ✓ Only one farm was visited and assessed per day.
- ✓ Any equipment to catch and store fish belonged to, and was used and stored on the farm itself.
- ✓ Dissecting equipment used between farms was disinfected using a chlorine solution of approximately 5g/L, and stored in in F10® Rust inhibiting sterilizing solution
- ✓ Personal handling of farm aquaculture system water or farm tools was avoided.
- ✓ All procedures were performed in separate area away from the aquaculture system.

- ✓ Water samples were collected in bottles that were cleaned and sterilized with 5g/L chlorine solution, and well rinsed.
- ✓ Cleaned clothing was used between farm visits.

3.4.3 Farm Management and Husbandry assessment

The following details were recorded via a questionnaire submitted to each farmer, with respect to farm management and husbandry practices: *(SEE APPENDIX 3)*

Farm Details:

- ✓ Province Code used

Gauteng province	**G**
Northwest province	**NW**
Limpopo province	**L**

- ✓ Name of farm
- ✓ Name of owner
- ✓ Physical address
- ✓ GPS coordinates (if available)
- ✓ Telephonic details
- ✓ Email address

Aquaculture System

- ✓ Duration in operation
- ✓ Total water capacity of the system
- ✓ Type of system: RAS, Pond, Raceway
- ✓ Capacity of the tank sampled
- ✓ Presence of aquaponics
- ✓ Green vs clear water system
- ✓ Fish life stages farmed
- ✓ Water source: recorded as borehole, municipal or natural.
- ✓ Heating methods for water
- ✓ Fish handling protocol

Stocking rate

- ✓ Stocking density of sample tank

Species farmed

- ✓ Monoculture or polyculture
- ✓ *O. niloticus, O. mossambicus,* hybrids, or other

Sex reversal

- ✓ Use of YY Super-male Brood-stock
- ✓ Testosterone use in fry

Feed

- ✓ Feed type and source used
- ✓ Storage protocol and management

Vector management

- ✓ Presence of freshwater snails
- ✓ Accessibility to birds
- ✓ Water as a source of disease or vector introduction

Disease history and management

- ✓ Morbidity and mortality rates
- ✓ Life stage most affected
- ✓ Growth rate of sampled fish
- ✓ History of previous infectious diseases

Movement of fish

- ✓ Life stages purchased
- ✓ Supplier/s of fish
- ✓ History of purchases: local/ interprovincial/ international
- ✓ Level of health or movement certification with purchases

General level of biosecurity

- ✓ Water source

- ✓ Management of outflow water
- ✓ Quarantine protocol for new introductions
- ✓ Equipment/Tank management: (Separate system equipment disinfection protocol, handwash stations/ footbaths)
- ✓ Signage and labelling
- ✓ Disposal protocol of dead fish

3.4.4 Recording

Data collected on each farm was recorded in a Health and Biosecurity Farm Questionnaire *(APPENDIX 3)*, a Water Quality Analysis table, and individual Fish Microscopy and Necropsy Record sheets.

3.4.5 Data Analysis

Data was analysed using various statistical models in an attempt to identify key suboptimal independent and dependant variables through the assessed populations. Correlations were then assessed between these key independent and dependant variables. Models used included:

- ✓ Excel spreadsheets: a farm and fish level comparison
- ✓ "R" Statistical software: Chi-squared tests, Fitting generalized linear model
- ✓ Regression Analyses, with stepwise regression and binary logistic regression
- ✓ Tree Analyses
- ✓ Frequency Tables
- ✓ Chaid method
- ✓ Canonical correlations

- ✓ Pearson's Correlations

With recognition of the many variables that form part of the host, pathogen, environment triad, focus was placed on those key variables with greatest potential to impact health. These were assessed in an attempt to determine individual impact and the existence of relationships affecting fish health. The measured water parameters, nutrition and fish stocking density comprised the key independent variables of the study. The dependant variables were determined from the physical macro- and microscopic examination of the listed fish tissues. In an attempt to allow for statistical analysis, a basic model quantifying each dependant variable was developed based on the macro-/ microscopic characteristics of each and correlations drawn with independent variables.

3.5 PERMISSION TO UNDERTAKE RESEARCH

Ethical approval was granted from the University of Pretoria Animal Ethics Committee.

Certificate No: V015-17**.**

Permission to do Research under Section 20 of the Animal Diseases Act (Act No 35 of 1984) was granted by the Dept. of Agriculture, Fisheries and Forestry (DAFF) as well as Provincial Directorates of Veterinary Services for each province visited.

Reference No: 12/11/1/1

CHAPTER 4 - RESULTS

4.1 FARM OVERVIEW

4.1.1 Distribution

Seventeen farms, with nineteen systems in total, were visited and assessed through the three provinces (*Fig.4-1)*: eight in Gauteng, seven in Northwest Province, and two in Limpopo Province (one of the initial eighteen farms was excluded as it was found to have only *Tilapia rendalli*).

Fig. 4-1 Overview of provincial distribution of farms visited in Gauteng, Northwest and Limpopo provinces (www.googlemaps.co.za)

4.2 ASSESSMENT OF HEALTH PARAMETERS

4.2.1 Water quality parameter assessment

4.2.1.1 DO

Water DO levels ranged between a minimum of 0.96mg/L and 10.2mg/L, with a mean of 4.16mg/L, and a minimum of 12.6% saturation to a maximum of 132.7% saturation with a mean of 57.9%.

Table 4-1 Comparison of farm DO measurements (mg/L and % saturation), with those highlighted red, falling below the optimum DO level of 5mg/L (Timmons and Ebeling, 2013.c) and/or suggested minimum saturation of 80% (Boyd, 2000.b)

Farm:	DO (mg/L)	DO (% saturation)
GA	1.58	25.30%
GB	4.98	72.90%
GC	6.68	109.30%
GD(a)	3.18	49.30%
GD(b)	2.68	40.60%
GE	2.28	35.90%
GF(a)	3.14	45.60%
GF(b)	0.96	15.40%
GG	2.14	31.60%
GH	6.01	89.60%
NA	8.28	111.4%
NB	10.2	132.70%
NC	2.68	36.50%
ND	1.1	14%

NE	0.97	12.70%
NF	8.53	115.10%
NJ	2.28	30.40%
LB	4.55	51.40%
LC	6.85	80.90%

36.8% of farms measured ideal DO levels close to or above 5mg/L, while 63.2% fell below.

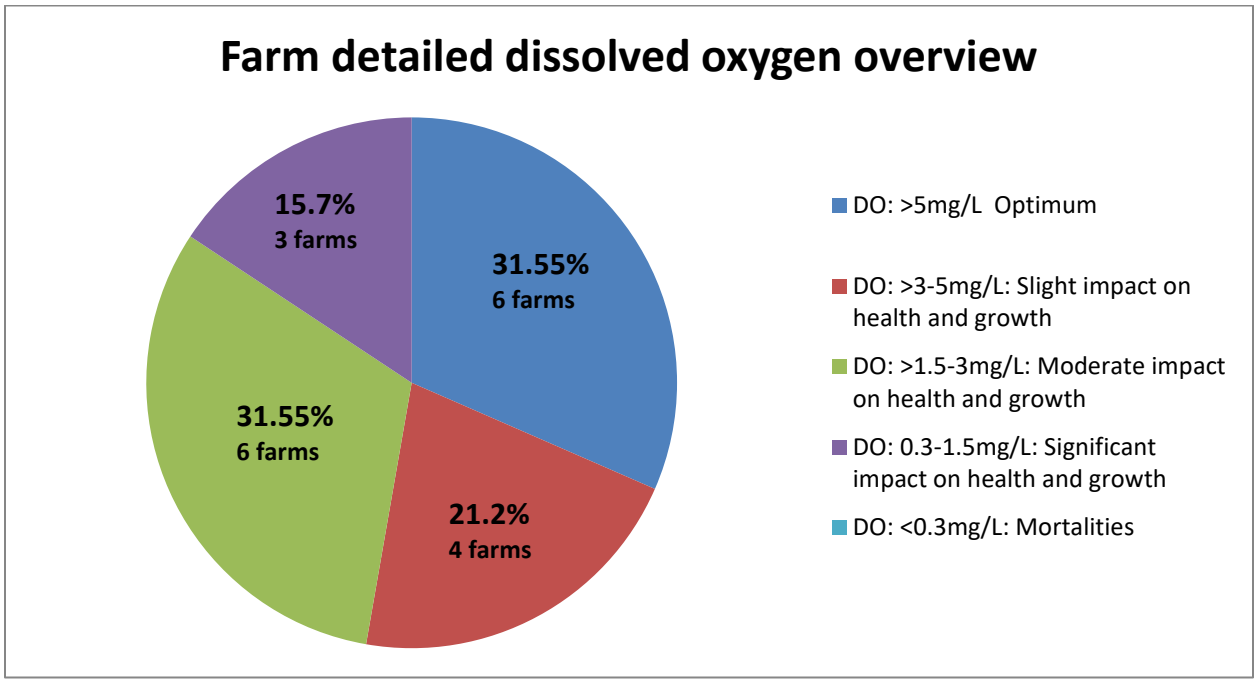


Fig. 4-2 Percentage distribution of farm DO ranges with associated impact upon fish population (Andrews et al., 1973; Boyd, 2000.b; Boyd, 2004; DeLong et al., 2009; Hollerman and Boyd, 1980; Noga 2010.d; Swann, 1997)

Table 4-2 Colour-highlighted comparison of farm DO ranges (mg/L)

Farm:	DO Range (mg/L)
GA	>1.5-3
GB	>3-5
GC	>5
GD(a)	>3-5
GD(b)	>1.5-3
GE	>1.5-3
GF(a)	>3-5
GF(b)	0.3-1.5
GG	>1.5-3
GH	>5
NA	>5
NB	>5
NC	>1.5-3
ND	>0.3-1.5
NE	>0.3-1.5
NF	>5
NJ	>1.5-3
LB	>3-5
LC	>5

4.2.1.2 Temperature

Water temperatures in systems tested varied between 17.8˚C and 32.2˚C, with a mean of 25.4 ˚C. Despite tunnel and additional heating applications, system water temperatures showed correlation with seasons, with definite lower water temperatures through Autumn and Winter.

Table 4-3 Comparison of farm water temperatures (^{0}C) and season of sampling. Systems colour-highlighted according to ranges in Fig. 4-3

Farm:	Water temperature (°C)	Season
GA	32.2	Mid-Summer (December)
GB	26.2	Mid-Summer (December)
GC	31.4	Mid-Summer (January)
GD(a)	27.9	Late Summer (February)
GD(b)	26.5	Late Summer (February)
GE	29.2	Late Summer (February)
GF(a)	26.6	Late Summer (February)
GF(b)	31.9	Late Summer (February)
GG	26.7	Late Summer (February)
GH	26.3	Autumn (April)
NA	23.4	Autumn (May)
NB	22.8	Autumn (May)
NC	23.6	Autumn (May)
ND	21.1	Winter (July)
NE	23.4	Winter (July)
NF	23.3	Winter (July)
NJ	23.5	Winter (August)
LB	17.8	Winter (July)
LC	19.1	Winter (July)

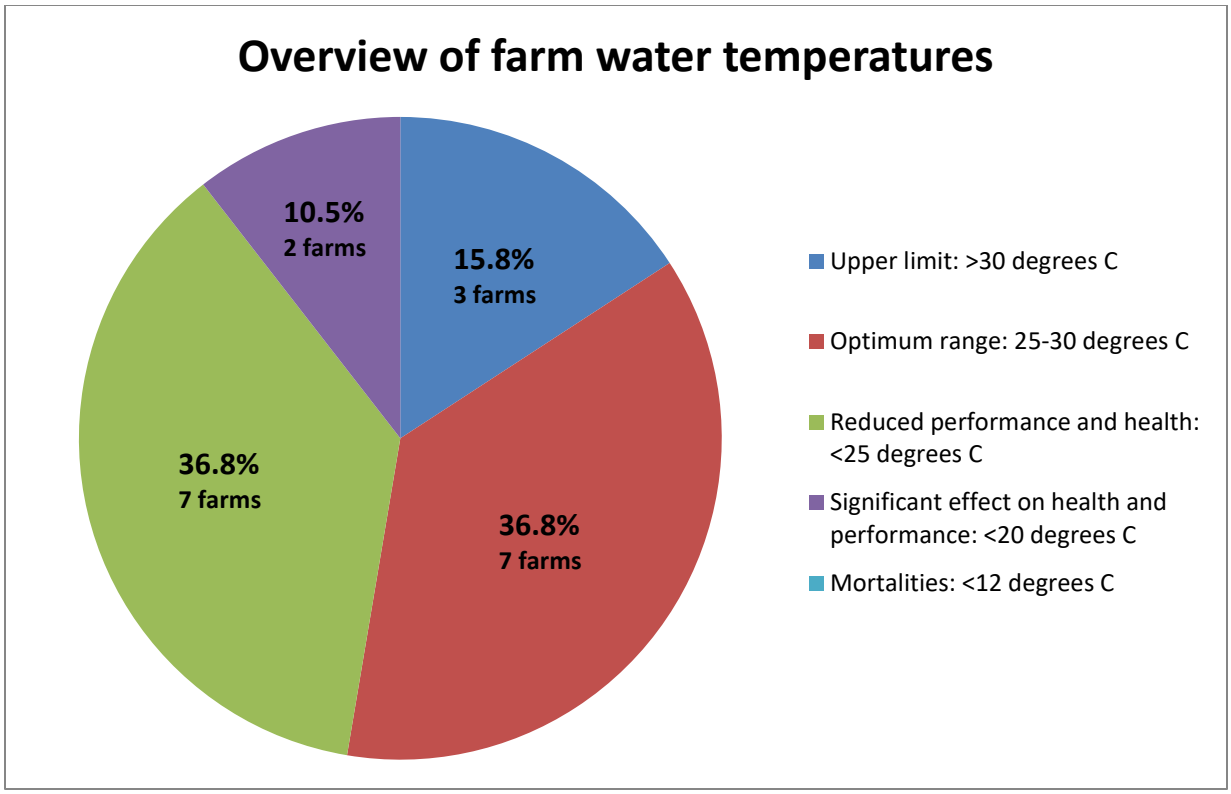


Fig. 4-3 Percentage distribution of farm temperatures with associated potential impact upon fish populations (Aquatic Network, 2012; Boyd, 2004; El-Sayed, 2006.b; El-Sayed and Kawanna, 2008; James, 2014; Towers, 2005; Watanabe et al., 1993)

4.2.1.3 CO_2

All farm water samples showed elevated CO_2 levels, many within extremely high readings.

Readings varied between a minimum of 29mg/L to well over a maximum of 150mg/L, and a mean of 83.45mg/L .

Table 4-4 Overview of farm CO_2 readings: all measured over the ideal maximum of 20mg/L (Southgate, 2005)

Farm:	
GA	114.4
GB	117.8
GC	31.8
GD(a)	66
GD(b)	50
GE	51
GF(a)	116.8
GF(b)	50.2
GG	150
GH	104.4
NA	45
NB	150
NC	101.4
ND	100
NE	76.4
NF	48.6
NJ	60
LB	29
LC	122.8

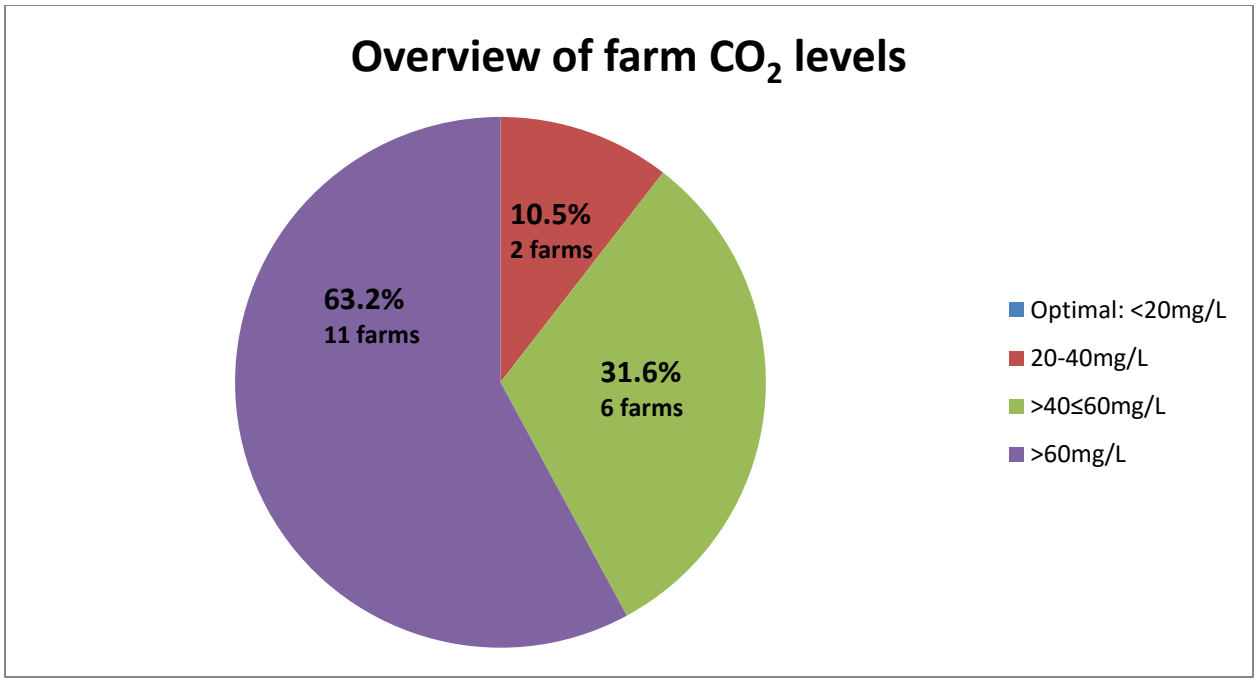


Fig. 4-4 Percentage distribution of farms with respect to water CO_2 ranges: (Aquatic Network, 2012; Fish, 1956; Loh and Landos, 2011.e; Timmons and Ebeling, 2013.c)

4.2.1.4 Ammonia (NH_3-TAN and NH_3-UIA)

Total ammonia nitrogen (TAN) readings varied between 0 and 3mg/L with a mean of 1.395mg/L. Un-ionized form of ammonia (UIA) varied between 0mg/L and 0.37mg/L, with a mean of 0.06mg/L .

Table 4-5 Farm TAN and NH_3-UIA readings, with TAN readings ≥ 3mg/L and NH_3-UIA readings ≥ 0.05mg/L, highlighted in red as the upper maximum recommended levels (Noga, 2010.d; Timmons and Ebeling, 2013.c)

Farm:	NH_3-N: TAN (mg/L)	NH_3-UIA (mg/L)
GA	0.2	0.005
GB	0.8	0.036
GC	1	0.089
GD(a)	0.4	0.009
GD(b)	3	0.072
GE	2	0.1
GF(a)	3	0.0195
GF(b)	2.4	0.0192
GG	3	0.0063
GH	0.5	0.11
NA	0.2	0.011
NB	0	0
NC	3	0.37
ND	3	0.06
NE	0	0
NF	0.2	0.04
NJ	3	0.18
LB	0.7	0.01
LC	0.1	0.005

The conversion chart by Emerson *et al.,* 1975 was used to calculate NH_3-UIA, from TAN, taking into account water temperature and pH readings.

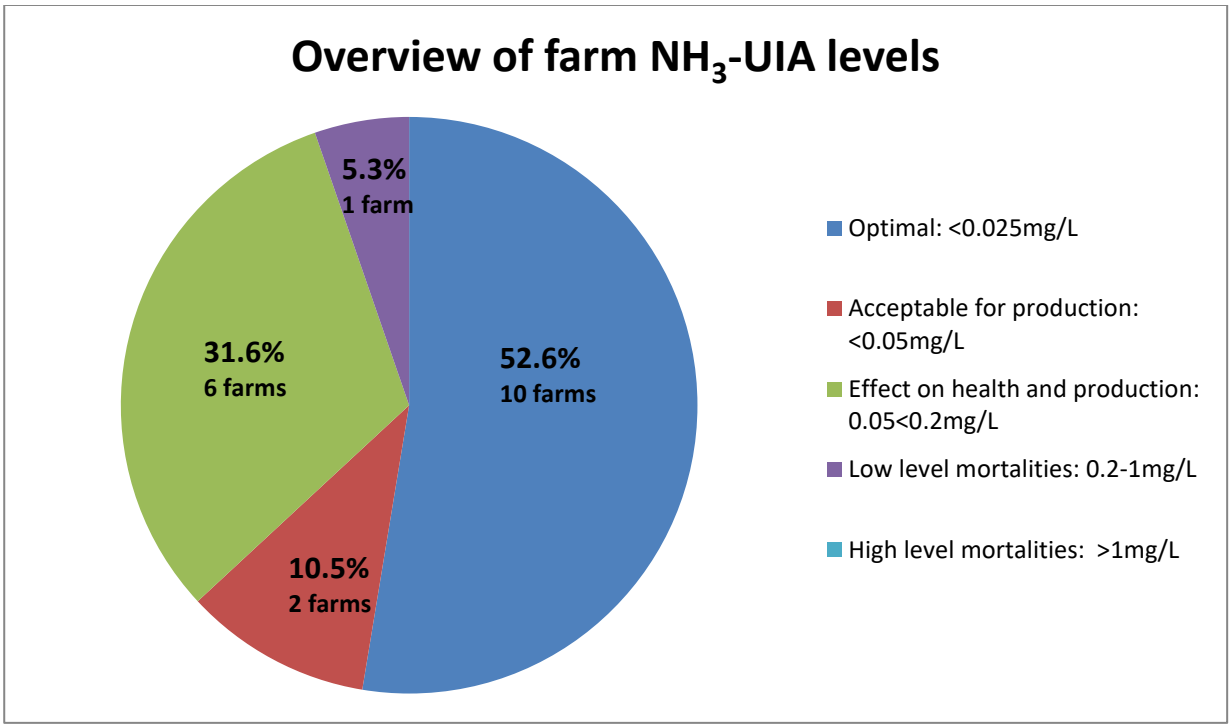


Fig. 4-5 Percentage distribution of farms through a scaled health- and production-related range (Boyd, 2000.c; Loh and Landos, 2011.e; Noga, 2010.d; Timmons and Ebeling, 2013.c).

52.6% of farms assessed measured UIA levels below the optimal 0.025mg/L level, while a further 10.5% fell just short of the 0.05mg/L mark *(Table 4-5).*

However, a large percentage, 31.6%, fell within the 0.05-2mg/L range. One farm, farm NC, presented with extremely high levels between 0.2-1mg/L (0.37mg/L) *(Table 4-5).*

4.2.1.5 Nitrite (NO_2^-)

Nitrite levels assessed varied from 0 to 1.32mg/L, with a mean of 0.576mg/L, post conversion from nitrite-nitrogen levels *(Fig. 4-6).*

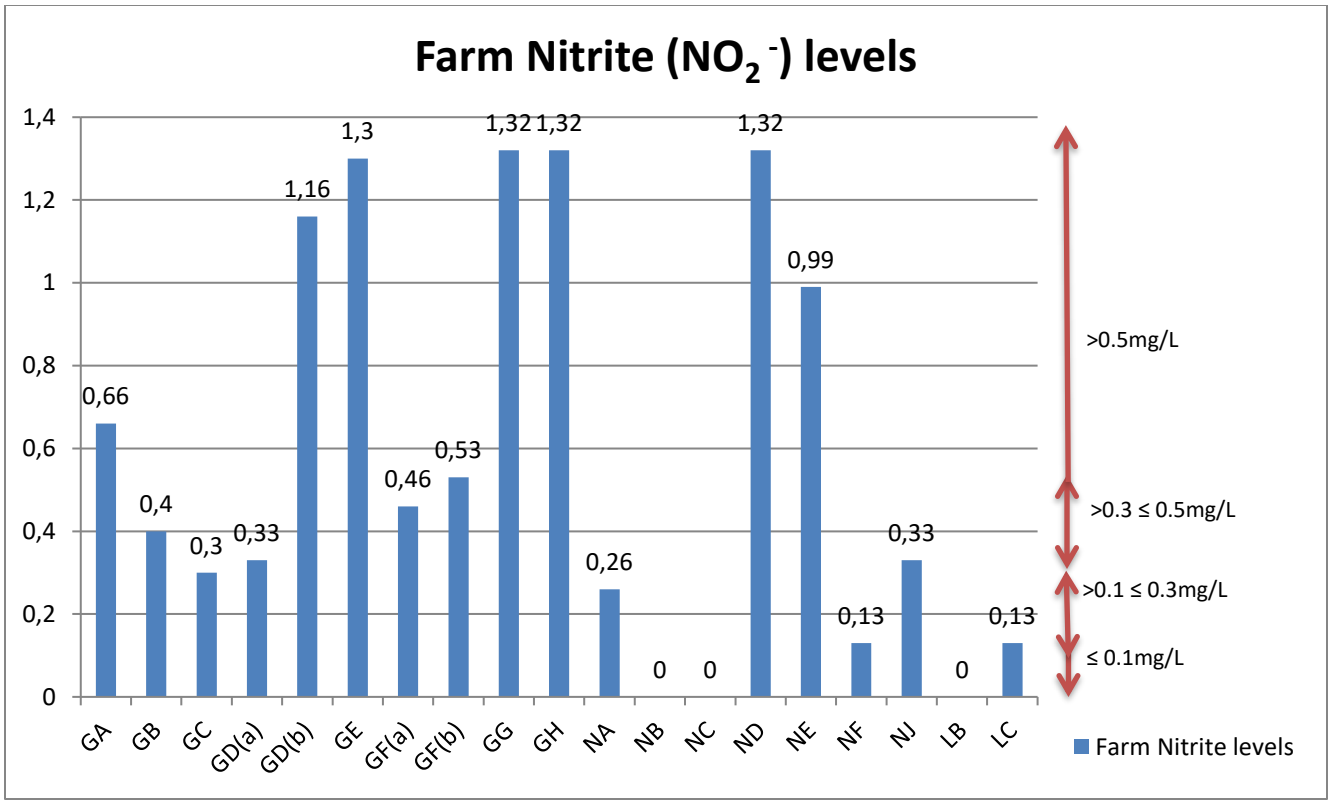


Fig. 4-6 Comparative overview of farm NO_2^- readings, with readings >0.5mg/L likely to reflect significant production stress and secondary disease, readings >0.3 ≤ 0.5mg/L: production stress, lethargy and anorexia, readings >0.1 ≤ 0.3mg/L considered acceptable, and readings ≤ 0.1mg/L ideal (Aquatic Network, 2012; Huchzermeyer, 2015; Loh and Landos, 2011.e)

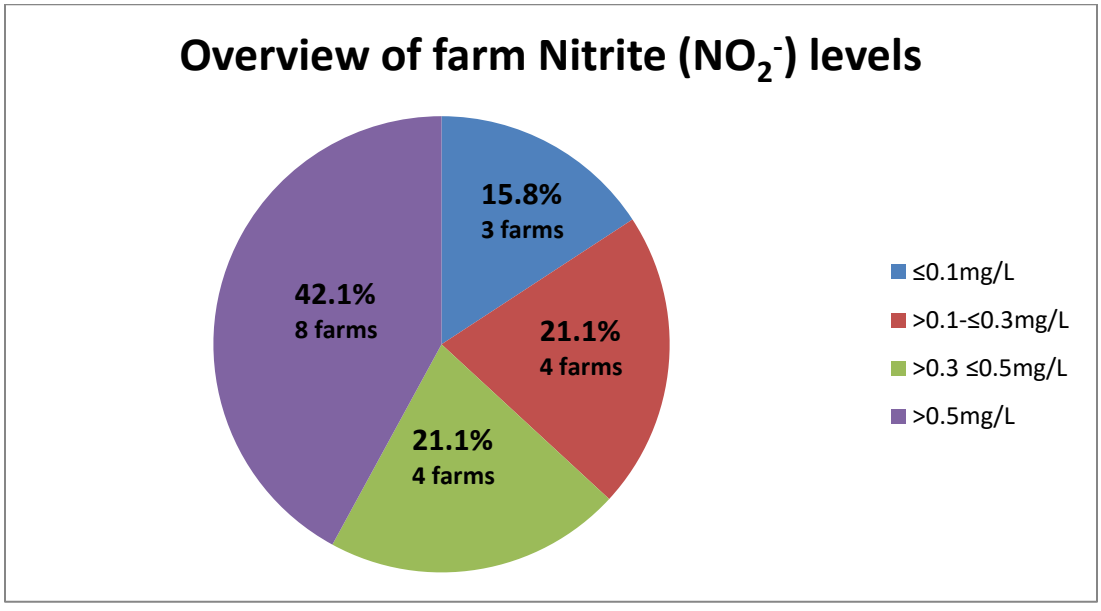


Fig. 4-7 Percentage distribution of farms through the NO_2^- production-related ranges

4.2.1.6 Nitrate (NO_3^-)

Nitrate was assessed in all systems, but results were inconclusive due to the limited test range (0-4.4mg/L) of the of the test kit used. Those farms with maximum readings (≥ 4.4mg/L), were advised to independently test water and confirm that readings were within the safe margin ≤ 27mg/L (Broders *et al.,* 2005).

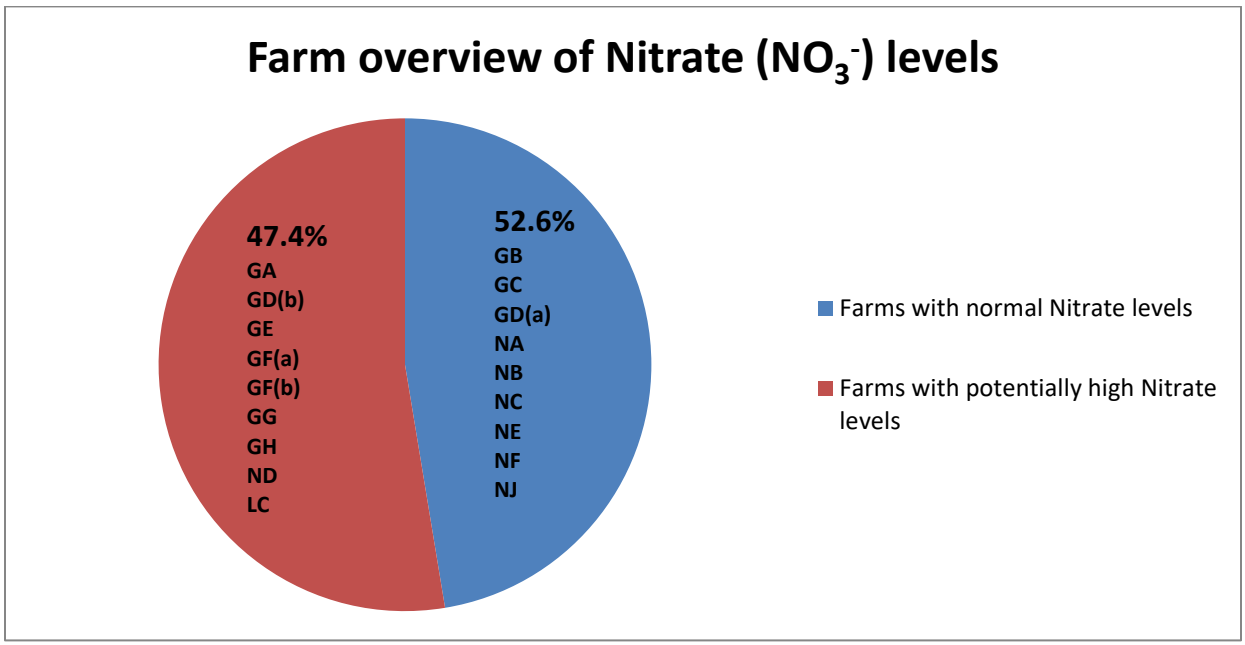


Fig. 4-8 Percentage distribution of farms with respect to NO_3^- readings, with those ≤ 4.4mg/L considered normal, and those > 4.4mg/L potentially too high (Broders et al., 2005; Loh and Landos, 2011.e)

4.2.1.7 pH

Water pH readings varied between 6 and 8.5, with a mean of 7.785 .

95% of water readings fell within a normal range of 6.5-9 (Boyd and Tucker, 1998).

Table 4-6 Overview of farm pH readings

Farm:	Water pH
GA	7.5
GB	7.8
GC	8
GD(a)	7.5
GD(b)	7.5
GE	7.8
GF(a)	6
GF(b)	6.8
GG	6.5
GH	8.5
NA	8
NB	8.5
NC	8.5
ND	7.5
NE	8
NF	8.5
NJ	8
LB	8.5
LC	8

4.2.1.8 Hardness, Alkalinity, and CH:TA ratio

Water hardness and alkalinity levels tended to be quite high throughout all systems.

Hardness fluctuated between a minimum of 73mg/L and a maximum of 586mg/L, with a mean of 252.8mg/L, while alkalinity showed greater range with minimums of 14 and a maximum of 730mg/L. Mean was 203.68mg/L. Acceptable readings for both were defined as 50-150mg/L (Boyd, 2000.e; Petrie-Hanson *et al.*, 2004.b; Shelton, 2010).

Ideal CH: TA ratio was defined as 1, acceptable as >1<5, and problematic <1 and ≥5 (De Holanda Cavalcante *et al.*, 2014).

Table 4-7 Comparative overview of farm complete hardness (CH), total alkalinity (TA), CH:TA ratio readings and proposed effect upon system and fish

Farm	**Complete Hardness (CH)(mg/L)**	**Total Alkalinity (TA) (mg/L)**	**CH:TA ratio**	**Potential significance**
GA	300	126	2.3	Acceptable
GB	204	171	1.19	Acceptable
GC	176	78	2.3	Acceptable
GD(a)	182	250	0.73	Low buffering capacity, reduced growth
GD(b)	73	90	0.8	Low buffering capacity, reduced growth
GE	237	130	1.8	Acceptable
GF(a)	174	14	12.4	Osmotic stress, signif.reduced growth, low buffering capacity

GF(b)	390	35	11.1	Osmotic stress, signif. reduced growth, low buffering capacity
GG	117	47	2.49	Acceptable
GH	460	320	1.4	Acceptable
NA	89	100	0.89	Low buffering capacity, reduced growth
NB	205	120	1.7	Acceptable
NC	450	400	1.13	Acceptable
ND	231	94	2.5	Acceptable
NE	586	650	0.9	Low buffering capacity, reduced growth
NF	106	175	0.61	Low buffering capacity, reduced growth
NJ	100	165	0.61	Low buffering capacity, reduced growth
LB	450	730	0.62	Low buffering capacity, reduced growth
LC	273	175	1.56	Acceptable

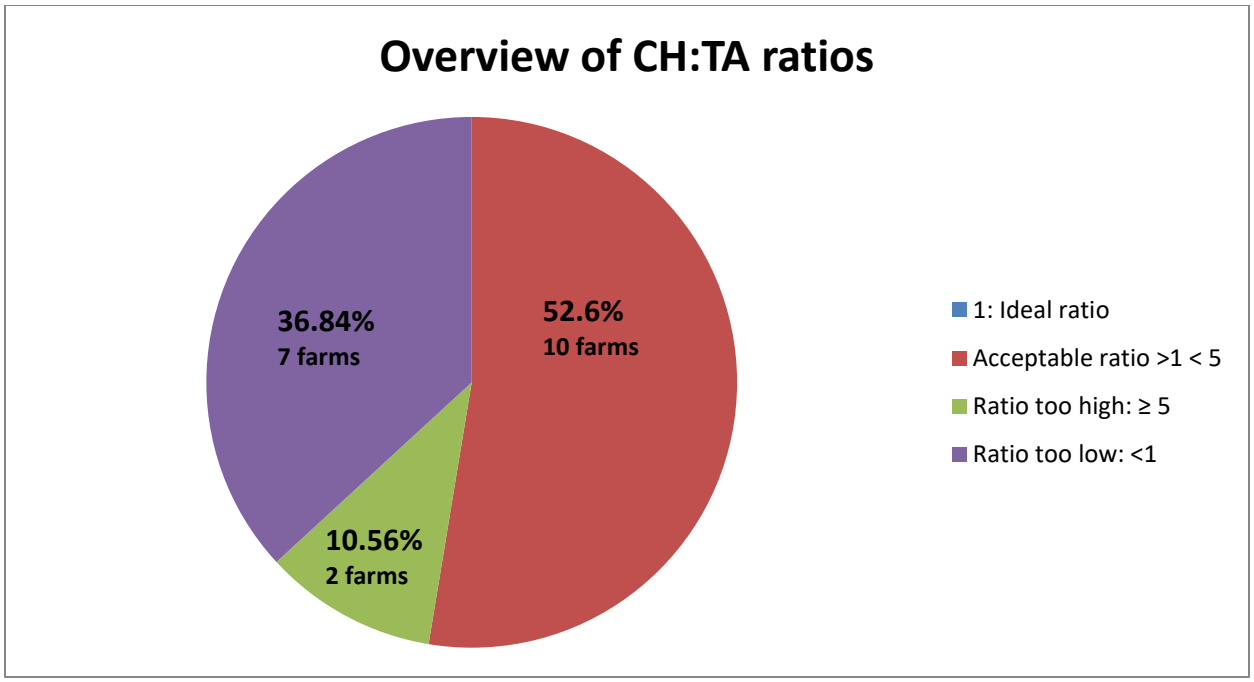


Fig. 4-9 Percentage distribution of farms with respect to their CH:TA ratios, with interpretation based on De Holanda Cavalcante et al., 2014

4.2.1.9 Other

Pre-system water holding tanks

The majority of farms (58%) made use of holding tanks to hold water for a period of time, prior to entering the system.

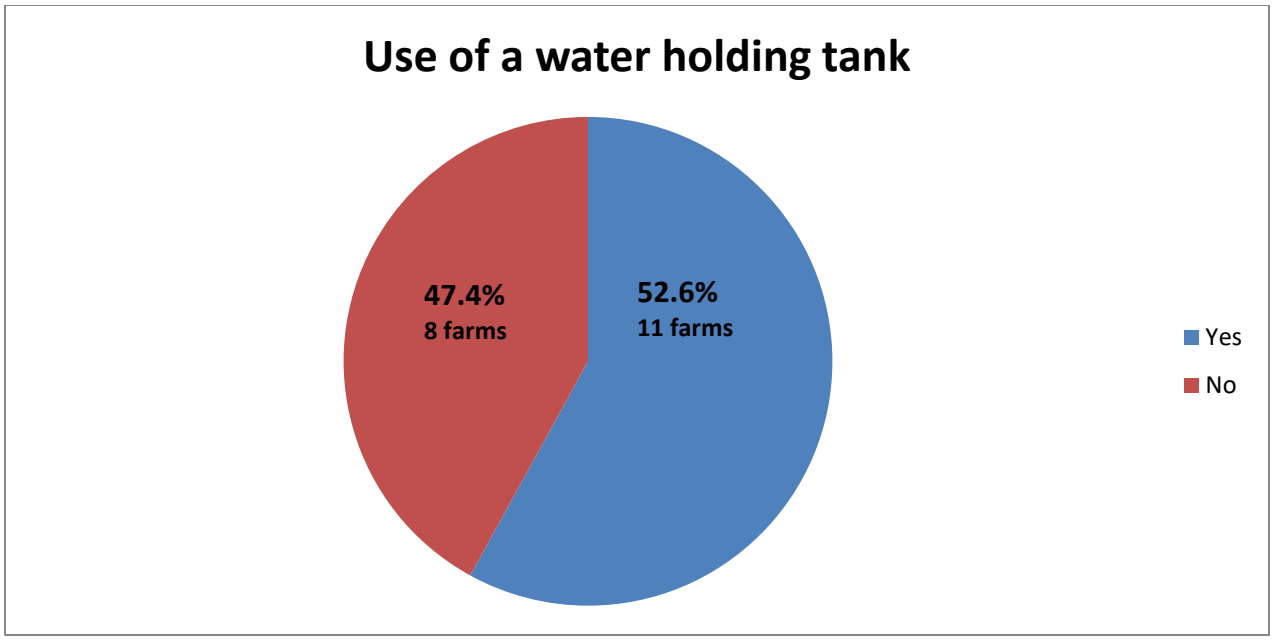


Fig. 4-10 Relative percentage of farms making use of pre-system water holding tanks

Green water systems

36.8% of farms (GA, GB, GC, GDa, GDb, NC, NF) had visibly high phyto/zooplankton levels *(See Table 4-30, PLATE 19:D and E.*

Hydrogen Sulphide Presence

Only one farm (NC) had suspicion of presence of hydrogen sulphide gas (odour).

4.2.2 Fish Assessment

4.2.2.1 Fish Behaviour

Piping behaviour was noted on 4 of the 19 systems assessed, accounting for 21.1% of the farms.

No other behavioural abnormalities were noted in fish.

4.2.2.2 Morphometrics and Age

Table 4-8 Overview of average fish morphometrics: length, weight and age, for each farm sample group. Comparison is drawn between actual average fish weight per farm and projected potential weight at an acceptable water temperature of 26°C, and at actual system water temperature (Til-Aqua Tilapia Hatchery Management System, 2016). Farm ave. fish weight comparison >100% of projected: highlighted green; <100% ≥75%: highlighted orange, <75%: highlighted red.

Farm	Average weight (grams)	Average length (cm)	Age (months)	Projected weight (g) for a water temp. of 26° C	Weight comparison to a 26° C growth curve	Actual water temp. (0 C)	Weight comparison to projected similar temp. growth curve
GA	165	21.2	11	>750g	Below 22% of potential weight	32.2	22% of potential weight
GB	237	23.7	4	160g	148% of potential weight	26.2	148% of potential weight
GC	126	19.7	4	270	84% of potential weight	31.4	47% of potential weight

GD(a)	118	20.7	7	500	78% of potential weight	27.9	63% of potential weight
GD(b)	156	20.2	4	180	104% of potential weight	26.5	87% of potential weight
GE	203	22.8	6	550	68% of potential weight	29.2	37% of potential weight
GF(a)	112	23.4	6	300	37% of potential weight	26.6	37% of potential weight
GF(b)	111	22.4	9	>750	17.6% of potential weight	31.9	15% of potential weight
GG	119	19.2	12	>750	Below 16% of potential weight	26.7	16% of potential weight
GH	178	20.6	6	300	59% of potential weight	26.3	59% of potential weight
NA	149	20.6	15	300	Below 20% of potential weight	23.4	50% of potential weight
NB	195	23.8	Mixed		Mixed ages	22.8	Mixed ages
NC	111	20.1	Mixed		Mixed ages	23.6	Mixed ages
ND	286	23.2	Mixed		Mixed ages	21.1	Mixed ages
NE	246	22.3	12	300	Below 33% of projected weight	23.4	82% of potential weight

NF	179	22.5	Mixed		Mixed ages	23.3	Mixed ages
NJ	174	19.7	6	90	58% of projected weight	23.5	193% of potential weight
LB	157	19.9	12	300	Below 21% of projected weight	17.8	52% of potential weight
LC	123	20.2	Mixed		Mixed ages	19.1	Mixed ages

(Farms NB, NC, ND, NF, and LC) with mixed age populations could not be assessed)

4.2.2.3 Taxonomic differentiation *(PLATE 2)*

Using the ten fish sampled as a representation of each farm's grower phase , 35.3% of farms visited were farming with only *O. niloticus*, 17.6% were farming with only *O. mossambicus,* and 58.8% had hybrid populations or combinations of *O. niloticus* and *O. mossambicus.*

Plate 2: Farmed fish species

A: Oreochromis mossambicus *adult male, farm LC;* ***B:*** O. mossambicus *"Red 5" strain adult male, farm GF;* ***C:*** Oreochromis niloticus *adult male, farm NB;* ***D:*** O. niloticus *adult male, farm GC;* ***E****: Hybrid adult male, farm NF;* ***F****: Hybrid adult male, farm LB;****G****: Hybrid adult female, farm GE*

Only two of the 19 systems assessed were farming with other freshwater aquaculture species:

Farms GD(a) with *Tilapia rendalli* and rainbow trout *(Oncorhynchus mykiss*), and GE with catfish (*Clarias gariepinus*) and rainbow trout. On both farms, these species were kept separate in their own systems and tanks, yet housed in the same tunnels and in close proximity. No fish with appearances typical of *O. aureus* or *O. andersonii,* were seen.

4.2.2.4 External Examination

Body Condition

The majority of fish assessed, had low to average body condition scores.

Table 4-9 Overview of average fish body condition scores (BCS) and percentage composition through sample population (poor BCS highlighted red, average: orange, and good: green)

Farm:	Average farm body condition score	Percentage distribution
GA	1	100% poor BCS

GB	2.3	10% poor BCS
		50% moderate BCS
		40% good BCS
GC	1.3	70% poor BCS
		30% moderate BCS
GD(a)	3	100% good BCS
GD(b)	1	100% poor BCS
GE	1.7	30% poor BCS
		70% moderate BCS
GF(a)	3	100% good BCS
GF(b)	3	100% good BCS
GG	1.7	40% poor BCS
		50% moderate BCS
		10% good BCS
GH	2.3	10% poor BCS
		50% moderate BCS
		40% good BCS
NA	1.2	80% poor BCS
		20% moderate BCS
NB	1.8	20% poor BCS
		80% moderate BCS
NC	1	100% poor BCS

ND	2.3	30% poor BCS
		10% moderate BCS
		60% good BCS
NE	2.3	70% moderate BCS
		30% good BCS
NF	1.3	70% poor BCS
		30% moderate BCS
NJ	2.8	20% moderate BCS
		80% good BCS
LB	1.9	10% poor BCS
		90% moderate BCS
LC	1.6	40% poor BCS
		60% moderate BCS

15.8% of farms presented with poor average fish body condition scores, 42.1% with moderate body condition scores, and 42.1% with good body condition scores.

However, an overview of general body condition at fish level presented a different picture:

Table 4-10 Overview of fish BCS through full sampling population

Poor condition	**38.7%**
Average condition	37.5%
Good condition	23.8%

External pathology

In the visual assessment of fish prior to microscopy and necropsy, most were free from macroscopically visible abnormalities with some exceptions:

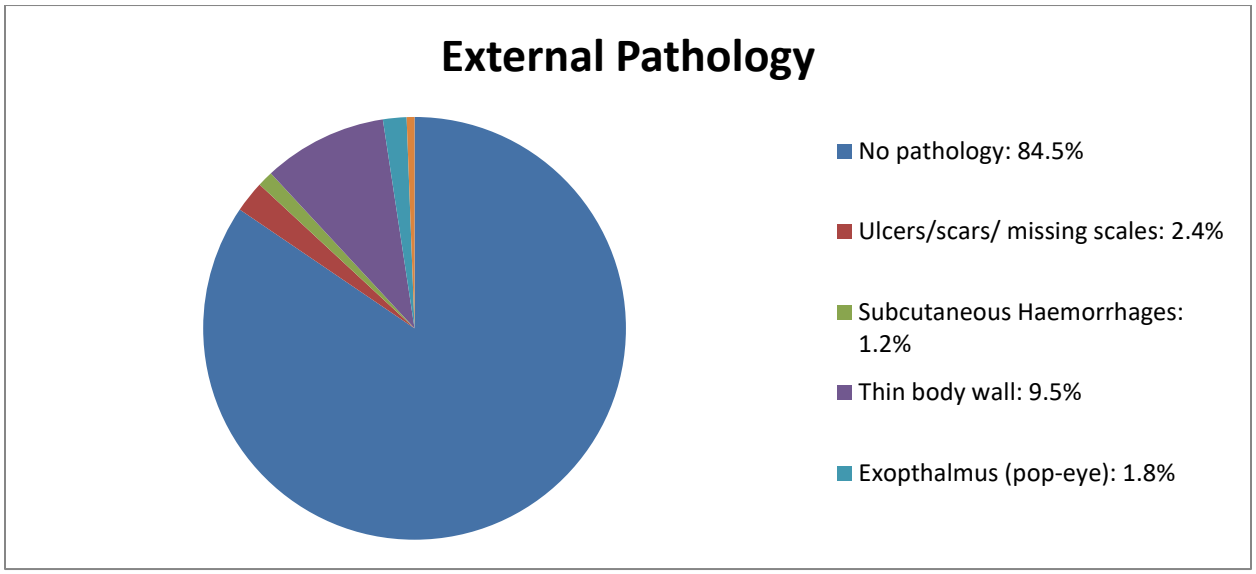


Fig. 4-11 Percentages of sampled fish presenting with various macroscopic external pathological abnormalities

Fig. 4-12 External macroscopic pathology: severe skin erosions and fin and tail fraying

Fig. 4-13 External macroscopic pathology: thin muscle walls, subcutaneous haemorrhage and A.schubertii *septicaemia*

Fig. 4-14 External macroscopic pathology: severe exophthalmos and bulbar cellulitis secondary to a combined Aeromonas hydrophila*,* Staphylococcus pseudo-intermedius *and* Lactococcus garviae *septicaemia. Note the lack of other macroscopic lesions and moderate BCS.*

Fig. 4-15 External macroscopic pathology: severe traumatic fraying of tail

Gender

Sexing fish on external examination proved an unreliable tool. Suspected gender was recorded and then confirmed on necropsy *(See 4.2.2.6 b).*

Following the physical examination of the fish that included macro- and microscopic tissue examination, a list of detected abnormalities could be compiled that served as the key dependant variables in the study. These factors could be semi-quantified and identified as follows:

1. Average *Trichodina* spp. infestation rate
2. Average Gyrodactylidea spp. infestation rate
3. Average Dactylogyridae spp. infestation rate
4. Average *Ambiphrya* spp. infestation rate
5. Average *Ichthyobodo necator* complex infestation rate
6. Average total parasitic burden
7. Degree of gill lamellar epithelial hyperplasia
8. Degree of gill lamellar goblet cell hyperplasia
9. Degree of gill eosinophilic granular cell (EGC) infiltration at the base of the gill arches
10. Hepatocellular lipid content
11. Hepatocellular lipofuscin content
12. Hepatocellular nuclear activity
13. Degree of gastritis
14. Presence of septicaemic disease
15. Growth

4.2.2.5 On farm direct light microscopy

Ectoparasites on gills and skin were a very common finding. The dominating groups encountered were the motile ciliate, *Trichodina* spp., the sessile ciliate, *Ambiphrya* spp., the flagellate, *Ichthyobodo necator* complex*,* and the monogenean orders of Gyrodactylidea and Dactylogyridae *(APPENDIX 4 TABLE 1: Data sheets).* A few fish with *Chilodonella* spp. and *Epistylus* spp. infestations were encountered.

***Trichodina* spp.**

This motile ciliate, was by far the most prevalent parasite encountered, detected on 68.4% of farms, and 57% of all fish. Great variation in infestation rates was often observed on the same farm, with levels ranging from 1 parasite per 10X microscopic field, to > 15. Variation was also encountered at a fish level between the various sampling sites. In some cases, equal parasite number representation between gill clips, gill scrapes and skin scrapes were found, yet in other fish, parasites could only be detected at one of the diagnostic wet prep locations. However, they were most often found on skin scrape examination, with 50.6% of positive fish presenting with parasites in skin mucous, 35.1% in gill clip examination, and 23.8% in gill scrape examination. *Trichodina* spp. were often encountered in association with monogenean and/or *Ambiphrya* infestations. *Ichthyobodo necator* was rarely encountered together with *Trichodina*, but most often in isolation. Interestingly, *Trichodina* was noted by its absence in farms where *Ichthyobodo necator* complex dominated. There was no distinct species predilection for *Trichodina.* Although 62% of fish testing positive for *Trichodina* were *O. niloticus, (*14% hybrids

and 21% *O. mossambicus),* overall species differentiation reflected general species differentiation of all sampled fish.

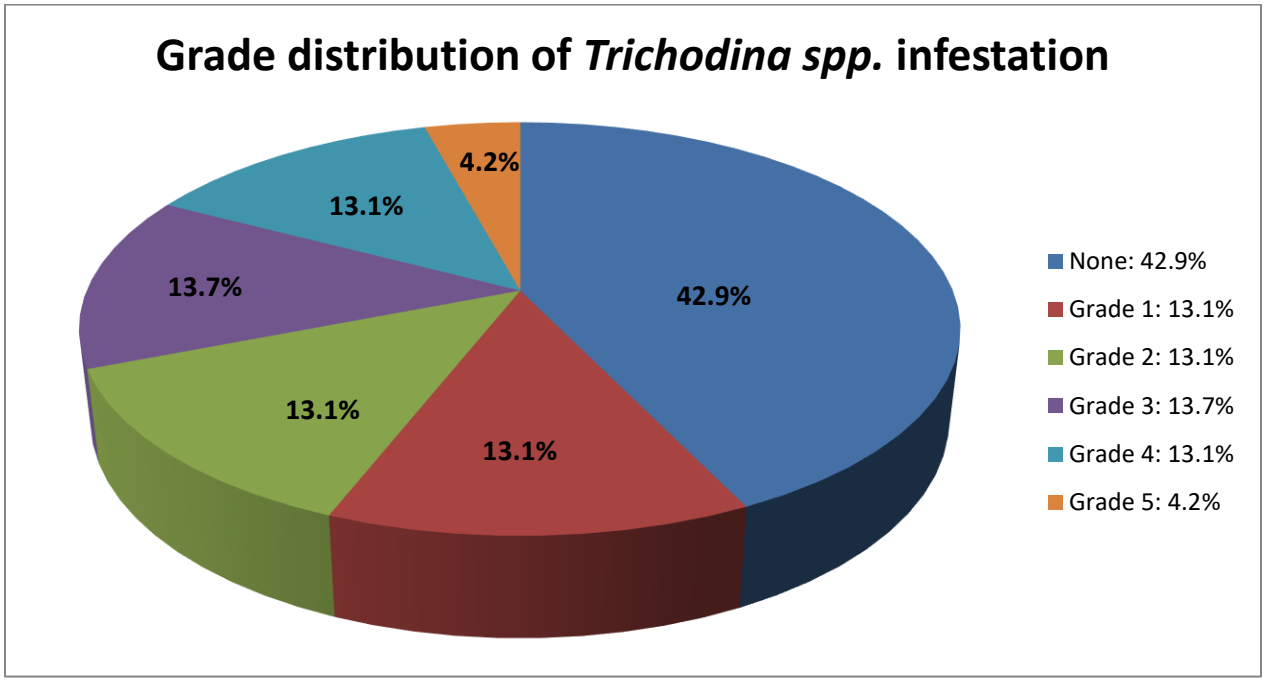


Fig. 4-16 Percentage representation of various infestation grades of Trichodina *spp. (See Table 3-7) through total fish population sampled (fish grade was taken as the highest encountered grade between all three wet mounts per fish)*

Only 42.9% of fish were free of *Trichodina* spp. Parasite grades were very equally represented between grades 1 to 4 with all featuring between 13.1-13.7%. Grade 5 parasite infestation levels were significant at 4.2%.

Statistical data analysis, using a Pearson correlation model of the effect of key independent variables on average *Trichodina* spp. prevalence revealed the following: (negative values indicate a negative correlation, positive values indicate a positive correlation).

Table 4-11 Pearson correlation model of the effect of independent variables on fish average Trichodina *spp. burdens*

Stocking rate (kg/m^3)	Water temp(°C)	Water pH	Water DO (mg/L)	Water CO_2 (mg/L)	NH_3 toxic: UIA (mg/L)	Nitrite NO_2^- (mg/L)	CH:TA ratio	
0.220	-0.014	-0.125	-0.028	-0.301	-0.470	-0.103	0.326	

Red: High correlation; **Orange**: moderate correlation; **Green:** Low correlation

There is a strong significant negative correlation ($p \leq 0.05$) with a predictive value of -0.470 between *Trichodina* spp. and NH_3-UIA, using this model. CH:TA ratio offers a significant moderately positive predictive correlation ($p \leq 0.05$) with prevalence of 0.326, CO_2 a moderately significant negative predictive correlation ($p \leq 0.05$) with prevalence value of -0.301, and stocking rate a low positive correlation ($p \leq 0.05$) with predictive value of 0.220. This suggests that *Trichodina* spp. infestation would be predisposed to increase where water CH:TA ratio increases, and stocking rate/ density increases, while a high NH_3 or CO_2 level in the water body would suppress levels of *Trichodina* spp.

A stepwise regression model corroborated these correlations and placed the independent variables in order of decreasing significance as: NH_3-UIA, CH:TA ratio, CO_2, stocking rate.

Pearson's canonical correlations between *Trichodina* spp., other gill parasites and gill pathology yielded the following results:

Average *Trichodina* spp. prevalence shows a significant positive correlation ($p \leq 0.01$) with gill goblet cell hyperplasia, average *Ambiphrya* prevalence , and average total parasite score. It showed a significant negative correlation ($p \leq 0.01$) with *Ichthyobodo necator* complex

prevalence. A positive correlation ($p \leq 0.05$) was found with average Dactylogyridae levels and gill epithelial hyperplasia. No correlation with EG cell infiltration in the base of the gill arches could be found.

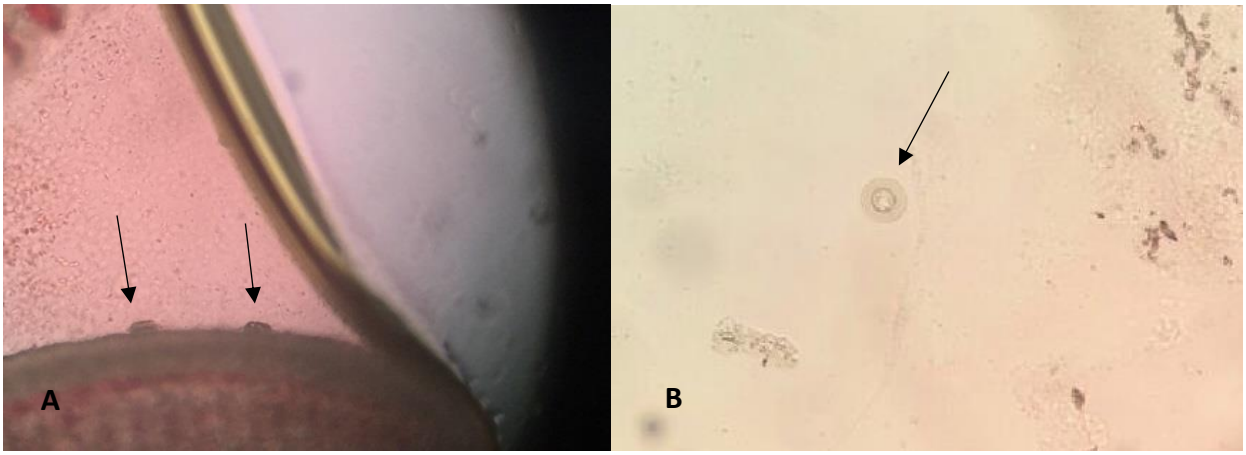


Fig. 4-17 Light microscopy images: Trichodina *spp. A-* Trichodina *moving and feeding on gill lamellum (100X), B-motile* Trichodina *spp. in skin mucus prep, with typical circular shape seen in dorsal view (100X)*

Monogeneans

Monogeneans from both orders of Gyrodactylidea and Dactylogyridae, were fairly frequently encountered, yet for the most part in low numbers, and not consistently through all fish on a farm. Gyrodactylid species ranged in number between 0 to 5 parasites per 40X magnification (low power field (LPF)), and the dactylogyrid species between 0 and 6 parasites per LPF. Interestingly, in addition to occurrence on skin, the gyrodactylids were also detected on gills scrapes and clips, which is an uncommon finding (Reed *et al.,* 2009), while the dactylogyrids showed a definite predilection for the gills, never appearing on skin mucus scrapes. Both were often encountered together with high *Trichodina* spp. parasite burdens.

Gyrodactylid species appeared in 47.4% of farms assessed, but only 14.3% of all the fish assessed. They were generally present in low numbers, and on occasional fish within the sample group, with the exception of farm NA, with an 80% infestation rate. Although present in gill clips and gill scrapes, numbers were consistently higher in skin mucus preparations, with 62.5% of positive fish reflecting parasites on skin mucous scrapes, while only 41.6% showed evidence on gill scrapes and 29.2% on gill clips. A parasite level of grade 1 dominated through all wet-mount preparations. No grade 4 levels of parasite infestation were encountered. Although distribution through fish species showed predilection for *O. niloticus* and hybrids, with 60% of affected fish being *O. niloticus,* 27% hybrids, and only 13% of affected fish *O. mossambicus ,* again, this closely followed general fish species differentiation.

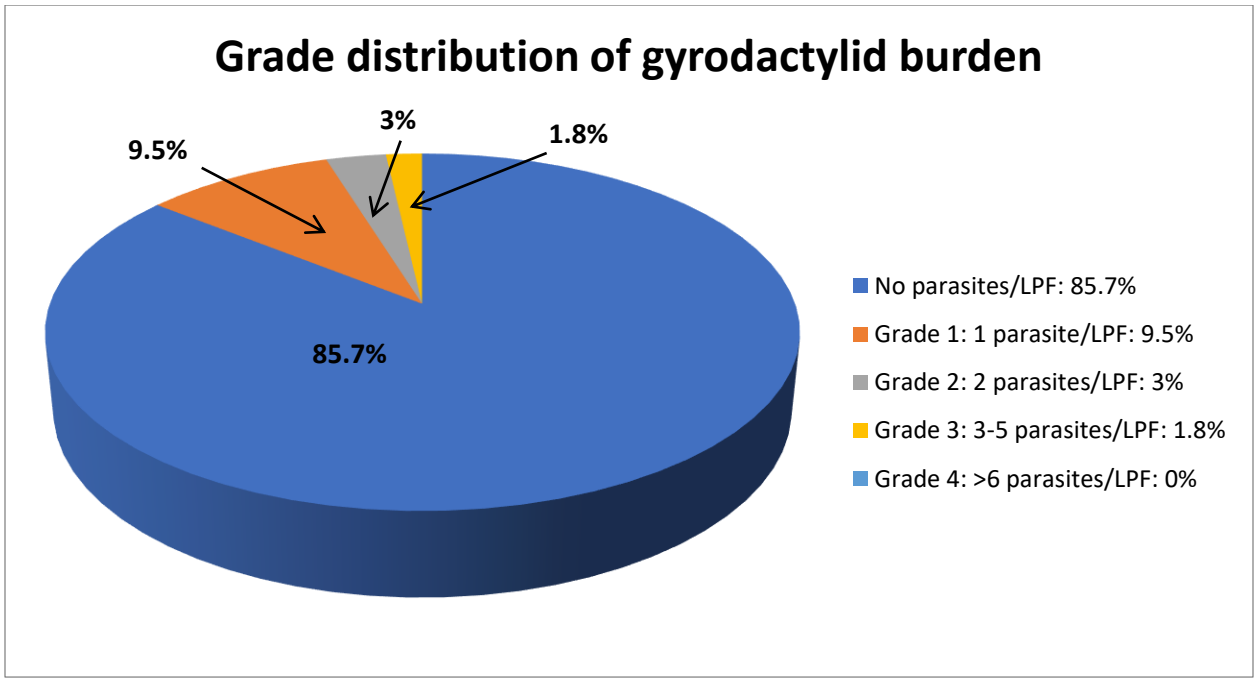


Fig. 4-18 Percentage distribution of various grades of gyrodactylid parasite burdens through total fish population (fish grade was taken as highest encountered grade between all wet prep mounts per fish)

Statistical data analysis, using a Pearson correlation model, of the effect of independent variables on average Gyrodactylidea prevalence revealed the following: (negative values indicate a negative correlation, positive values indicate a positive correlation)

Table 4-12 Pearson correlation model of the effect of independent variables on average gyrodactylid burdens on fish

Stocking rate (kg/m^3)	Water temp(°C)	Water pH	Water DO (mg/L)	Water CO_2 (mg/L)	NH_3 toxic: UIA (mg/L)	Nitrite NO_2^- (mg/L)	CH:TA ratio
0.034	0.054	-0.011	-0.183	-0.041	-0.172	-0.097	0.134

Red: High correlation; **Orange**: moderate correlation; **Green:** Low correlation

No correlations of significance were reflected. Water parameters and stocking density appears to exert no significant effect upon gyrodactylid burdens.

Pearson's statistical data correlation analysis between average Gyrodactylidea levels and other gill ecto-parasites or gill pathology, showed only a positive correlation of 0.18 ($p \leq 0.05$) between average Gyrodactylidea score and total parasite score. No significant correlations existed between this group of monogeneans and gill pathological changes or other ecto-parasites.

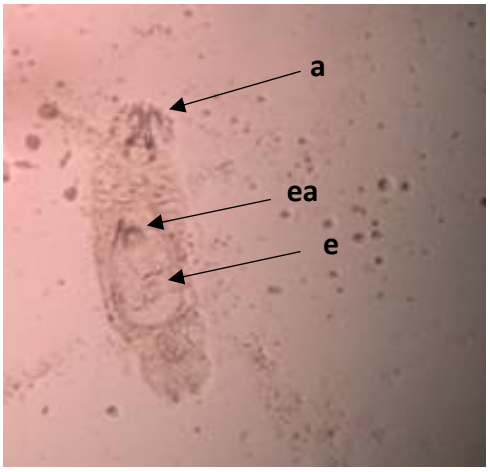


Fig. 4-19 Light microscope image: gyrodactylid in skin mucus prep (40X): note the lack of eyespots, the large developing embryo (e) with anchors (ea), and the prominent single pair of tail anchors (a)

Dactylogyrids were slightly more represented than the gyrodactylid species, with 57.9% of farms, and 19% of all fish, positive for this group of parasites. There was no overwhelming high infestation on any of the farms. Parasites were found on occasional fish only and in low numbers. Gill scrapes yielded higher infestation rates when compared to gill clips in assessing these parasites, and it was never detected on skin mucous scrapes on any of the fish. Only 7.8% of fish showed evidence on gill clips and 13.1% on gill scrapes. When present, a parasite level of grade 1 dominated through both preparations, with clips reflecting a 4.8% and scrapes a 11.3% prevalence. No grade 3 or 4 levels of parasite infestations were encountered. Interestingly, dactylogyrids were most commonly encountered on *O. niloticus,* with only the occasional hybrid or *O. mossambicus* carrying a detectable parasite. In fact, of the 32 fish carrying dactylogyrids, 82% were *O. niloticus,* 7% hybrids, and 11% *O. mossambicus.* Farms where *O. mossambicus* dominated as the farmed species, were largely unaffected by this parasite.

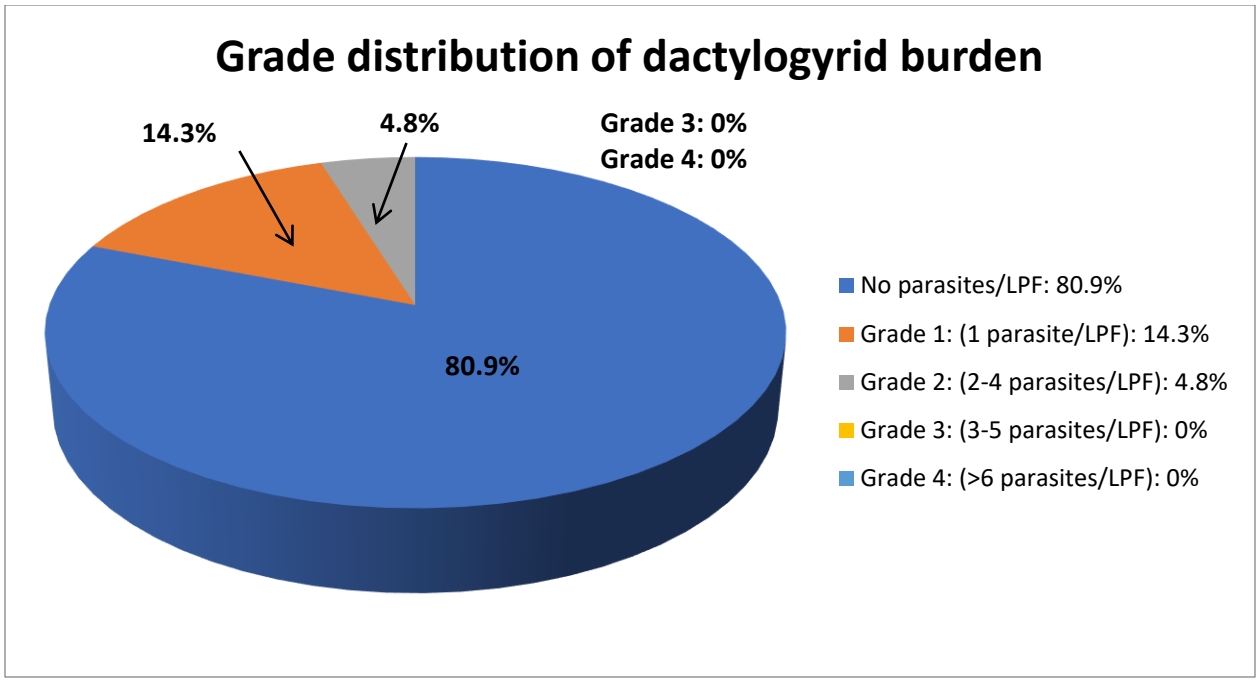


Fig. 4-20 Schematic representation of grade distribution of dactylogyrids through total fish population (fish grade was taken as highest encountered grade between all wet prep mounts per fish)

Statistical data analysis, using a Pearson correlation model, of the effect of independent variables on average dactylogyrid prevalence revealed the following: (negative values indicate a negative correlation, positive values indicate a positive correlation)

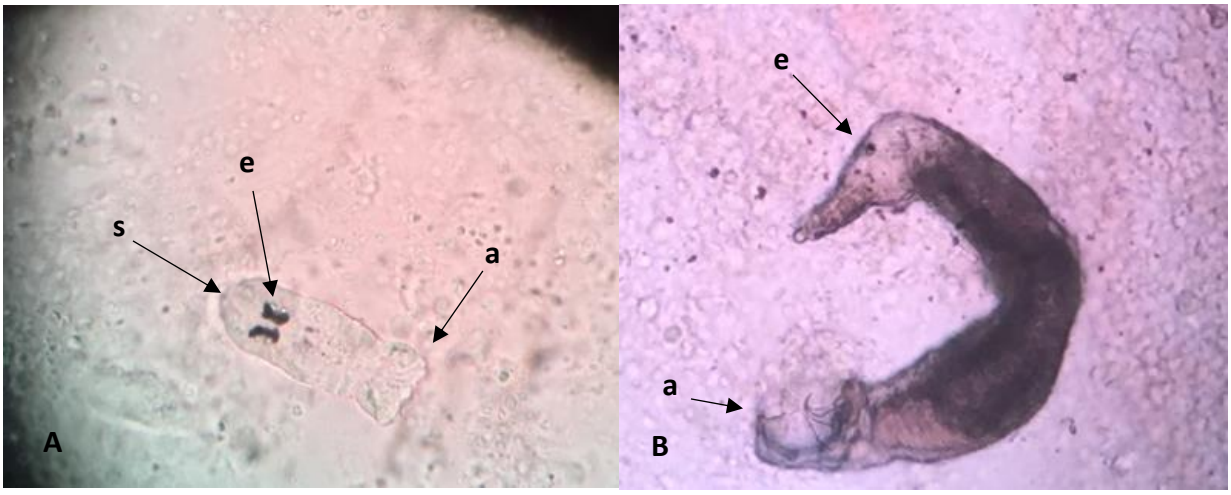
Table 4-13 Pearson correlation model of effect of independent variables on average dactylogyrid fish burdens

Stocking rate (kg/m^3)	Water temp(°C)	Water pH	Water DO (mg/L)	Water CO_2 (mg/L)	NH_3 toxic: UIA (mg/L)	Nitrite NO_2^- (mg/L)	CH:TA ratio
-0.111	-0.041	-0.175	-0.060	-0.239	-0.087	-0.067	0.090

Red: High correlation; **Orange**: moderate correlation; **Green:** Low correlation

Dactylogyrids correlated poorly with water parameters, with CO_2 being the only parameter showing a low-level significant negative correlation of -0.239 ($p \leq 0.05$). This was corroborated by a step-wise regression model.

Pearson's canonical correlation analysis between average dactylogyrid levels and other gill ecto-parasites or gill pathology, showed significant positive correlation of 0.183 ($p \leq 0.05$) between average dactylogyrid score and average *Trichodina* spp. score, and a significant positive correlation of 0.227 ($p \leq 0.01$) with total parasite score. No significant correlations existed between dactylogyrids and gill pathological changes.



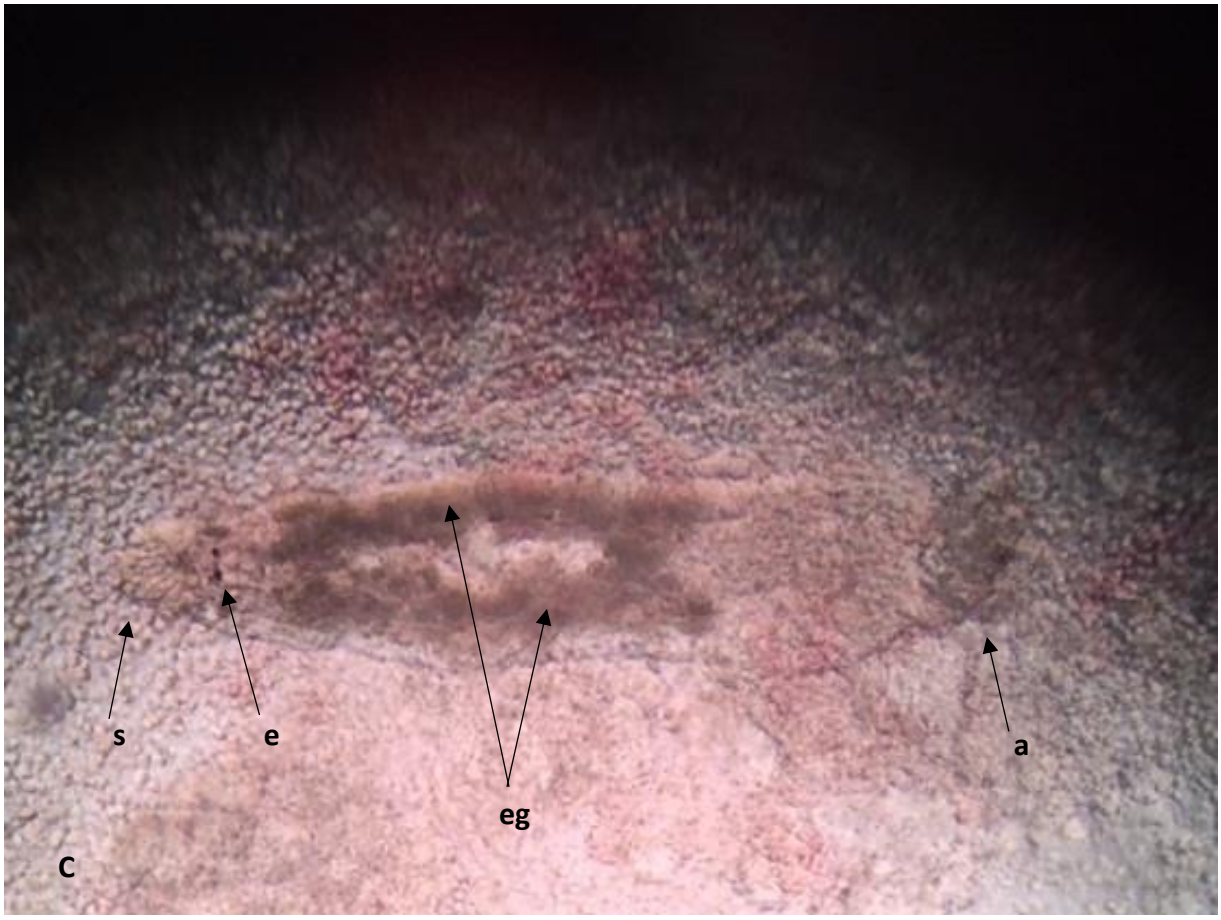


Fig. 4-21 Light microscope images: dactylogyrids: A: Adult in gill scrape prep(100X), B: Adult in gill scrape prep (100X), C: Adult in gill scrape prep (100X). Note e (eyespots), eg (eggs), a (attachment anchors), s (scalloped head)

***Ichthyobodo necator* complex**

Ichthyobodosis was encountered fairly frequently (21% of the farms, and 10.7% of the fish), and when present, occurred in high numbers. Fish in affected populations showed varying parasite infestation grades between 1 and 4, with most fish reflecting grade 2 or 3 levels. Parasites were often more easily detected, in their feeding stage on stained histopathology tissue sections, rather than wet-prep observation of the free-living stages, and numbers in these sections often reflected a grade 4 level. Interestingly, when encountered on a farm, they were generally

prevalent through all fish examined, and most often to the exclusion of any other ecto-parasites. These parasites were detected on only *O. niloticus spp.* and hybrids, never on *O. mossambicus.* 90% of positive fish were *O. niloticus.*

They were seen equally distributed between all wet mount preparations, with 7.8% of fish positive for parasites in mucous scrapes and gill clips respectively, and 7.7% in gill scrapes.

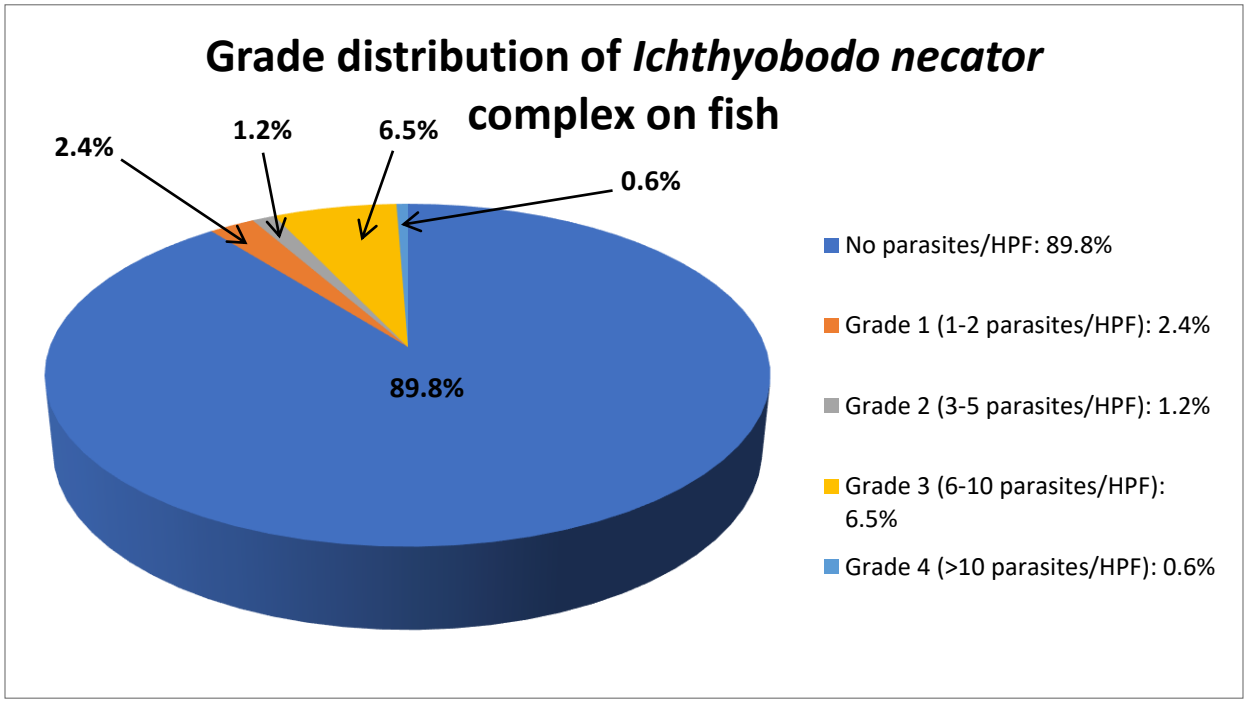


Fig. 4-22 Schematic representation of distribution of various infestation grades of Ichthyobodo necator *complex through the sampled fish population (fish grade was taken as highest encountered grade between all wet prep mounts per fish)*

Statistical data analysis, with a Pearson correlation model, of the effect of independent variables on average *Ichthyobodo necator* complex prevalence revealed the following: (negative values indicate a negative correlation, positive values indicate a positive correlation)

Table 4-14 Pearson correlation model of the effect of independent variable parameters on fish average Ichthyobodo necator complex burdens

Stocking rate (kg/m^3)	Water temp(°C)	Water pH	Water DO (mg/L)	Water CO_2 (mg/L)	NH_3 toxic: UIA (mg/L)	Nitrite NO_2^- (mg/L)	CH:TA ratio
0.038	0.055	-0.053	0.184	0.309	0.337	0.156	-0.272

Red: High correlation; **Orange**: moderate correlation; **Green:** Low correlation

Average *Ichthyobodo* levels showed moderate positive correlations of 0.309 ($p \leq 0.05$) and 0.337 ($p \leq 0.05$) with water CO_2 and water NH_3-UIA, respectively, and a low negative correlation of -0.272 ($p \leq 0.05$) with water CH:TA ratio. This reflects an increase in *Ichthyobodo* burdens with higher water CO_2 and NH_3 levels, and reduced levels in the presence of high CH:TA ratios. A step-wise regression model corroborated these findings, placing NH_3 as the factor with greatest impact.

Pearson's canonical correlation analysis between average *Ichthyobodo* levels and other gill ecto-parasites or gill pathology reflected a significant correlation ($p \leq 0.01$) between average *Ichthyobodo necator* complex levels and average *Trichodina* spp. levels. This was a negative correlation of -0.346. Another significant positive correlation of 0.2 ($p \leq 0.01$) was also reflected between average *Ichthyobodo necator* complex and total parasite score. No correlations of significance could be drawn between this parasite and gill pathology.

Sessile Ciliates

Ambiphrya spp. were uncommonly encountered (10.5% of farms assessed, and 8.3% of fish), but when present, existed in high numbers within the sampled fish in a system. *Ambiphrya* spp.

was found in all wet prep slide preparations, ranging in number from 0-6 parasites per primary gill lamellum, and often high numbers embedded in skin mucus. Numbers on the primary gill lamellae were counted to evaluate levels as parasites were difficult to accurately assess in the skin mucus samples. All grade levels of parasites were equally represented, with 0.8% of fish reflecting grade 1 infestation, 1.8% grade 2 infestation, 0.8% grade 3 infestation and 1% a grade 4 infestation. Again, although *O. niloticus* dominated as the most common affected species, differentiation between species followed general population species differentiation closely, with 64% *O. niloticus,* 21% hybrids, and 14% *O. mossambicus* testing positive.

Epistylus sp. was detected on only one fish on one farm (Farm GG).

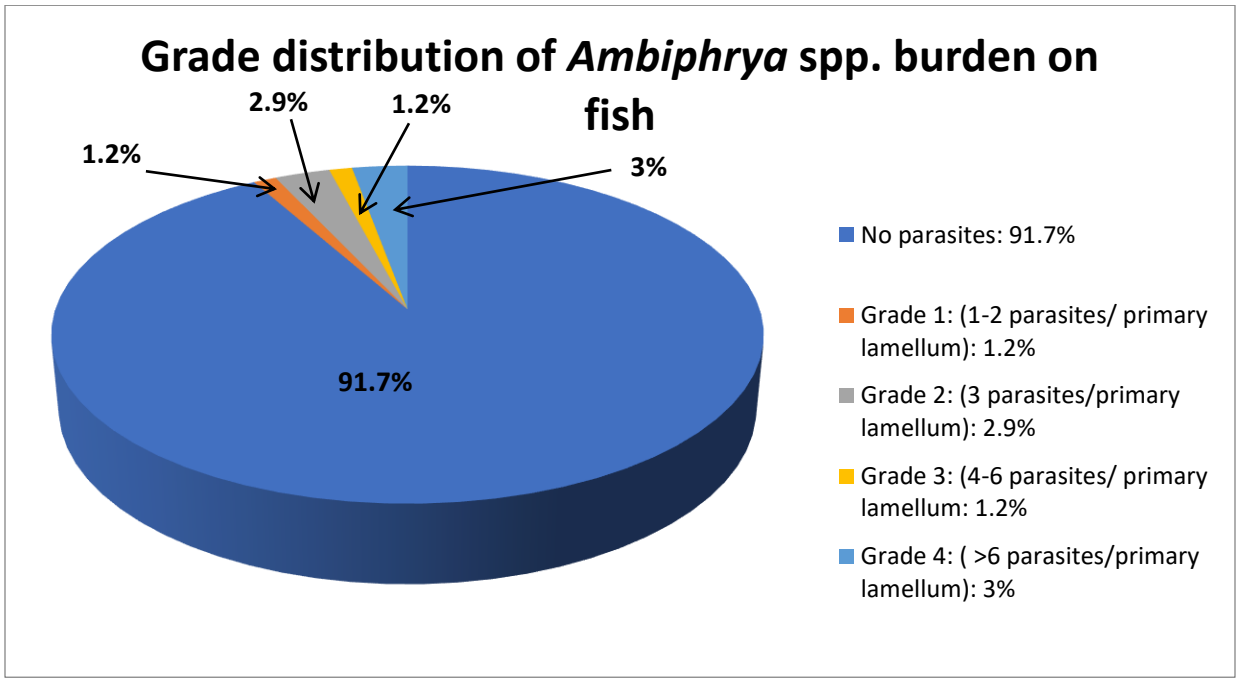


Fig. 4-23 Schematic representation of grade distribution of Ambiphrya *spp.burdens through sampled fish population (fish grade was taken as highest encountered grade between all wet prep mounts per fish)*

Statistical data analysis, using a Pearson correlation model, of the effect of independent variables on average *Ambiphrya* spp. prevalence revealed the following: (negative values indicate a negative correlation, positive values indicate a positive correlation)

Table 4-15 Pearson correlation model of effect of independent variables on average fish Ambiphrya *spp. burdens*

Stocking rate (kg/m^3)	Water temp(^oC)	Water pH	Water DO (mg/L)	Water CO_2 (mg/L)	NH_3 toxic: UIA (mg/L)	Nitrite NO_2^- (mg/L)	CH:TA ratio
0.493	0.388	-0.225	0.007	-0.160	-0.194	-0.309	0.331

Red: High correlation; **Orange**: moderate correlation; **Green:** Low correlation

This model highlighted a number of significant correlations between average *Ambiphrya* spp. and underlying water parameters or husbandry, with a high positive correlation of 0.493 ($p \leq 0.05$) with fish stocking density, moderate positive correlations of 0.388 ($p \leq 0.05$) and 0.331 ($p \leq 0.05$) with water temperature and CH:TA ratios respectively, a moderate negative correlation of -0.309 ($p \leq 0.05$) with water NO_2^- , and a low negative correlation of -0.225 ($p \leq 0.05$) with water pH. This reflects a significantly increasing *Ambiphrya* burden with increasing stocking density, as well as warmer water temperatures and higher CH:TA ratios. *Ambiphrya* spp. burdens appear to be negatively impacted primarily by higher nitrite, and to a lesser degree by higher water pH. A step-wise regression model also placed stocking density as the most significant impacting variable.

Pearson's canonical correlation analysis between average *Ambiphrya* spp. burdens and other gill ecto-parasites or gill pathology showed significant positive correlations of 0.407 ($p \leq 0.01$)

with average *Trichodina* spp. score, 0.529 (p≤ 0.01) with total parasite score, and 0.261 (p≤ 0.01) with gill lamellar goblet cell hyperplasia. Higher *Ambiphrya* spp. burdens seem to closely associate with higher *Trichodina spp.* burdens, contribute significantly to total parasite representation, and to exert significant pathological influence on gill structure, with increasing levels of goblet cell infiltration seen as *Ambiphrya* spp. burdens increase.

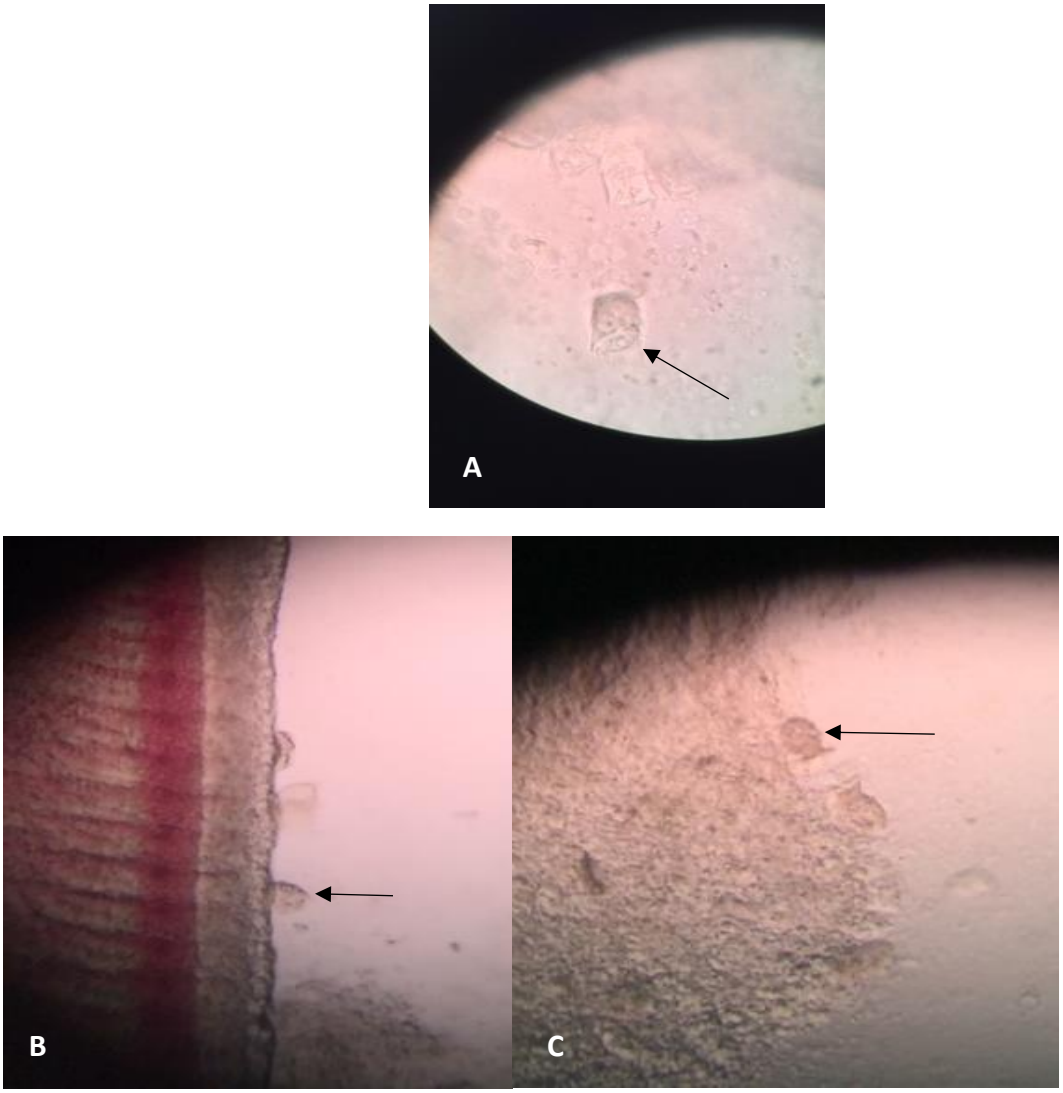


Fig. 4-24 Light microscope images of sessile solitary ecto-commensal ciliates: Ambiphrya *spp. A: gill clip prep: note the cylindrical to conical body shape, ring of oral cilia- the equatorial ring of cilia cannot be clearly seen at this magnification (400X), B: gill wet prep: note attachment to primary gill lamellae (100X), C: skin mucus prep: the conical body shape can be clearly seen (100X)*

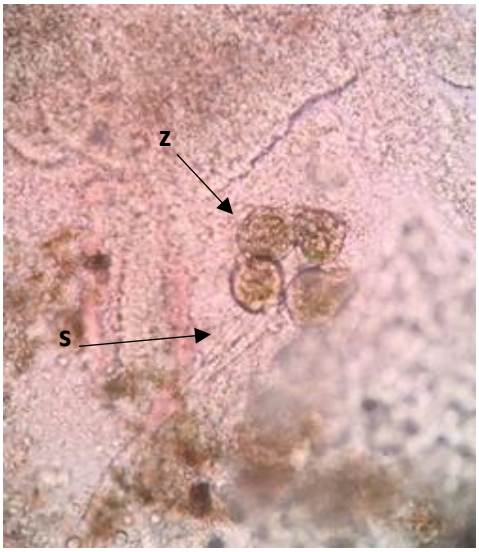


Fig. 4-25 Light microscope image of sessile colonial ectocommensal infestation: Epistylus *sp. Gill scrape prep (100X): Note z: zooids (cilia not visible at this magnification, s: connecting branched stalks*

Total parasitic analysis

Other than the impact of *Trichodina* spp., affecting 57% of fish, all other parasites were prevalent in less than 20% of the sampled fish population. If one looks at farm level, however, prevalence was much higher, with more than 45% of farms reflecting presence of *Trichodina* spp., Gyrodactylidea, or Dactylogyridae. Distribution of parasite grades followed similar patterns with all parasites assessed, with grades 1 and 2 being most the prevalent, and percentage fish affected with higher infestation burdens, tapering off.

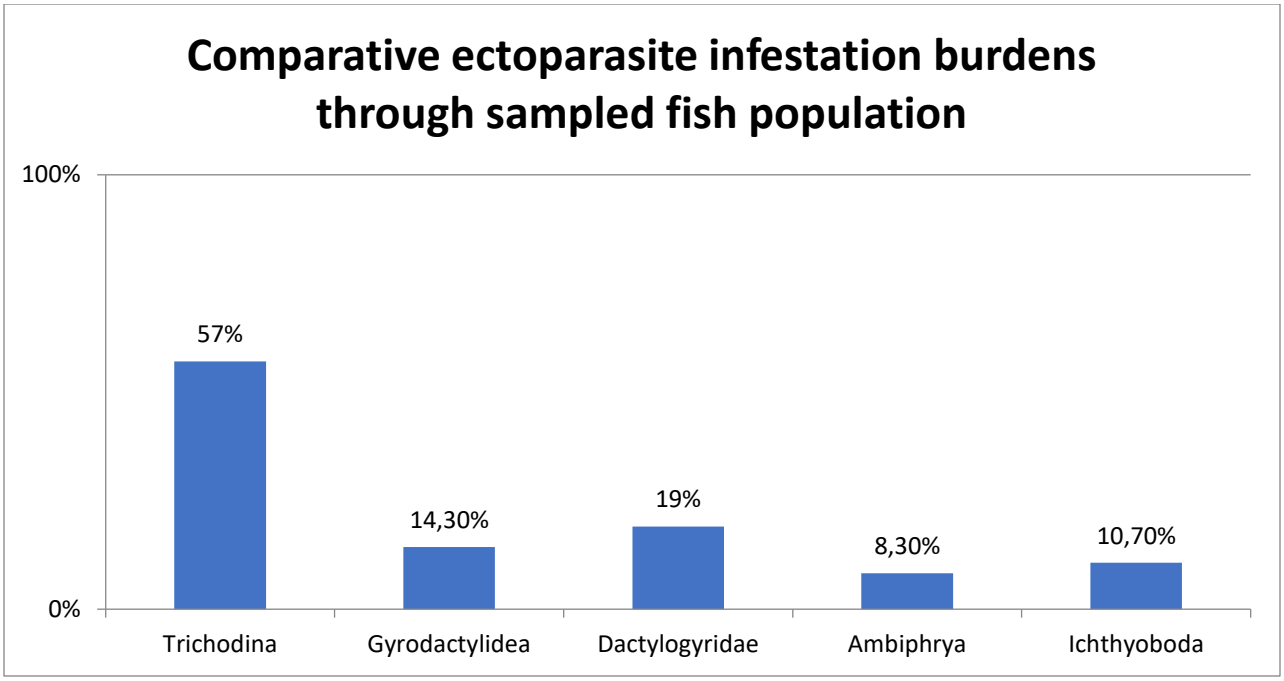


Fig. 4-26 Comparative ectoparasite burdens through the sampled fish population showing percentage of fish population infested

Statistical data analysis using a Pearson correlation model of the effect of independent variables on average total parasite counts showed a significant low positive correlation of 0.22 ($p \leq 0.05$) with stocking density, and significant low negative correlations of -0.309 and -0.221 to water pH and NH_3 respectively ($p \leq 0.05$). In other words, parasitic prevalence increases with higher fish stocking densities and decreases with higher water pH or NH_3 levels.

Pearson's canonical correlations show significant ($p \leq 0.01$) correlation between total parasite score and average *Trichodina* spp., average Dactylogyridae, average *Ambiphrya* spp., and average *Ichthyobodo necator* complex. There was a correlation ($P \leq 0.01$) to gill lamellar goblet cell hyperplasia. Epithelial cell hyperplasia and gyrodactylid intensity correlate less well ($p \leq 0.05$).

4.2.2.6 Necropsy

The most pronounced macroscopic observations during necropsy were the large variation in macroscopic appearance of fish livers in terms of colour, size and friability *(PLATE 3),* variation in visceral fat content *(Table 4-16)*, and variable hyperaemic appearing stomachs *(PLATE 4A)***.** Macroscopically spleens appeared normal, unlike the white nodular appearance commonly associated with *Francisella* spp. or *Edwardsiella* spp. infections (Soto, 2015). Gills appeared healthy, with no macroscopic pathology. Classification of abnormalities in these tissues were performed at histological level (see below).

Other macro-pathological abnormalities were rare apart from the following: (*PLATE 4)*

Subjective impression of a thin body wall in 16 fish (9.5%)

Peritonitis in 2 fish (1.2%)

Retro-bulbar cellulitis in 1 fish (0.6%)

Intestinal perforations in 3 fish (1.8%)

A macroscopic visible parasitic helminth (nematode) in the pericardial cavity of 1 fish (0.6%)

Plate 3: Fish Necropsy: Hepatic macroscopic appearance

Note the varying appearance of the liver (L) in terms of colour, size, and rounding of margins

Plate 4: Fish Necropsy: macroscopic pathology

***A**: Severe diffuse gastric hyperaemia; **B**: Retrobulbar cellulitis and sepsis with exophthalmos; **C**: Suspected parasitic intestinal larval migratory perforations; **D**: Nematode (suspected* Contracaecum *spp.) in pericardial cavity*

The following changes were macroscopically characterised: *(SEE APPENDIX 4: TABLE 2 Data sheets)*

a. **Visceral fat quantification:**

Fish visceral fat scores varied considerably between farms, resulting in fairly even representation of most grades throughout the study population. Grades reflected in the following way: grade 0 (14.9%), 1 (17.9%), 2 (19.6%), 3 (23.8%), 4 (16.1%) and grade 5 (3.6%). 4.2% of results were not recorded. Although sampled fish of the same locality generally presented with similar visceral fat grades, still variability existed in some systems. Although more fish were classified within the extremely low to low range (0 and 1 = 32.8%) than high range (4 and 5 = 19.7%), most of the fish (43.4%) exhibited normal visceral fat stores within the 2 to 3 grade range.

Table 4-16 Overview of average fish visceral fat scores and body condition for each farm assessed (See Plate 1, Fig. 3-7 and APPENDIX 4 TABLE 2 Data sheets)

Farm	Ave. visceral fat score	Average condition score of fish
GA	2	Poor
GB	2	Good
GC	2	Moderate
GD(a)	3	Good
GD(b)	2	Poor
GE	2	Moderate
GF(a)	4	Good
GF(b)	3	Good
GG	2	Moderate
GH	3	Good
NA	1	Moderate
NB	1	Moderate
NC	1	Poor
ND	4	Good
NE	4	Good
NF	0	Moderate
NJ	4	Good
LB	2	Moderate
LC	4	Moderate

b. **Sexual diversity:**

Of the fish farms sampled, 42.1% showed male only populations, with the remaining 57.9% mixed male and female fish in varying ratios.

The total fish population was composed of 72.6% males, 26.2% females, and 1.2% hermaphrodites with both male and female gonads. Where the dominating sex (>50% of sample population) was female, average fish growth was always moderate to poor, never good *(See Tables 4-8 and 4-32).*

c. **Heart to spleen ratios:**

In most fish (44%), the spleen and heart were identical in size (volume). Slight variability existed, with some spleens as small as half of the total heart size (in 6% of fish), and others as large as five times heart size (in 4% of fish). Spleens showed definite correlation between significant enlargement (>4:1 spleen to heart ratio) and septicaemia. Ratios below 4, were not suggestive of infectious disease. Farms with highest ratios, cultured positive *for Lactococcus garviae, Aeromonas hydrophila, Aeromonas schubertii, Staphylococcus epidermidis* and *Shewenella putrifaciens*.

4.2.2.7 Bacterial Culture and Sensitivity

Following histological assessment of the sampled fish, 11 cases were identified as possible candidates for bacterial disease. Anterior kidney swabs from these specimens, were submitted for bacterial culture. In addition, swabs of exudate from the retrobulbar regions and cranial cavities of ND7 and ND8, were submitted. The following bacterial isolations were obtained:

Table 4-17 Fish targeted for bacterial isolation, and results of positive cultures

(MO: Myroides odoratum*; AH:* Aeromonas hydrophila*; AS:* Aeromonas schubertii*; B:* Bacillus*; BV:* Brevimundus vesicularis*; SP:* Shewenella putrifaciens*; StP:* Staphylococcus epidermidis*; LG:* Lactococcus garviae*; A:* Acinetobacter *sp.)*

Fish No.	**Bacteria isolated**
GD10	MO
GE6	AH
GF1	AS
GF5	B
GG7	BV
GG9	AS
GG10	AS
ND1	SP (Ant.kidney, orbit , brain) AH (Ant.kidney)
ND7	SP and AH (Ant.kidney, orbit), StP (orbit)
ND8	SP and AH (Ant. Kidney, brain), StP (Ant. Kidney), LG (brain)
LC10	A and StE

*Fig. 4-27 Bacterial septicaemia (*A. hydrophila *and* S. putrifaciens*) in fish ND1. Note the lack of macroscopic abnormalities other than the non-specific signs of exophthalmos and darkening of skin. Body condition is also moderate.*

4.2.2.8 Histological assessment of organ systems

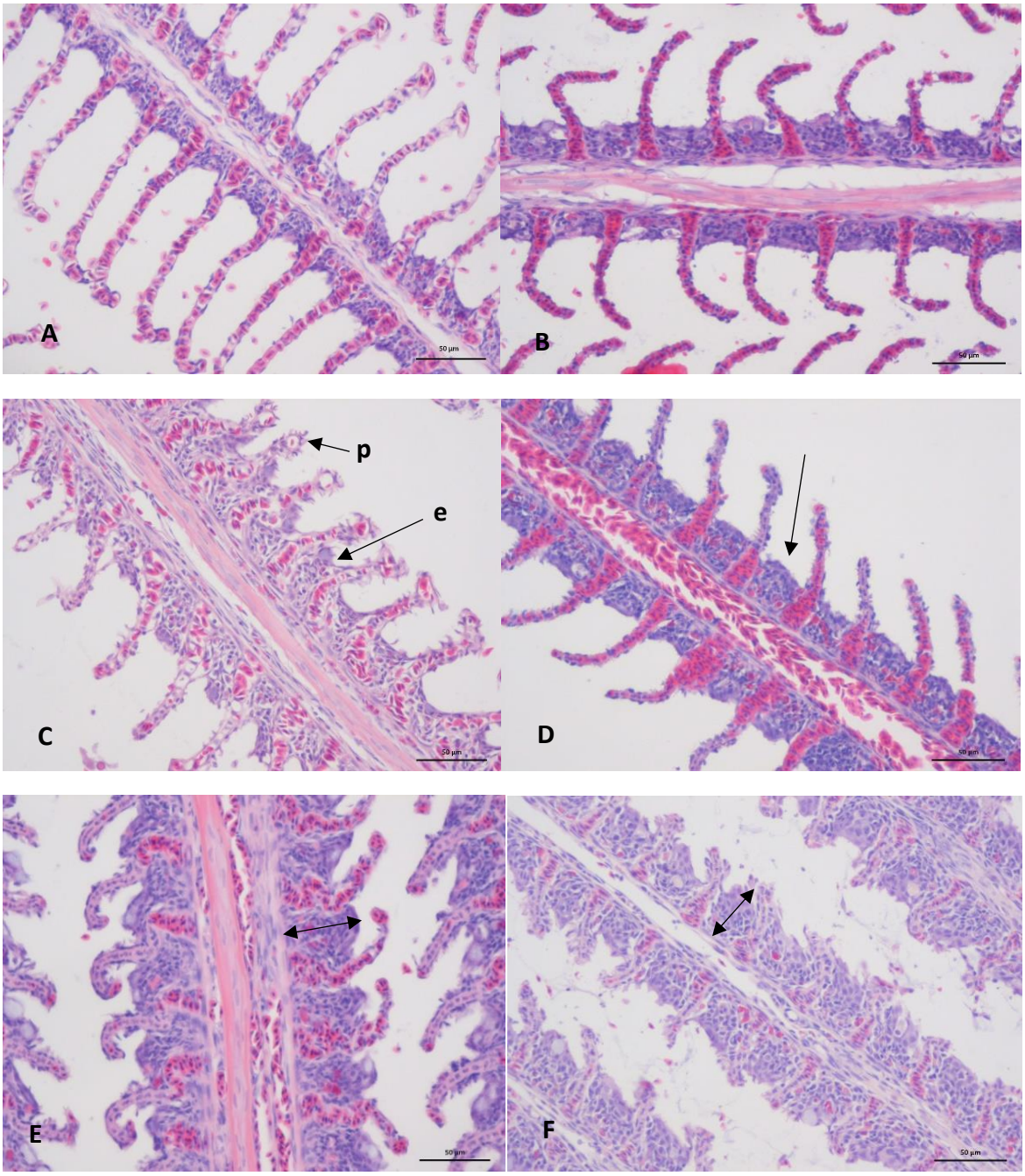
1. Gill Pathology *(SEE APPENDIX 4: TABLE 3 Data sheets)*

a. Gill epithelial hyperplasia: *(PLATE 5)*

Epithelial hyperplasia of the primary and secondary gill lamellae was a common finding, affecting 70.8% of the fish assessed. However, low to moderate degrees predominated, with only 6.6% of fish showing severe epithelial hyperplastic change.

Plate 5: Gill epithelial cell hyperplasia

***A**: Normal healthy gill, fish GA1 (200X); **B**: Grade 1 hyperplasia, fish GA10 (200X). Note the slight peak formation as irregularities of the secondary lamellar surface; **C**: Grade 2 hyperplasia, fish NB9 (200X). Note the severe peaking (p) and increase of epithelial layer (e); **D**: Grade 3 hyperplasia, fish GD3 (200X) with marked epithelial expansion (arrow); **E**: Grade 4 hyperplasia with epithelial expansion (arrow) up to half of secondary lamellar height, fish GE1 (200X); **F**: Grade 5 hyperplasia with secondary lamellae almost enveloped by epithelial tissue, fish GB5 (200X) and resultant lamellar fusion.*



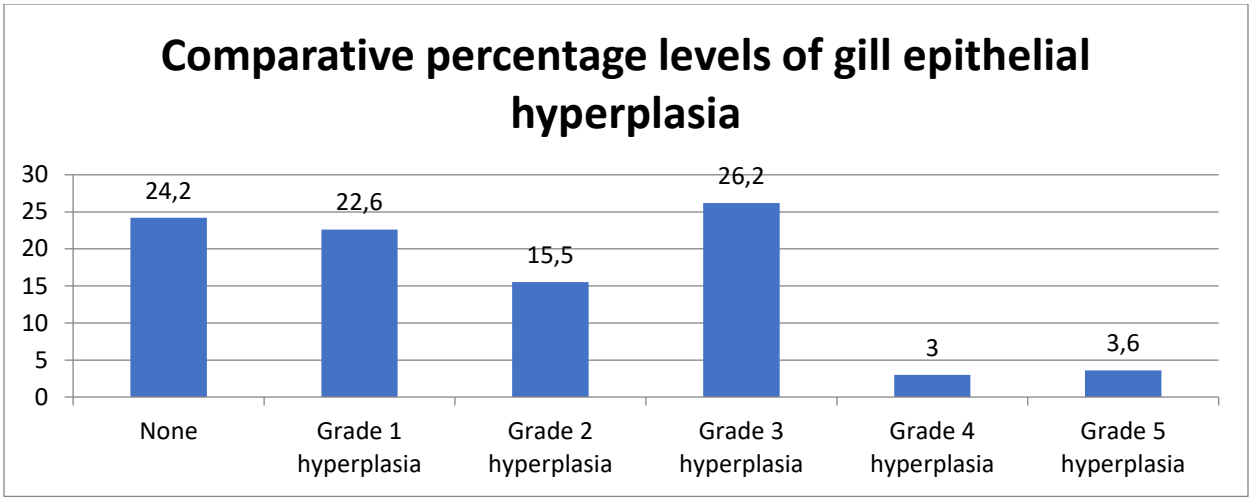


Fig. 4-28 Comparative representation of percentages of sampled fish displaying various grades of epithelial hyperplasia

(5.2% of samples could not be analysed due to sectioning artefacts or missing data)

Table 4-18 Statistical data analysis (Pearson correlation model) of the effect of independent variables on gill epithelial hyperplasia levels (negative values indicate a negative correlation, positive values indicate a positive correlation)

Stocking rate (kg/m^3)	Water temp. (°C)	Water pH	Water DO (mg/L)	Water CO_2 (mg/L)	NH_3 toxic: UIA (mg/L)	Nitrite NO_2^- (mg/L)	CH:TA ratio
-0.001	0.090	-0.041	-0.170	-0.176	-0.252	-0.082	0.082

Red: High correlation; **Orange:** moderate correlation; **Green**: Low correlation

Gill epithelial hyperplasia showed only a low negative correlation of -0.252 with NH_3 ($p \leq 0.05$).

Table 4-19 Pearson's canonical correlation analysis of gill lamellar epithelial hyperplasia with average parasite scores

		Ave Trichodina score	Ave Gyrodactylus score	Ave Dactylogyrus score	Ave Ambiphrya score	Ave Ichthyobodo score	Fish total parasite score
Histopath: Gill epithelial hyperplasia	Pearson Correlation	**.165***	0.025	0.002	0.150	-0.018	**.156***
	Sig. (2-tailed)	0.032	0.744	0.984	0.053	0.819	0.043
	N	168	168	168	168	168	168

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

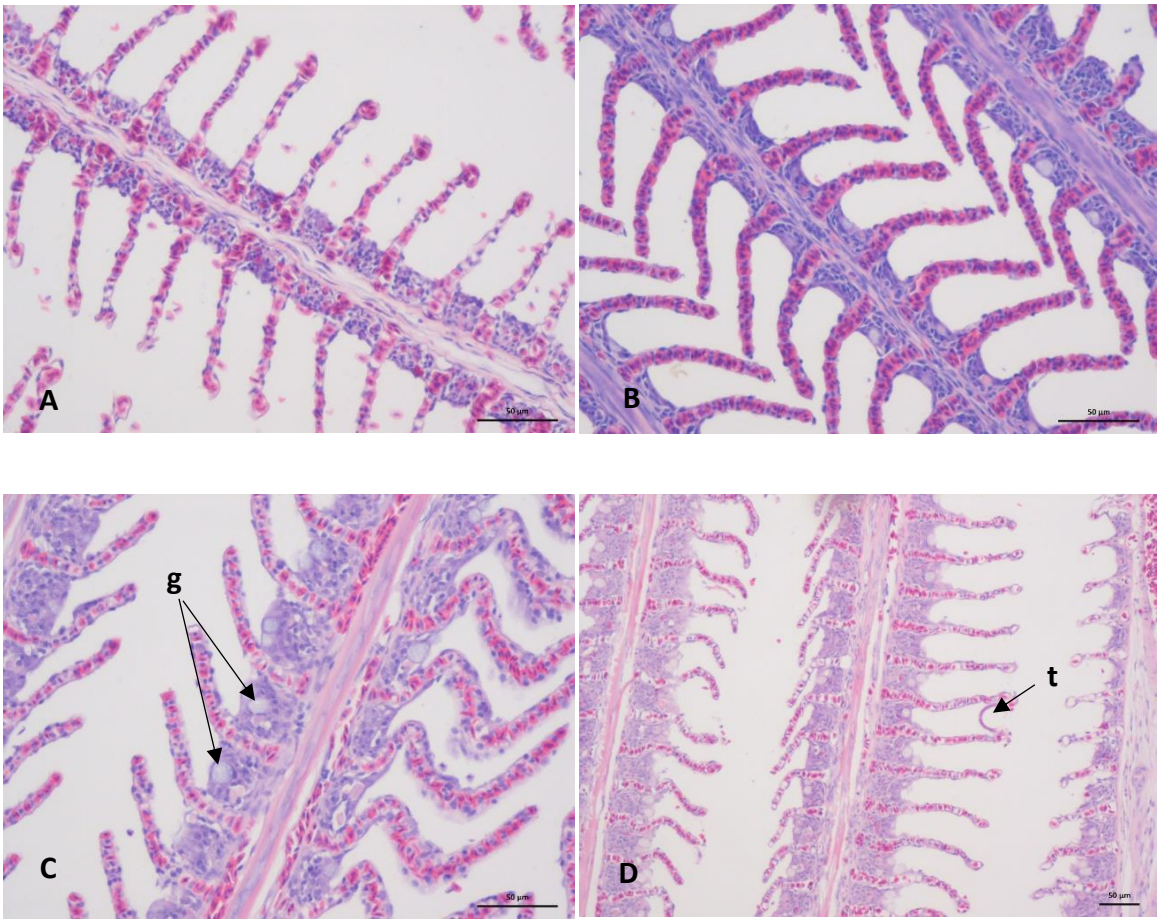
This model reflects a positive correlation of gill epithelial hyperplasia and average *Trichodina* spp. score of 0.165 ($p \leq 0.05$), as well as a positive correlation with of 0.156 with total parasite score ($p \leq 0.05$). In other words, epithelial hyperplasia increases with increased *Trichodina* spp. burdens, and higher total parasite burdens.

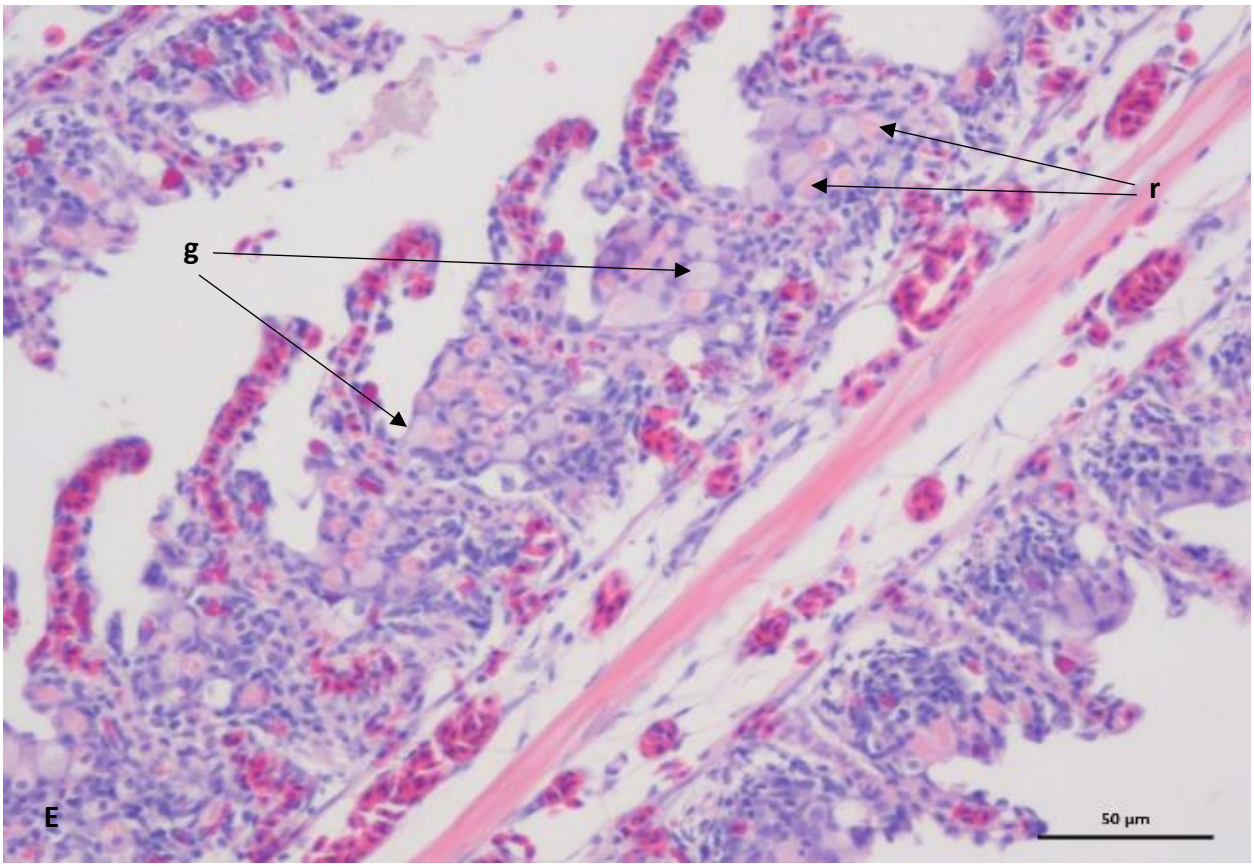
b. Gill lamellar goblet cell hyperplasia: *(PLATE 6)*

Goblet cell hyperplasia of the lamellae featured as less prominent, evident in 32.7% of fish, and largely as a grade 2 (mild) level of change. Only 1.2% of fish presented with a high level of goblet cell proliferation in primary and secondary lamellae.

Plate 6: Gill goblet cell hyperplasia

***Note colour difference between goblet cells (g) and rodlet cells (r)**. **A**: Normal gill, fish GA6 (200X); **B**: Grade 1, fish NJ1 (200X)*:<5 goblet cells/ 5 interlamellar spaces(ILS)*; **C**: Grade 2, fish ND3 (200X)*:5-10 goblet cells/ 5 ILS*; **D**: Grade 3, fish LB8*: 11-15 goblet cells/5 ILS*. Note the Trichodina spp. parasites (t) between the secondary lamellae (100X); **E**: Grade 4, fish GE5 (200X)*: 16-20 goblet cells/5 ILS. *No grade 5 levels were encountered*.





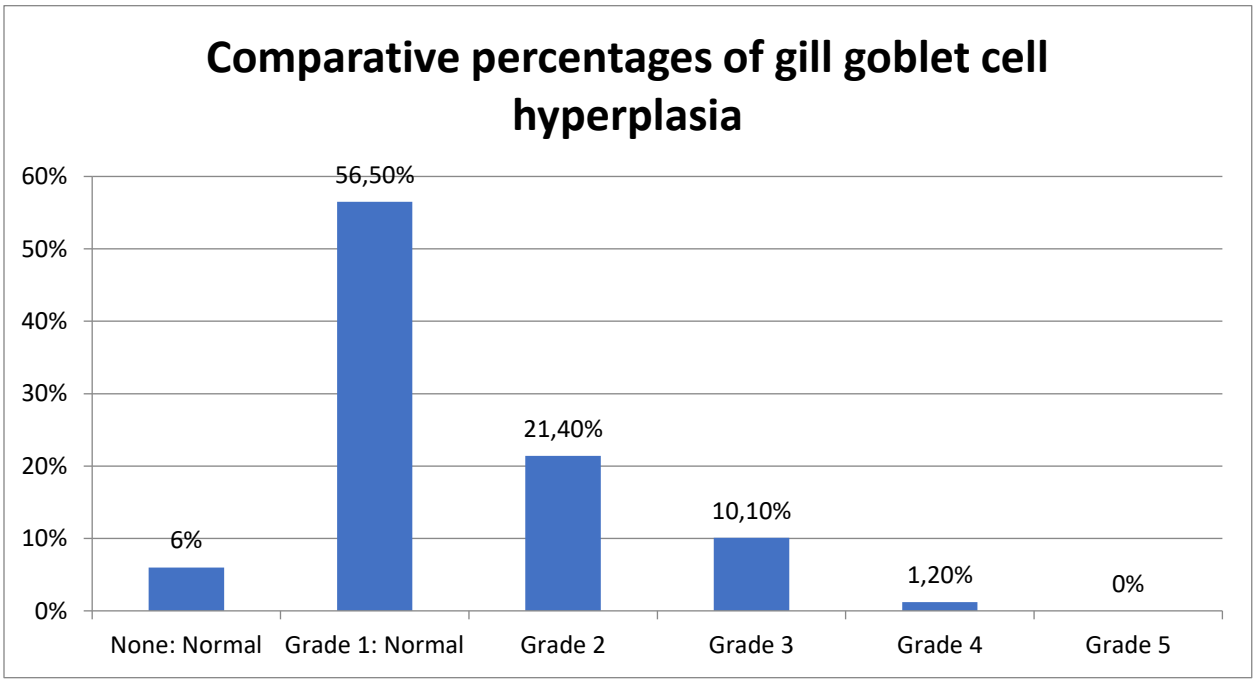


Fig. 4-29 Comparative percentages of sampled fish displaying various levels of goblet cell hyperplasia (5.2% of samples were excluded due to sampling error or sectioning artefacts).

Table 4-20 Statistical data analysis (Pearson correlation model) of the effect of independent variables on gill goblet cell hyperplasia levels (negative values indicate a negative correlation, positive values indicate a positive correlation)

Stocking rate (kg/m^3)	Water temp(° C)	Water pH	Water DO (mg/L)	Water CO_2 (mg/L)	NH_3 toxic: UIA (mg/L)	Nitrite NO_2^- (mg/L)	CH:TA ratio
-0.058	-0.022	-0.174	-0.179	-0.270	-0.085	0.061	-0.082

Red: High correlation; **Orange**: moderate correlation; **Green:** Low correlation

Goblet cell hyperplasia showed only a significant low negative correlation of -0.270 with water CO_2 ($p \leq 0.05$).

Table 4-21 Pearson's canonical correlation analysis of gill lamellar goblet cell hyperplasia with average parasite scores

		Ave Trichodina score	Ave Gyrodactylus score	Ave Dactylogyrus score	Ave Ambiphrya score	Ave Ichthyobodo score	Fish total parasite score
Histopath: Gill goblet hyperplasia	Pearson Correlation	.208**	0.100	0.036	.261**	0.088	.376**
	Sig. (2-tailed)	0.007	0.198	0.642	0.001	0.258	0.000
	N	168	168	168	168	168	168

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

This model reflects three significant positive correlations between gill goblet cell hyperplasia and average *Trichodina* spp. score (0.208) ($p \leq 0.01$), average *Ambiphrya* spp. score (0.261) ($p \leq 0.01$), and total parasite score (0.376) ($p \leq 0.01$). In other words, increases in both parasite

levels, as well as total parasite score reflect as increased levels of goblet cell hyperplasia in gill lamellae.

c. Eosinophilic granular cell (EGC) infiltration at the base of the primary gill lamellae:

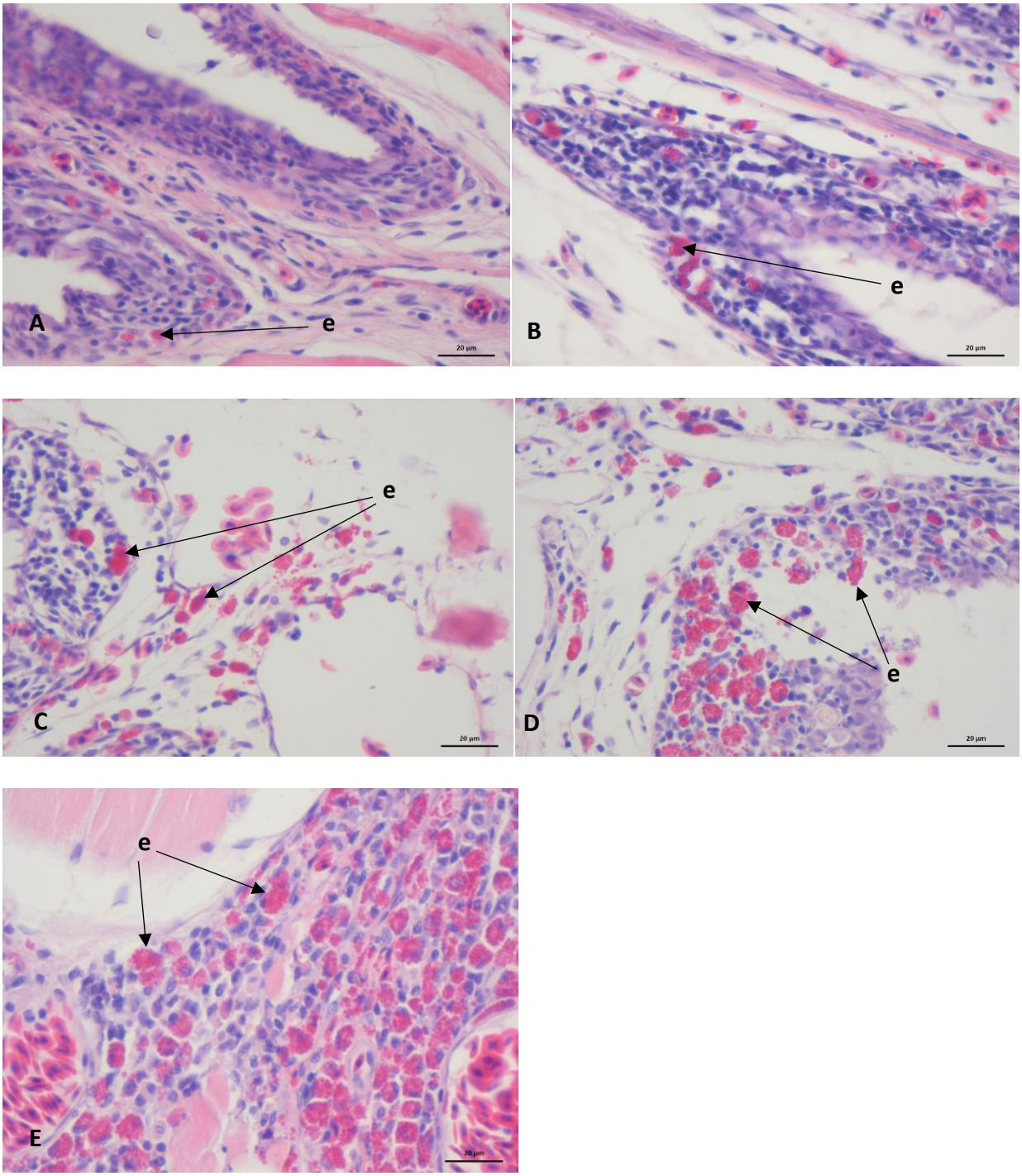
(PLATE 7)

Presence of eosinophilic granular (EG) cells at the base of the gill arches, was a common finding with EG cell counts in most tissue sections ranging from very low (<5 cells / 400X magnification field (HPF)) to moderate levels (11-20 cells / HPF). In 26.8% of tissues sections, however, significantly elevated EG cell numbers with counts above 21 cells / 400X magnification (HPF) were detected.

Plate 7: EGC infiltration at base of gill primary lamellae

Note EGC's (e) with highly eosinophilic cytoplasmic inclusions.

***A**: Grade 1/ Normal, fish GE7 (400X); **B**: Grade 2, fish GF5 (400X); **C**: Grade 3, fish GA2 (400X); **D**: Grade 4, fish GB3 (400X); **E**: Grade 5, fish ND3 (400X)*



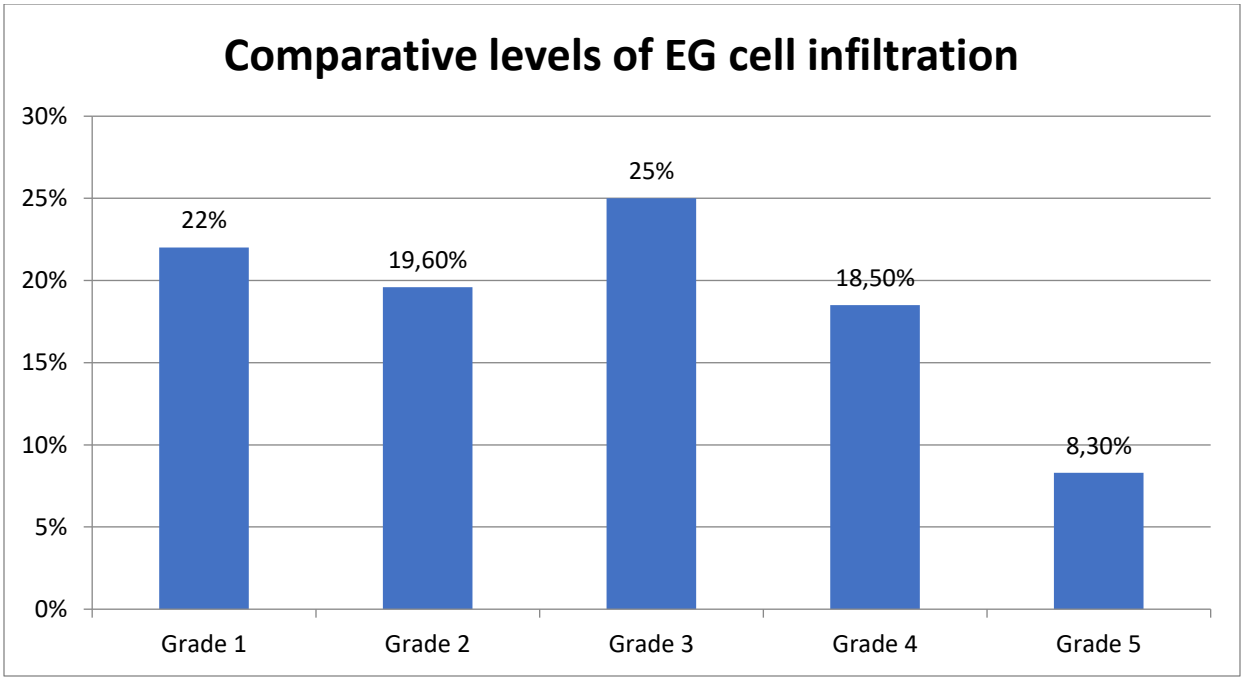


Fig. 4-30 Comparative percentages of sampled fish exhibiting various grades of EGC infiltration at the base of the primary lamellae (6.6% of samples were excluded due to sampling error or sectioning artefacts).

Table 4-22 Statistical data analysis (Pearson correlation model) of the effect of independent variables on levels of EG cells at base of gill arches (negative values indicate a negative correlation, positive values indicate a positive correlation)

Stocking rate (kg/m^3)	Water temp(°C)	Water pH	Water DO (mg/L)	Water CO_2 (mg/L)	NH_3 toxic: UIA (mg/L)	Nitrite NO_2^- (mg/L)	CH:TA ratio
-0.043	0.087	0.140	0.129	-0.038	0.010	0.142	-0.010

Red: High correlation; **Orange:** moderate correlation; **Green:** Low correlation

There were no correlations of significance between EG cell infiltration at base of gill arches and water parameters or stocking density.

Table 4-23 Pearson's canonical correlation analysis of the EG cell infiltration at the base of the gill arches, with average parasite scores

		Ave Trichodina score	Ave Gyrodactylus score	Ave Dactylogyrus score	Ave Ambiphrya score	Ave Ichthyobodo score	Fish total parasite score
Histopath: Gill branchitis	Pearson Correlation	-0.005	-0.054	0.027	0.150	0.139	0.120
	Sig. (2-tailed)	0.951	0.483	0.725	0.053	0.073	0.121
	N	168	168	168	168	168	168

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

EG cell infiltration at the base of gill arches showed no correlation with individual or total parasites scores.

d. Other gill pathology: *(PLATE 8)*

Fusion of the secondary lamellae was frequently seen and varied in distribution. Of the 53.5% affected, 23.8% were focal lesions , 20.2% multifocal, and in 9.5%, generalized lamellar fusion could be detected.

The following other pathological changes within the gill lamellae were also noted:

An increase in EG cells, subjectively assessed relative to healthy gill sections with no evidence of pathology (24% of fish affected)

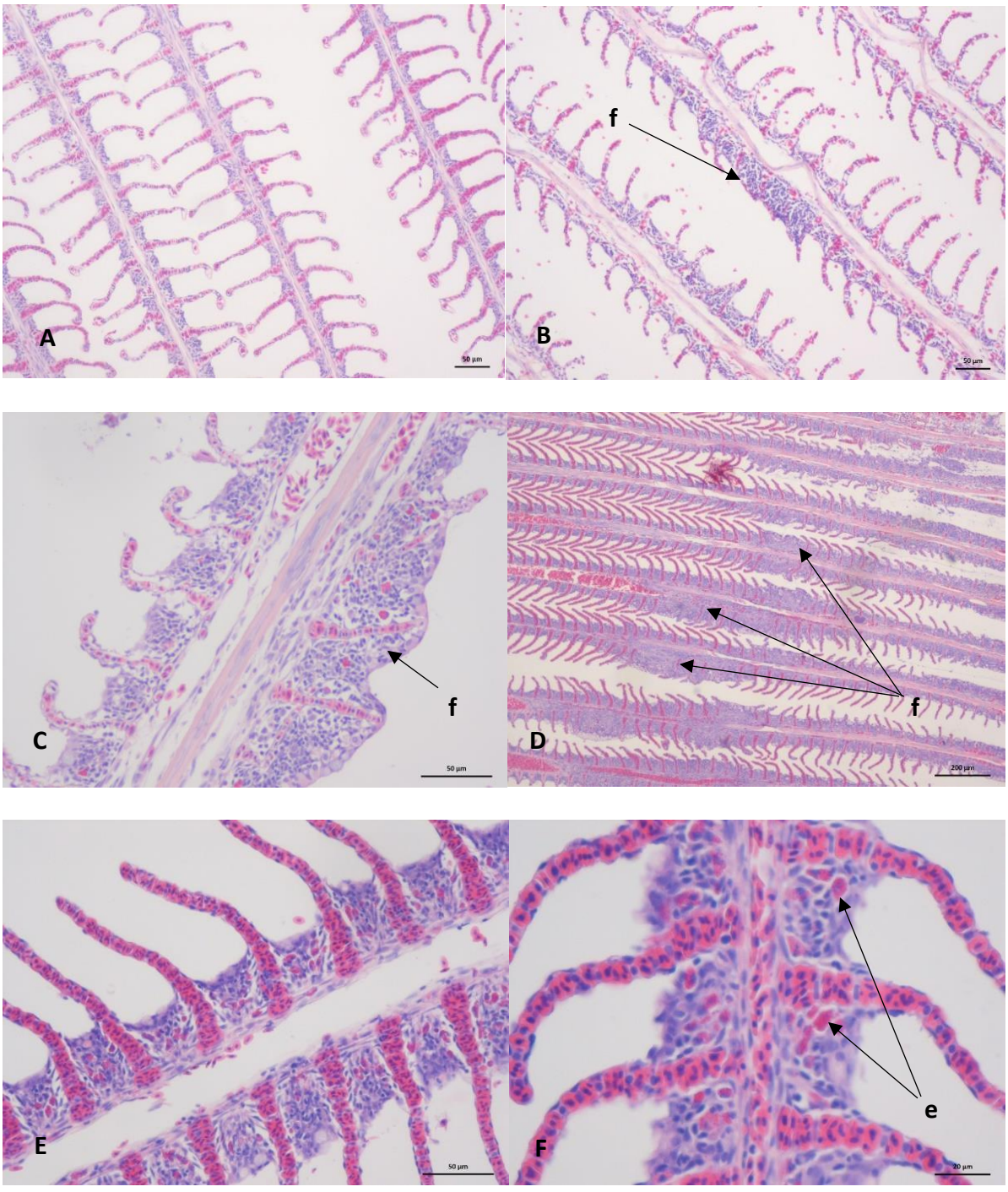
An increase in rodlet cells, subjectively assessed relative to healthy gill sections with no evidence of pathology (16% of fish affected)

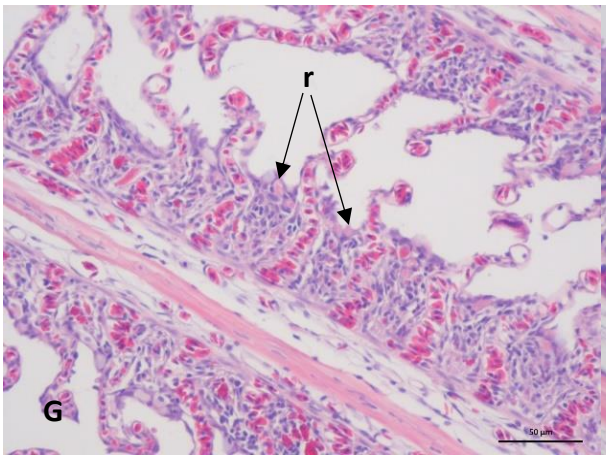
Presence of telangiectasis (13% of fish affected)

Presence of epitheliocystis (16% of fish affected)

Plate 8: Other gill pathology

***A**: Normal gill, fish GA9 (100X); **B**: Mild lamellar fusion (f), fish GA4 (100X); **C**: Moderate lamellar fusion (f), fish GB7 (200X); **D**: Severe lamellar fusion (f), fish NJ4 (40X) **E and F:** Increased EG cells (e) in primary lamellum: fish GG3 (200X) and GG2 (400X); **G and H**: Increased rodlet cells (r) in primary lamellum: fish NB6 (200X) and (400X); **I and J**: Telangiectasis (t): fish NJ9 (200X) and GA8 (100X); **K**: Epitheliocystis (E): fish GB1 (100X)*

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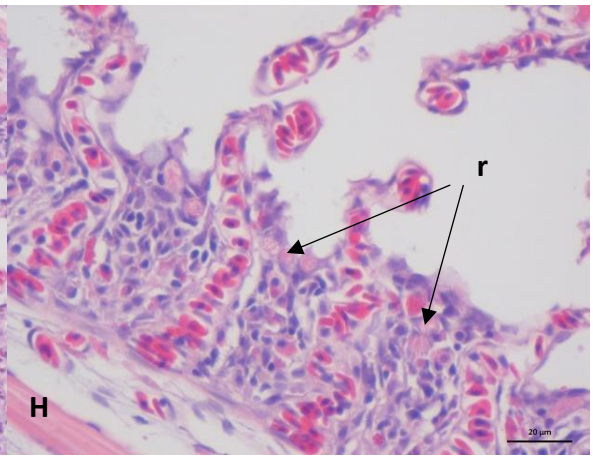
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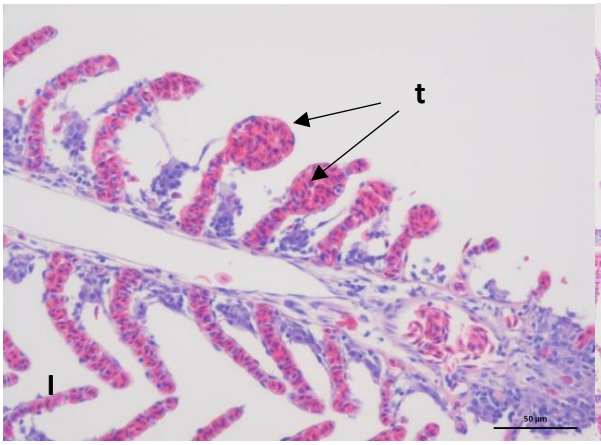
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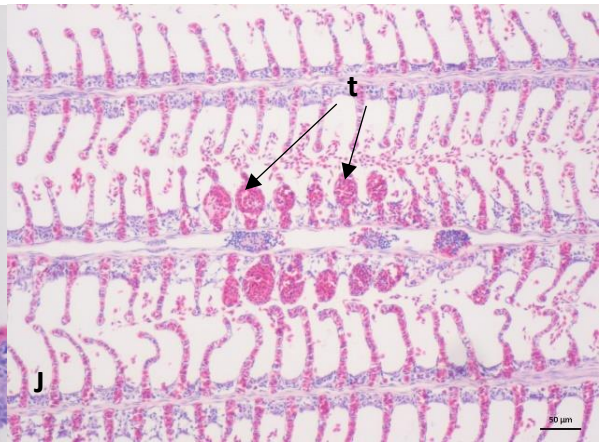
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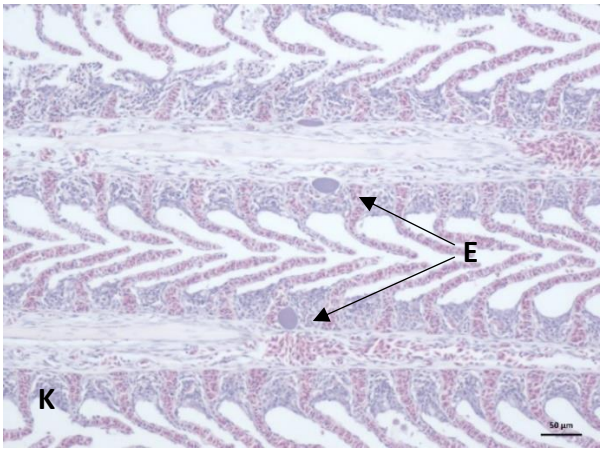
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2. Hepatic pathology *(SEE APPENDIX 4 TABLE 4 Data sheets)*

a. Hepatic lipid content *(PLATE 9)*

Hepatocellular lipid content showed significant variation between fish. Most liver sections (68.4%) examined, exhibited very low relative lipid levels, with cytoplasmic lipid vacuole to nuclear size ratios below 10:1. Only a small percentage, (7.2%), of liver sections were lipid rich, showing the classic "signet ring" hepatocytes (Reimschussel, 2008).

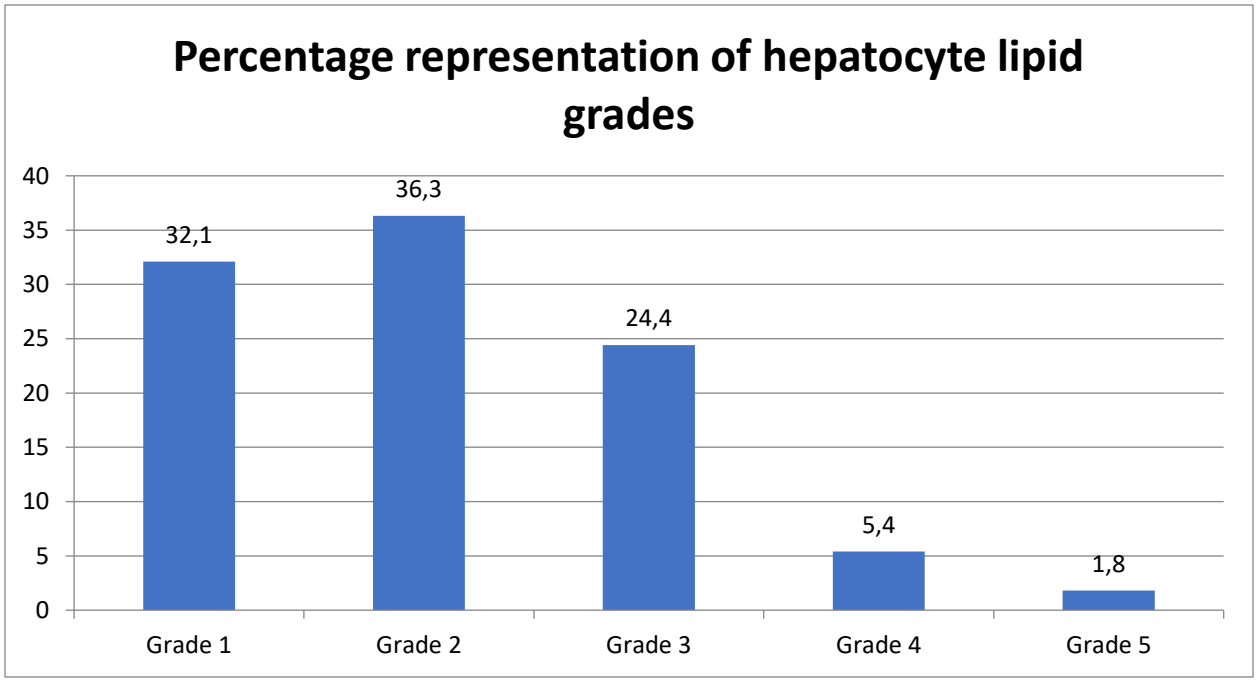


Fig. 4-31 Percentage representation of the various lipid grades through the sampled fish population

Table 4-24 Statistical data analysis (Pearson correlation model) of the effect of independent variables on hepatocyte lipid content, with negative values indicating a negative correlation and positive values indicating a positive correlation

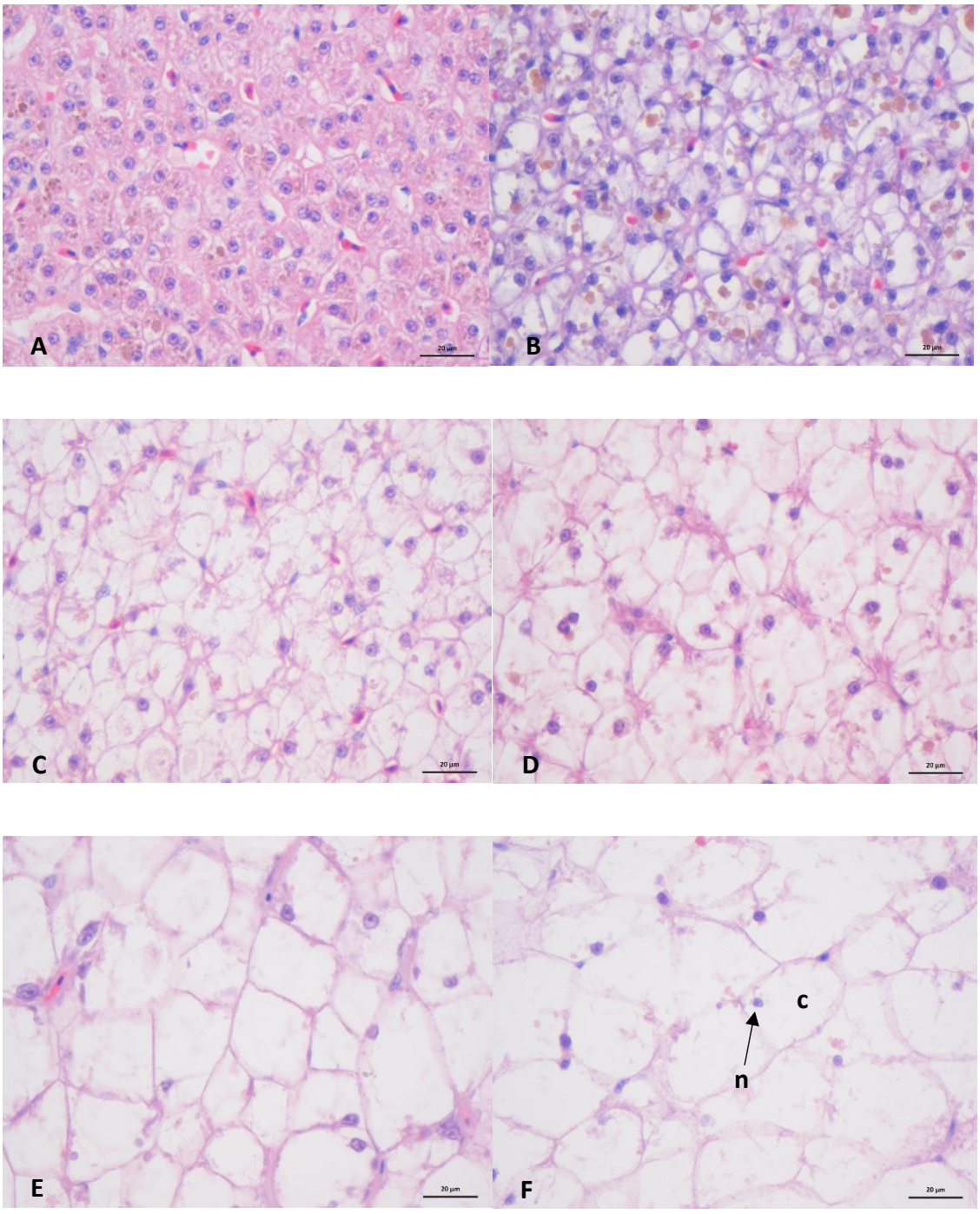
Stocking rate (kg/m^3)	Water temp(°C)	Water pH	Water DO (mg/L)	Water CO_2 (mg/L)	NH_3 toxic: UIA (mg/L)	Nitrite NO_2^- (mg/L)	CH:TA ratio
0.202	0.155	0.005	0.294	0.104	0.053	0.107	0.334

Red: High correlation; **Orange**: moderate correlation; **Green:** Low correlation

Hepatocyte lipid shows moderate significant positive correlation to CH:TA ratio of 0.334 ($p \leq 0.05$), and low significant positive correlations to both stocking density (0.202) ($p \leq 0.05$), and water DO (0.294) ($p \leq 0.05$). In other words, hepatic lipid increases with increasing CH:TA ratios, higher water DO levels and higher fish stocking densities.

Plate 9: Hepatocyte lipid

***A**: No lipid, fish NF4 (400X); **B**: Grade 1, fish GF8 (400X); **C**: Grade 2, fish NB1 (400X); **D**: Grade 3, fish NC1 (400X); **E**: Grade 4, fish ND3 (400X); **F**: Grade 5, fish NF5 (400X). Note the nuclei (n) and cytoplasm (c) and increasing ratios of cytoplasm to nuclear size through grade 0 to 5*



b. Portal adipose tissue

Deposits of adipocytes (fat cells), within the portal triad regions, were noted as a varying histological feature. This was not a consistent finding through all fish, and showed no correlation with hepatocyte lipid.

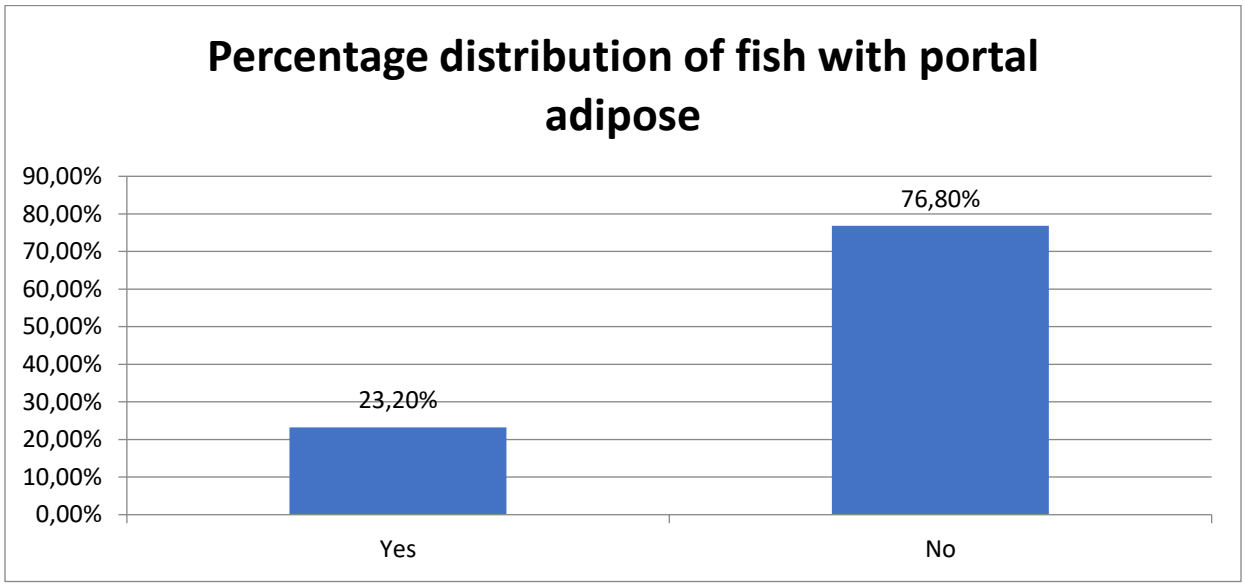


Fig. 4-32 Percentage representation of fish showing evidence of portal adipose deposits

Of the 23.2% of fish showing portal adipose deposits, the majority correlated with low grade hepatocyte lipid: 18% correlated with grade 1 hepatocyte lipid levels, 41% with grade 2, 26% with grade 3, and 15% with grade 4. No fish showing grade 5 hepatocyte lipid levels, showed evidence of portal adipose.

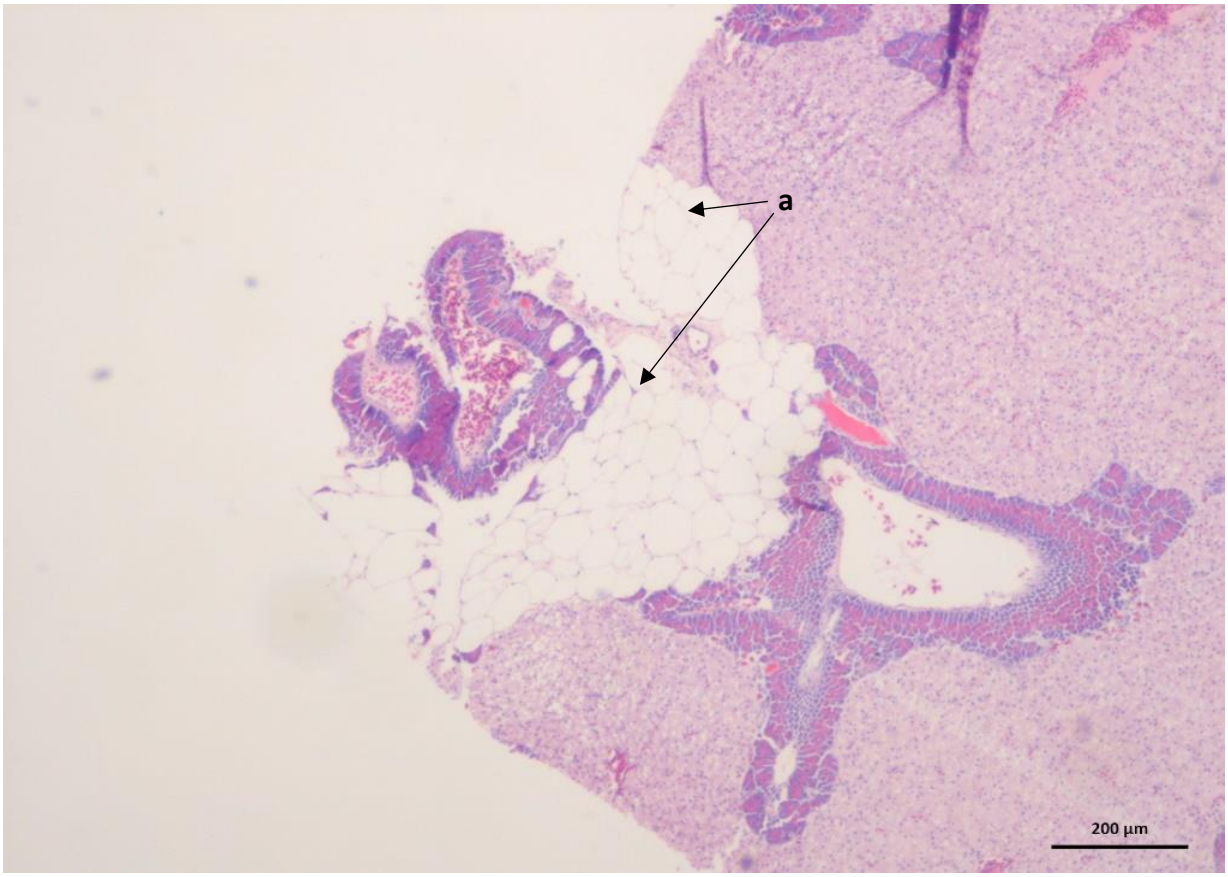


Fig. 4-33 Histological evidence of portal adipose deposit, fish GC6 (100X). Note adipocytes (a)

c. Hepatic lipofuscin content : *(PLATE 10)*

Lipofuscin / ceroid was frequently encountered, deposited as golden yellow, granular cytoplasmic precipitates of varying size within hepatocytes. Grades 1 to 5 showed fairly equal representation through fish, however, a significant percentage (51.8%) of fish presented with grade 4 levels and higher. These fish commonly showed widespread lipofuscin deposits within other parenchymatous tissues as well, with highest prevalence in anterior kidney and spleen, but also encountered in posterior kidney, gonads, adipose tissues and heart.

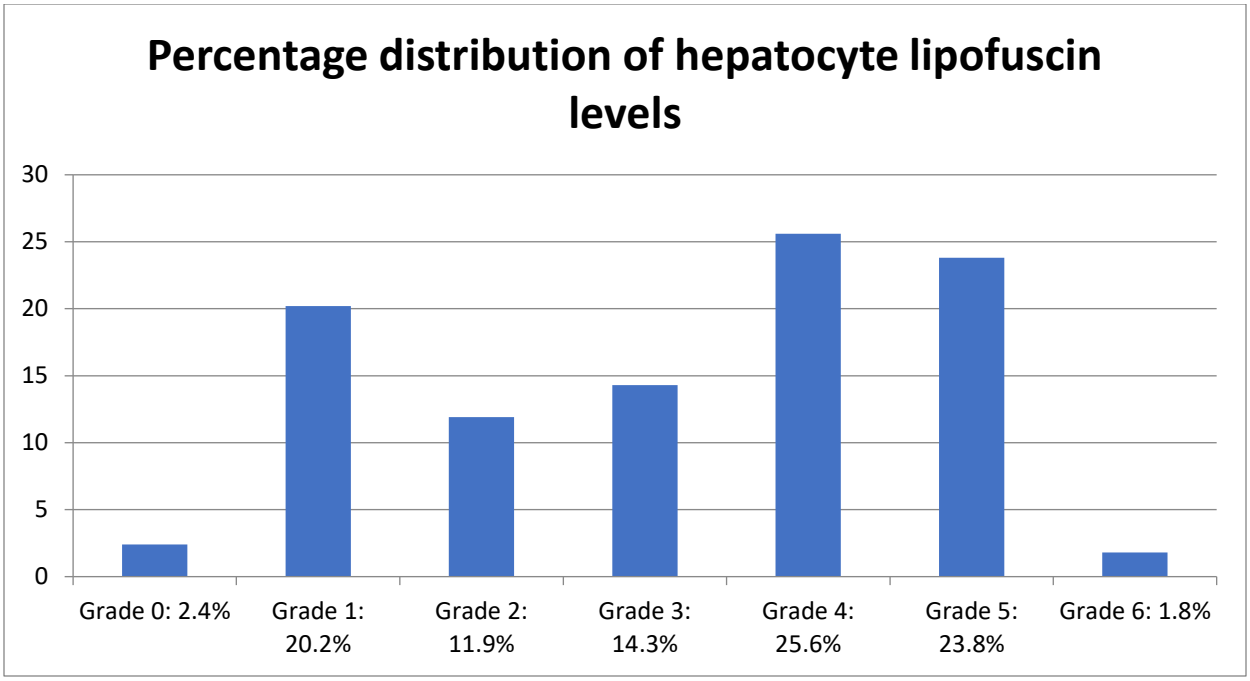


Fig. 4-34 Percentage representation of the various grades of lipofuscin within hepatic parenchyma

Table 4-25 Statistical data analysis (Pearson correlation model) of the effect of independent variables on liver lipofuscin content, with negative values indicating a negative correlation and positive values indicating a positive correlation

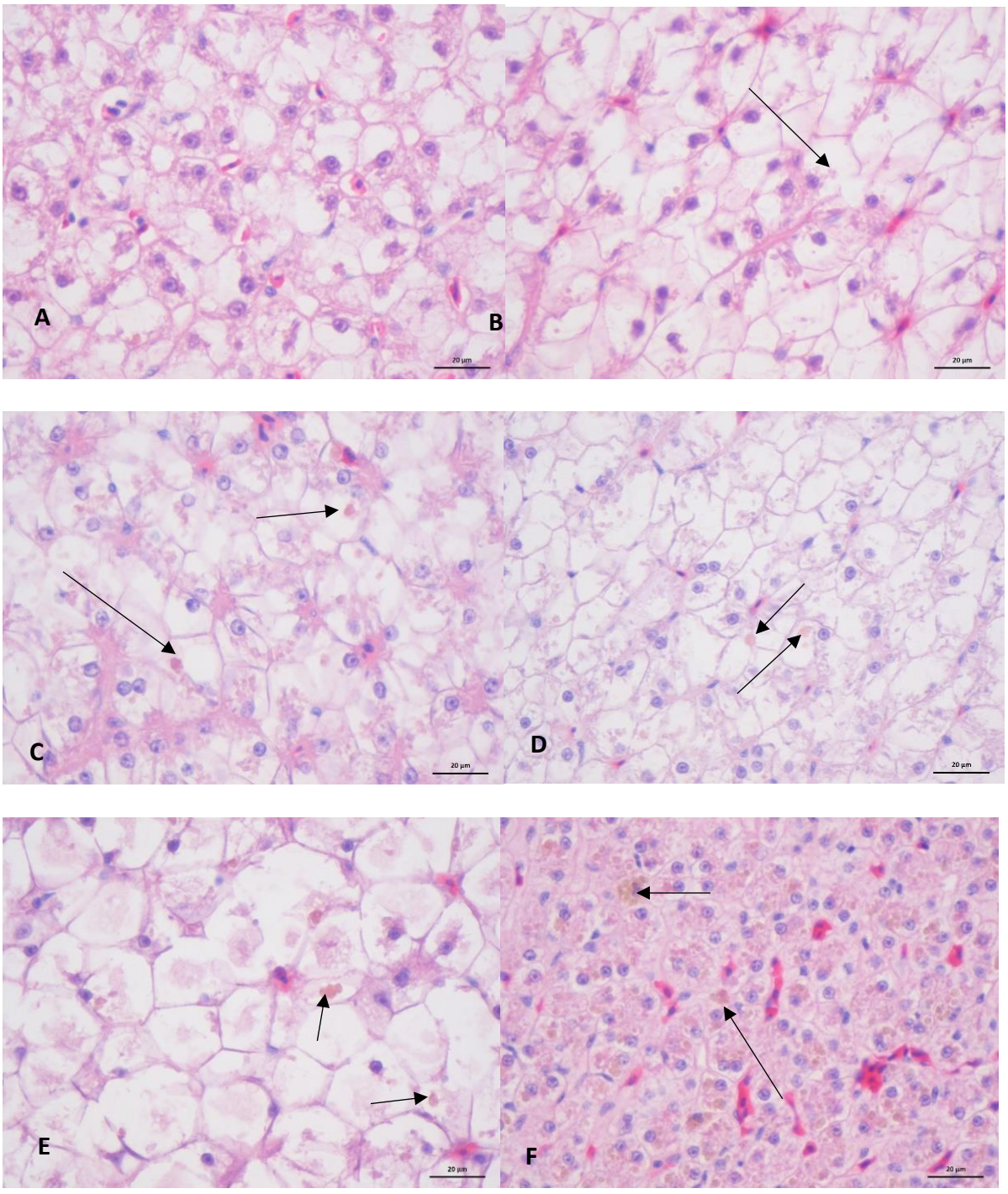
Stocking rate (kg/m^3)	Water temp (^{0} C)	Water pH	Water DO (mg/L)	Water CO_2 (mg/L)	NH_3 toxic: UIA (mg/L)	Nitrite NO_2^- (mg/L)	CH:TA ratio
0.061	-0.226	-0.052	-0.017	0.386	0.187	-0.134	-0.086

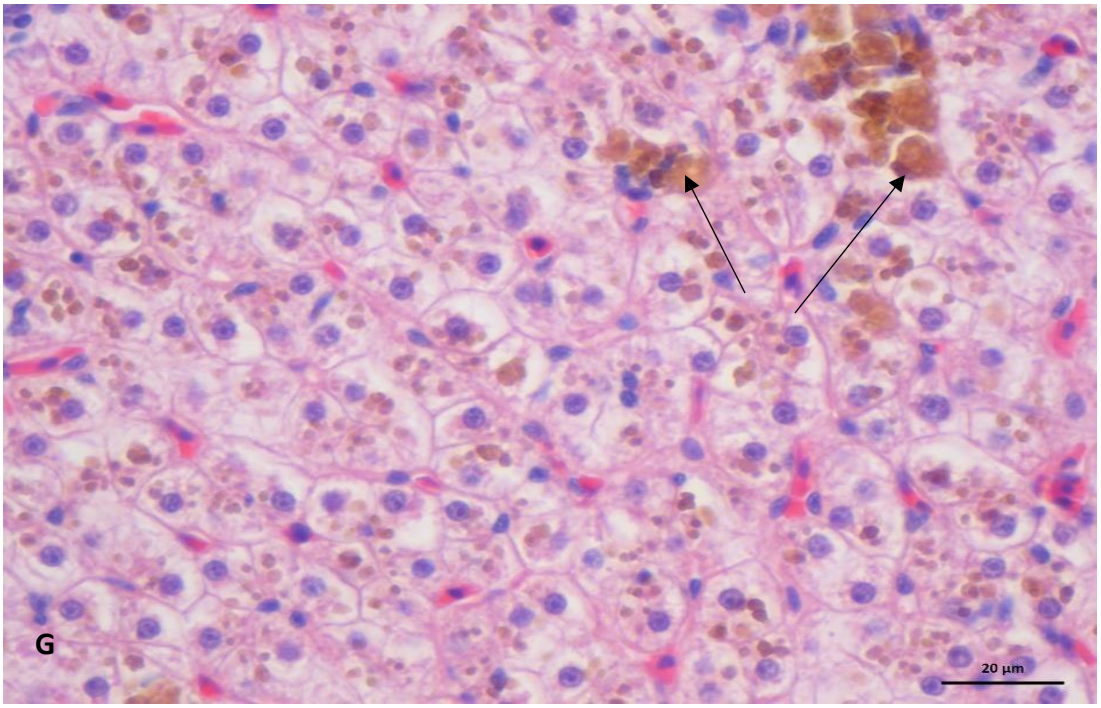
Red: High correlation; **Orange**: moderate correlation; **Green:** Low correlation

A significant moderate positive correlation of 0.386 ($p \leq 0.05$) was drawn between liver lipofuscin and water CO_2 levels, while water temperature correlated negatively to the value of -0.226 ($p \leq 0.05$). In other words, liver lipofuscin shows increase with increasing CO_2 and decreasing temperatures.

Plate 10: Liver lipofuscin

***A**: Grade 0, fish LC10 (400X); **B**: Grade 1, fish LC2 (400X); **C**: Grade 2, fish NJ7 ((400X); **D**: Grade 3, fish GB3 (400X); **E**: Grade 4, fish NA3 (400X); **F**: Grade 5, fish NB6 (400X); **G**: Grade 6, fish NC2 (400X). Note lipofuscin precipitates (arrows)*





d. Portal Lipofuscin: *(PLATE 11)*

Hepatocyte and portal triad lipofuscin deposits correlated moderately in terms of comparative distribution.

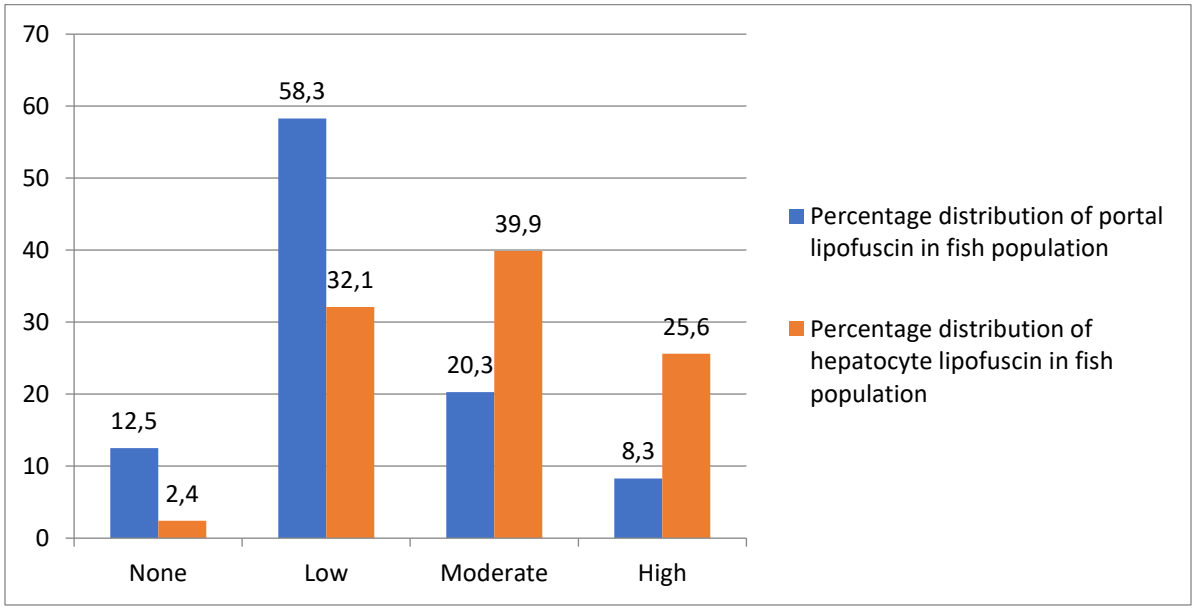
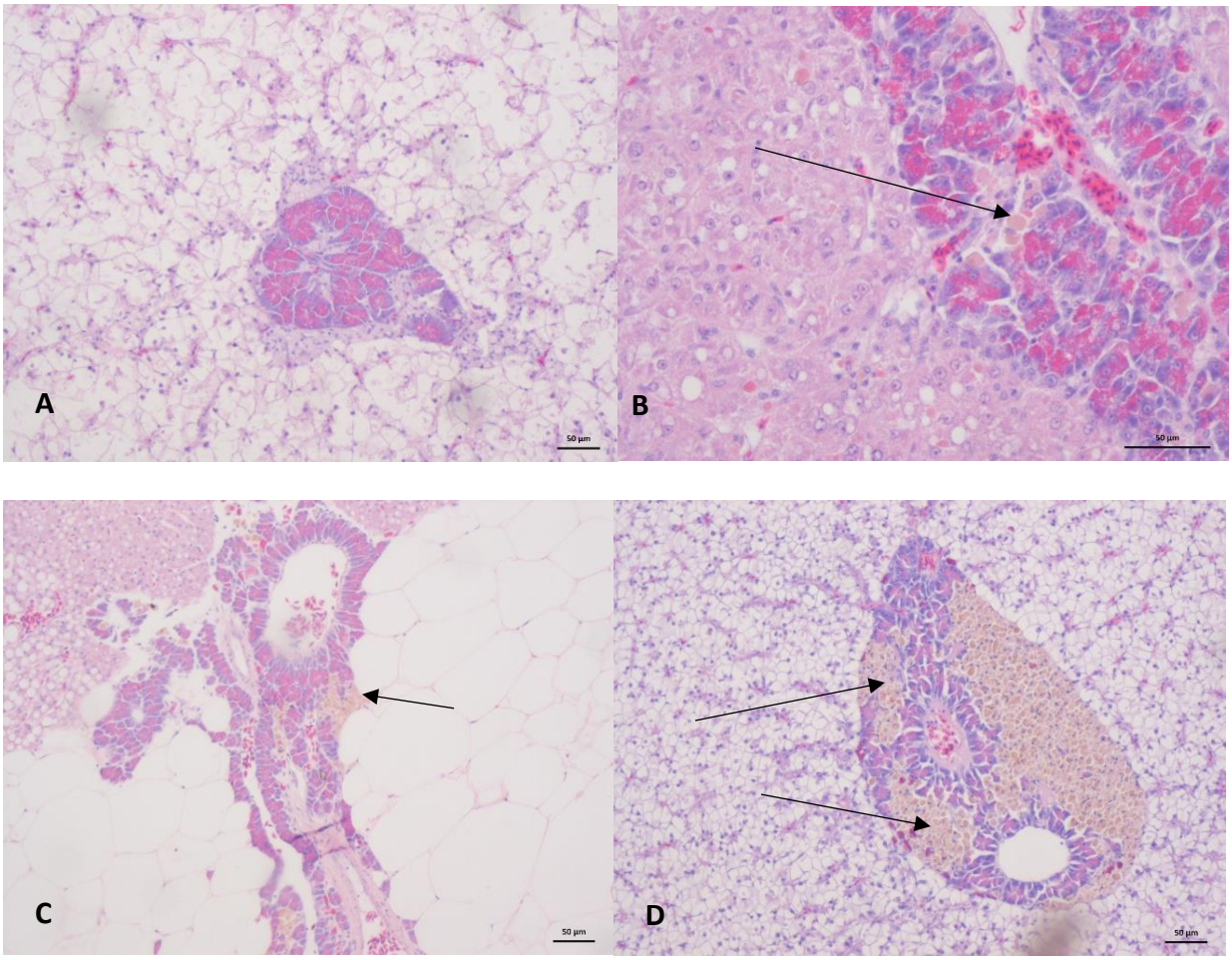


Fig. 4-35 Comparison of hepatocyte and portal lipofuscin levels

Plate 11: Portal triad lipofuscin

***A**: None, fish NE1 (100X); **B**: Low grade, fish ND8 (200X); **C**: Moderate grade, fish GA10 (100X); **D**: High grade, fish GH1 (100X). Note lipofuscin deposits (arrows)*



e. Hepatocellular nuclear activity:

Activated versus inactive hepatocyte nuclei, were almost equally represented.

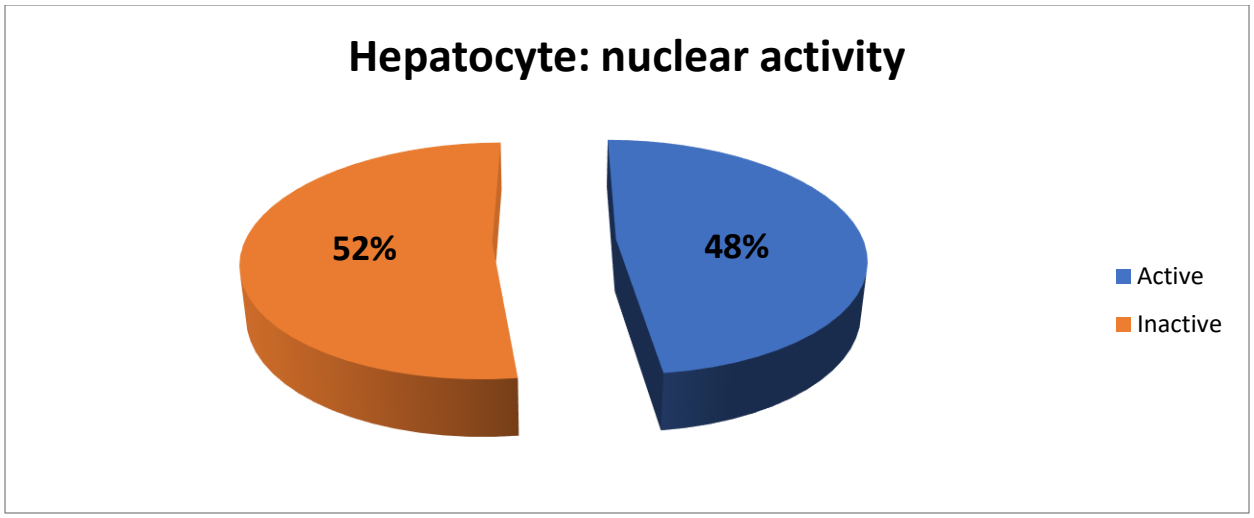


Fig. 4-36 Percentage representation of hepatocyte nuclei activity through fish population

Table 4-26 Statistical data analysis (Pearson correlation model) of the effect of independent variables on liver nuclear activity, with negative values indicating a negative correlation and positive values indicating a positive correlation

Stocking rate (kg/m^3)	Water temp (^{0} C)	Water pH	Water DO (mg/L)	Water CO_2 (mg/L)	NH_3 toxic: UIA (mg/L)	Nitrite NO_2^- (mg/L)	CH:TA ratio
0.001	0.238	0.097	-0.021	0.028	-0.085	-0.147	0.215

Red: High correlation; **Orange**: moderate correlation; **Green:** Low correlation

Two significant low positive correlations were drawn between liver nuclear activity and both CH:TA ratio (0.215) ($p \leq 0.05$) and water temperatures (0.238) ($p \leq 0.05$). In other words, as water temperature or CH:TA ratios increase, so does hepatocyte nuclear activity.

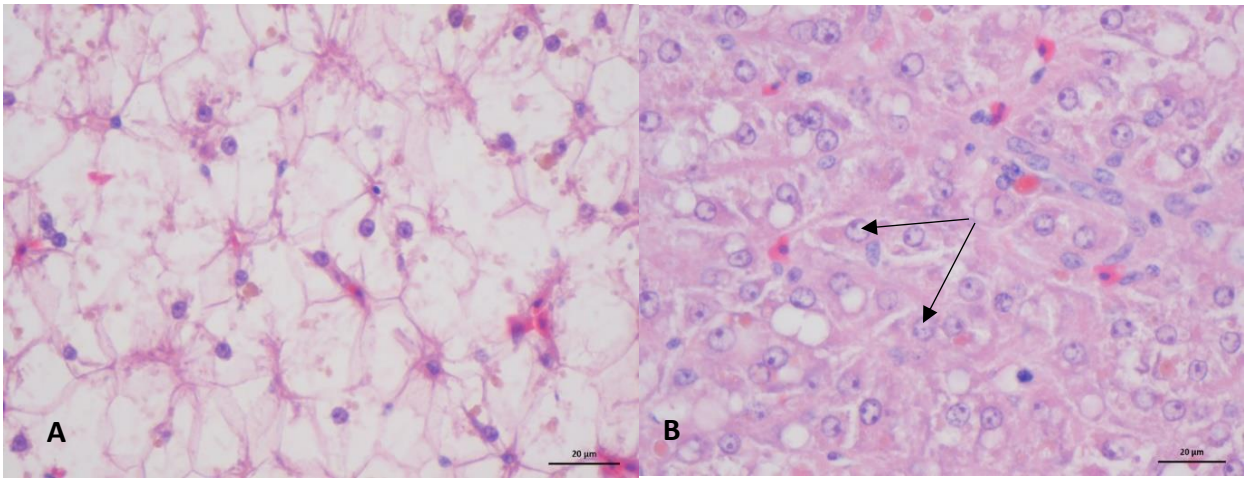


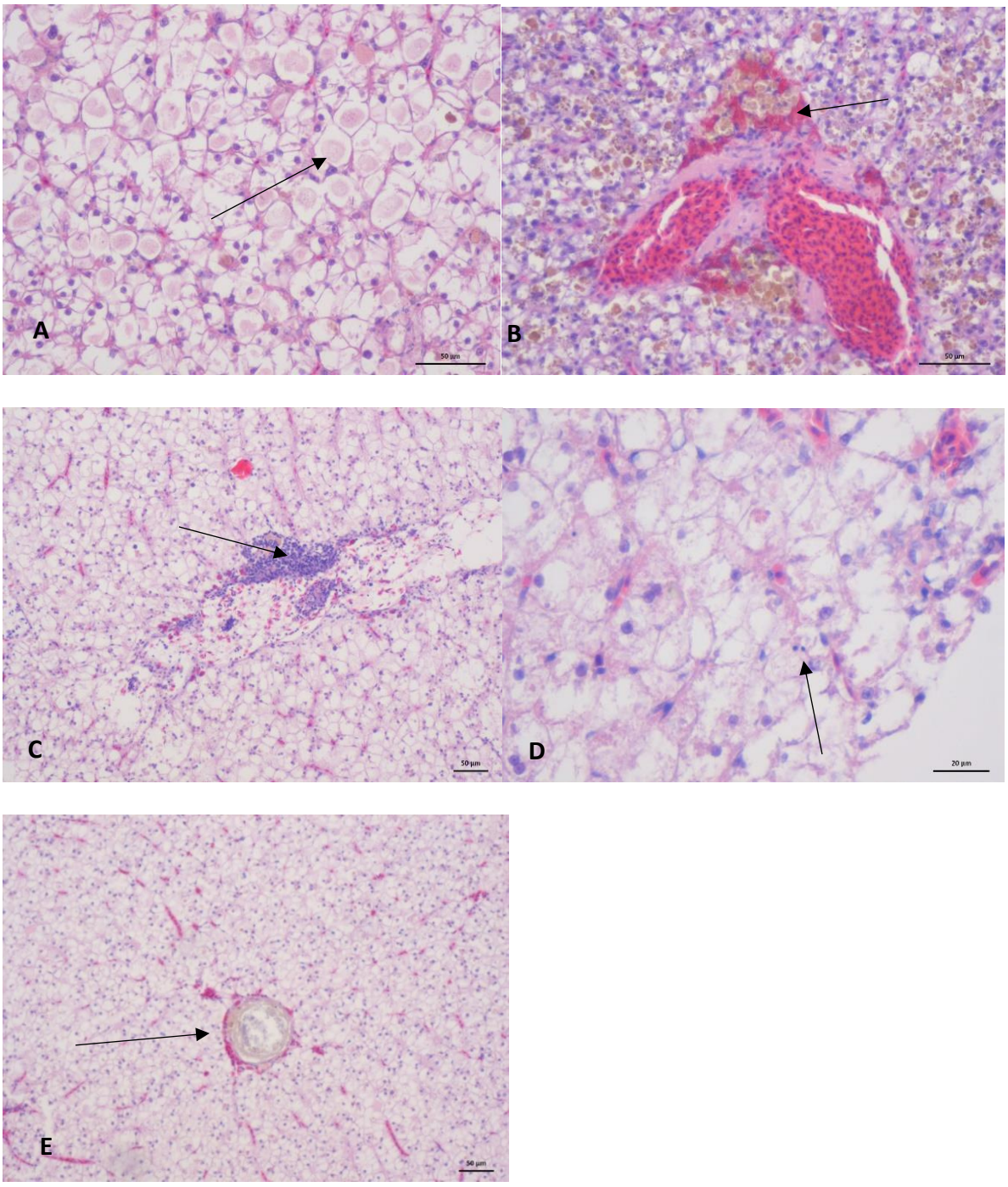
*Fig. 4-37 Hepatocyte nuclear activity: **A**: Inactive nuclei, fish NC1 (400X); **B**: Active nuclei, fish ND8 (400X). Note the nuclear karyomegaly, anisokaryosis, and euchromatism in B.*

Table 4-27 Other pathological changes noted (PLATE 12)

Other liver histo-pathological changes seen:	**% of fish affected:**
Hepatocellular cytoplasmic eosinophilic laking	9%
Eosinophilic granular cell infiltration	8%
Inflammatory cell infiltration	3%
Parasitic cysts/ granulomas	2%
Apoptosis	1%

Plate 12: Liver: other histo-pathological changes

***A**: Cytoplasmic-"laking", fish NA5 (200X)- note the centrally pooled cytoplasm; **B**: EG cell infiltration, fish GH8 (200X); **C**: Inflammatory cell infiltration, fish GF5 (100X); **D**: Apoptosis, fish NJ9 (400X); **E**: Parasitic cyst, fish NE2 (100X)*



3. Gastric pathology *(SEE APPENDIX 4 TABLE 4 Data sheets; PLATE 13)*

Gastritis, as characterized in *CHAPTER 3: MATERIALS AND METHODS*, was a very prominent finding through many of the fish samples analysed. Only 1.2% of fish were free of inflammatory cell infiltrates and typical histopathological evidence of a gastritis. The majority of fish displayed grade 1 level of eosinophilic granular cell infiltrate, but 7% presented with a level 5 gastritis, showing severe gastric erosions and ulceration.

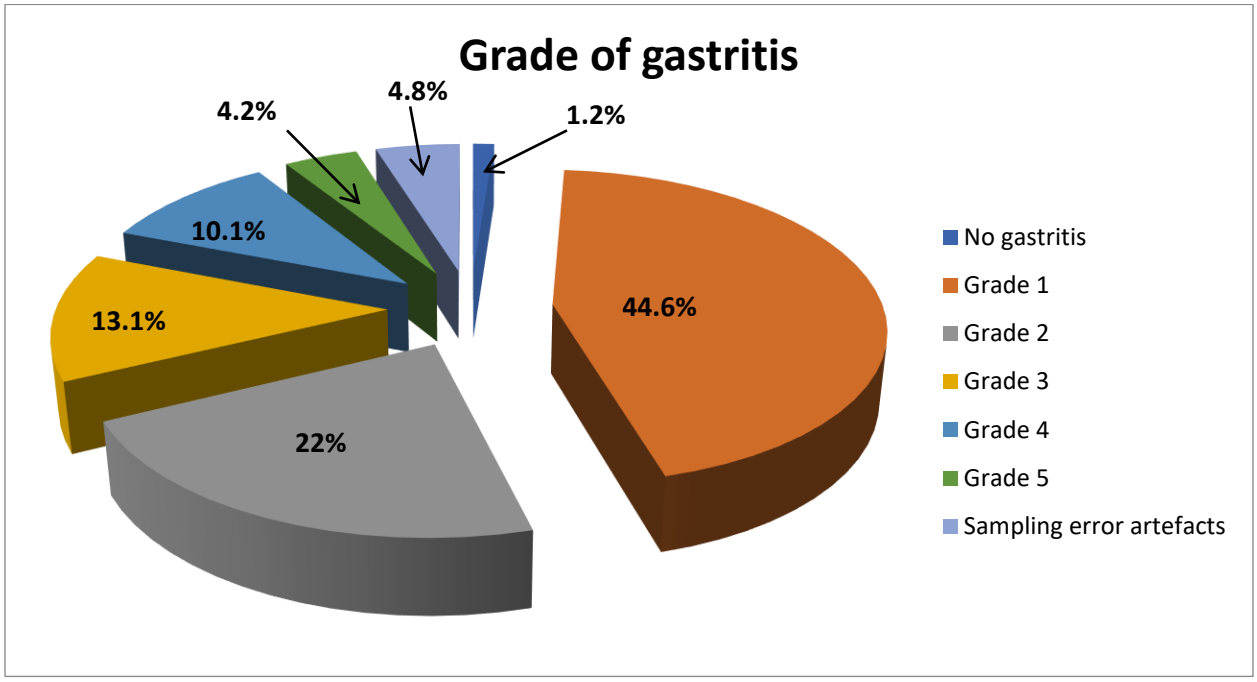


Fig. 4-38 Percentage distribution of various grades of gastritis through sampled fish population

Table 4-28 Statistical data analysis (Pearson correlation model) of the effect of independent variables on gastritis, with negative values indicating a negative correlation and positive values indicating a positive correlation

Stocking rate (kg/m3)	Water temp (^{0}C)	Water pH	Water DO (mg/L)	Water CO_2 (mg/L)	NH_3 toxic: UIA (mg/L)	Nitrite NO_2^- (mg/L)	CH:TA ratio
-0.318	0.104	-0.039	-0.245	-0.226	0.050	0.145	-0.225

Red: High correlation; **Orange**: moderate correlation; **Green:** Low correlation

Gastritis only correlated negatively with water and husbandry factors, showing significant moderate correlation of -0.318 with stocking density ($p \leq 0.05$), and significant low correlations with water DO (-0.245) ($p \leq 0.05$), water CO_2 (-0.226) ($p \leq 0.05$) and CH: TA ratio (-0.225) ($p \leq 0.05$). This would suggest that gastritis increases predominantly in lower stocking density situations, and with decreasing water DO, CO_2 or CH:TA ratios.

Plate 13: Gastritis

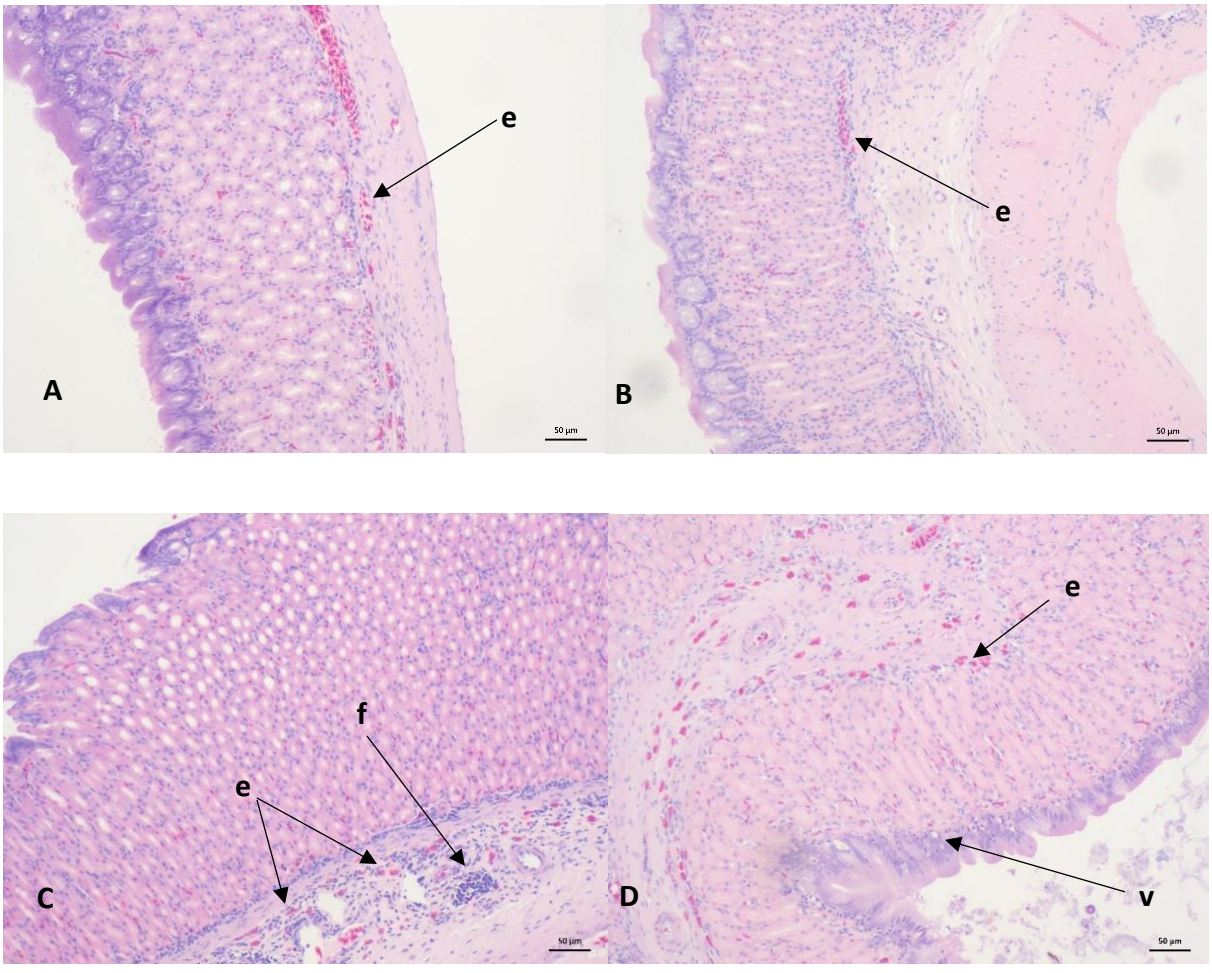
***A**: Grade 1, fish ND3 (100X); **B**: Grade 1, fish GB8 (100X)- note EGC's in submucosa (e)*

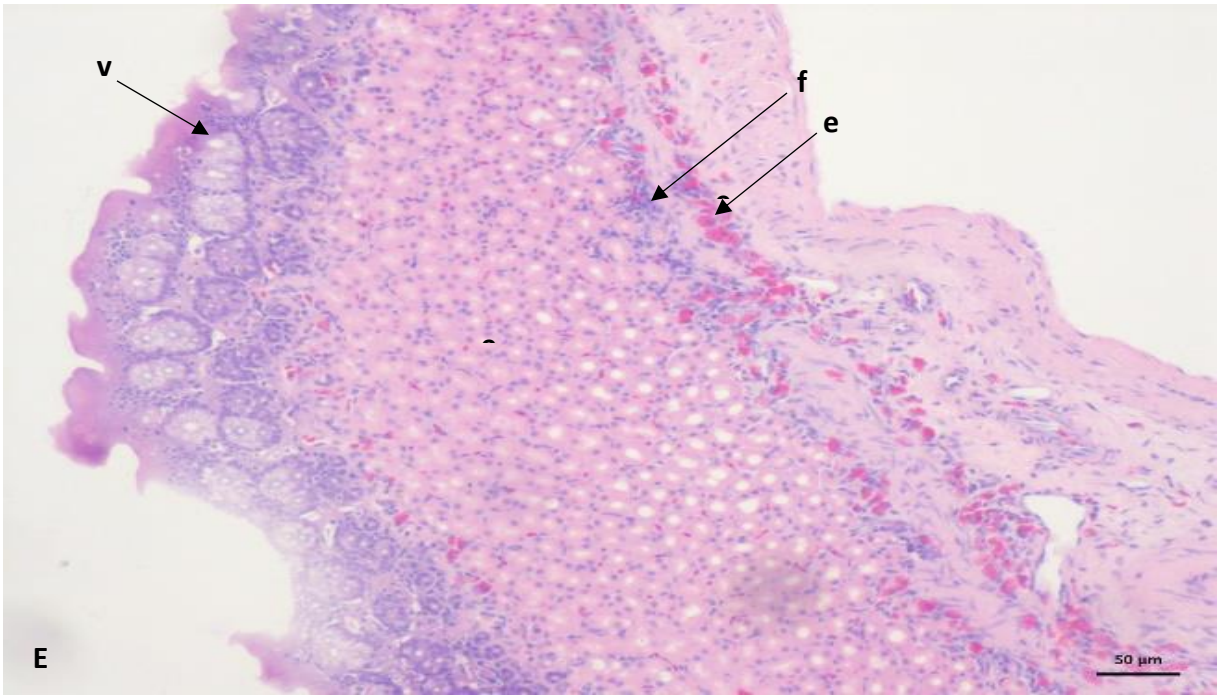
***C**: Grade 2, fish NE3 (100X); **D**: Grade 2, fish NE4 (100X)- note increased EGC's in submucosa (e), some vacuolation (v) and lymphocytic foci (f)*

***E**: Grade 3, fish NF1 (100X)- note increased EGC's in submucosa (e), increased lymphocytic infiltration (f), increased vacuolation (v)*

***F**: Grade 4, fish GH2 (100X); **G**: Grade 4, fish LC3 (100X)- note increased EGC's with infiltration into mucosa (e), severe lymphoid infiltrate (f)*

***H**: Grade 5, fish LC10 (100X)- note gastric ulcer/ erosion (u)*




v
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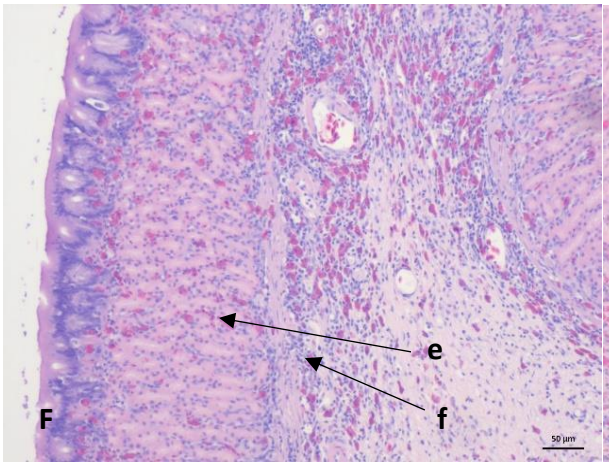
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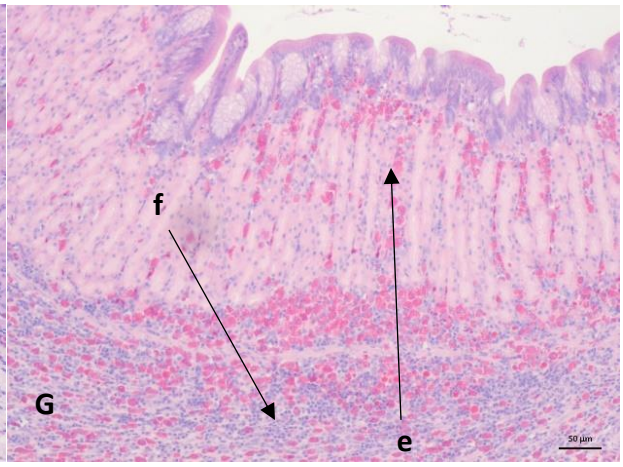
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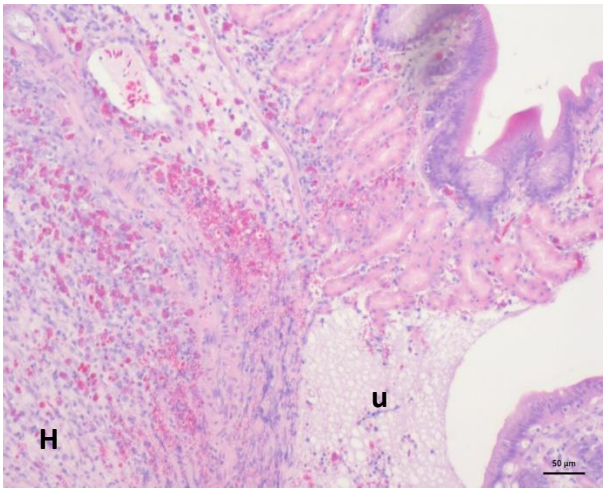
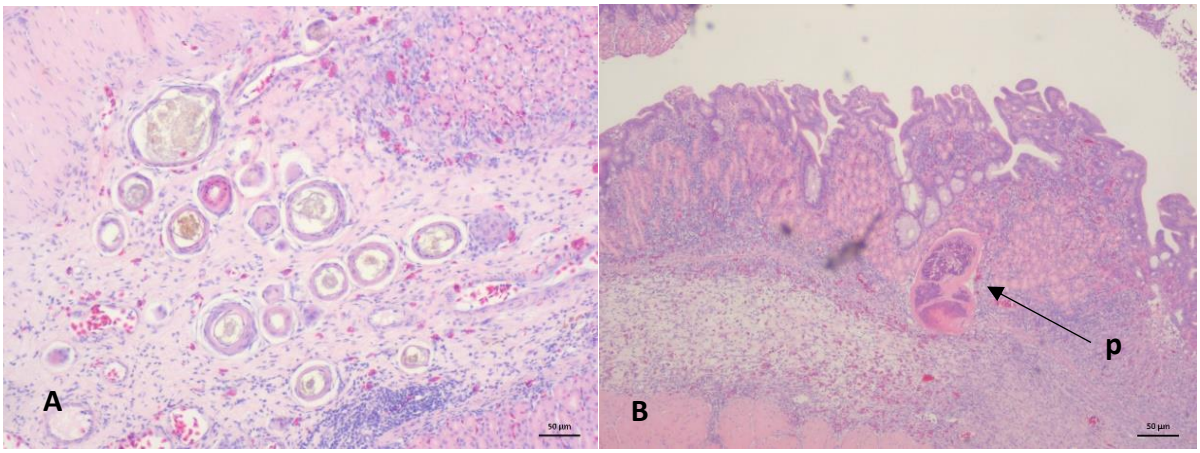

u
H
50 µm

Other gastric pathology noted included gastric cystic granulomas (8.3% of fish). Some gastric sections showed evidence of suspected Metazoan parasites in the gastric lumen and mucosa *(PLATE 14)***.**

Plate 14: Other gastric pathology

***A**: Gastric wall cysts, fish GE2 (100X); **B**: Parasite (p) attached to gastric mucosa, fish LC10 (40X)*



4. Histopathological changes affecting other organs

a. Pancreas:

Variability in pancreatic activity was noted. In 85.1% of fish, the pancreatic activity was normal, evidenced by exocrine acinar cells with prominent zymogen granulation. 14.9% showed atrophied (inactive) acinar cells with low zymogen activity.

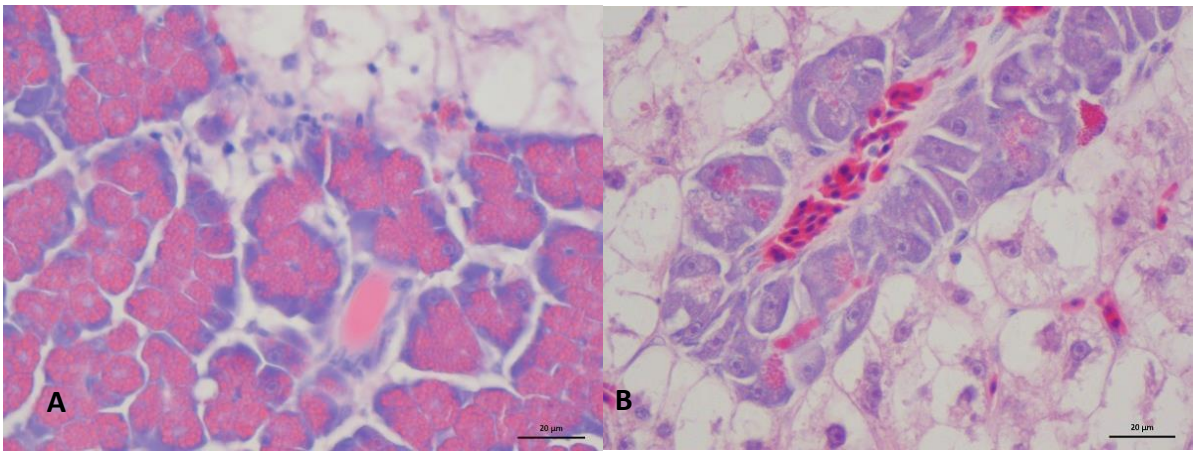


*Fig. 4-39 Varying levels of pancreatic activity: **A**: Active pancreas, fish NJ6 (400X); **B**: Inactive pancreas, fish LB6 (400X)- note the colour difference in acinar cells due to loss in number and size of cytoplasmic zymogen granules*

b. Spleen *(PLATE 15)*

Melanomacrophage centres (MMC) comprised various degrees of phagocytosed melanin and lipofuscin. Lipofuscin precipitates in the spleen tended to be deposited as discrete focal aggregates of varying size, rather than widely disseminated throughout the tissue. MMC's were in all instances affected by a degree of lipofuscinosis. Lipofuscin always occurred as phagocytosed lipid breakdown product in macrophage cytoplasm. Large accumulation resulted in eccentric nuclear compression, obscuring the intracellular nature of the pigment. No other inflammatory cell infiltrate/s were associated with this process.

Splenic lipofuscin content was directly proportional to hepatic lipofuscin levels, with 47.4% of fish showing moderate to high splenic lipofuscin grades in comparison to 51.8% of livers.

High melanin pigment levels in melanomacrophages were less often encountered, with only 6.6% of fish exhibiting moderate to high deposits, and with poor correlation to MMC lipofuscin levels.

Table 4-29 Comparison of MMC composition through fish population

	Lipofuscin in MMC's	**Melanin in MMC's**
Low level	49.4% of fish	89.9% of fish
Moderate level	32.7% of fish	6% of fish
High level	14.7% of fish	0.6% of fish

Lymphoid infiltrates in splenic tissue presented as small multifocal foci of round cell hypercellularity. The prevalence of this finding was 42.3% with little variation from mild to moderate in degree.

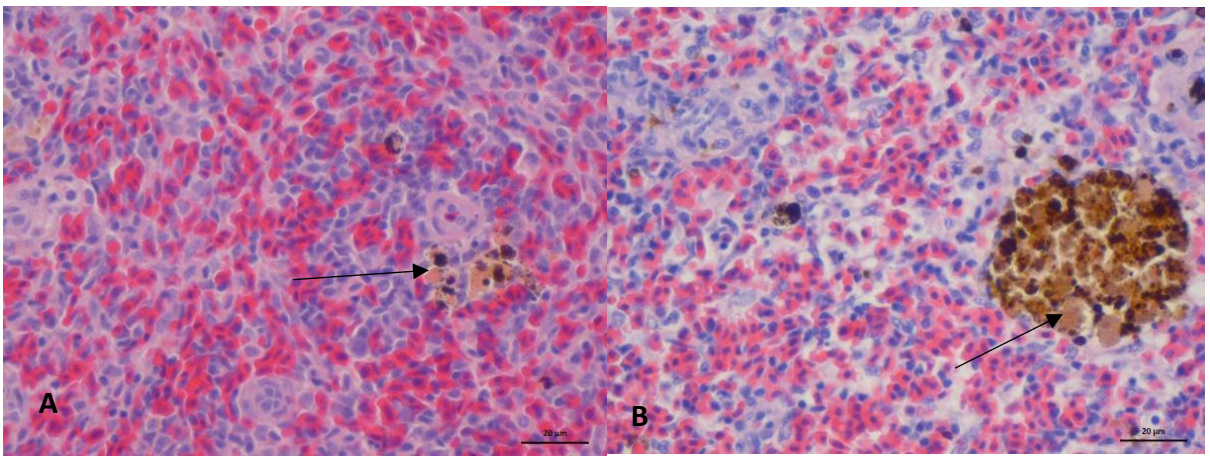
Cystic granulomas of varying size (60-100 µm) were a frequent finding, seen in 11% of all splenic sections. These small cystic structures were characterised by a thin fibrous wall surrounded by scant macrophage infiltration. Central content/s could not be identified as most seemed to have been lost during processing. In one anterior kidney tissue section, contents of a similar cystic structure appeared to be parasitic (metazoan) in origin.

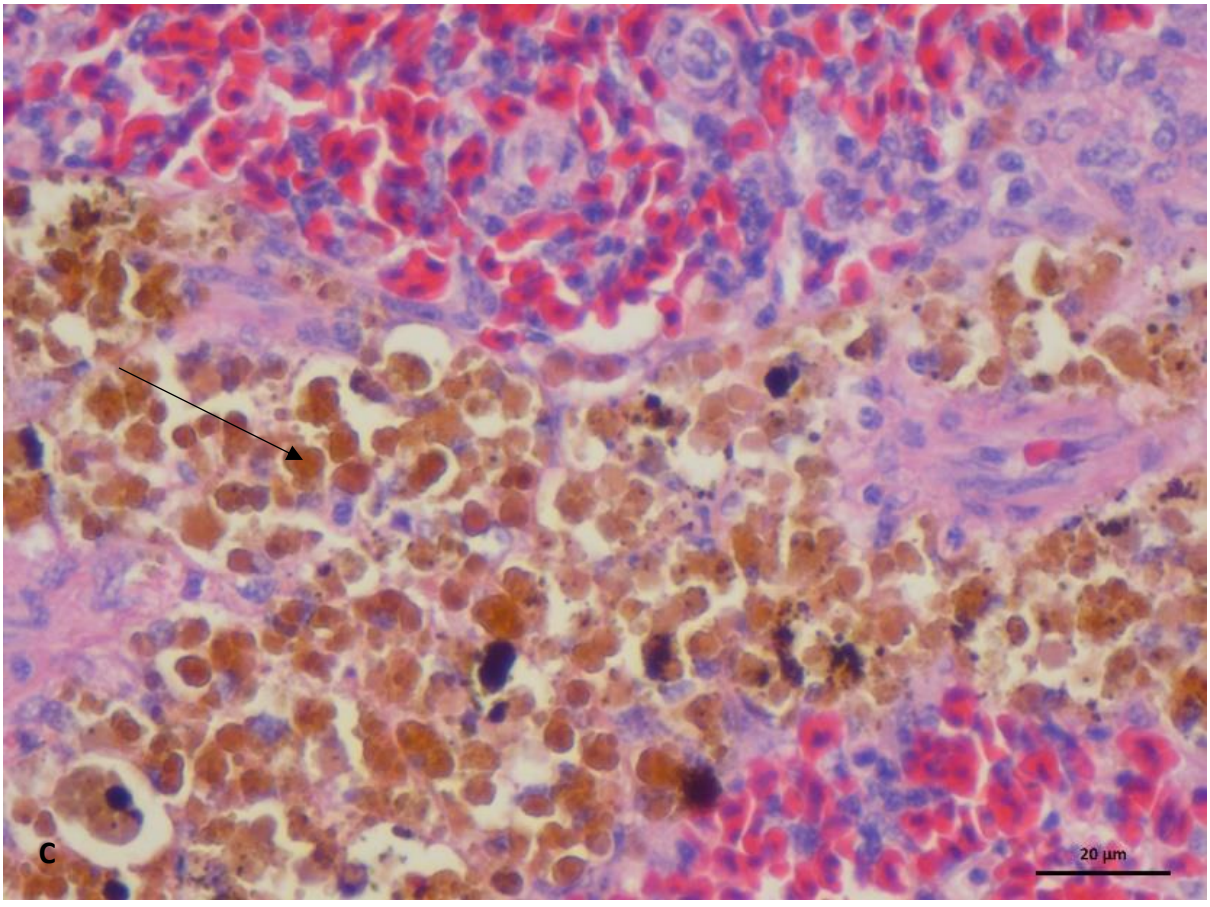
Generalised red pulp macrophage infiltration was also infrequently encountered (9% of tissues sections).

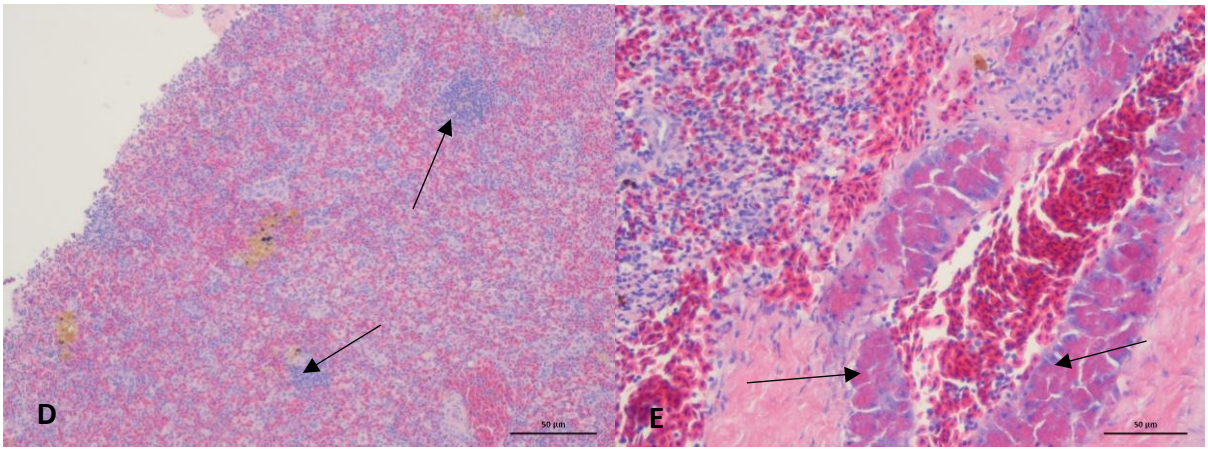
No typical necrotizing or granulomatous lesions in the spleen with intracellular bacteria typical of *Edwardsiella* spp. or *Francisella* spp. were evident (Soto *et al.*, 2012; Soto, 2015).

Plate 15: Splenic pathology

***A**: Low MMC lipofuscin, fish NB1 (400X); **B**: Moderate MMC lipofuscin, fish GH3 (400X); **C**: High MMC lipofuscin, fish NC9 (400X)- note yellow/ brown lipofuscin precipitates; **D**: Multifocal lymphoid infiltrates (arrow), fish GB5 (100X); **E**: Focal EG cell infiltrates (arrows), fish GE2 (200X); **F**: Generalized red pulp macrophage infiltrates, fish GG3 (100X)- note "starry-sky" appearance; **G**: Cystic granuloma (arrow), fish GH3 (100X); **H**: Cystic granuloma (arrow), fish GF7 (400X)*



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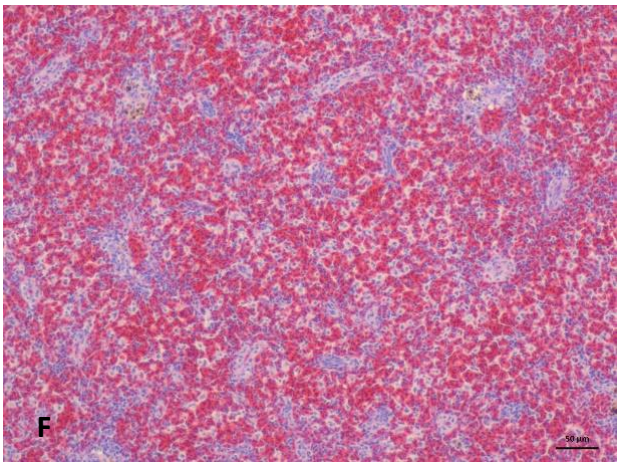
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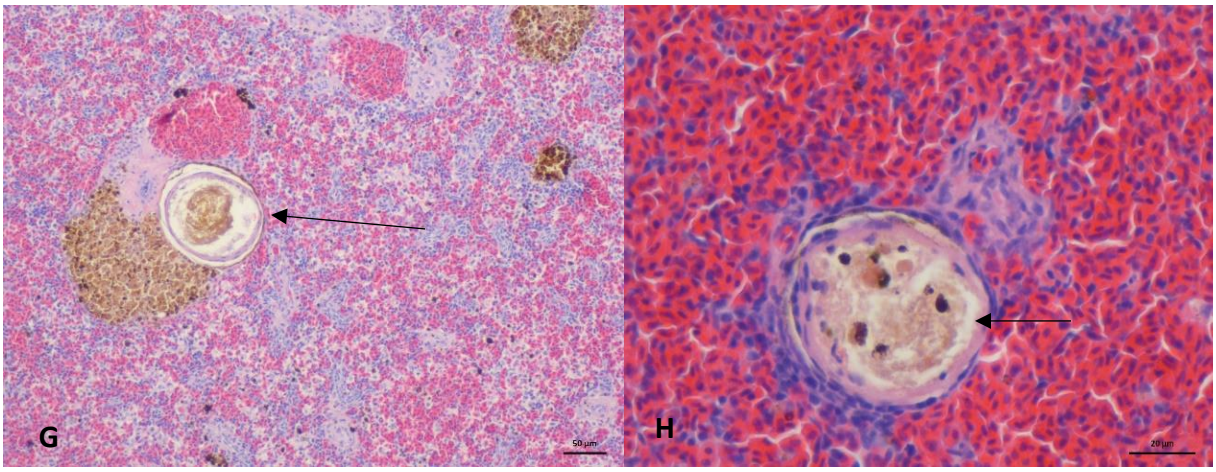

G
H

c. Kidney *(PLATES 16 and 17)*

The anterior kidney was sampled and examined for lesions. Variable lipofuscin levels were noted and were generally low (52.4% of fish, with a small percentage (4.2%) showing high levels of deposits associated with the MMC's. MMC melanin pigment levels also showed mild variation in quantity and were also generally low (60.7% of fish), with only 1.2% of sections qualifying in the high range. Other minor findings included the presence of cysts in 11% of sections, one containing an intact suspected metazoan parasite, interstitial mononuclear leucocytosis (lymphocytes and macrophages), mild myeloid cellular degeneration (apoptosis) and occasional EGC infiltration. No granulomatous lesions typical of *Edwardsiella* spp. (Soto *et al.,* 2012) or *Francisella* spp. (Soto, 2015) were seen.

Unfortunately, a large percentage of anterior kidney samples (26.2%) were not available for examination due to sampling and sectioning artefacts.

Samples collected from the posterior kidney, showed a small percentage of fish displaying amorphous basophilic interstitial mineral deposits. As with the anterior kidney, sampling or sectioning error resulted in a number of cases (25%) not available for examination.

Other minor findings included lipofuscin deposits in 8% of sections, the presence of cystic granulomas in 3% of sections, occasional EGC infiltration (2%), MMCs (2%) and hyaline deposits (1%).

Plate 16: Anterior kidney pathology

***A**: Low Lipofuscin (arrow), fish GB1 (400X); **B**: Moderate lipofuscin (arrow), fish GE6 (200X); **C**: High lipofuscin (arrow), fish NC3 (200X); **D**: Apoptosis (arrows), fish GH6 (400X); **E**: Suspected parasitic cyst (c), fish ND10 (100X); **F**: Cyst (wall marked with c) with suspected parasite (p), fish GF5 (200X)*

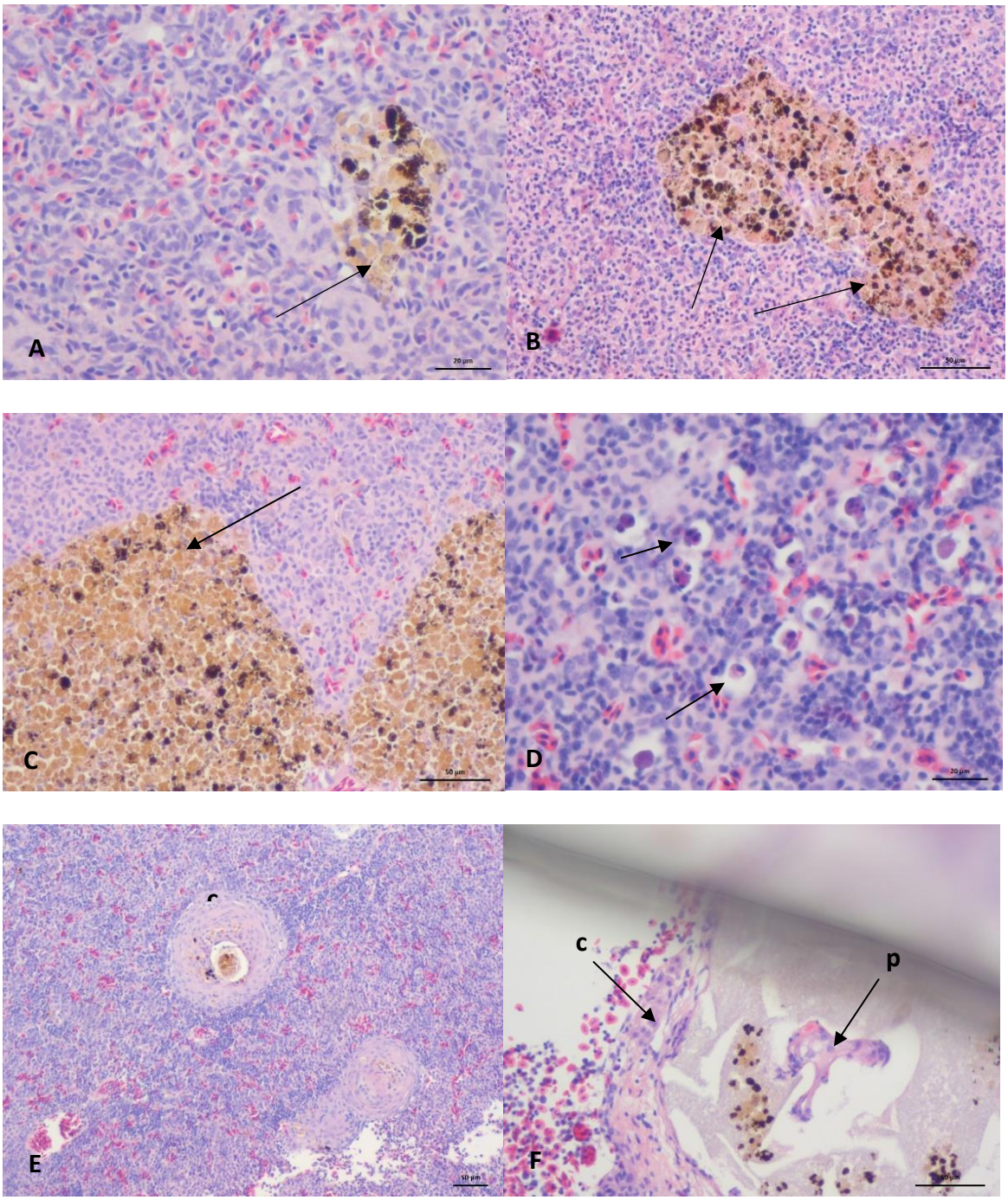
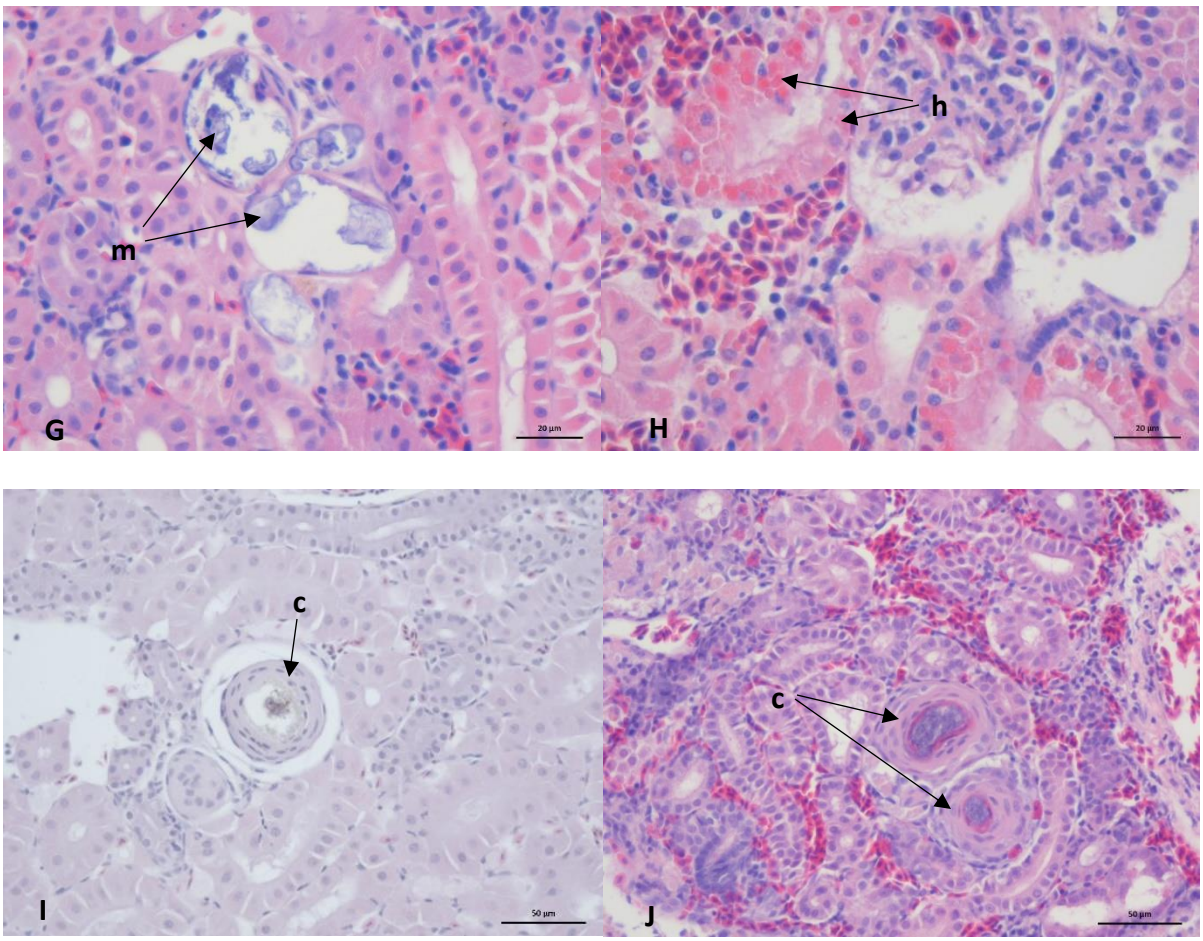


Plate 17: Posterior kidney pathology

***G**: Mineralization (m), fish GG6 (400X); **H**: Hyaline necrosis (h), fish GG7 (400X); **I**: Cyst (c), fish GB7 (200X); **J**: Cysts (c), fish GG10 (200X)*



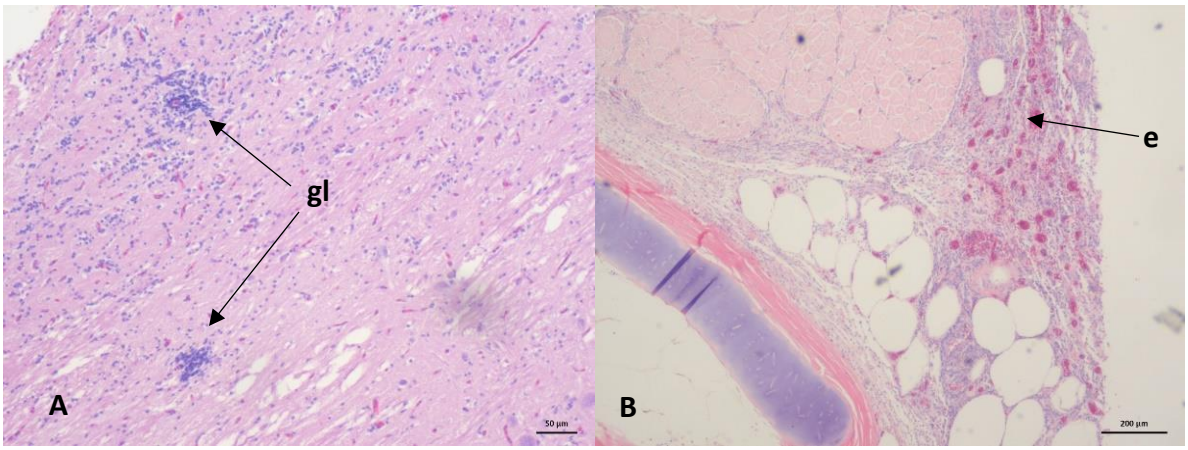
d. Lesions observed in other tissues *(PLATE 18)*

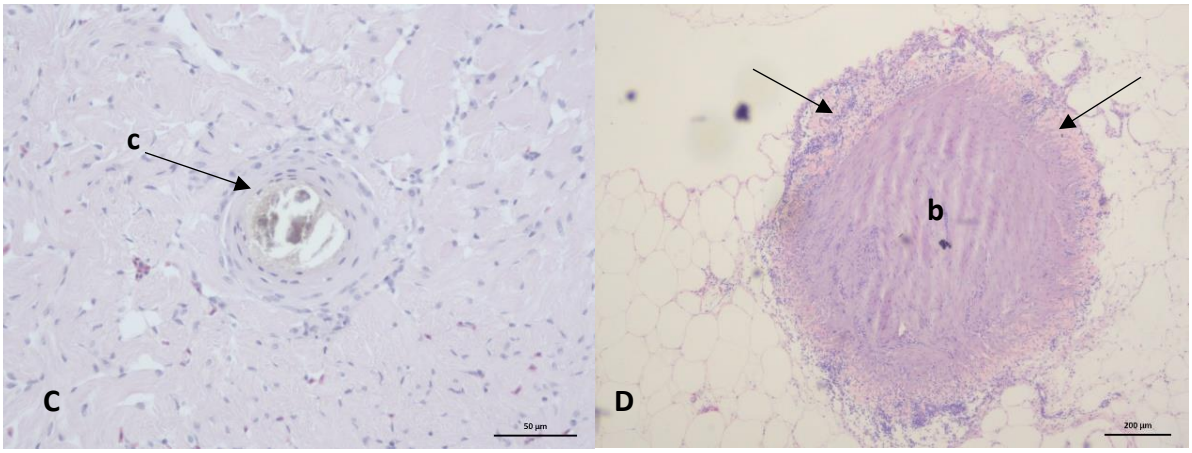
Myocarditis, pericarditis and/ or bulbar arteritis (28%), Cysts in brain, multifocal gliosis/ lymphocytic encephalitis (18.5%), Peritonitis, Steatitis, Parasitic cysts in visceral fat, Retrobulbar cellulitis.

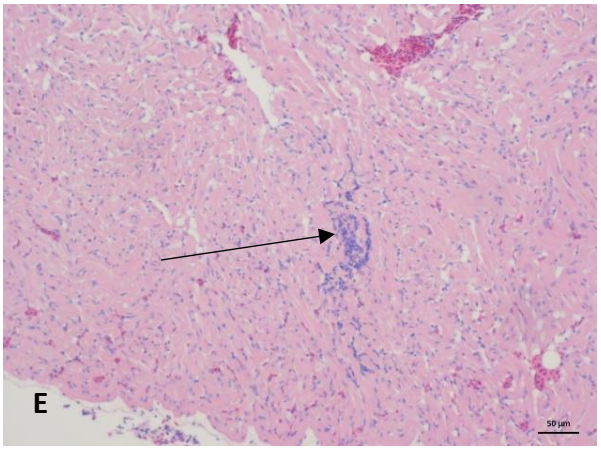
No intestinal pathology of significance was noted

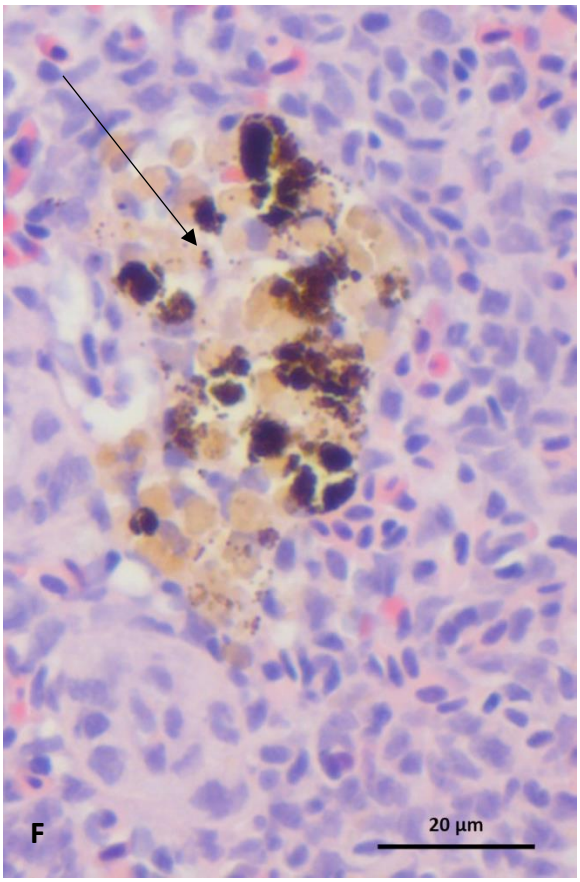
Plate 18: Other pathology

***A**: Multifocal gliosis/ lymphocytic encephalitis (gl), fish GG3 (100X); **B**: Retrobulbar cellulitis with high EG cell infiltrates (e), fish ND7 (40X); **C**: Myocardial cyst (c), fish GB7 (200X); **D**: Heart bulbar arteritis with high lymphocytic and EG cell infiltrates (arrow) surrounding the bulbar arteriosus (b), fish GG5 (40X); **E**: Myocarditis, fish GG3 (100X); **F**: Posterior kidney MMC with scattering of the melanin granules, fish GB1 (200X)*





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20 µm

4.3 QUESTIONNAIRE ASSESSMENT OF FARM SYSTEMS, MANAGEMENT AND HUSBANDRY

4.3.1 Tilapia culture systems

Aquaculture systems assessed, varied in total capacity between 10 000 litres and 750 000 litres, with a mean of 18 705 litres. Variation was seen in capacity of sampling units, with water volumes ranging between 2000 litres in the plastic and concrete tank design systems, to 360 000 litres in the earthen ponds systems.

The predominant aquaculture production system encountered was the recirculating aquaculture system (RAS), in 89% or 17 of the 19 systems, where sourced water was continuously recirculated through filtration systems of varying design and complexity, and water changes performed at varying intervals and volumes. The only differing systems were farms NB and NF. NF was a through-flow design, with a regular input of fresh water from the source (borehole), and output onto vegetable gardens, with no recirculation or reuse of water in the system. Farm NB was semi-recirculating with river water routinely pumped into a holding dam then recirculated through the system. Virtually all farms (other than three), relied on additional passive heating of the water through the green-house effect in PVC-lined tunnels, however, supplemental forms of heating like heat pumps, wood-burning donkey boilers, heating elements, gas heaters, solar heaters and even heat generated as a by-product from a crematorium, were also used.

Filtration methods included the use of mechanical modalities like cyclone solid sludge removers, solid drains, vortex filters, sand swimming pool filters, settling chambers, drum micro-screen filters, and swirl-separators in the Cornell-dual-drain design. Bio-filtration was

supplemented through the use of floating bead filtration, moving bed bioreactors, trickle towers, and other high surface area media ranging from mesh netting to industrial high surface area media. One farm relied on water changes alone. Another had a really interesting bio-filter with an additional small crop of water hyacinth acting as a floating macrophyte on its water surface- a mini aquaponic facility to facilitate nitrate removal.

UV sterilizers were also often in use.

Systems varied in age from 6 months to 14 years, with most having been in operation for around 4 years.

Green water (algae-rich) systems were encountered in 36.8% of farms and aquaponics accounted for 15.8% of farms. No farms were employing polyculture with any species other than *O. niloticus, O. mossambicus, a*nd hybrids thereof.

Table 4-30 General overview of farms assessed with respect to systems

Farm	**System**	**Heating**	**Total capacity (cubic meters)**	**Sample tank capacity (cubic meters)**	**Age (years)**	**Green water system**	**Aquaponics**
GA	RAS Plastic tanks	Tunnels Solar heaters	40	23.5	3	Yes	No
GB	RAS Concrete tanks	Tunnels Incinerator	720	9	4.5	Yes	No
GC	RAS	Tunnels	200	10	4	Yes	No

	Plastic tanks	Heat pumps Elements					
GD(a)	RAS Plastic tanks	Tunnels	30	7.5	14	Yes	Yes
GD(b)	RAS Plastic tanks	Tunnels	40	5	14	Yes	Yes
GE	RAS Plastic tanks	Tunnels Heat pumps	142	6	5	No	No
(GF(a)	RAS Plastic tanks	Tunnels	34	7	3	No	Yes
GF(b)	RAS Plastic tanks	Tunnels	90	5	3	No	No
GG	RAS Plastic tanks	Tunnels Wood and gas heaters	95	4.5	1.5	No	No
GH	RAS Plastic tanks	Tunnels	53	20	2.5	No	No
NA	RAS Plastic lined earthen pond	None	10	10	4	No	No
NB	Semi-RAS/	Tunnels	Missing data	Missing data	4	No	No

	through flow Concrete tanks						
NC	RAS plastic lined earthen pond	Tunnel	360	360	2.5	Yes	No
ND	RAS concrete tanks	Tunnel, heat pump, donkey	188	40	4	No	No
NE	RAS Plastic tanks	Tunnel, boiler	750	46	1.5	No	No
NF	Flow through, plastic lined earthen pond	Tunnel	300	260	2	Yes	No
NJ	RAS Concrete tanks	Plastic tarpaulin	180	50	0.5	No	No
LB	RAS Concrete tanks	Tunnel (broken)	24	2	4	No	No
LC	RAS Plastic tanks	Tunnel	104	8	3	No	Yes

Plate 19: Systems overview

***A**: RAS system, commercial city venture, tunnel heating, innovative square wooden plastic-lined tanks, 2000l each (farm GG)*

***B**: RAS system, large scale commercial venture, tunnel heating, round metal, plastic-lined tanks, 5000l each (farm GE) (Photograph: C. Milburn)*

***C**: RAS system, small scale rural farm commercial venture, tunnel heating, round plastic tanks, 20 000 l each (farm GH)*

***D**: RAS system, rural small-scale farm venture, no passive heating, rectangular earthen pond, plastic-lined 10 000l (farm NA)*

***E**: RAS system, rural large plastic-lined earthen pond with minimal management, tunnel heating, 260 000 l capacity (farm NC), green-water*

***F**: RAS/ semi throughflow system using gravity-fed river water, large scale commercial venture, tunnel heating, small concrete tanks, 2000 l each (farm NB)*

***G**: RAS system, small scale rural commercial venture, tarpaulin passive heating, long rectangular concrete tanks, 50 000l each (farm NJ)*

***H**: RAS system, small scale rural commercial venture, Cornell dual-drain system drain, tunnel heating, metal round tanks, 5000 l each (farm LB)*

***I**: RAS system, large scale rural venture, tunnel heating, large plastic round tanks, 46 000l each, automatic feeders (farm NE)*

***J**: RAS system, large scale commercial rural venture, concrete "Mixed-cell-raceway" (MCR) design, 40 000l each, tunnel heating (farm ND)*

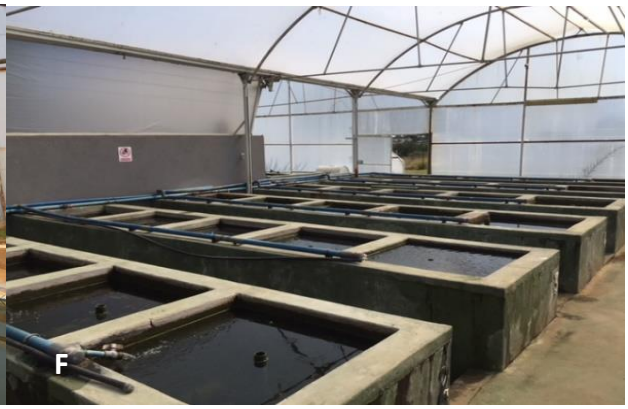

A


B


C


D


E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G

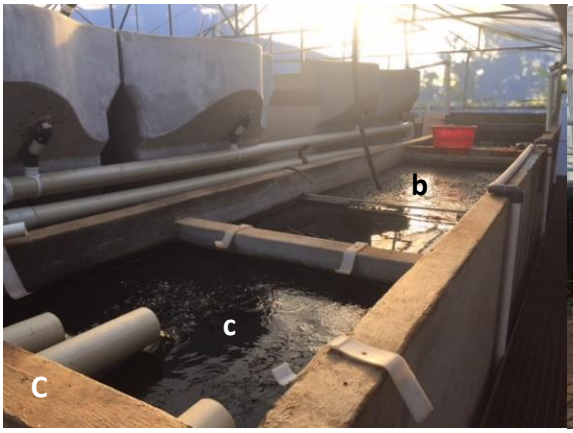

H

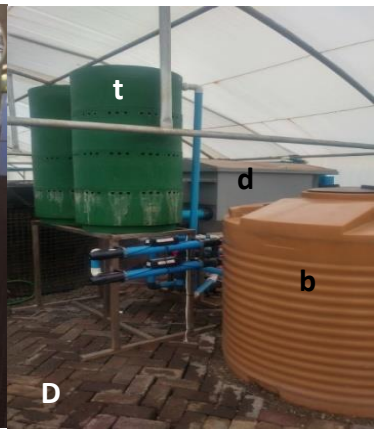

I

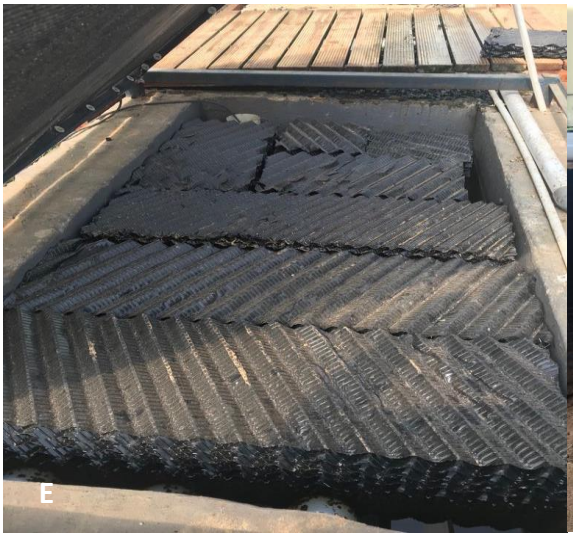

J

Plate 20: Examples of filtration practices

***A**: Sand swimming pool mechanical filter (s); **B**: High surface-area packed shade-cloth raceway biofiltration; **C**: Gravity-fed settling chambers (c) with moving bead biofiltration (b); **D**: Mechanical drum micro-screen filter (d), media-filled trickle towers (t) for degassing and biofiltration, biological moving bed filtration (b); **E**: High surface-area packing media biofiltration; **F**: Cyclone solid sludge remover*







4.3.2 Water management

Water quality parameters were monitored on most farms to various degrees. Frequency of monitoring varied considerably from multiple times per day to no monitoring at all. Farmers made use of various testing modalities including high-end probes for DO or temperature, to simple test strips, test drop kits and even just fish behaviour.

Water was never drained from any system back into natural waterways. Any discharge from a system was onto on-farm vegetable gardens or land.

a. Water source

Water for the production units was sourced as follows:

Borehole: 76.4%

Municipal: 17.6%

River: 0.06%

There was no pre-treatment or filtration of water prior to entering the system, in any production units.

b. Water treatment protocols:

Farms varied in their use of routine water treatments.

Table 4-31 Water treatment protocols used by farms

Farm:	Routine water treatments:
GA	Potassium Permanganate
GB	None
GC	Water changes
GD(a)	None
GD(b)	None
GE	None
GF(a)	None
GF(b)	None
GG	None
GH	None
NA	Water changes
NB	Water changes
NC	None
ND	None
NE	None
NF	Water changes
NJ	None
LB	Water changes
LC	None

c. Aquaponics

Of the 4 farms utilizing an aquaponic system, all were producing a variety of vegetables including lettuce varieties, radish, strawberries, spring onions, duckweed, and herbs like fennel, basil, comfrey, amongst others. These were for retail as well as feeding fish in the systems. Various aquaponic designs were seen, including gravel bed systems, floating rafts, and alternative systems based on the nutrient –film technique principles.

*Fig. 4-40 Aquaponic systems: **A**: nutrient-film and gravel bed aquaponic farm, using a 15 000 litre RAS system with* O. mossambicus*; **B**: A small-scale dual purpose rural enterprise, using a 24 000 litre system to produce* O. niloticus *and nursery plants; **C**: A large scale dual-purpose commercial rural enterprise, of 160 000 litre capacity, with floating raft-bed lettuce production and* O. niloticus *grow-out fish; **D**: Example of a gravel bed system*

4.3.3 Handling

Most farms implemented minimal-handling policies of fish, other than routine morphometric sampling, brood-stock egg and fry harvesting, movement between fingerling and grow-out systems and adult harvesting for market. There was history of increased but low-level mortalities in many systems, post harvesting.

Routine use of gloves was not common practice. Harvesting was never automated in any systems evaluated and all employed the use of manual labour-intensive netting of various designs.

Fig. 4-41 ***A****: Pully hapa nets used for easy harvesting from a plastic-lined earthen pond;* ***B****: Example of hand nets used for routine morphometric sampling (Photographs T.Kersten-UP 4th year Veterinary student)*

4.3.4 Breeding management

Of the 19 systems evaluated, 9 (47.4%) had their own hatchery systems separate from the grow out tanks. Two farms had hatcheries under development, four had plans to establish their own on–farm hatcheries, one had no intention, and two were in the process of closing the system. Systems varied dramatically in set-up and design *(PLATE 21).* 47.4% of the population groups were treated with methyltestosterone for sex reversal, as fry, and 15.8% were bred with a combination of hormone sex reversal and YY super-male brood-stock. Temperature was never used as a means of manipulating gender. Where both modalities were used, the male % of sampled fish was consistently 100%, one modality (methyl-testosterone alone) produced varying results between 40-100% males, and where neither modality was employed, great variation resulted with males forming 20-100% of the sample group.

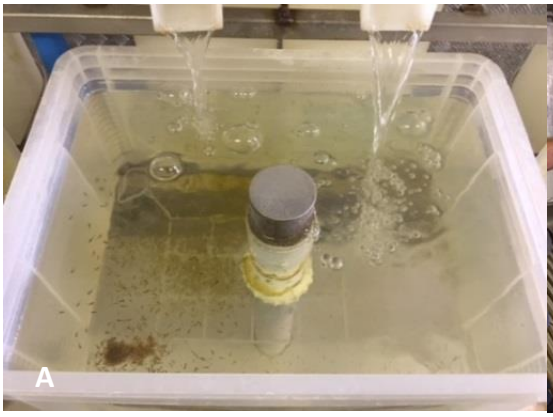
Eleven of the 19 systems had mixed sex populations with ongoing breeding occurring and resulting poor assessment of known stocking density. This was particularly apparent in the two pond systems which had essentially been stocked and left. Netting revealed dominating small fingerling populations in-amongst the adult fish. Four of these systems were using methyl-testosterone treatment of fry to regulate the gender of the population, with seemingly poor results. Farm GF had a 40% :60% female to male ratio in the populations sampled in each of its systems, farms ND and NA each had a 60%: 40% female to male ratio within their populations. The remaining farms had all either purchased in supposed male fingerlings for grow-out, again reflecting poor or absent breeding management at source, or had no sex reversal program implemented on site.

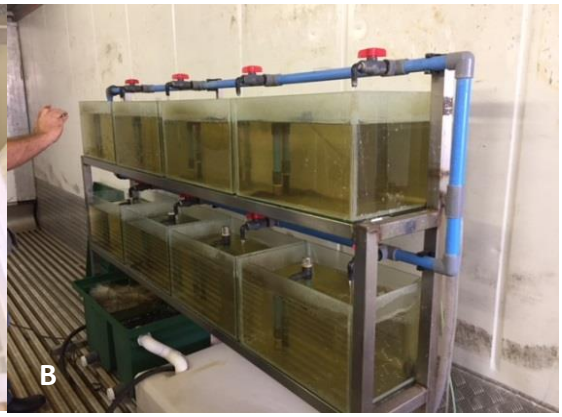
Table 4-32 Comparison of farm percentage of male fish in relation to gender manipulation practice

Farm:	% males	Methyltestosterone use in hatchery	YY super-male brood-stock
GA	100	Y	N
GB	100	Y	Y
GC	100	Y	Y
GD(a)	100	Y	Y
GD(b)	100	Y	N
GE	50	N	N
GF(a)	80	Y	N
GF(b)	80	Y	N
GG	60	N	N
GH	20	N	N
NA	40	Y	N
NB	100	No data	No data
NC	70	N	N
ND	40	Y	N
NE	80	N	N
NF	30	N	N
NJ	100	N	N
LB	80	N	N
LC	100	N	N

Plate 21: Breeding management and hatchery systems

***A**: Newly-hatched fry (farm GA); **B**: Multi-unit RAS system for first-stage fry, post yolk-sac absorption. It is at this stage, as soon as feeding, that methyl-testosterone feed treatment would be applied. Note the supporting mechanical and biological filtration system beneath the unit (farm GA); **C and D**: Alternative design of sorting and separating eggs (in upper chambers) from hatched fry, which are washed into lower chambers (farm NB); **E**: RAS grow-out units for fingerling stage (farm ND); **F**: Example of poor breeding management with large numbers of small fingerlings in amongst grow-out fish (farm NF)*





4.3.5 Stocking rate/ density

Fig. 4-42 A highly-stocked (33.3kg/m^3) RAS system with "Red 5" strain O. mossambicus

Stocking densities in sample tanks varied dramatically between 1.5kg/m^3 to 39.25kg/m^3, with a mean of 18kg/m^3. 63.2% of fish were stocked in the lower-risk range below 20kg/m^3, however a prominent percentage of 15.8% were in the high-risk range of 31-40kg/m^3.

Table 4-33 Overview of farm stocking densities, with systems <10kg/m^3 highlighted blue, >10 ≤20kg/m^3 yellow, >20≤ 30kg/m^3 orange, and >30kg/m^3 red

Farm:	**Stocking rate (kg/m3)**
GA	18
GB	21
GC	20
GD(a)	15
GD(b)	15
GE	20

GF(a)	13.5
GF(b)	33.3
GG	15
GH	4.5
NA	22.5
NB	11.14
NC	31
ND	22.5
NE	21.4
NF	1.5
NJ	7
LB	39.25
LC	11.5

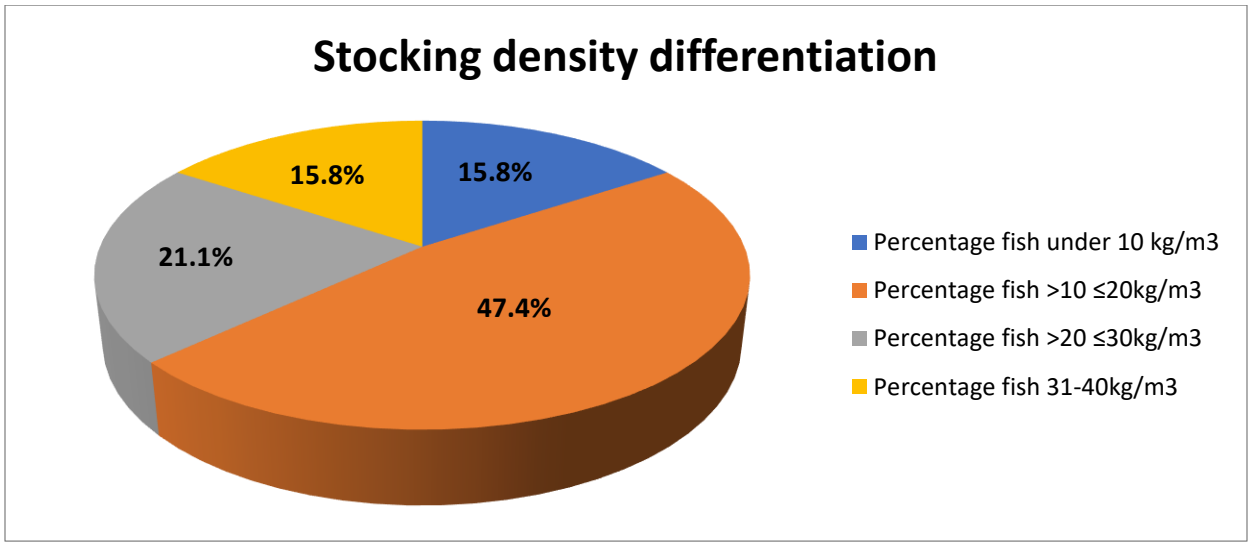


Fig. 4-43 Percentage distribution of fish through different stocking density ranges

Table 4-34 Overview of water DO, CO_2, NH_3 and NO_2^- readings through the highest stocked systems

Farms with high stocking density	Stocking density (kg/m^3)	DO (mg/L)	CO_2 (mg/L)	NH_3 (mg/L)	NO_2^-(mg/L)
GFb	33.3	0.96	116.8	0.0192	0.53
NC	31	2.68	150	0.37	0
LB	39.25	4.55	60	0.01	0
GB	21	4.98	114.4	0.036	0.4
NA	22.5	8.28	104.4	0.011	0.26
ND	22.5	1.1	101.4	0.06	1.32
NE	21.4	0.97	100	0	0.99

4.3.6 Nutrition

All fish populations assessed were fed on a commercial floating fish pellet from the same manufacturer: AVI Aqua-Plus ™, with exceptions of farm NB (commercial dog food), and NC (combination commercial dog food and fish pellets).

47.1% of the fish population groups fed only fish feed, were on a “grower” tilapia pellet, and 52.9% on a “finisher” tilapia pellet. The finisher pellet had a marginally lower protein content and a higher fat content.

All farms, bar one (Farms NA and NE), fed fish manually at varying intervals through the day, ranging from once every few days to multiple feeds per day. Farm NE was employing automatic feeders.

Feed was stored under cover on all farms, and according to farmers, protected from heat and moisture, and used within expiry dates.

There was no record from any farmer of nutrition-related poor health of fish.

Duckweed was used as a supplemental feed in many systems with O*. mossambicus and T. rendalli.* Comfrey was fed as a food supplement in farm ND(b).

4.3.7 Vector involvement

Systems were assessed in terms of two important vectors of parasitic disease i.e. freshwater snails and birds (Noga, 2010.c), as well as water source as a potential source of introduction. Correlation was drawn with prevalence of suspected parasitic cysts in histological sections.

Of the 12 systems, where presence of tissue cysts occurred, 42% had evidence of snails in the system, and highest numbers of affected tissue sections were seen in those systems where both presence of snails and high accessibility to birds existed. High accessibility and use of river water, as factor alone or combined, showed very poor correlation to presence of cysts.

Table 4-35 Comparison of presence of cysts/ granulomas in histological sections and vector risk-factors

Farm	Snails present	Open access to birds	Use of river water	No of tissue sections with cysts/granulomas
GA	Yes	Low	No	0
GB	No	Low	No	0
GC	No	Low	No	4
GD(a)	No	High	No	0
GD(b)	No	High	No	0
GE	Yes	High	No	13, plus live nematode
GF(a)	Yes	Mod	No	4, plus gastric lumen parasite
GF(b)	Yes	Low	No	3
GG	No	Low	No	0
GH	No	Low	No	7
NA	No	High	Yes	0
NB	No	High	Yes	0
NC	No	Mod	No	6
ND	No	Low	No	3
NE	No	Low	No	3
NF	No	Low	No	3
NJ	No	High	No	1
LB	Yes	High	No	10
LC	Yes	Low	No	4, plus intestinal migratory perforations

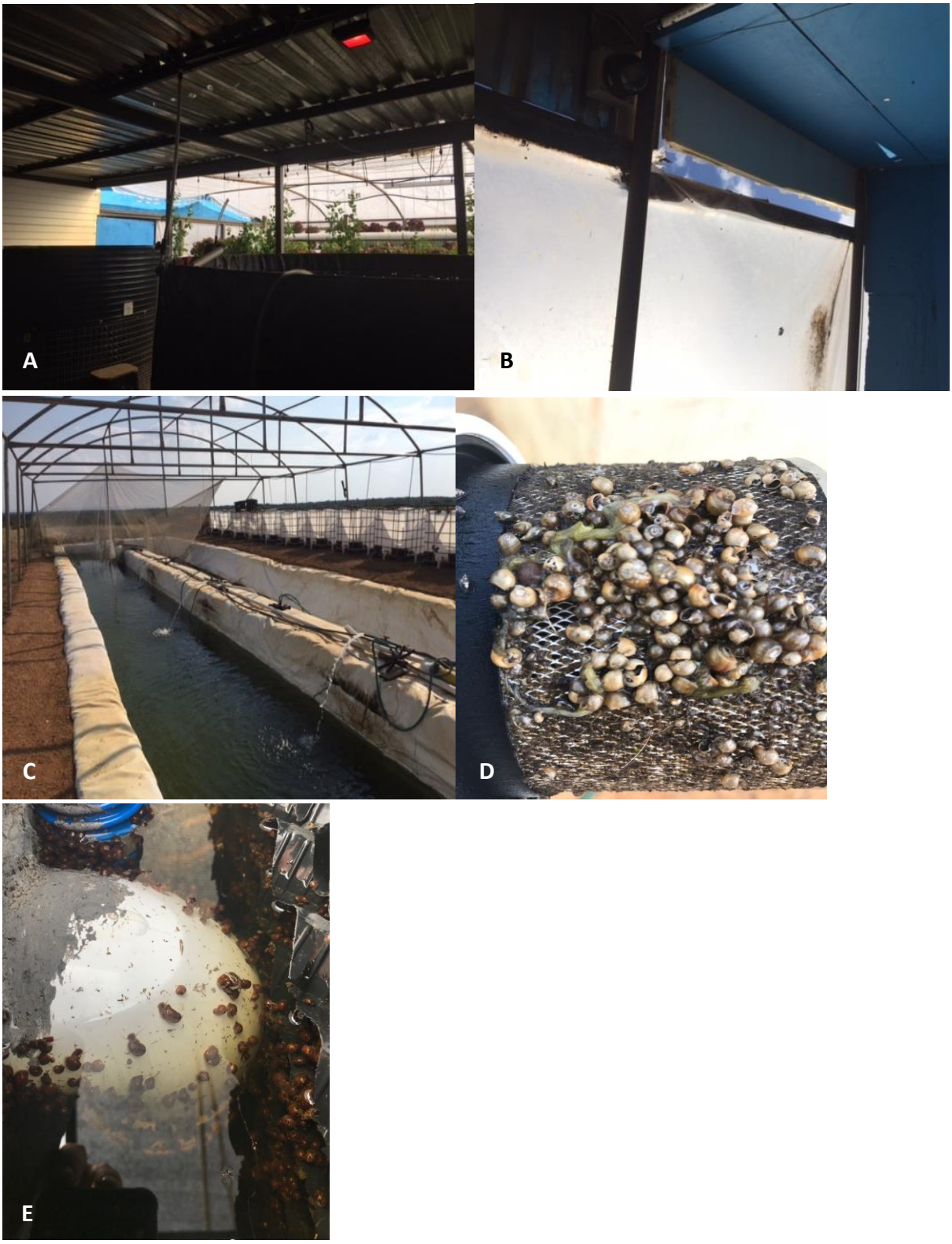


*Fig. 4-44 Vectors: **A, B and C**: Open access to bird vectors through broken roofing/ torn or absent tunnelling;*

***D and E**: Massive infestation and biofouling of freshwater snails in systems*

4.3.8 Fish movement

Widespread unregulated movement of fish between farms was a common finding, with indiscriminate movement interprovincially. International sources of fish included the United Kingdom and Thailand. 77% of surveyed farms in South Africa sourced brood-stock or fingerlings originally from farm GB and another farm in Northwest Province (not included in the survey). These farms originally sourced their brood-stock from the UK, as well as fingerlings from Mozambique. Farm GB, in turn, outsourced to farms GA, GDa, GDb, NJ and LC. Farms GG, NA, NB, ND, NJ and LB received stock from the Northwest source, and another unknown local Gauteng source supplied to farms GH, NC and NE. Thus, distribution has been widespread and uncontrolled.

4.4 HEALTH MANAGEMENT : THE FARMER'S PERSPECTIVE

Farmers were asked to identify their key health challenges with fish and systems. Chief concerns were:

a. Maintaining adequate DO levels
b. High CO_2 levels
c. Chronic low-grade mortality rates (from fry to adult stage- but largely fingerling mortalities)
d. Poor rates of fish growth
e. Risk with poor biosecurity, particularly vector-related (snails)
f. Infections from *Streptococcus* spp., Tilapia Lake Virus, fungal infections, gill parasites, "brown-blood disease"/ Methemoglobinemia (High Nitrite)
g. Maintaining water temperatures at adequate levels
h. Poor quality feed
i. Lack of knowledge or information on fish health

Preventative or treatment protocols were rare, other than occasional use of non-iodized salt (NaCL) or potassium permanganate ($KMNO^4$), which were used empirically without veterinary diagnostics or treatment protocol. No farm had any history of regular use of antimicrobials.

On all farms assessed, the reasons for on-going chronic fish mortalities had not been investigated and were therefore a concern for farmers. Farmers were unsure of potential diseases, improper husbandry practices, or water or feed-related factors that could pose as a threat to the health and viability of their stock.

CHAPTER 5 - DISCUSSION

The health of a large representative number of South Africa's farmed tilapia (*O. niloticus, O. mossambicus,* and hybrids) and their associated farms, was evaluated through the assessment of key independent variables (physical, chemical, biological or management stressors) with potential to impact fish and population health, and their potential impact upon key dependant variables that were likely to reflect sub-optimal health.

Taking into account the many confounding variables that exist in a dynamic aquatic system, and the fact that many can only be assessed subjectively, yet, the canonical correlation, which is the maximum linear correlation between the two sets of independent (Stocking density, DO, CO_2 , Temperature, CH:TA ratio, pH, NH_3-UIA, Nitrite) and key dependant variables (Average *Trichodina*, Average Gyrodactylidea , Average Dactylogyridae, Average *Ambiphrya,* average *Ichthyobodo*, Total parasites, Lamellar epithelial hyperplasia, Lamellar goblet cell hyperplasia, EG cell infiltration at base of the gill arches, Liver lipid levels, Liver lipofuscin levels, Liver nuclear activity, and Gastritis levels), was 81.1%. Although one must take into account that not all variability in the dependant data set can be explained by variability in the independent data set, the linear correlation still illustrates that the independent set of variables, as a whole, is significantly able to explain the variability of the dependant variables, as a whole.

5.1 KEY STRESSORS

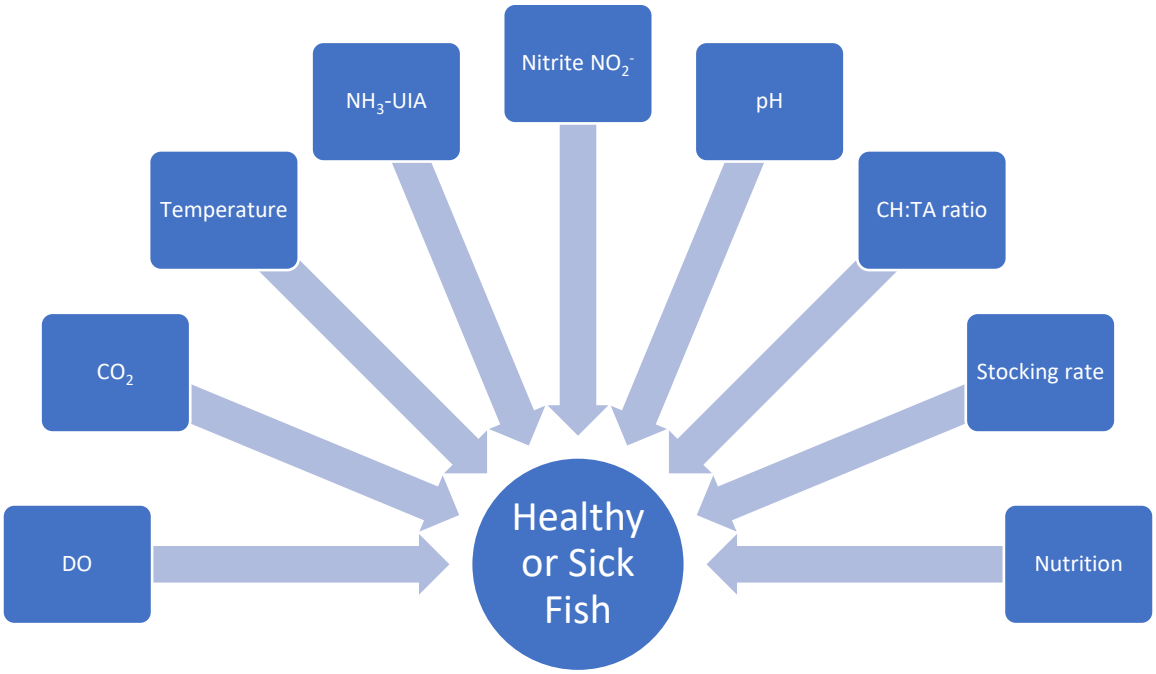


Fig. 5-1 Schematic representation of key independent variables impacting the health of a fish

5.1.1 DO

Environmental hypoxia was the fourth most significant water quality problem seen through all the farms assessed, with only 36.8% of farm DO readings measuring within preferred ranges, with the rest in sub-optimal ranges below 5mg/L (Boyd 2000.a; Loh and Landos, 2011.e; Timmons and Ebeling, 2013.c) *(Table 4-1)*. This is a significant finding, considering that DO is regarded as the most critical water parameter determining productivity and health of intensive aquaculture systems (Boyd, 2000.c; Timmons and Ebeling, 2013.b). The effect of this upon the fish populations is one of chronic stress (Noga ,2010.d). This results in a cascade of secondary

physiological effects and reduced defence against infection (Francis-Floyd *et al.*, 1992), manifesting as regressive levels of growth, opportunistic infections and mortalities as DO levels approach the 1.5mg/L mark (Noga, 2010.d). Swann's, 1997, observations of the tilapia species' high tolerance to low DO levels was corroborated in this study. Even in those fish populations where DO fell between 1.5-3mg/L, minimal mortalities were observed, and evidence of secondary disease was rare . Only one farm, ND (one of the lowest DO levels of 1.1mg/L), showed multiple signs of secondary septicaemia, with exophthalmos, retrobulbar cellulitis, splenic enlargement and high evidence of lymphoid infiltrates**.**

Low DO not only has a direct effect on aerobic fish respiration (Boyd, 2000.b) and other processes, but also affects the biofilter health, with aerobic bacteria within the biofilter unable to function competently in their oxidative capacity, with resultant accumulation of nitrogenous compounds like NH_3-UIA and nitrite in the water body (Boyd, 2000.b). Increased anaerobic decomposition processes further elevate NH_3-UIA , nitrite, H_2S and other compounds (Boyd, 2000.a). In this study, all systems with DO readings below 1.5mg/L, had corresponding high nitrite (NO_2^-) levels above 0.5mg/L, which would be expected to predispose to high production stress and presence of secondary opportunistic infection (Aquatic Network, 2012; Huchzermeyer, 2015). In 83.3% of systems with DO readings between 1.5-3mg/L, corresponding high nitrite > 0.3mg/L were measured. NH_3-UIA did not correlate as similarly. This poses the question: are the ammonia –oxidizing bacteria (*Nitrosomonas - , Nitrosococcus -.,* and other spp.) within system biofilters more tolerant of low DO levels or require less oxygen than the nitrite-oxidizing bacteria (*Nitrobacter - , Nitrococcus* - and *Nitrospira* spp.)?

Although oxygen solubility decreases with increasing water temperatures, no clear association was seen through systems tested. Of the three farms with lowest DO readings, water temperature varied between 21.2 ^{o}C and 31.9 ^{o}C. This finding can be explained by high but variable stocking rates in all three systems , ranging between 21.4 kg/m^3 and 33 kg/m^3. Ineffective aeration practises were an additional factor contributing to low DO readings.

Of the seven green-water systems, only two (28.6%) had evening DO readings > 5mg/L, another two measured between 3-5mg/L, but the majority (43%) measured between 1.5-3mg/L. Three of the farms with DO < 3mg/L were green water systems with visible phytoplankton levels. It is important to note that water readings were taken in the late afternoon, when photosynthetic processes have been actively adding to the DO water levels through the day, and oxygen levels are expected to be at their highest (Noga, 2010. d). Following the typical diurnal variation in DO concentration, the reverse process of respiration overnight, would drop these levels significantly (Romaire *et al.*, 1978), posing dramatic levels of stress upon the fish in the early mornings. Interestingly, none of these systems had history of such events occurring, again speaking to tilapia's high tolerance levels to low DO. Piping behaviour seen on farms, is a common behavioural change associated with low DO levels. Additional aeration techniques to support fish and avoid a mortality event would be necessary on these farms.

Regular handling was not a common practice on any farms and thus not a contributing factor to low DO levels. Interestingly, all farms running regular water changes (GC, NA, NB, NF, and LB measured with good DO levels >3mg/L.

The common use of borehole water is another potential contributing factor to the low DO levels.

Despite tilapia's apparent resilience to disease and mortality, low DO seems to exert its greatest impact on the fish through its effect on growth. Of seven systems where DO readings fell below 3mg/L, six fish populations exhibited extremely slow growth below target *(Table 4-8)*. Only one system showed good growth. Hypoxic conditions in systems, for whatever reason, would impact fish growth through slowing of their basal metabolic rates and reducing feeding behaviour (Huchzermeyer, 2015), slowing functional digestive process and effective nutrient absorption (Wedemeyer *et al.,* 1976.b), as well as contributing dramatically to the deteriorating well-being of the fish through increased general stress response, acidosis from suppressed CO_2 release (Fievet *et al,* 1988; Wedemeyer *et al.,* 1976.b), increased osmoregulatory load (Wedemeyer *et al.,* 1976.b) and increased potentiated toxic impact of NH_3. A toxic compound of potential significance in this study would be hydrogen sulphide (H_2S), seen on farm NC. H_2S has impact upon fish by inhibiting oxidative respiratory processes, increasing blood lactate and predisposing to a physiological hypoxic state, which is further exacerbated in low DO situations (Boyd, 2000.g). This was a serious finding due to its high toxicity to aquatic spp., and warrants immediate intervention with increased aeration and long term improved filtration.

Although growth rate is multifactorial , low DO is undoubtedly playing an important role in many systems and is a factor that farmers will need to address to optimize production.

Farms were summarized as:

Farms with sub-optimal DO (mg/L and % saturation) readings:

GA, GD(a),GD(b),GE,GF(a), GF(b),GG, NC, ND, NE, NJ, LB

Farms with adequate DO (mg/L and % saturation) levels:

GB, GC, GH, NA, NB, NF, LC

Analysing these further according into specific DO ranges according to *Table 3-1*(Aquatic Network, 2012; Boyd, 2000,b; Boyd, 2004; Huchzermeyer, 2015; Noga, 2010.d; Timmons and Ebeling, 2013.c), the farms were graded into the following risk categories:

RISK ASSESSMENT OF FARMS WITH RESPECT TO OXYGEN LEVELS:

NO RISK: Farms GC,GH,NA,NB,NF,LC	(DO above 5mg/L): Optimum
LOW RISK: Farms GB, GD(a), GF(a), LB	(DO: >3-5mg/L): Slight impact on health/ growth
MOD RISK: Farms GA,GD(b), GE, GG, NC, NJ	(DO: >1.5-3mg/L): Moderate impact on health/growth
HIGH RISK: Farms GF(b), ND, NE	(DO: ≤1.5mg/L): Significant impact on health

Remedial actions to address low DO levels include the following:

Additional aeration or oxygenation; temporary reduction of feeding; reduction of stocking densities to match aeration capacity; maintaining water temperatures within ideal ranges; and

addition of NaCl to the water, which has been shown to improve both fish-tolerance to upper and lower level temperature ranges (Wedemeyer *et al.,* 1976.e).

Other risk factors include: using borehole water with low DO levels at source, and high phytoplankton levels resulting in serious DO and CO_2 fluctuations.

5.1.2 CO_2

CO_2 was the most consistently abnormal water parameter assessed through the farms.

It is a water parameter that is not commonly assessed, especially on-farm, and most farmers were unaware of their water CO_2 levels, the significance thereof, and how to test.

Using 20mg/L as an ideal upper limit (Southgate, 2005; Timmons and Ebeling, 2013.c), all the farms showed levels exceeding this threshold *(Table 4-4).* However, the literature differs regarding the upper ideal limit with 72.6mg/L also reported (Fish, 1956). If this threshold is used, 52.6% of farms still exceeded the limit. In addition, considering that most measurements were taken towards the end of the day, when CO_2 measurements should be at their lowest due to the photosynthesis effect (Boyd, 2000.f), the values obtained were likely to be the lowest for that system during a 24h period, with the mean, in all probability being much higher.

The key factors likely to be implicated in the consistently high CO_2 are multifactorial and vary between systems, but certain factors dominated and showed cumulative effect. These were the use of borehole source water (Timmons and Ebeling, 2013.c), presence of green water with high photosynthetic contribution to diurnal CO_2 levels, and overfeeding of fish and high

stocking densities contributing to an organic overload of the system (Southgate, 2005). Of the 11 farms with CO_2 readings over 60mg/L, 10 (91%) were using borehole water, and one municipal. Obviously, water CO_2 readings would need to be measured at the source before entering systems, to weigh the real impact upon each system, which was not performed for this study. Six of the 11 farms (55%) had obvious green water systems; 3 (27%) were overfeeding the fish: 2 (18%) were intensively stocked at > 30kg/m^3 and a further 3 (27%) highly stocked between 20-30kg/$m^{3.}$ Of these 11 systems, 36% had three of these risk factors playing a role, 46% two factors, and 18% one factor. Yet a high concentration of CO_2 does not seem to preclude adequate growth, as seen in farm GB. Again, demonstrating the multifactorial aetiology on fish health and growth.

From this study it would appear that borehole water is one of the highest risks associated with elevated CO_2 supported by the fact that it was the only common predisposing factor in the two systems with highest readings of 150mg/L. The normal pH range seen through most systems indicates that carbonate dissociation under acidic conditions in hard water was not an important source of high CO_2 measurements (Wedemeyer *et al.,* 1976.d.).

Farms were also assessed to see if there was any apparent link between use of water holding tanks and high CO_2 levels. Of the 8 farms with no holding tanks and water being pumped directly from source to system, 7 made use of borehole-sourced water, with potentially high CO_2 levels. From these, 5 (71.4%), had high CO_2 readings of over 60mg/L. This indicates the potential importance of using holding tanks to lower CO_2 when borehole water is used. Use of

regular water changes as a management tool did not appear to improve CO_2 readings, with all farms regularly changing water, still measuring CO_2 >40mg/L.

Chronic elevated levels act as yet another chronic stressor upon the fish, with additional specific subclinical physiological effects like hypercapnia, metabolic acidosis , and secondary physiological hypoxia due to reduced oxygen uptake, through the Bohr or Root effect, or directly (Heath, 1995.c; Noga, 2010.g). These physiological changes would predispose to the piping behaviour often seen. Of the three farms that presented with the greatest cumulative evidence of underlying secondary disease (in terms of macroscopic lesions, massively enlarged spleens, positive bacterial cultures, and splenic lymphoid foci (Farms GFa, GG, and ND: all had underlying high CO_2. No typical clinical signs of narcosis were seen in any fish, with loss of equilibrium and poor response to gross stimuli. This is to be expected, as a dose of at least 200-1500 mg/L is generally needed to induce anaesthesia (Ackerman *et al.,* 2005*).*

Curiously**,** of the 11 farms with extremely high measured CO_2 levels (> 60mg/L), 9 of these (82%) had evidence of posterior renal interstitial mineralization, supporting a link that has been demonstrated in salmonids (Southgate, 2005). The two remaining farms that showed mineralization, had CO_2 levels around 50mg/L. None of the farms with levels below 35mg/L showed any mineralization in the fish. With low prevalence in affected fish, impact is unknown but probably minimal. No evidence of calcified granulomas in the stomach walls, as described by Harrison and Richards, 1979, as another sequalae to high CO2, were seen.

Farms were graded into the following risk categories with reference to *Table 3-3:*

RISK ASSESSMENT OF FARMS WITH RESPECT TO CO_2 LEVEL:

NO RISK:	NONE	<20mg/L
LOW RISK:	Farms GD(a), LC	20-40mg/L
MOD RISK:	Farms GE, GF(a), GG, NB, NJ, LB	>40 ≤ 60mg/L
HIGH RISK:	Farms GA, GB, GC, GD(b), GF(b), GH, NA, NC, ND, NE, NF	>60mg/L

Looking at the combined factors of low DO with high CO_2, highlights farms: GF(b), ND, and NE at highest risk.

Table 5-1 Combination Risk Effect of high CO_2 (level 3) and low DO (Bohr or Root effect)

Farm with Level 3 CO_2 Risk Level	Farms with Level 3 DO risk	Farms with Level 2 DO risk	Farms with Level 1 DO risk
GA			
GB			
GC			
GF(b)			
GH			
NA			
NC			
ND			
NE			
NF			

Mitigating practises to reduce CO_2 levels through various gas-exchange techniques were suggested, like open packed columns with gravity-fed water, with the optional use of blowers to assist in stripping water of CO_2 (Timmons and Ebeling, 2013.g). Farmers were cautioned against simply increasing aeration or oxygenation, which serves to exacerbate the situation because of slowing rate of ventilation and reducing offloading of CO_2 from the fish (Southgate, 2005).

5.1.3 Temperature

Farm water temperatures were fairly evenly distributed between acceptable and risky ranges, with around 50% over and 50% up to and below 25^{o}C *(Table 4-3).*

Temperature variation was largely seasonally regulated. As the study progressed into winter months, there was a marked trend for water temperatures to cool, despite passive supplemental heating through use of greenhouse tunnels in most systems. A common reason for this was economic feasibility of using additional active heating methods. It was evident that tunnels alone, could not passively maintain water temperatures within desirable ranges through the colder months of the year**.** All farms with water temperatures under 25^{o}C, were visited in the Autumn and Winter months of the year, while those with high water temperatures over 30^{o}C, were assessed in the mid-Summer months. Ironically, as tunnels were used to increase temperatures during winter months, they had the opposite effect of dramatically increasing ambient and water temperatures through the hotter summer months, and some farmers indicated difficulties to regulate water temperatures below 30^{o}C , with

water temperatures sometimes reaching the 40^{o}C range. This undoubtedly, would play a role as a significant stressor, further exacerbate existing low DO levels, and predispose to pathogen proliferation. Water depths were generally in the range of 1-1.5 meters, offering very little depth buffering against water temperature fluctuations Most tunnel systems also offered no ventilation control.

There was no distinct parallel between systems with high temperatures and low DO levels, probably because of the interplay of so many other variables like stocking rate, phytoplankton contributions to diurnal DO levels and differing levels of supplemental aeration.

The lower temperatures in all the Northwest and Limpopo systems, were low enough, to have slowed nitrification processes and impact NH_3 and NO_2^- levels within these systems. Again, correlations can't be seen comparing these parameters alone, due to the multiple other factors impacting the nitrification process, with DO probably being the most limiting factor (Boyd, 2000.c).

Slower fish metabolic rates in cooler water are also known to suppress immune function and may have played a contributory role in negative impact upon health (Huchzermeyer, 1993).

There was no evidence of secondary Oomycete infections like *Saprolegnia* spp., which are important pathogens in systems with low water temperatures (Noga, 2010.e) and common in African aquaculture systems (K. Veverica, personal communication, 14th March, 2016). No distinct correlations could be drawn between farms with temperatures in the extreme ranges and high evidence of secondary disease. None of the farms with high temperatures, showed high NH_3 levels, so the danger of potentiated toxicity of this compound would have been

negligible. With heavy metals not having been evaluated in this study, the potential impact and increased toxicity associated with their increased uptake and solubility with warmer water temperatures (Munro, 1978; Olsson, 1998), would be another unknown variable and possible important parameter to assess.

Similar to El-Sayed and Kawanna, 2008 and Roberts, 2012.a's findings, effect upon growth was probably the most important effect seen in systems. It was interesting to note that very poor growth levels were seen in two of the three systems with high temperatures (GA and GFb). Of the three systems with overall good levels of fish growth (GB, GC, GDb), two had water temperatures in the ideal range, and one was high. The greatest impact upon growth rates appears to occur with temperatures in the lower ranges. All farms (except NJ) with water temperatures below 25^{o}C showed consistently poor growth rates. With temperature regarded as the most important environmental stressor affecting fish (Roberts and Ellis, 2012), these findings are concerning. Again, interpretation needs to be done with respect to the dynamic fluctuation of water temperatures in some systems and taking into account that readings in this study were taken at a single point in time only.

Farms were graded into the following risk categories with reference to *Table 3-2:*

RISK ASSESSMENT OF FARMS WITH RESPECT TO TEMPERATURE:

NO RISK:	Farms GB, GD(a) GD(b), GE, GF(a), GG, GH	25-30^{o}C
LOW RISK:	Farms GA, GC, GF(b)	>30^{o}C
MOD RISK:	Farms NA, NB, NC, ND, NE, NF, NJ	≥20^{o}C <25^{o}C
HIGH RISK:	Farms LB, LC	<20^{o}C

The study emphasized the importance of controlling water temperature year-round to maintain health and optimize growth. It was clear that there is great need to research alternative affordable heating methods for closed system aquaculture for the South African winter conditions.

5.1.4 NH_3-UIA

Using the recommended upper TAN limit of 1mg/L (Timmons and Ebeling, 2013.c) and upper NH_3-UIA limit of 0.05mg/L (Noga, 2010.d), the health effects of high NH_3-UIA were assessed as per *Table 3-4.*

The un-ionized form of ammonia was not as overwhelming a factor within systems assessed, yet 36.9% measured high enough levels to impact health, growth and production. Taking into account the effect of water temperature and pH through the Emerson's conversion table (Emerson *et al.*, 1975), NH_3-UIA levels in most systems (63.1%), were below 0.05mg/L *(Table 4-5).*

52.6% of farms assessed measured optimal NH_3-UIA levels below the 0.025mg/L level. A further 10.5% fell just under 0.05mg/L which was suboptimal, but within an acceptable range for intensive production. However, a large percentage, 31.6%, fell within the 0.05-2mg/L range, which is believed to have an effect on growth and health (Noga, 2010.d). There was no pattern of direct correlation with stocking rate, other than the one system (Farm NC) where highest stocking rate of 31kg/m^3, correlated with highest NH_3-UIA level of 0.37mg/L. Other factors like inadequate biofiltration would very likely be playing an additional role here.

There were varying correlations seen between lower DO readings and higher NH_3-UIA, but in most systems with NH_3-UIA between 0.05mg/L and 1mg/L, DO levels were very low (<3mg/L). In these instances, DO was undoubtedly playing a role through effect upon bacterial oxidative processes in the biofilters (Speare, 2008), as well as reduced feeding activity and nutrient absorption by the fish, with resultant organic load build-up. Lower temperatures commonly encountered through systems, would also be negatively impacting biofilter nitrification processes (Speare, 2008), yet also predisposing to ionization of the ammonia molecule.

High organic load from overfeeding fish was probably a minor contributing factor. Of the three farms where fish were overfed *(Table 5-4)*, two had marginally raised NH_3 levels within the 0.05-0.2mg/L range. High NH_3 did not seem to be a significant factor in affecting growth of fish. Of the three systems displaying healthy levels of growth, two had raised NH_3, albeit in the 0.05-0.2mg/L range. However, when the combined effect of elevated NH_3 in the presence of low DO (alkalosis in the fish creates a demand for more oxygen) is considered, poor fish growth was recorded in all systems, bar farm GDb, who managed to attain good levels of growth in the face of low DO and high NH_3 *(Table 4-8)*.

No correlations could be drawn between elevated NH_3 alone and high prevalence of secondary disease, other than farm ND.

There were three key factors in most systems assessed, that were also probably playing a role in keeping NH_3 levels lower: low feeding rates, thus lower organic load; higher CO_2 levels and the lower water temperatures, both facilitating the conversion of ammonia to ionized NH_4^+. There was no seasonal variation in NH_3-UIA levels.

Farms were graded into the following risk categories with reference to *Table 3-4*:

RISK ASSESSMENT OF FARMS WITH RESPECT TO NH_3-UIA:	
NO RISK: Farms GA, GB, GD(a), GF(a), GF(b), GG, NA, NB, NE, NF, LB, LC	<0.05mg/L
LOW RISK: Farms GC, GD(b), GE, GH, ND, NJ	0.05 ≤ 0.2mg/L
MOD RISK: Farm NC	>0.2 ≤ 1mg/L
HIGH RISK: NONE	>1mg/L

To avoid the risks associated with elevated NH_3, short term mitigation like water changes or reducing feeding rates is suggested, and long-term mitigation like addressing stocking rates and increasing aeration to support the biofilter bacteria, as well as improving biofiltration to match the fish biomass, is advocated. Those farms already changing water regularly (GC, NA, NB, NF, LB) all measured NH_3< 0.2mg/L.

5.1.5 Nitrite (NO_2^-)

With reference to *Table 3-5* potential impact of NO_2^- on fish health, elevated nitrite levels were a serious water quality abnormality encountered through systems with 63.2% of systems measuring levels in excess of 0.3mg/L, potentially adversely impacting on health and production (*Fig. 4-6).*

A correlation was observed between systems with high nitrite and those with low DO, with 87.5% of systems with extremely high nitrite levels over 0.5mg/L correlating with DO readings < 3mg/L. Both processes of conversion of ammonia to nitrite, and nitrite to nitrate are oxygen and temperature dependant within the biofilm, requiring a minimum system oxygen level of

2mg/L and an optimal temperature range between 28-36 ^{o}C (Loh and Landos, 2011.d). High nitrite prevalence indicates ineffective biofilter nitrification conversion of nitrite to nitrate by the *Nitrobacter*, *Nitrococcus* and *Nitrospira* groups, with resultant accumulation of toxic nitrite levels. Bacteria are either functioning sub-optimally in the oxygen-deficient water or suffering from die-off (Huchzermeyer, 2015). Considering that high NH_3-UIA was not as much of a problem, it may be that these species of bacteria are less tolerant to lower DO levels or lower temperatures in the water body than the *Nitrosomonas* and *Nitrosococcus* species, responsible for conversion of ammonia to nitrite. These organisms are also known to adhere less strongly to the biofilter media (Loh and Landos, 2011.e), so high water flow rates through the biofilter could also possibly be a contributing factor to an impaired second stage nitrification process. Correlation with lower water temperature and its potential impact on biofilter nitrification, was not encountered and farms with pH levels outside the optimal 7-8 range was also not a strong predictor for high nitrite concentrations. It would thus appear that DO is the stronger limiting factor on bacterial nitrification process.

The impact of low alkalinity, as an energy supply for the nitrifying bacteria (Loh and Landos, 2011.e), was also an interesting observation. Not all systems with high nitrite correlated with a low alkalinity, yet all three systems with total alkalinity readings below 50mg/L reflected high nitrite readings over 0.3mg/L. This could reflect a more targeted impact of low alkalinity again upon the *Nitrobacter* group, rather than the *Nitrosomonas* bacteria, and also illustrates the possible underlying multifactorial aetiology to the elevated nitrite levels.

Seasonal variation appeared to play a role, with a greater percentage of farms assessed through the warmer months, presenting with high nitrite levels. This is probably associated with increased fish metabolic activity and higher feeding rates in these months with resultant increased organic load, as well as poor biofilter capacity.

Assessing effect upon fish, no macroscopic evidence of methemoglobinemia, visible as dark brown gill discolouration, could be identified, even with extremely high nitrite levels. No obvious lethargy was seen, with fish lying on the bottom, and farmers had not noticed any anorexic behaviour- although this would be practically really hard to assess. Links with secondary disease were scant: of the eight systems with high nitrite >0.5mg/L, only two had evidence of secondary septicaemic disease (GG and ND). Bearing in mind the variation in susceptibility and tolerance levels that exist between species and population groups, this poses the question on the value of visual assessment unless nitrite are significantly high >1mg/L.

Farms were graded into the following risk categories with reference to *Table 3-5:*

RISK ASSESSMENT OF FARMS WITH RESPECT TO NITRITE (NO_2^-):

NO RISK:	Farms NB, NC, LB	≤0.1mg/L
LOW RISK:	Farms NA, NF, GC, LC	>0.1 ≤ 0.3mg/L
MOD RISK:	Farms GB, GD(a), GF(a), NJ	>0.3 ≤ 0.5mg/L
HIGH RISK:	Farms GA, GD(b), GE, GF(b), GG, GH, ND, NE.	>0.5mg/L

Table 5-2 Compounding Risk Effect of high nitrite with low DO, low temperature, or low pH, with farms with two or more risk factors highlighted red, those with 1 risk factor highlighted orange

Farm with High NO_2^-	Low DO	Low Temperature	Low pH
GA	Yes	No	No
GD(b)	Yes	No	No
GE	Yes	No	No
GF(b)	Yes	No	No
GG	Yes	No	Yes
GH	No	No	No
ND	Yes	Yes	No
NE	Yes	Yes	No

Once again, effect upon growth of fish seems to be much more apparent. Of the eight high nitrite systems, six exhibited very poor growth.

It is interesting that Farms GG, and NE were of the group of farms with poorest growth levels in fish and this poses a question of potential underlying suppressive impact of high nitrite levels on fish growth. With macro and microscopic evidence of secondary bacterial disease on both farms GG and ND, further research into the potential immunosuppressive effect of high nitrite, would be of interest.

The possibility exists that fish in many systems with high nitrite may have adapted to some degree resulting from recurrent or chronic exposure (Shelton, 2010).

To reduce the risk of nitrite toxicity, NaCl can be added to the system. The NaCl molecule actively competes with nitrite transport across the gill lamellae cells, thereby reducing systemic

absorption of nitrite (Boyd, 2000.c; Loh and Landos, 2011.i; Noga, 2010.d). Other palliative measures like increased water changes and supportive aeration will also help to decrease nitrite concentration in aquaculture systems. Once again, regular water changes appeared to be a useful management tool in reducing $NO2^-$ levels, with all farms using this protocol, measuring NO_2^- ≤0.3mg/L.

5.1.6 Nitrate

Because of lack of conclusive results due to use of a nitrate-test kit with limited parameter range (upper range of 4.4mg/L) and general higher tolerance levels to nitrate (Broders *et al.*, 2005; Shelton, 2010), this was not included as a variable of significance in impacting fish health. Nitrate is believed to have almost no toxicity (Atwood *et al.,* 2001; Heath, 1995.b). No clinical signs of tan gills, reflective of a methemoglobinemia, and possible high nitrate levels, were seen. One would expect to see an association between green water systems and high nitrate, yet farms with top range (>4.4mg/L) NO_3^- readings showed poor correlation with higher phytoplankton levels. Only two farms (GDb and NC) of the nine with visibly green water measured at upper range. Interestingly, all three systems showing evidence of secondary septicaemic disease, measured at the upper limit for nitrate. For future NO_3^- evaluation, a test kit with upper range of 100mg/L would be advisable.

5.1.7 pH

Recirculating systems have a tendency to become acidic with time (Huchzermeyer, 2015), because of consumption of HCO_3^- and CO_3^- by the biofilter (Loh and Landos, 2011.d). In this study however, pH measurements did not demonstrate a general tendency to acidification with most systems falling within a normal range of 6.5-9 (Boyd and Tucker, 1998) *(Table 4-6)*. What is of significance, however, is that 84% of systems had alkaline pH readings over 7, which facilitate the conversion of NH_4^+ to its toxic un-ionized form with potential impact upon fish (Roberts and Ellis, 2012). However, the general low NH_3 prevalence, would indicate that low temperatures and higher CO_2 levels are probably playing a role in countering this conversion. This would be a factor needing close monitoring, in systems where water is adequately heated, and CO_2 levels corrected.

Considering the diurnal fluctuation of pH because of photosynthetic processes (Noga, 2010.d), it is important to acknowledge that the one-off pH readings in this study would have been at the highest level, thus potentially masking more acidic conditions that may have existed earlier in the day. This would have been of greatest significance in those systems with hard water but low alkalinity (GFa and GFb), and thus reduced buffering capacity for the diurnal / nocturnal pH swings. The high CO_2 levels consistently encountered would be expected to acidify the water through conversion to carbonic acid, and again, considering that overnight respiration would elevate the already high CO_2 levels, one may find that morning pH is considerably lower.

Potentiating pathological effects of pH changes upon toxicity of H_2S and metals like copper, cadmium, zinc or aluminium, could not be demonstrated with routine histological examination.

However, toxicity-specific evaluation was not performed in this study and requires further investigation.

Taking into account the compromised buffering in many systems because of abnormal hardness or alkalinity measurements, morning pH readings would offer more information on daily fluctuation ranges which could be compromising fish health and posing an additional stress upon fish.

Farms were graded into the following risk categories:

RISK ASSESSMENT OF FARMS WITH RESPECT TO pH *(See 2.3.3.5)*

NO RISK: None	(pH:7)
LOW RISK: Farms GA, GB, GC, GDa, GDb, GE, GFb, GG, GH, NA, NB, NC, ND, NE, NF, NJ, LB, LC	(pH>6 <9, excl. 7)
MOD RISK: Farms GFa	(pH: 5-6 or 9-10)
HIGH RISK: Farms None	(pH<5 or >10)

5.1.8 CH:TA ratio

Normal ranges for both complete hardness and total alkalinity were reported as 50-150mg/L (Boyd, 2000.e; Loh and Landos, 2011.e; Shelton, 2010).

Using De Holanda Cavalcante *et al.*, 2014, findings as a reference, acceptable CH : TA ratio ranges lie between 1: 1 and 5: 1. When the ratio exceeds 5, osmotic stress significantly reduces growth. Reduced buffering capacity with tendency to low afternoon pH readings and a predisposition to increased total-ammonia-nitrogen (TAN) can be expected. When the ratio falls

below 1, reduced buffering capacity with tendency to an afternoon pH spike, mild impact on growth and predisposition to increased TAN can be expected. According to De Holanda Cavalcante *et al.*, 2014, a ratio of 1:1 was ideal, and showed best FCR until trial conditions.

Both high ratio readings (11.1 and 12.4) were measured on the same farm (Farm GF), within different systems, but using the same water source (municipal) *(Table 4-7)*. In each case, they were associated with low alkalinity readings in water that is considered hard to very hard. The low alkalinity can result from geological exposure of the water to non-limestone deposits or highly leached soils (Boyd, 2000.e), or be associated with chronic removal through the biofilter nitrification process (Loh and Landos, 2011.e). It would result in a system with potential poor buffering capacity, a tendency to acidify, and experience massive pH swings, with resultant further stress upon fish (Loh and Landos, 2011.e).pH readings in the late afternoon concurred with De Holanda Cavalcante *et al.*'s findings, with systems with ratios over 5, displaying the lowest pH readings of 6 and 6.8, and those systems with ratios below 0.65, showing elevated pH readings of 8 and 8.5. Both situations would be adding to the stress component on fish populations.

Effect upon growth was not as clear. Those farms showing the best levels of fish growth (GB, GC, GDb) *(Table 4-8)* had ratios ranging between 0.8 and 2.3 and close to the 1:1 target *(Table 4-7)*, however other farms with similar CH :TA readings showed very poor growth levels in fish- again a reflection of the multifactorial causes of poor growth.

Those systems with low ratios below 1 where total alkalinity is greater than complete hardness, reflected waters containing a large amount of sodium and potassium relative to calcium and

magnesium (Boyd, 2000.d). These would be considered lower risk systems, as they are constituted to balance themselves with time (De Holanda Cavalcante *et al.,* 2014), while those systems with ratios over 5:1 would be high risk, as they will continue to increase unless corrective measures are taken.

No clinical evidence of "Hole-in-the-head" disease of Cichlids, thought to be associated with low CH levels, or cataracts, associated with high CH levels, were seen (Loh and Landos, 2011.e) in any systems.

Correlations between renal mineralization and high CH levels (Loh and Landos, 2011.e) were not clear enough to draw conclusions. Of the systems with high CH over 300mg/L, 67% showed evidence of renal mineralization. Three of these four systems had concurrent high CO_2, possibly indicating a synergistic mechanism between CH and CO_2.

High TAN levels in systems could possibly be a repercussion of the abnormal (<1 or >5) CH:TA ratios, with 4 of the 9 systems (GDb, GFa, GFb and NJ) with abnormal ratios showing elevated TAN readings. Ammonia toxicity would be potentiated in these systems, through suppression of the fish's normal ammonia excretion mechanisms (Huchzermeyer, 1993). High nitrite levels above 0.3mg/L were reflected in those systems where alkalinity was below 60mg/L, possibly from impact upon the biofilter nitrification processes.

Going forward, it would be necessary to perform comparative assessment of the diurnal/ nocturnal pH swings in all systems to conclude if those with low buffering capacity reflected a greater degree of pH change.

With respect to CH:TA ratios and low alkalinity, farms were graded into the following risk categories: (with zero risk: 1, Low risk: 0.5-2 (excl. 1), mod risk <0.5 or >2 ≤ 5, high risk: >5)

RISK ASSESSMENT OF FARMS WITH RESPECT TO CH:TA ratio

Zero risk:	None
Low risk:	Farms GB, GDa, GDb, GE, GH, NA, NB, NC, NE, NF, NJ, LB, LC
Mod risk:	Farms GA, GC, GG, ND
High risk:	Farms GFa, GFb

Low alkalinity in systems could benefit from the addition of $CaCO_3$ or $MgCO_3$ to elevate the carbonate level and ameliorate a poor buffering state. Optimal biofilter health would, in addition, increase buffering ability in a RAS.

5.1.9 Fish Stocking Density

Stocking density is thought to be one of the biggest factors impacting growth of fish in an intensive system (El-Sayed, 2006.a), probably because of chronic social and hierarchical stress (Barcellos *et al.*, 1999). Farms assessed were fairly evenly distributed above and below the 20kg/m^3 stocking density level *(Fig. 4-43).*

Numerous factors in a RAS will determine the optimal stocking density for the particular system, primarily water quality, fish species, and fish size (El-Sayed, 2006.a; Timmons and Ebeling, 2013.i).

Feedback from the South African tilapia farming community suggested the following 'rules of thumb':

A simple system with no additional pure oxygen could be safely stocked at 15-20kg/m^3 (D. Fincham- "Rydawi Farms"). TAASA's farming manual, *A guideline to Tilapia Aquaculture in SA,* suggests safe stocking densities of 10-25kg/m^3 (100-250g fish), 12.5-22.5kg/m^3 (250-450g fish) and 8.1-10.8kg/m^3 (450-600g fish).

A grading system was designed, using the above information, and subjectively taking into account the average fish weight of 165g (with variation between 111g and 286g) and general prevalence of low DO in systems (63%):

Ideal (Grade 0 risk):	Stocking Rate 1-10kg/m^3
Acceptable (Grade 1 risk):	Stocking Rate 11-20kg/m^3
Higher risk (Grade 2 risk):	Stocking Rate 21-30kg/m^3
Potentially dangerous (Grade 3 risk):	Stocking Rate 31-40kg/m^3

High stocking rates decrease the adaptive capacity of a system against any stressor.

Using the above, 40% of farms stocked fish at level 2 and 3 risk. Considering the number of potential stressors prevalent amongst farms, with particular reference to low DO levels, the expected impact in such systems would include clustering stress, deteriorating water quality and suboptimal fish growth and health (Abdel-Tawwab *et al.*, 2014). Within the study, all three systems with the highest stocking rates, had DO levels below 5mg/L, one with DO at 0.96mg/L. This combination will profoundly inhibit growth and system yield (El-Sayed, 2006a; Yousif, 2002). Direct correlations with growth were not so clear-cut through the systems surveyed,

with poor growth evident in some low-stocked systems. But, systems with good fish growth, were always associated with stocking densities below 25kg/m^3, and those systems highly stocked at over 30kg/m^3 always produced fish with very poor growth rates, most likely because of concurrent associated low DO levels in these systems (El-Sayed, 2006a; Yousif, 2002).

With the high number of females encountered, as well as large size-discrepancies and evidence of juveniles in some systems, it appears that poor breeding management was a factor playing a role within systems assessed, with breeding occurring in grow-out systems. Poor effectivity or implementation of methyl-testosterone hormone treatment of fry seemed to be a likely component in many cases, highlighting potential underlying poor sex-reversal technique or inaccurate dosage of hormone.

There were no clear correlations in our study between high stocking density and starvation-associated low visceral fat quantity (Abdel-Tawwab *et al.*, 2014) or low liver lipid. This is a reflection of other factors other than merely stocking density at work in determining body condition. It seems the factor most likely to be affecting both visceral and hepatocellular fat is underfeeding, or anorexia secondary to non-specific stressors.

Although fraying of fins was not uncommon, the typical unilateral loss of a pectoral fin tissue and unilateral eye trauma associated with feeding frenzies in overstocked systems, was not seen (Huchzermeyer, 2015). Macroscopic evidence of tail and fin-fraying showed no correlation to high stocking densities and cannot be used as a measure of association, with highest evidence seen in a system stocked at 1.5kg/m^3. Only system ND showed signs of secondary

septicaemic disease under over stocked conditions of 22 kg/m^3, but as previously discussed, a multitude of contributing factors were playing a role in this system.

As expected *(See Table 4-34)*, specific water parameter indicators that were measured were predictive of dangerously high stocking densities. Farms GFb, ND and NE showed greatest correlation, with very low DO, accumulating CO_2 and nitrogenous products.

If one uses Timmons and Ebeling, 2013.d, guide on potential stocking density under optimal water conditions, based on average fish length, it becomes very apparent that the gap between potential and actual farm stocking densities, in assessed systems, is dramatic *(Table 5-3)*, and reflective of deficits in underlying poor filtration capacity, water quality, poor breeding management and husbandry.

Table 5-3 Farm overview of actual and potential stocking densities, with reference to Timmons and Ebeling calculations based on fish length (Timmons and Ebeling, 2013.d)

Farm:	Average fish length (cm)	Potential stocking density (kg/m^3)	Actual stocking density (kg/m^3):
GA	21.2	88.3	18
GB	23.7	98.75	21
GC	19.7	82	20
GD(a)	22.4	93.3	15
GD(b)	20.2	84.2	15
GE	22.8	95	20
GF(a)	23.4	97.5	13.5
GF(b)	22.35	93.1	33.3
GG	19.15	79.8	15
GH	20.6	85.8	4.5
NA	20.6	85.8	22.5
NB	23.8	99.1	11.14
NC	20.1	83.75	31
ND	23.2	96.7	22.5
NE	22.3	92.9	21.4
NF	22.5	93.8	1.5
NJ	19.7	82	7
LB	19.9	82.9	39.25
LC	20.2	84.2	11.5

Farms were graded accordingly into the following risk categories based on the stocking density grading system outlined:

RISK ASSESSMENT OF FARMS WITH RESPECT TO STOCKING DENSITY:

NO RISK:	Farms GH, NF, NJ	(1-10kg/m^3)
LOW RISK:	Farms GA, GC, GD(a), GD(b), GE, GF(a), GG, NB, LC	(11-20kg/m^3)
MOD. RISK:	Farms GB, NA, ND, NE	(21-30kg/m^3)
HIGH RISK:	Farms GF(b), NC, LB	(31-40kg/m^3)

5.1.10 Nutrition

There were many concerning indicators with respect to both low quantity and poor quality of feed used through the systems assessed.

Using Timmons and Ebeling's formula, using fish length and a species-specific condition factor (K), a projected average fish weight could be calculated and compared with a real average weight (Timmons and Ebeling, 2013.a) to assess adequacy of feeding.

Table 5-4 Assessment of farm-level feeding adequacy, based on Timmons and Ebeling, 2003.a calculations

Farm	Ave Length of fish(cm)	Projected weight(grams): (using a tilapia-specific K factor of 2.08)	Real weight	Interpretation:
GA	21.2	198	165	Underfeeding
GB	23.7	276.9	237	Underfeeding
GC	19.7	159	126	Underfeeding
GD(a)	22.4	233.8	235.3	Adequate
GD(b)	20.2	171.4	156	Underfeeding
GE	22.8	246.5	202.6	Underfeeding
GF(a)	23.4	266.5	111.5	Underfeeding
GF(b)	22.4	233.8	111.1	Underfeeding
GG	19.2	147.2	119.4	Underfeeding
GH	20.6	181.8	178.4	Adequate
NA	20.6	181.8	149.1	Underfeeding
NB	23.8	280.4	195	Underfeeding
NC	20.1	168.9	111	Underfeeding
ND	23.2	259.7	286	Overfeeding
NE	22.3	230.7	246	Overfeeding
NF	22.5	236.9	179	Underfeeding
NJ	19.7	159	174	Overfeeding
LB	19.9	163.9	157	Adequate
LC	20.2	171.4	123	Underfeeding

Using this, 64.8% of systems assessed, reflected an inadequate volume of feed eaten by fish.

There was corroborating evidence of starvation/ cachexia and associated stress in the

microscopic pathological findings *(See 5.2.2 and 5.2.3)*, including low hepatocellular lipid levels (Ellis *et al.*, 1978; Evenson, 2006; Heath, 1995.a), prolific lipofuscin deposits in the melanomacrophage centres (MMCs) (Ellis *et al.*, 1978; Roberts and Rodger, 2012), low pancreatic activity, increased number and size of MMCs, increased hepatocyte nuclear activation, gastritis with evidence of apoptosis(Roberts and Rodger, 2012), as well as overwhelming poor growth (Gatlin, 2008), poor body condition, low visceral fat, and other indicators of chronic stress**.**

Farmers were frequently uncertain about actual biomass of fish in each tank, and would have had to estimate the correct volume of feed necessary for an estimated tank biomass. In addition, although farmers adjusted feed volumes seasonally, no modification seemed to be made according to water DO levels. Tilapia's small stomach size also make frequent feeding a very important part of the daily regime in maintaining weight and growth. Excess feeding reflected using the above calculations, on farms ND, NE and NJ would contribute to poor water quality due to decomposition of uneaten feed, and pose additional environmental stress upon fish. Interestingly, excess feeding of fish seemed to increase visceral fat and improve condition, without boosting growth *(Table 4-16, Table 5-4, Table 4-8)* . It is interesting to note that all farms exhibiting good growth were underfeeding fish, highlighting the multiple other factors at play affecting growth.

The high prevalence of compounding factors like low DO and high CO_2 would further predispose to poor metabolism and feed intake, and exacerbate the problem further.

Poor levels of fish growth in association with high lipofuscinosis, point to inferior quality and composition of feed with relation to carbohydrate : protein ratio, deficiencies of fatty-acids and amino-acids (El-Sayed, 2006c; Fitzsimmons, 1997; Huchzermeyer, 2015), mineral deficiencies like Ph, K, Zn and Cu (Timmons and Ebeling, 2013.h), and low inclusion levels of Vitamin E and other protective anti-oxidants (Gatlin, 2008; Huang and Huang, 2004; Wedemeyer *et al.,* 1976.d).

Potential impact of protein variability with excessively high levels as a possible associative factor with renal mineralization, and low levels with poor growth (El-Sayed, 2006.c; Wedemeyer *et al.,* 1976.b) would need to be taken into account. Source and quality of protein would also play a role in terms of potential contribution to anorexia and poor growth (El-Sayed, 2006.c; Huchzermeyer, 2015).

Lipid deficiency, in addition, could potentially have an impact on growth (El-Sayed, 2006.c) as well as reduced capacity to deal with additional stress challenges (Wedemeyer *et al.,* 1976.d).

There was good correlation between visceral fat and external condition scoring of fish *(Table 4-16)*, so it appears that condition scoring is a useful non-lethal tool for fairly accurately assessing visceral fat levels, which would give farmers a rudimentary means of assessing whether fish are receiving adequate nutrition.

Because detailed nutritional analysis did not form part of this study, farms were subjectively graded into the following risk categories based on the above findings and farm questionnaire results: with zero risk =adequate feed offered (*As per Table 5 4)* of appropriate quality, low risk:

excess feed of appropriate quality, moderate risk: inadequate feed of appropriate quality or adequate/excess feed of poor quality, High risk: inadequate feed of poor quality.

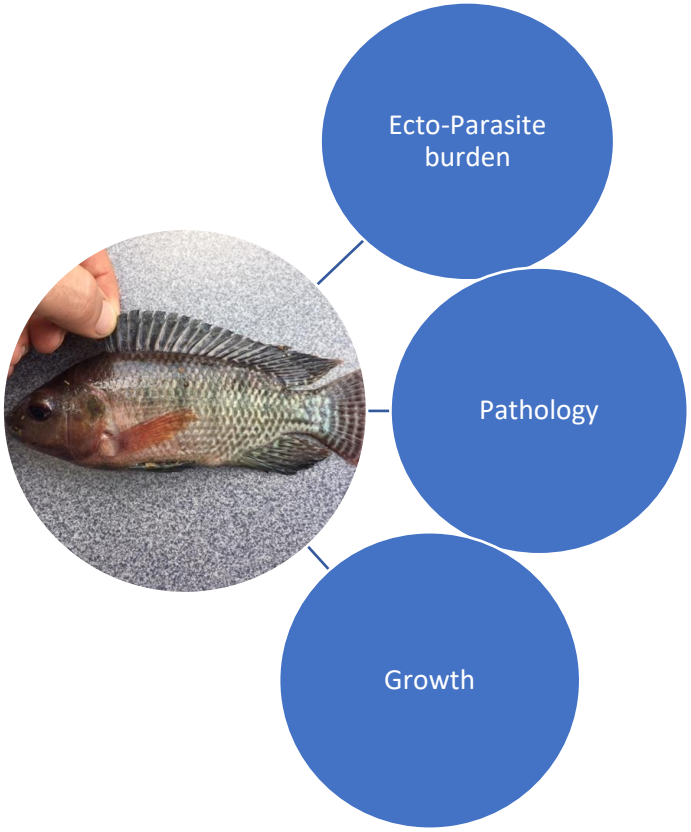
RISK ASSESSMENT OF FARMS WITH RESPECT TO NUTRITION:

NO RISK:	None
LOW RISK:	None
MODERATE RISK:	Farms GDa, GH, ND, NE, NJ, LB
HIGH RISK:	Farms GA, GB, GC, GDb, GE, GFa, GFb, GG, NA, NB, NC, NF, LC

Interpretation of the impact of all underlying independent variables, in particular, water parameters, always needs to be done cautiously because of the complexity of analysing a data set with a single time-scale assessment. Nutrition quantities or quality may vary with batches or farm management practice. The impact of stocking density changes with fish growth, water flow rates, and farm management and breeding practice, and water parameters are by nature, dynamic and can fluctuate dramatically within a short period of time. Interpreting direct cause and effect is challenging.

5.2 HEALTH ASSESSMENT

The greatest impact of the independent variables on fish health discussed earlier, have been broadly categorized into three areas:



Without discounting the multitude of ways in which sub-optimal health can manifest within the dynamic aquatic ecosystem, these factors dominated as key recurrent findings.

5.2.1 Ecto-parasite burden

An average farm burden was calculated as an initial average grade (1-5) for each fish between all wet preparations, and then an average between fish to achieve a farm score *(APPENDIX 4, TABLE 1 Data sheets)*.

5.2.1.1 *Trichodina* spp.

Trichodina spp. was detected in varying grades (1-5) in 57.2% of all fish *(Fig. 4-16)* and was the most common ectoparasite encountered, tying in with global and African trends (A. Shinn, personal communication, 29th May 2017; K. Veverica, personal communication, 14th March, 2016). Although all wet mounts were, on average, equally representative, on many occasions the parasites were only seen in a single wet mount diagnostic test. A skin scrape proved, generally, the most reliable test. Monogeneans were frequently found together with high burdens of *Trichodina* spp.*,* which is an expected finding (Barker *et al.*, 2002; Noble *et al.,* 1963). The varying numbers encountered on individual sampled specimens within a system, highlight the impact of varying individual immune responses to stress. However, what was very clear, was the rapid horizontal transmission that must occur within systems when underlying predisposing factors are present as common system stressors. When *Trichodina* was present, generally 90-100% of sampled fish were positive, many with high grade infestations. All species of tilapia seemed to be equally susceptible. This is supported by literature reports of infestations in *O. niloticus, O. mossambicus,* hybrids and other tilapia spp. (El-Sayed, 2006.d). Histories of chronic low-grade mortalities in many systems *(See 4.4)*, would tie in well with these *Trichodina* burdens (Noga, 2010.e).

Increased mucus on fish skin or gills was not a reliable parameter to assess underlying *Trichodina* spp. burdens. Even fish with high-grade infestation often showed no obvious "greying" in appearance or visible high mucus levels. No macroscopic or microscopic haemorrhages, as suggested by Colorni, 2008, were visible on skin or gills.

Known associations between *Trichodina spp.* and poor water quality (Huchzermeyer, 2015; Lewbart, 1998.a; Loh and Landos, 2011.h; Shinn, 2016) are not supported in this study, with all data analytical models revealing a predisposition for *Trichodina* spp. burdens to increase significantly in the presence of decreasing NH_3-UIA levels, or conditions with lower CO_2 *(Table 4-11)*. Highest burdens were associated with NH_3 below the 0.05mg/L mark (70.1% of systems), which is the goal of a healthy aquaculture system, and questions whether *Trichodina* spp. may in fact be averse to organic rich water, which would by definition be high in NH_3 or NO_2^-. No similar documented findings could be found. It is more likely that the one-off water parameter analysis is not accurately reflecting the dynamic changes that occur within a water body over time. The apparent predilection for harder water with lower alkalinity, appears to be a false positive, largely influenced by the extremely high CH:TA ratios of farms GFa and GFb. 50% of systems with high *Trichodina* burdens had CH:TA ratios below 1.

A significant positive correlation existed with stocking density *(Table 4-11)*, which was to be expected with close contact between fish predisposing to horizontal transfer of parasites, and all the associated immunosuppressive impacts of overcrowding (El-Sayed, 2006.d; Lewbart, 1998a; Loh and Landos, 2011.h; Reed, *et al.,* 2009; Wedemeyer *et al.,* 1976.c).

Although the study results showed poor correlation with DO and temperature, the negative correlation between DO and *Trichodina* spp. burdens is supported by previous documentation (Wedemeyer *et al.,* 1976.c) which associates these parasites with water low in DO. One would need to take into account the nocturnal-diurnal DO fluctuation that occurs within an aquatic system, and bear in mind that readings were assessed in late afternoon at their DO peaks. The

early mornings lowest readings would have been extremely low in many systems. To really assess significance of this relationship, it would be valuable to repeat early morning readings as well. Similar to this study, Ramadan, 1991, also showed a negative correlation with water temperature but with an apparent preference for the ideal 25-30 ^{o}C range. This is not typical of what one would expect with most protozoal infestations where multiplication is faster at higher water temperatures (Noga, 2010.e). Lewbart's, 1998.a, association between *Trichodina* spp. and warmer water temperatures, is supported as the 25-30^{0}C range is an ideal range for tropical fish species, so it seems this correlation needs to be carefully interpreted.

Interestingly, Wedemeyer *et al.,* 1976, drew a correlation between high *Trichodina* spp. burdens and excessive size variation among fish. This was certainly a factor playing a role in the systems assessed, with evidence of large variation in fish weights within systems, as well as presence of fingerlings due to poor breeding management.

The association of *Trichodina* spp. with other parasites showed a high predilection to cohabit with *Ambiphrya* spp. and monogeneans, particularly Dactylogyridae, with a significant reluctance to cohabit with *Ichthyobodo necator complex.*

The parasite's most significant effect upon fish health was found to be through stimulation of goblet cells hyperplastic changes and to a lesser degree epithelial hyperplastic change, in the gill lamellae**.** Non-specific hyperplastic changes are supported by Lightner, *et al.,* 1988's findings as well as Loh and Landos, 2011.h. These gill filament changes will, in turn, impact vital physiological functions like respiration, osmoregulation and excretion, tolerance of low water

DO levels, with impact upon fish health and immune response, and increased susceptibility to higher parasite burdens- the basis of a compounding negative cycle.

Two of the three farms with highest level secondary bacterial disease, exhibited high *Trichodina* burdens and this aligns with similar findings by Lio-Po and Lim, 2002 *(Table 4-17)*. This supports the believed impact of the immunosuppressive effect and epithelial trauma from the parasites' mode of attachment and feeding (Macmillan, 1991; Noga, 2010.e; Paperna, 1996.a).

The known association between poor nutrition, fish debilitation and high *Trichodina* spp. burdens (Loh and Landos, 2011.h) is supported in this study, with sub-optimal nutrition being highlighted as a key factor playing a multifactorial role in compromised fish health. High parasite-burden positive correlation with anorexic behaviour (El-Sayed, 2006.d) would further compound the impact of inadequate meeting of nutritional needs and the impact of this upon fish health and growth. Another circle of negative impact would result.

RISK ASSESSMENT OF FARMS WITH RESPECT TO AVERAGE *Trichodina* FARM GRADE: *(APPENDIX 4 TABLE 1 Data sheets)*

(With No Risk=0 grade, Low risk≤ grade 2, moderate risk> grade 2 <grade 4, high risk ≥ grade 4)

NO RISK: Farms GA, GC, GH, NC, ND, NJ

LOW RISK: Farms GB, GE, GFa, GFb, GG, NA, NB , NE, NF, LC

MOD RISK: Farms GDa, GDb and LB

HIGH RISK: None

5.2.1.2 Gyrodactylidea

Gyrodactylidea featured as an insignificant parasite in this study, with low representation (14.3%) of the fish *(Fig. 4-18)*, no pathological correlations of significance and no predictive water quality parameters of significance *(Table 4-12)*.

With only occasional fish testing positive within system sample batches, and levels on fish being largely low, this parasite appears to carry little significance within systems currently and infestation rates represented what one would expect to find in a healthy population (Whittingdon and Chisholm, 2008). Mode of reproduction may be the reason for this, with transfer of live young between fish less easily achieved than dispersal of eggs, as seen with Dactylogyridae spp. No stage of the life-cycle exists free of the host (Loh and Landos, 2011.h), thus transfer of parasites between fish would require direct contact. Larvae, being larger than eggs, may also be more susceptible to trapping within the mechanical filtration systems and removed. With a small local pool of captive farmed fish within the country, it may also simply be that the population is a fairly "clean" one, and without parasite introduction from wild-caught fish, may not prove to be a serious problem within farming systems. In fact, farm NA, which was the only system reflecting high Gyrodactylidea numbers, had sourced some fish from river systems.

Despite believed associations between Gyrodactylidea and low water DO (Svobodová, 1993), no correlation was seen with high stocking density of fish or underlying water stressors. There were no seasonal correlations, with parasites encountered through summer and winter sampling. As with *Trichodina* spp., presence on fish showed no species predilection. It was

interesting that it was detected on gill clips and scrapes as well, which is uncommon, but documented in literature (Loh and Landos, 2011.h), again highlighting the importance of multiple diagnostic modalities.

RISK ASSESSMENT OF FARMS WITH RESPECT TO AVERAGE Gyrodactylidea FARM GRADE: *(APPENDIX 4, TABLE 1 Data sheets)*

(With No Risk=0 grade, Low risk≤ grade 2, moderate risk> grade 2 <grade 4, high risk ≥ grade 4)

NO RISK: Farms GA, GB, GDb, GE, GFb, GH, NB, NC, NE, NJ

LOW RISK: Farms GC, GDa, GFa, GG, NA, ND, NF, LB, LC

MOD RISK: None

HIGH RISK: None

5.2.1.3 Dactylogyridae

Dactylogyridae spp., featured more significantly as a parasite, present on 19% of the fish *(Fig. 4-20)*., however, most parasite levels were low on positive fish. They showed a greater tendency to cross-infect, with a larger percentage of the sample fish testing positive within a system, in comparison to Gyrodactylidea. This is most likely attributed to the egg-laying reproductive practice of the Dactylogyridae group, as a more effective mode of transmission. Eggs laid settle to the bottom of the systems and are more likely to escape being captured in the filtration processes and the hatched motile oncomiracidia facilitate the process of horizontal transmission (Noga, 2010.e). Finding dactylogyrids on gill clips and scrapes was to be expected. The fact that no juvenile stages were seen on skin scrapes, further supports evidence of low infestation rates. It is to be noted that that these parasites predominantly infested *O. niloticus* , albeit at low levels, and warrants a more comprehensive analysis of the susceptibility of the

species. Similar to Gyrodactylidea spp., no clear association could be drawn between high mucus levels or evidence of focal haemorrhages on gill filaments, probably because of low infestation levels encountered (Noga, 2010. e).

Despite believed associations with low DO, warmer water temperatures, and overcrowding (Noga, 2010.e), and presence of these factors within systems assessed, the only predictor for Dactylogyridae, in the Pearson's correlation model, proved to be CO_2, with a low negative correlation *(Table 4-13)*. No seasonal correlation could be drawn with higher parasite burdens which were seen in summer as well as mid-winter sampling. The step-wise regression model corroborates this, highlighting CO_2 as a primary predictor. As discussed with gyrodactylids, the small size and fairly closed nature of the farmed population within South Africa, may have lent itself to this with lack of introduction of these parasites yet.

Pearson's canonical correlations show a positive correlation ($P<0.05$) between Dactylogyridae and *Trichodina,* and a positive correlation ($P<0.01$) with total parasite score. The fact that both parasite groups show preference for lower CO_2 water parameters may be a factor supporting their common cohabitation. Interestingly, dactylogyrids were most often seen on gill scrapes, where samples were taken from the base of the primary lamellae. When seen on histology gill tissue sections, monogeneans were also often encountered attached at the base or angle of the primary lamellae. *Trichodinids,* on the other hand, were most frequently encountered gliding along the secondary lamellae surfaces and within skin mucus. It may be that there is a lack of competition for feeding areas, and thus a tolerance to co-habit together. A symbiotic relationship has been proposed between the two parasites (Barker *et al.*, 2002; Colorni and

Diamant, 2005) but it may simply be that they both prefer a similar water environment and do not interfere with one another.

Despite dactylogyrids known propensity to induce gill pathology (Lewbart, 1998c; Loh and Landos, 2011.h), no significant correlations could be drawn. However, varying degrees of gill lamellar fusion were a fairly frequent finding on histopathology sections, often associated with Monogenean (gyrodactylid or dactylogyrid) parasites. Thus, their effect, at the levels encountered, seems to be more one of focal irritation at points of attachment and feeding, rather than generalized lamellar changes or cellular infiltrates.

RISK ASSESSMENT OF FARMS WITH RESPECT TO AVERAGE Dactylogyridae FARM GRADE
(APPENDIX 4 TABLE 1 Data sheets)

(With No Risk=0 grade, Low risk≤ grade 2, moderate risk> grade 2 <grade 4, high risk ≥ grade 4)

NO RISK: Farms GA, GDb, GFa, GFb, GH, NC, ND, NE

LOW RISK: Farms GB, GC, GDa, GE, GG, NA, NB, NF, NJ, LB, LC

MOD RISK: None

HIGH RISK: None

With the two monopisthocotylean “superfamilies”: the Gyrodactylidea and Dactylogyridae, regarded as those monogeneans with greatest potential negative impact on farm ecomonics (Noga, 2010.e), it was encouraging that these featured so insignificantly.

5.2.1.4 Ichthybodo necator complex

Ichthybodo necator, although not often encountered *(Fig. 4-22)*, when it was present, tended to be a significant problem with high infection rates and indication of easy transmission between fish, emphasizing its danger within the closed recirculating system design**.** This is also probably associated with its flagellated stage that facilitates movement between fish in close contact. The comparative difficulty in often detecting it on wet mounts rather than histopathology, demonstrates the value of dual-method diagnostics to detect this particular small parasite. Despite histopathological processing, high numbers were often encountered on gill sections, despite not being obvious on wet mount preparations, and I would suggest this as a necessary tool to assess infestation. This parasite was not detected on any *O. mossambicus* fish*,* and showed a clear preference for *O. niloticus* and hybrids*.* This would benefit from further research. Despite supposedly showing a predilection for younger fish (Shinn, 2016), this was seen in high numbers in fish ranging from 6-15 months, and possibly even older in the mixed populations. Presence of anticipated heavy mucus accumulation on gill and skin, or associated macroscopic pathology (Lewbart, 1998d; Loh and Landos, 2011.h) was not evident. As with other ecto-parasites assessed, this reinforces the poor value in interpreting disease based on macroscopic pathology, and the need to assess to microscopic level.

Pearson's canonical correlations showed a significant negative correlation ($p<0.01$) between *Ichthyobodo necator* and *Trichodina* spp.*,* as well as with the total parasite score. Despite being considered a very pathogenic parasite (A. Shinn, personal communication, 29th May, 2017), no other significant correlations existed between *Ichthybodo necator* and gill pathology or other

parasites. This contradicts some literature where infestation is supposedly associated with gill epithelial hyperplasia, secondary lamellar fusion and necrosis of the gill lamellae (Loh and Landos, 2011.h; Lom and Dykova, 1992), however, accounts exist of mortalities despite little pathology (Noga, 2010.e).

High NH_3-UIA (organic-rich) water dominated as the chief significant positive predictor for *Ichthyobodo necator* infestation, with higher CO_2 also correlating significantly *(Table 4-14)*. Both of these parameters fit the picture of an overstocked, organic-rich, stressed system. A stepwise regression model corroborated these findings placing them in order of significance as NH_3, CO_2, and then CH:TA ratio. NH_3 readings over 0.05mg/L and CO_2 readings over 100mg/L correlated the best with higher parasite infestations. Although lower water temperatures < 25^0C (Noga, 2010.e) and low DO (Wedemeyer *et al.,* 1978) are believed to be contributing factors, no correlations could be drawn.

The significant negative correlation seen between *Trichodina* spp. and *Ichthyobodo necator* is an interesting observation. Competition for attachment or feeding space on tissue, or simply differing water conditions predisposing to different parasites could both potentially play a role. But considering the complete opposite preferences of both parasites with *Trichodina* showing preference for low NH_3, low CO_2 water, and *Ichthyobodo necator* preferring higher NH_3, higher CO_2 water, it appears more likely that this is an issue of water parameter preference. This is potentially a very useful tool as it would allow one to predict potential underlying parasite proliferation by simply evaluating water parameters. Farmers with no access to microscopy as

a diagnostic modality, could anticipate potential parasite threats by the existing parameters in the watery environment.

A low negative correlation with water CH:TA ratio may indicate that this parasite prefers softer water composition or higher alkalinity. I would suggest that this is another water parameter worth investigating further, as 50% of farms with high *Trichodina* burdens, were associated with harder water and lower alkalinity.

Despite high cross-infectivity of this parasite and believed associations with highly stocked systems (Lewbart, 1998.a; Shinn, 2016), links with high stocking density could not be drawn, with only one of the heavily stocked systems over 30kg/m^3 showing evidence of infestation. However, as this parasite needs to be introduced from natural waterways or infected fish, it may be that, once again, as with the observations with monogeneans, our population of farmed fish has been reasonably protected.

No clear correlations could be drawn between *Ichthyobodo necator* complex and secondary bacterial disease *(Table 4-17)*.

RISK ASSESSMENT OF FARMS WITH RESPECT TO AVERAGE *Ichthyobodo necator complex* FARM GRADE *(APPENDIX 4, TABLE 1)*

(With No Risk=0 grade, Low risk:≤ grade 1, moderate risk : > grade 1<grade 3, high risk: ≥ grade 3)

NO RISK: Farms GA, GB, GC, GD(a), GD(b), GE, GF(a), GF(b), GG, NC, NE, NF, NJ, LB, LC

LOW RISK: Farms NA, NB, ND

MOD RISK: None

HIGH RISK: Farm GH

5.2.1.5 *Ambiphrya* spp.

Ambiphrya, although rarely encountered (8.93% of fish) *(Fig. 4-23)*, was however, significant in its level of infestation on each fish and its generalized prevalence through the sample batch, confirming literature observations of rapid reproduction rates and transmission between fish (Colorni, 2008). Again, corroborating with literature (Colorni, 2008), no species preference was noted, and all *Oreochromis* spp. and hybrids evaluated showed equal susceptibility. Parasites were equally distributed through skin and gill tissue, thus both areas of sampling should prove diagnostic in a positive fish. However, infestation quantification is difficult in skin mucus wet mounts as parasites are difficult to visualize. Gill clips and scrapes proved more valuable.

Stocking density served as a high positive predictor for *Ambiphrya* levels, with Pearson's correlation model *(Table 4-15)*. Nitrite (NO_2^-) act as a moderate negative predictor, temperature as a moderate positive predictor, CH:TA ratio as a moderate positive predictor, and pH as a low negative predictor. A step-wise regression model corroborates stocking rate as primary and CH:TA as a secondary predictor.

Despite belief that *Ambiphrya* exerts little impact on its host other than an attachment vehicle, or causes mild impairment of gaseous exchange (Noga, 2010.e), Pearson's canonical correlations show a significant positive correlation ($p<0.01$) between *Ambiphrya* and gill goblet cell hyperplasia. Significant correlations ($p<0.01$) between *Ambiphrya* and *Trichodina* levels, and *Ambiphrya* and total parasite score were also found. No other correlations of significance can be drawn with the other parasites and gill pathology.

With only two systems positive for this parasite, and thus data limited, caution needs to be exercised in its interpretation. High stocking rate serves as the best predictor through most analytical models used, with higher stocking rate favouring higher *Ambiphrya* infestations. Of the two farms testing positive, (NE and LB) stocking density varied between 21.4kg/m^3 and 39.5kg/m^3 *(Table 4-33)*. This is a wide variability, however, it is interesting to note that where stocking density was the highest on LB, infestation was most severe. This aligns itself with documented findings (Colorni, 2008).

A positive correlation with higher CH:TA ratios was documented. But both positive systems had CH:TA ratios<1. I suspect this is more a case of preference of hard water with high alkalinity. In fact, both systems had extremely hard water with GH> 450mg/L, and high alkalinity >650mg/L- in fact approaching lethal levels, which in itself, would be acting as an extreme stressor upon fish. Interestingly, *Trichodina* spp. showed positive correlation with harder water as well. Positive correlation with water temperature and negative correlation with pH are probably biased because of small farm sample size: highest parasite prevalence was found in systems with water < 25^0C, and extremely alkaline pH's over 8. Despite references to *Ambiphrya's* predilection for feeding in organic-rich water (Colorni, 2008; Noga, 2010.e), no positive correlation with NH_3-UIA (organic rich water) could be found. The negative correlation with NO_2^- needs to be carefully interpreted because of the small data base. One system, (farm NE) had extremely high NO_2^- levels.

A predilection to cohabit with *Trichodina* existed. It is possible that *Trichodina* spp. primarily disturb the immune barrier and damage the epithelial layer, predisposing to secondary

Ambiphrya infestations (Colorni, 2008). It may be that their joint preference for highly stocked systems or hard water, play a role.

Despite secondary bacterial disease being a commonly associated finding (Esch *et al.,* 1976; Miller and Chapman, 1976; Noga, 2010.e), no correlations with secondary bacterial infections could be drawn *(Table 4-17)*.

Epistylus, seen on farm GG on one fish only, suggested a low level of relevance in tilapia RAS systems.

RISK ASSESSMENT OF FARMS WITH RESPECT TO AVERAGE *Ambiphrya* FARM GRADE *(APPENDIX 4 TABLE 1 Data sheets)*

(With No Risk=0 grade, Low risk≤ grade 2, moderate risk> grade 2 <grade 4, high risk ≥ grade 4)

NO RISK: Farms GA, GB, GC, GDa, GDb, GE, GFa, GFb, GG, GH, NA, NB, NC, ND, NF, NJ, LC

LOW RISK: Farms NE, LB

MOD RISK: None

HIGH RISK: None

5.2.1.6 Total Parasite Analysis

Total ecto-parasite levels were, generally, low through fish and farms assessed, but significant in terms of particular parasites encountered, their levels on individual fish and their potential pathological impact. Total score was chiefly composed of *Trichodina* spp., to a lesser degree of Dactylogyridae, and *Ichthyobodo necator*. *Ambiphrya* and Gyrodactylidea comprised a minor proportion *(Fig.4-26)*. Apart from the single account of *Epistylus,* no other parasitic infection could be demonstrated in this study.

Surprisingly, Pearson's model proposes stocking density as only a low positive predictor for total parasite count, and pH and NH_3 as low negative predictors.

Using Pearson's canonical correlations, total parasite score shows high positive correlation ($p<0.01$) with gill goblet cell hyperplasia, as well as with *Trichodina* count, Dactylogyridae*,* *Ambiphrya*, and *Ichthyobodo*. There is a positive correlation ($p<0.05$) with gill epithelial hyperplasia and Gyrodactylidea, but no correlation with EG cell infiltration at the base of the gill arches.

No seasonal correlations were seen with any parasites. An interesting observation was the absence of any parasites on farm GA, where water was routinely treated with $KMNO_4$, suggesting the possible value of this preventative protocol.

Interpreting these findings, one needs to bear in mind that, with different parasite species preferring differing water conditions or stocking densities, conflictive results will yield a questionable summarised value. This highlights the value in correct parasite species identification without grouping and interpreting parasites as a whole.

One must bear in mind that sensitivity of most of the diagnostic tools used, was likely to be low and low parasitic burdens may have been missed. But with greatest impact on population health and overall system productivity generally associated with high parasitic burden, tests used would have been sensitive enough for the purposes of the study. Inclusion of multiple practical modalities like macroscopic examination, three wet prep mount techniques and histopathology helped provide a broad assessment of overall burdens and avoid false negatives.

5.2.2 Pathology

5.2.2.1 Gill pathology

Lamellar epithelial hyperplasia *(PLATE 5)*

Lamellar epithelial hyperplasia can be described as an increased number and size of unspecialised epithelial cells lining primary or secondary lamellae (Daoust and Ferguson, 1983).

Widespread low-grade epithelial hyperplasia was a common finding**,** with grades 1-3 out of 5 dominating through 64.3% of the sample population *(Fig. 4-28)*.

In Pearson's model, only NH_3-UIA served as a predictor of epithelial hyperplasia *(Table 4-18)*. It is a low negative correlation. A step-wise regression model corroborated this result. Epithelial hyperplasia has been associated with higher organic load and NH_3 levels in water, with reduced ammonia excretion through the fish gills, and resultant gill epithelial hyperplastic change (Hawkins *et al.,* 2002; Huchzermeyer, 2015). A study by Daoust and Ferguson (1984), however, challenged this finding in their study where they found no correlation either between chronic high un-ionized ammonia and gill lesions or pathology in intensively farmed trout. They proposed that fish susceptibility to high NH_3 increases in conditions of low DO only. In this study *Trichodina* spp. infestation levels also show association with low NH_3-UIA water (as does *Ambiphrya*). It thus stands to reason, that low NH_3-UIA may not be directly resulting in lamellar hyperplasia, but, as a result of these conditions being favourable for high *Trichodina* spp. infestations, the hyperplastic epithelial response is likely to be due to direct mechanical injury from the feeding action and attachment of high numbers of these parasites. This is supported by the significant Pearson correlation ($p<0.05$) between lamellar epithelial hyperplastic change

and *Trichodina* spp. *(Table 4-19).* Similar links between high *Trichodina* spp. and epithelial hyperplastic reaction are documented (Lightner *et al.,* 1988; Loh and Landos, 2011.h).

Using Pearson's canonical correlations, a positive correlation ($p<0.01$) exists between gill epithelial hyperplasia and goblet cell hyperplasia. In addition, positive correlations ($p<0.05$) exist between epithelial hyperplasia and EG cell infiltration in the base of the gill arches. This may thus suggest that all these cellular changes carry links with high *Trichodina* spp., and *Ambiphrya* spp. infestations. When one considers the close physical relationship and the network of immune function existing between goblet cells, EGC's and rodlet cells (Leino, 2001), all interspersed within the gill tissue, closely associated with epithelial cells, and exposed to influence of inflammatory mediators and migrating EGC's (Reite, 1998; Reite and Evenson, 2006) it is no wonder that parasitic triggers would stimulate a host of cellular changes.

There was no link established with higher pH levels in the water and no correlations could be drawn with green water systems either, despite proposed links (Huchzermeyer, 2015). No clear association could be drawn between *Ichthyobodo necator complex* and epithelial hyperplasia, despite literature references to the contrary (Loh and Landos, 2011.h).

Soliman and Wilson, 1992, found a link between epithelial hyperplasia and pantothenic acid deficiency, in *Oreochromis aureus.* Vitamin and nutrient levels in feed currently used, would be worth investigating further. Exposure to toxicants in the water or feed like heavy metals or pesticides would be additional factors that could be implicated (Heath, 1995.d; Heath, 1995.f; Olsson, 1998; Reimschussel, 2008).

Thus, in summary, this pathological finding seems primarily to be associated with the mechanical effect of high *Trichodina* spp. burdens, and their associated low NH_3 watery environment. It seems to form part of a triad of pathological changes including goblet cell hyperplasia and EG cell infiltration.

RISK ASSESSMENT OF FARMS WITH RESPECT TO AVERAGE FARM GRADE EPITHELIAL HYPERPLASIA *(APPENDIX 4, TABLE 3 Data sheets)*

(With No Risk=0 grade, Low risk: ≤ grade 2, moderate risk :> grade 2 < grade 4, high risk: ≥grade 4)

NO RISK: Farm GDb

LOW RISK: Farms GA, GC, GDa, GE, GFb, GG, GH, NA, NB, NC, NJ

MOD RISK: Farms GB, ND, NE, NF, LB, LC

HIGH RISK: Farm GFa

Lamellar Goblet cell hyperplasia *(PLATE 6)*

Goblet cell hyperplasia of the lamellae, defined as an increase in number of goblet/ mucous cells in primary lamellae, was not as prevalent a finding as epithelial hyperplasia, and never with high grade severity *(Fig. 4-29)*. It seems to follow similar correlation relationships as epithelial hyperplasia, with significant positive correlations with *Trichodina* spp. ($p<0.01$) and total parasite scores ($p<0.01$). However, significant association with the sessile *Ambiphrya* spp., is a key difference *(Table 4-21)*. Association with water parameters also differ with a low negative correlation with CO_2 ($p< 0.05$) highlighted with Pearson's correlation model *(Table 4-20)*.

The negative association with CO_2 is most likely reflective of the water conditions most suitable for *Trichodina* spp. and *Ambiphrya* spp. proliferation rather than any direct chemical effect. Of those fish exhibiting over 30% prevalence of goblet cell hyperplasia, 80% were in systems with water CO_2 levels below 60mg/L. Recognizing the function of mucous production in fish as an important part of the immune barrier, one could hypothesize that reduction in number of goblet cells may reflect the immunosuppressive effect of cumulative stressors, including high CO_2.

It is interesting that epithelial hyperplastic response shows significant association to only *Trichodina* spp. and total parasite score *(Table 4-19).* It appears that a mucus/ goblet cell proliferation is more significantly triggered by or associated with the presence of *Ambiphrya* spp. Epithelial cells are not considered part of the immune system ,whereas goblet/ mucus cells are considered a first line of defence and their proliferation may reflect the primary function of the goblet cells as part of the immune cellular triad of mucus, rodlet and EG cells, secreting a glycoprotein-rich mucus containing humoral immune factors with biostatic / -cidal properties (Sung Jung, 2017), in response to the increased micro-organism or parasite levels. This reflection of a higher level of immune activation may be reflective of *Ambiphrya's* mode of attachment, its propensity to multiply rapidly, presence of concurrent secondary bacterial disease (Esch *et al.,* 1976; Miller and Chapman, 1976), or simply reflect a severely compromised immune barrier (Colorni, 2008) open to secondary parasitic invasion. *Trichodina* spp. lack of association with goblet cell proliferation may reflect more of a symbiotic relationship with the host fish, and *Ambiphrya,* a more pathogenic impact.

There was no direct correlation with NH_3-UIA, despite references to its irritant effect on fish, with stimulation of goblet cells (Roberts and Rodger, 2012). It may be that NH_3-UIA levels were too low in systems tested, to draw any correlations. But it appears that primary triggers for this pathological change are more likely parasite immune stimulation rather than direct chemical irritation from abnormal water parameters. No association with high pH, as proposed by Boyd,2000.e, could be demonstrated.

As with epithelial hyperplasia, no association could be drawn between *Ichthyobodo necator* and goblet cell hyperplastic change *(Table 4-21)*. Other factors like dietary nutrient deficiencies and toxicants like cadmium in the water, would need to be investigated as contributors (Olsson, 1998; Reimschussel, 2008).

RISK ASSESSMENT OF FARMS WITH RESPECT TO AVERAGE GRADE GOBLET CELL HYPERPLASIA *(APPENDIX 4 TABLE 3 Data sheets)*

(With No Risk: ≤ grade 1, Low risk: > grade 1≤ grade 2, moderate risk: > grade 2 < grade 4, high risk: ≥grade 4)

NO RISK: Farms GA, GC, GDa, GFb, NC, NJ

LOW RISK: Farms GB, GDb, GE, GFa, GG, NA, NB, ND, NE, NF

MOD RISK: Farms GH, LB, LC

HIGH RISK: None

Eosinophilic granular (EG) cell infiltration *(PLATE 7)*

This finding was common through all systems *(Fig. 4-30)* but at a low to moderate grade, indicating that a low-level cellular infiltration may be normal and part of ongoing immune defence of the host fish to challenges in the water body or part of normal osmoregulatory

function. However variation did exist, between systems and even between fish within a single system, highlighting possible reaction to different stimuli within different systems, differing levels of immune response within fish, or differing physiological responses of fish. With the osmoregulatory function of the EGC's to actively pump in Na^+ and Cl^-, in exchange for NH_3 and CO_2/HCO_3 (Wedemeyer *et al.*, 1976.b), it stands to reason that any physiological disturbance leading to a hypercarbia or hyperammonaemia in fish tissues, may trigger an EG cellular infiltration in an effort to stabilise blood parameters.

Although there were no significant independent variable predictors for EG cell infiltration in Pearson's model *(Table 4-22)*, all systems where severe to extreme level infiltrates at the base of the gills existed, were in water bodies with CO_2 >60mg/L. These fish would certainly be candidates for a hypercapnic state. Most of these systems had zero to low risk NH_3 levels, and this was unlikely to be playing a role. EG cells in gill lamellae have been shown to increase in response to higher NO_2^- levels as a compensatory mechanism to ensure adequate uptake of chloride (Heath, 1995.e). This finding could be supported in this study since 67% of systems with severe to extreme EG cell infiltration showed NO_2^- levels exceeding 0.2mg/L. Because of the high energy expenditure involved in the active osmoregulatory function, it is also possible that the stress imposed by hypoxic conditions result in a compensatory increase in numbers of EG cells at the base of the primary lamellae to maintain the intake of Cl^- and Na^+, as well as ammonia and bicarbonate excretion. However, this statistical correlation proved to be poor, with only 42% of systems with high level EG infiltration showing DO levels below 3mg/L.

Using Pearson's canonical correlations, a positive correlation ($p<0.05$) exists between EG cell infiltration in the base of the gill arches and both gill epithelial hyperplasia and goblet cell hyperplasia. These three changes within the gill tissue seem to exist largely as a triad response to challenge. Although EG cell infiltration shows no direct link with high parasite burdens *(Table 4-23)*, it links indirectly through its association with the above two changes, and appears to be the second line of defence following initial mobilization of mucus, rodlet and epithelial cells and in response to tissue damage. This ties in well, with the understanding of EG cells as an intricate part of fish immune function (Reite and Evenson, 2006), with parasite loads triggering their immune recruitment into the base of the gills, where many parasites often hide. This pathological change could be used as a measure of immune challenge to the fish, or indicator of both acute and chronic tissue injury in fish.

Exposure to potential toxicants in the water or food, were not included as part of the study and are also known to induce lamellar pathology, including hyperplastic change, fusion of lamellae, mucus cell proliferation, and inflammatory cell infiltration (Heath, 1995.d; Heath, 1995.f). However, typical signs for these challenges like necrotic change, epithelial desquamation, and oedema were not noted. It would be of value to assess potential impact of these factors on gill pathology. Gill surface dynamics between CO_2, pH and ammonia toxicity are another factor that comes into play. CO_2 levels at gill surfaces are known to be higher than surrounding water levels, with resultant pH drop and potentiated ammonia toxicity (Heath, 1995.c). It may be possible that high water CO_2 levels or hypercapnic states may exacerbate this toxic effect. Infectious causes like bacteria (e.g. *Edwardsiella* spp.*)* and viruses are also known to cause

inflammatory cell infiltration of the gills with secondary lamellar hyperplasia, but no evidence for these were detected during this survey study.

RISK ASSESSMENT OF FARMS WITH RESPECT TO AVERAGE GRADE EG CELL INFILTRATION AT BASE OF GILL ARCHES *(APPENDIX 4 TABLE 3 Data sheets)*

(With No Risk: ≤ grade 1, Low risk: > grade 1 ≤ grade 2, moderate risk:> grade 2 < grade 4, high risk: ≥ grade 4)

NO RISK: None

LOW RISK: Farms GE, GFb, NC, NF

MOD RISK: Farms GA, GB, GC, GDa, GDb, GFa, GG, GH, NA, NB, ND, NE, NJ, LB, LC

HIGH RISK: None

Other gill pathology *(PLATE 8)*

Pathology like increased rodlet and EG cells within the primary and secondary gill lamellae in fish are probably a reflection of tissue response to high mechanical or chemical irritation (Leino, 2001; Reite and Evenson, 2006) and activation of the immune system with initial rodlet activation and secondary EG cell activation. Key identified stressors like high parasite burdens, high levels of NH_3, NO_2^- and CO_2, low DO and presence of potential water pollutants like copper (Olsson, 1998) would be most likely to be implicated. Considering that these cellular changes were affecting 16-24% of fish, these underlying factors potentially reflect serious impact upon gill physiology and stress upon fish immune systems.

Fusion of gill lamellae was a regular finding, but generally presented as a low to moderate grade pathological change. Its presence is always a concern, however, because of its potential negative impact on functional lamellar surface area. Most of the areas of fusion seen were focal

or multifocal, with large extensive generalized fusion rare. On many occasions, monogeneans were seen attached still, or closely associated with the areas of generalized fusion, and were most likely implicated. Farms with most severely impacted fish often showed mixed parasite loads: some with high *Trichodina* spp. only, some with high Dactylogyridae, and some with combined *Trichodina* spp.*,* Dactylogyridae and *Ambiphrya* spp. populations.

The emerging disease (Blandford *et al.,* 2018), Epitheliocystis, was recorded at a prevalence of 16% in this study with low infection rates on individual fish. It has been shown that juvenile tilapia are able to maintain the condition as a benign infection even in adverse growth conditions (Paperna, 1996.c), thus questioning its significance. However, possible implications of infection may include stressed fish from exposure to environmental influences beyond tolerance levels (Blandford *et al.,* 2018) with depressed immune function and increased susceptibility to secondary infections. Although no vector association has been proven, links with parasitic vectors could be an underlying possibility, or simply reflective of opportunists in an immunosuppressed population of fish.

With little known about the disease, it requires further investigation.

Telangiectasis was occasionally encountered. It may have been associated with hypothermic shock from the killing method used in the study (Endo and Oguri, 1995.a; Roberts and Rodger, 2012) however chemical pollutants and toxins would be potential differential diagnosis (Reimschussel, 2008). Hydrogen-sulphide has also been implicated (Wedemeyer *et al.,* 1976.c) as well as prolonged exposure to high NH_3 levels (Wedemeyer *et al.,* 1976.d), both of which could be playing a role in systems assessed. Physical handling trauma to fish through

percussive stunning, would not be a contributing factor in this study, however, gill tissue removal (for microscopic examination during clinical examination in this study) has been associated and would have to be considered. Physical irritation from parasites would be another possible trigger. Telangiectasis has also been associated with *Yersinia ruckeri* septicaemia, due to bacterial induced haemorrhage (Noga, 2010.b), however no other typical gross lesions or positive cultures could be demonstrated.

Gill lamellar changes like oedema and necrosis, which are typical of either high level chemical pollutants like heavy metals and pesticides, or acute *Aflatoxicosis*, were not encountered (Roberts and Rodger, 2012). However, low levels of such irritants in the water, would present normally with a hyperplastic or proliferative response, and with the high level epithelial and mucus cell hyperplastic responses, these pollutants need consideration that warrants further investigation.

No evidence of commonly reported disease conditions of tilapia such as *Branchiomyces, Flavobacterium columnare* or *Saprolegnia* were evident (Soto, 2015).

All lamellar gill pathology, irrespective of aetiology, that results in an increased diffusion distance, will compromise respiratory, excretory and osmoregulatory health of the fish, with resultant physiological compromise, increased stress, poor health and growth (Huchzermeyer, 1993). Compounding factors like low DO and inappropriate water temperatures further exacerbate the stress challenge upon the host fish, with potential mortalities (Roberts and

Rodgers, 2012). The piping behaviour often encountered is reflective of this state of physiological compromise, but is often multifactorial in aetiology.

5.2.2.2 Hepatic pathology

Hepatocellular lipid content *(PLATE 9)*

In Pearson's analytical model, liver lipid shows a moderate positive correlation with CH:TA ratio, and low positive correlations with stocking rate and water DO levels *(Table 4-24)*.

Stepwise regression corroborates CH:TA ratio as the most significant predictor with a positive correlation, while DO served as the second-best predictor also with a positive correlation.

High hepatocellular lipid is a common feature of intensively farmed fish (Strussmann, 1995). In this study, generally poor body condition scores were recorded *(Table 4-9, Table 4-10)*, supported by histories of inadequate and low frequency feeding. Thus, the low hepatocellular lipid levels in the majority of fish (68.4%) *(Fig. 4-31)*, (together with the widely seen elevated lipofuscin in the liver), can most likely be primarily ascribed to starvation or anorexia secondary to chronic underlying stressors (Ellis *et al.,* 1978; Heath, 1995.a; Roberts and Rodger, 2012). Underfeeding as a management problem was highlighted using Timmons and Ebeling's KC calculations *(Table 5-4).* KC is used routinely within the aquaculture industry as a measure of total impact of key underlying stressors, including stocking density, nutrition, sub-optimal water parameters and toxins. Cumulative stress is known to reduce the KC, visceral fat and liver lipid. (Schlenk *et al.,* 2008).

The common low exocrine secretory activity (atrophy) seen in many pancreas sections was supportive of this fact. In addition, feed quality and nutritional deficits seemed likely to be a contributory factor in these findings that requires further investigation.

Correlations with underlying water parameters and stocking density need to be interpreted with caution. In this study, an increasing CH:TA ratio was associated with higher hepatic lipid levels *(Table 4-24)*. However, this correlation shows bias since the data was largely influenced by data from 2 farms i.e. farms GFa and GFb, where the highest CH:TA ratios >5 were found *(Table 4-7)*, in the presence of some fish with high lipid levels. Low prevalence of average farm level lipid levels <3.5 *(APPENDIX 4 TABLE 4: Data sheets)* contributed to the bias. Even so, hard to very hard water seemed to be a prevalent factor through systems with high hepatocellular lipid-rich fish with almost 88% of farms with lipid-rich fish prevailing in hard water over 150mg/L and 50% of systems reflecting these hepatic changes occurred in water with hardness above 350mg/L. Considering the CH:TA ratios of farms with highest lipid presence, 63%, although with low CH: TA ratios, were closest to the 1:1 ratio, with this ratio known to be conducive to optimum FCR.

Higher hepatocellular lipid levels seen with increasing DO levels correlated well with a study by Abdel-Tawwab *et al.,* 2014, who found a positive association between "body lipid" and DO levels. Favourable DO, temperature and pH levels, in particular, are also known to stimulate metabolism, feeding behaviour and nutrient absorption (Hollerman and Boyd, 1980; Huchzermeyer, 2015, Wedemeyer *et al.,* 1976.b). The low hepatocellular lipid levels seen

through the majority of fish (68.4%) were very likely also impacted by the low DO levels in the systems, acting as stressor and inhibiting appetites and metabolism.

Again, positive correlation with stocking density would have been skewed with farm GFb's high density of 33.3kg/m^3. Both other systems with highest average lipid (farms NE and GFa) stocked at 13.5 and 21.4kg/m^3.

Suspicion of severe oxidation resulting in rancidity of feed, evidenced by the high level of lipofuscinosis in parenchymatous organs, would very likely play a dramatic role in its effect on feed palatability, as well as its known negative impact on FCR (Huang and Huang, 2004).

Any potential toxins or pollutants within the water body or feed mycotoxins are unknown variables that could also play a role in hepatic lipid content with ability to cause reduction or accumulation of lipid (Wolf and Wolfe, 2005). Recognition of the fact that these and also other unevaluated factors in this study (Bruslé and Anadon, 1996) could have an impact on hepatic lipid content, is necessary to provide perspective of the correlations made here.

RISK ASSESSMENT OF FARMS WITH RESPECT TO AVERAGE HEPATOCELLULAR LIPID GRADE
(APPENDIX 4 TABLE 4 Data sheets)

Because of lack of available reference data on optimal hepatocyte cytoplasm to nuclear ratios in farmed tilapia, a zero-risk category was not included in this scoring.(Low risk: ≥ grade 3, moderate risk: ≥ grade 2< grade 3, high risk< grade 2). Note that values in the data sheet are lipid-scores, not grades, in recognition that high scores are reflective of an unhealthy system.

ZERO RISK: None

LOW RISK: Farms GFa, NE

MOD RISK: Farms GDb, GFb, GH, NA, NC, ND, NJ, LB, LC

HIGH RISK: Farms GA, GB, GC, GDa, GE, GG, NB, NF

Hepatic lipofuscin *(PLATE 10)*

Hepatic lipofuscinosis is a lipoid degenerative change reflecting exposure to free radicals from oxidized dietary oils or oxidative toxins like *Aflatoxin*, or pesticides. Accumulation in tissues may reflect excessive exposure or an underlying dysfunction in the ability of the body to neutralize the damaging free radicals and cations. The negative impact upon FCR, protein conversion and growth is high (Huang and Huang, 2004). Once high levels accumulate, restoration of normal function and FCR is unlikely (Cowey *et al.*, 1978; Hawkins *et al.,* 2002). This is significant, considering the high levels encountered in this study.

With 51.8% of fish affected, hepatic lipofuscin was a dominant finding in the study *(Fig.4-34)*. Together with varying hepatocellular levels, this resulted in great variation in macroscopic appearances of livers on necropsy, with higher hepatocellular fat predisposing to paler more friable livers, and higher lipofuscin, more orange-coloured livers.

The high levels of lipofuscin and lipoid degenerative changes raise the question of quality of feed used in the industry, particularly with respect to lipid/ unsaturated fatty acid quality, content and degree of oxidation (rancidity), and deficiencies of lipotrophic factors or antioxidants like tocopherols (Cowey *et al.,* 1978; Hardy, 2012; Roberts and Rodger, 2012), Vitamins E (Wedemeyer *et al.,* 1976.d) and C, Selenium (Gatlin, 2008), and amino-acid proportions. The source and causes of feed rancidity are variable, with correct storage protocol and exposure of raw materials and/or feed product to warmth and moisture throughout the entire value chain as one of the more important factors to consider.

Excess feed carbohydrate levels, often introduced in an attempt to spare protein percentage and thereby costs, are another factor that can contribute to lipoid degeneration (Wedemeyer *et al.,* 1976.b). Carbohydrate content of feed requires further investigation in an attempt to assess its role in predisposing to lipofuscin accumulation.

CO_2 showed a significant moderate positive correlation with liver lipofuscin levels, and temperature a significant low negative correlation, using Pearson's analytical model *(Table 4-25)*. This model proposes CO_2 levels as the best predictor of liver lipofuscin.

Increasing lipofuscin reflects an increase in accumulation of cellular debris (degraded cell membranes), secondary to deteriorating health and tissue damage. With elevated CO_2 levels and low temperatures featuring so strongly as key independent variable stressors in the study, their potential impact upon fish feeding behaviour is high. It is very likely that tilapia faced with cold water, low in oxygen and high in CO_2, suffer from anorexia. The high prevalence of poor hepatocellular lipid together with high lipofuscin strongly supports this. Roberts and Agius, 2003, also associate increased pigment levels in melanomacrophage centres with catabolic tissue breakdown processes in cachectic disease, environmental stress, low oxygen levels and chemical pollutants, which certainly relates well to the general low body condition of fish assessed, and generally suboptimal critical water quality parameters.

Lower water temperatures, with known negative effect upon metabolic processing (El-Sayed, 2006.b; Loh and Landos, 2011.e; Roberts, 2012.a), may also predispose to lower anti-oxidant enzyme function, with resulting increased lipid oxidation susceptibility. Lower temperatures are also known to potentiate the effect of toxins like pesticides upon fish, and this may be playing a

contributory role in the degree of lipofuscinosis seen (Loh and Landos, 2011.e). Environmental situations of low DO and/or high CO_2 have been shown to negatively impact multiple metabolic, respiratory and circulatory processes within the body and may play a significant contributing role. Increased anaerobic metabolism may occur in situations of higher CO_2, or low DO, with higher free radical production and hence, higher oxidative damage to tissues. A study by Strobel *et al.,* 2013, suggests a compensatory trade off where metabolic adjustments in the liver occur to allow for the hypercapnic-induced increased metabolic activity in red muscle and heart.

It is interesting that farms with high CO_2 >60mg/L featured as 52.8%, closely matching the % of fish with high lipofuscin, and all farms with high level lipofuscin in fish correlated with CO_2 readings ≥45mg/L.

RISK ASSESSMENT OF FARMS WITH RESPECT TO AVERAGE GRADE HEPATOCELLULAR LIPOFUSCIN *(APPENDIX 4 TABLE 4 Data sheets)*

(With Zero risk= none, Low risk: >0 ≤ grade 2, moderate risk: > grade 2 ≤ grade 4, high risk: > grade 4 ≤ grade 6)

ZERO RISK: None

LOW RISK: Farms GDa, LB, LC

MOD RISK: Farms GA, GB, GDb, GG, GH, ND,NE, NF, NJ

HIGH RISK: Farms GC, GE, GFa, GFb, NA, NB, NC

Hepatocellular nuclear activity *(Figs. 4-36, 4-37)*

Activation of the liver nuclei with resulting anisokaryosis and karyomegaly on histopathology, is a non-specific pathologic finding but reflects livers in an activated state of physiological

processing, often involved in detoxification processes (J. Steyl, personal communication, July 2018). Large pale nuclei reflect a state of active transcription while small basophilic nuclei suggest the opposite and low activity (Majno and Joris, 1996.b). Links have been proposed between hepatocellular nuclear pleomorphism and early liver neoplastic change, possibly due to chronic toxicity (Hinton *et al.,* 2008). Karyolysis, which is a process of nuclear disintegration or lysis (Reimschussel ,2008) was not seen.

Pearson's analytical correlations *(Table 4-26)* highlight two factors affecting hepatocyte nuclear activity: temperature and CH:TA ratio, both having a low predictive positive value on liver nuclear activity.

With ideal CH:TA ratio identified as 1:1 and ratios above this believed to exert increasing osmotic stress upon fish, and increased acidification of water, the associated liver nuclear activity may well reflect the dramatic physiological challenge that these conditions are subjecting fish to. The low buffering capacity of water associated with low alkalinity so often seen with these ratios, further acts as an additional stressor, often associated with dramatic pH swings over the 24-hour cycle. Impact of increased sulphates and chlorides in the water body, associated with increased hardness, could also play a role. CH:TA ratio may possibly have been highlighted in our data analysis because of the extremely high ratios seen on farm GFa and GFb, of over 12:1. Although the mechanism remains speculative, it is worth noting that lower ratios with their tendency to alkalinize water, do not have the same impact upon hepatocellular nuclear activity.

Positive correlation with temperature may reflect increased physiological processing associated with increased metabolic activity in warmer waters. In addition, there may be an indirect link between warmer water and higher environmental temperatures, with associated increased risk of rancidity and mycotoxins in feed. i.e. there may be a tendency for increased hepatic nuclear activity through summer months when water temperatures rise, not only because of increased metabolic rates but also as a result of increased risk to mycotoxins and oxidative stress (rancidity) of feed.

RISK ASSESSMENT OF FARMS WITH RESPECT TO AVERAGE GRADE HEPATOCELLULAR NUCLEAR ACTIVITY *(APPENDIX 4 TABLE 4 Data sheets)*

(With Zero risk=Farm average grade: 0 , Low risk= Farm average grade: >0 < 0.33, Moderate risk= Farm average grade: ≥ 0.33 < 0.66, High risk= Farm average grade ≥ 0.66)

ZERO RISK: Farms GE, GG, NC

LOW RISK: Farms GC, GDa, GH, LC

MOD. RISK: Farms GFa, GFb, NA, NE, NF

HIGH RISK: Farms GA, GB, GDb, NB, ND, NJ, LB

5.2.2.3 Gastric pathology

Gastritis *(PLATE 13)*

Microscopic cellular infiltrates (EG and lymphoid), typical of an inflammatory nature, were found through 98.8% of fish gastric walls. A high percentage (7%) showed grade 5 severity *(Fig. 4-38)*. This was macroscopically supported with many stomachs showing evidence of hyperaemia at necropsy *(PLATE 4.A)*.

Pearson's correlation model between water parameters, stocking density and levels of gastritis, shows a moderate negative correlation between stocking rate and degree of gastritis *(Table 4-28)*. Similarly, also DO, CH:TA ratios and CO_2 showed low negative correlations.

A stepwise regression model summary shows that the set of independent variables explain 41.2% of the variability of the gastritis ($p< 0.01$), which is a significant figure.

The strongest predictor was stocking density, in a negative role and with stocking densities less than or equal to 11.5kg/m^3 correlating the best with fish showing the most severe level of histo-pathological lesions (fish stocked at over 11.5kg/m^3 presented with gastritis at low levels only). This finding was somewhat surprising, and the explanation remains speculative, although it is possible that very low stocking densities may result in social stress in the fish population, similar to the study by Speare, 2008, where low stocking densities in captive Salmon populations resulted in increased territorial behaviour and aggression. The high prevalence of mixed sexes within population groups assessed and resultant breeding behaviour, may be playing a role with easier access to females in higher stocked systems. Other factors affecting the probability of gastritis other than the water parameters assessed in this study remain elusive and require further investigation.

Although EGC's are a known normal finding in intestinal salmonid connective tissue (Roberts and Ellis, 2012), the extreme variation encountered in the gastric mucosae and submucosa together with other inflammatory cell infiltrates, the degree of gastric epithelial degenerative changes (cellular vacuolation and apoptosis), and association with severe erosive lesions suggested an abnormal finding with high-level activation of the immune response (Reite and

Evenson, 2006). According to Roberts and Rodger, 2012, gastric pathology is uncommon in fish, but underlying stress is a potent potentiator of gastritis. The pathogenesis of stress-gastritis in mammals has been shown to involve three key mechanisms: increased gastric acid production, decreased mucosal resistance and reduced blood flow to the mucosa (Megha and Lopez, 2019). It may be that underlying triggers predisposing to similar changes in fish, are responsible for the resulting erosive gastritis seen. Both stress and gut stasis (associated with anorexia or starvation) have been shown to negatively impact protective gastric mucus production, exposing the mucosae to the potential digestion and ulceration from the luminal acid (HCl) and enzyme content (Smith, 1980). Although apoptosis was not as prevalent a finding as EG cell and lymphoid infiltration, it was however, often noted. Again, it may be yet another indication of sub-optimal nutrition and starvation (Roberts and Rodger, 2012) or effect of hypoxic water conditions (Nikinmaa, 2014). This would corroborate with Pearson's analysis where DO was the next most significant variable impacting upon prevalence of gastritis, with a negative correlation *(Table 4-28)*. Negative correlation with CO_2 needs to be interpreted with caution, as all CO_2 measurements in this study were above 25mg/L and classified as low to high risk. The resulting hypercarbia and acidosis associated with these elevated levels has been shown to act as a predisposing factor in the pathogenesis of erosive gastritis in humans (Silen, 1985), and mechanisms may be similar in fish. I would suggest this may be more likely a positive correlation, and warrants further research. Negative correlation with CH:TA ratio is most likely incidental. Those farms with most severe levels of gastritis, showed hardness readings between 100-460mg/L, while alkalinity ranged from 94-400mg/L. Resulting CH:TA ratios varied between 0.6-2.5. No patterns can be seen, and I would speculate that gastritis prevailing, is associated

with other aetiology. In this study a number of potential stressors, either featuring prominently and/or in combination with other low-level stressors, could culminate in the development of gastritis. It stands to reason that this condition may be a very important indicator of stress, albeit non-specific. The potential impact of structural disruption to the gastric wall could be dramatic in terms of affecting digestive function and optimal utilization of feed, and create a situation of functional starvation with resultant cachexia, weight loss and poor growth. In fact, in all farms where sub-optimal growth was recorded (NE, LB, NA, GG, GFa, GFb, GA, GDa, GE, GH, NJ) *(Table 4-8)*, gastritis was present. Farms GE, GH, and GA, which were three of the five farms with highest levels of gastritis (farms LC and NF had mixed age populations and growth could not be assessed), showed severely underperforming fish, with growth falling far below an optimum growth curve (*Table 4-8).*

In addition, many gastric histopathology sections showed evidence of intra-luminal parasites (150-200 µm length) *(PLATE 14)*, as well as regular occurrence of granulomatous cysts (suspected to be parasitic in origin) (25-200 µm length) within the mucosal and submucosal layers in many fish throughout many systems. Although uncommon in cultivated fish in the absence of intermediate hosts, these are most likely to be nematodes like *Contracaecum* spp. Other possible differentials would be adult digenean trematodes, however cysts encountered were smaller than typical digenean trematodes (1-5mm in length) (Noga, 2010.c; Shinn, 2016). Nematodes are known as highly invasive, often penetrating the gut wall and triggering an inflammatory response (Noga, 2010.c). The role of this finding in the pathogenesis of gastritis remains unclear, but could be playing an important role in the gastric inflammatory response observed, as a reflection of mobilization of the immune response (Leino, 2001). It should be

noted that the presence of cysts was always associated with a grade 4 or 5 gastritis, but gastritis was not always associated with presence of cysts. In intensive culture conditions, heavy gut parasitism is not uncommon with resulting gut pathology (Roberts and Rodger, 2012). Other potential causes of gastritis that could not be assessed within this scope of this project, but would have to be considered, would be infectious causes like viral diseases, bacterial disease like *Francisellosis* and *Edwardsiellosis* and, again, the effect of water pollutants or contaminants (Reimschussel, 2008). Evidence of apoptosis in gastric mucosal cells would support this (Reimschussel, 2008).

Irrespective of underlying aetiology, these changes are important in terms of their reflection of poor fish health, indicating acute tissue damage, EG cell degranulation, release of inflammatory mediators and cytokines and chronic inflammation (Reite and Evenson, 2006).

RISK ASSESSMENT OF FARMS WITH RESPECT TO AVERAGE GRADE GASTRITIS *(APPENDIX 4 TABLE 4: Data sheets)*

(With Zero risk: ≤ grade 1, Low risk: > grade 1 ≤ grade 2, moderate risk: > grade 2 < grade 4 , high risk: ≥ grade 4

ZERO RISK: None

LOW RISK: Farms GB, GC, GDa, GDb, GFb, GG, NA, NB, NC, ND, NE, NJ, LB

MOD RISK: Farms GA, GE, GFa, GH, NF

HIGH RISK: Farms LC

5.2.2.4 Other pathological observations

Adipocytes, as seen in some portal triads, are simply fat storage cells (Genten *et al.,* 2009.c) and considered a normal finding. Portal adipose deposits showed no clear correlation with body

condition of fish or hepatocyte lipid levels and seemed to be a random finding *(Fig. 4-33)*, while lipofuscin deposits in melano-macrophage centres (MMC) in the portal triads *(PLATE 11)* correlated moderately well with generalized hepatic lipofuscin levels *(Fig. 4-35)*, as well as lipofuscin disseminated through hepatocytes, and splenic and anterior kidney MMC lipofuscin. This finding indicates a systemic nature to lipofuscin deposition, suggestive of systemic oxidative injury instead of only affecting the liver.

Cytoplasmic eosinophilic "laking" within the hepatocytes was seen in 9% of all liver sections *(PLATE 12)*. This change indicates hyperplasia and expansion of the smooth endoplasmic reticulum and is commonly associated with exposure to xenobiotics (Maxie, 2015). Again, this hepatic reaction provides evidence of possible non-specific toxin exposure and highlights the need for further research in this area.

Infiltration of eosinophilic granular cells was quite prevalent: 8% of liver sections *(PLATE 12B)*, 25% of splenic sections *(PLATE 15E)* and 2% of posterior kidney sections, as well as in the anterior kidney. These reflect stimulation of the immune response (Reite and Evenson, 2006). They are either associated with chronic or chronic-active inflammatory processes (Reimschussel, 2008). Parasitic infestation may cause an increased number (Reimschussel, 2008).

Apoptosis was an uncommon finding, with greatest evidence seen in the gastric mucosa. Low evidence was seen in anterior kidney and liver sections, reflecting degenerative change associated with non-specific aetiology, inclusive of normal cell-death processes, infectious or chemical exposure or the common cachectic state.

Lymphoproliferative foci (follicular hyperplasia) were prevalent in many splenic sections (42.3%)*(PLATE 15.D)* , to a lesser degree in liver sections (4.2%). Interstitial lymphoid infiltration was observed in anterior kidney sections as well. They were recorded as present or absent. They are considered one of the key cells within the immune system and their presence reflects chronic inflammation and cell-mediated immune stimulation (Roberts and Rodger, 2012) in response to degenerating / injured tissue, on-going infectious processes (antigen presence), or immune mediated disease (Reimschussel, 2008). In the absence of significant degenerative pathology or signs of immune-mediated disease, infectious disease (antigen circulation) is the most likely inciting cause in these fish populations. Although widespread through the sampling population, splenic foci were small and of low prevalence within most splenic sections. With the absence of overt clinical signs, infections, if present, were subclinical in most fish, or indicated previous exposure and recovery. Serological, molecular and biological screening for underlying bacterial or viral aetiology is necessary to determine specific infectious disease presence and exposure.

Many fish showed evidence of cyst-like lesions in the parenchymatous organs, especially within the gastric submucosal layer *(PLATE 14A)*, anterior kidney *(PLATE 16E, F)*, and spleen *(PLATE 15G, H)*. Most were characterised by central cavity devoid of content (possibly a processing artefact) surrounded by a thin to moderately thick fibrous capsule and scant number of macrophages. These varied in size between 25-200 µm in diameter. One of these structures in the anterior kidney contained what appeared to be a parasite of metazoan origin *(PLATE 16F)*.The specific organism could not be identified but is suspected to be the intermediate / tissue stage of a parasitic species. With the high number of systems where freshwater snails

were present *(Table 4-35)*, digenean trematode infection forming these cystic structures in tissues should be considered an important differential diagnosis (Loh and Landos, 2011.h; Noga, 2010.c). The Digenean life-cycle always includes a mollusc or invertebrate like snails as intermediate hosts, and birds, fish or mammals as final hosts. Larval stages are very commonly seen encysted in fish in their metacercarial form, waiting to be ingested by the final host to complete the lifecycle (Needham and Wootten, 1978). But cysts tend to be larger (1-5mm) (Noga, 2010. c; Shinn, 2016) and macroscopically visible. These parasites are also uncommon in cultured fish (Shinn, 2016). Nematode infections e.g. *Contracaecum* would be another differential. Again, although uncommon in closed aquaculture systems (Shinn, 2016), where exposure to other host species (birds, mammals, or invertebrates) is possible, these parasites can become problematic. Molluscs like snails, often act as intermediate hosts and larvae encyst in fish (Noga, 2010, c). However, nematodes can complete a life cycle without a mollusc, where another fish acts as the intermediate host. Many species of nematodes are known to invade the gastric mucosa, inducing a marked inflammatory reaction (Noga, 2010.c), which would tie in well with our findings. Looking at those systems with high cystic prevalence through assessed fish, Farm GE had heavy snail burdens in the system and cysts were detected in liver, anterior kidney, spleen, posterior kidney and gastric walls *(PLATE 14A)*, and affected 70% of the fish assessed. A live suspected *Contracaecum* larvae (L4) was discovered in the pericardial cavity of one fish *(PLATE 4D)* (D. Huchzermeyer, personal communication, 9th January, 2019; Paperna, 1996.d). This particular farm had a history of introducing wild- caught fish as well as offering poor system biosecurity, with open access to birds.

Farm GF, also with high snail infestation *(Fig. 4-44D, E)* , had 50% of fish positive with cystic structures in anterior kidney, gastric wall, or spleen *(PLATE 15H)*. Farms LB and LC also presented with large numbers of cysts, and in addition, fish from farm LC showed suspected parasitic migratory perforations in intestinal walls as well *(PLATE 4C)*. These both had snail infestations. Both nematode and Acanthocephalan infestations have been associated with such perforative lesions (Noga, 2010.c). The fact that three of ten fish showed the same pathology, is interesting, and the long-term health implications in this population likely to be serious. Cystic structures were also seen on histopathological examination on fish in 6 other systems (NJ, NE, NF, GC, GH, NC, ND), where snails had not been observed. So, although there was strong correlation between some of the farms with widespread tissue cysts and known high levels of snails, the high prevalence of cysts in non-snail systems, make it more likely that nematodes are primarily involved. With most tunnels, being open, especially during the day, birds, acting as final hosts, would have had access to all systems.

Cryptobia iubilans would be another differential, with its predilection for *Cichlid* spp., and presentation with widespread cyst formation, particularly in the spleen and liver (Noga, 2010.c). Other differential diagnoses would be tissue coccidian spp. or bacterial granulomas. There was no correlation with open water systems (Farms NA and NB) where river water was routinely used.

Metacercarial stage cysts in gill lamellae, typical of *Centrocestus* spp*.,* were not seen.

There are many other accounts of granulomatous disease of cultured fish, with causes ranging from *Mycobacteria*, *Pasteurella multocida, Dermocystium*- like organisms, or *Rickettsia*- like

organisms, to non-infectious causes related to feed that had been stored for prolonged periods (Hawkins *et al.,* 2002). So, all these would have to be considered as differential diagnoses.

It is important to note that endoparasite assessment was not included as a focus of this study and techniques for assessment were not comprehensive enough to assess this group of parasites adequately. With the high prevalence of cysts encountered and high levels of gastritis, and potential zoonotic impact (Noga, 2010.c), further focused research on the pathological and economic impact of these parasites in farmed tilapia would be sensible. Despite the macroscopic endo-parasitic migratory tracts seen on farm LC, no supportive intestinal pathological changes or intra-luminal parasites were observed in any fish.

Similar to Huchzermeyer's, 2003, findings, between splenic size and immunologic activity in crocodiles, the positive correlation seen between extremely enlarged spleens, relative to the heart (>4:1), and presence of septicaemic disease could be used as a useful indicator of infectious challenge. Although small or moderate splenic enlargement may well be masked by stress or chronic disease (Huchzermeyer, 2003) or activity-induced splenic contraction (Suzuki and Yokote*,* 1995; Wedemeyer *et al.,* 1976.c) in the capture process, however protocol followed was identical through all fish, and all would have been equally impacted and chronic underlying multifactorial stress seemed to be such a widespread finding, impact was probably largely uniform through systems assessed. Red pulp hyperplastic changes *(PLATE 15F),* generally reflect proliferation of macrophages, which reflect a response to injury like chronic haemolytic disease or in response to a pathogenic exposure (Fry and McGavin, 2012). Common causes of

haemolysis in fish are *Vibrio* spp. or blood-borne protozoa (Roberts and Rodger, 2012). Assessment of these potential stressors did not form part of the study.

The increased number and size of the MMC's through haemopoietic parenchymatous organs were dominated more by the high lipofuscin deposits, rather than increased melanin, and are likely to be as a result of the same triggers causing higher hepatic lipofuscin levels. The high MMC lipofuscin *(PLATES 15C and 16C)* is associated with starvation in fish (Hardy, 2012). This was most evident in liver and splenic tissues. A number of other trigger factors could be associated. Splenic MMC's are known to increase in number and size in unhealthy or chronically stressed fish (Genten *et al.,* 2009.b), while anterior kidney MMC's have been shown to increase in environmental stress conditions (Genten *et al.*, 2009.b). Cachectic disease (Cowey *et al.*, 1978) or starvation (Hardy, 2012; Roberts and Rodgers, 2012) as well as dietary imbalances, age and sex of fish (Reimschussel, 2008) are other common underlying reasons for increased prominence of MMCs. Although a rare finding, the haemopoietic tissues (spleen and anterior kidney), also showed evidence of MMC rupture and scattering of the pigment granules, which suggest a toxaemic insult (Roberts and Rodger, 2012) *(PLATE 18)*.

The fairly high number of pancreas tissue sections showing acinar cell atrophy *(Fig. 4-39)* with low zymogen activity, reflected poor recent feeding activity in many fish (Hardy, 2012), and could be associated with inadequate feed or anorexia associated with feed quality, environmental stressors, or a clinical sign of poor health.

Hyaline deposits in renal tubules were an infrequent finding *(PLATE 17H)*, but their implications could be serious. Causes to be investigated further would be heavy metal toxicosis (Roberts and Rodger, 2012) or glomerular dysfunction (Endo and Oguri, 1995.b; Reimschussel, 2008).

Gas supersaturation or gas bubble disease was not an obvious prevalent problem through systems assessed, bar one fish (Farm GA), where a single gas bubble was observed in the gill lamellum under light microscopy. Because of tilapia's high tolerance for high oxygen saturation levels, this is most likely due to nitrogen or CO_2 supersaturation (Noga, 2010.e). The gas bubble seen is a pathognomonic clinical manifestation and reflects a total gas pressure in this system higher than barometric pressure (Noga,2010.e). Further investigation would be necessary to assess population prevalence and underlying aetiology. Chronic low supersaturation can exist sub-clinically just with grumbling low-grade mortalities, secondary opportunistic infections, and low stress-tolerance (Noga, 2010.e), and may well be an inapparent factor through farms. It would benefit from further research, with measurement of total concentration of gas in the water sources, and careful examination of systems for potentiating factors.

The high prevalence of posterior interstitial renal mineralization *(PLATE 17G)* is a concern as it reflects a degenerative process (Reimschussel, 2008). However, the degree encountered in each fish was low. Looking at the possible positive correlation between renal mineralization and high CO_2 levels (Southgate, 2005), 73% of all fish with evidence of mineralization, were in water with high CO_2 levels over 60mg/L. Yet, there were systems with CO_2 levels over 100mg/L where no evidence of mineralization was seen. Although a positive correlation is suggestive, a more focused study with larger sample size would be needed to investigate further. Despite the

proposed positive literature correlation (Loh and Landos, 2011.e), water hardness did not correlate as dramatically as CO_2**,** however, 55% of systems with affected fish, measuring hardness levels ≥ 176mg/L, showed evidence of renal mineralization. Protein (Wedemeyer *et al.,* 1976 d), calcium and magnesium (Gatlin 2008; Roberts and Rodger, 2012) imbalances in the feed could potentially be contributing factors and would benefit from further research.

5.2.2.5 Evidence of other disease: Bacterial, viral, water moulds of concern

Although anterior kidney aerobic cultures were only performed on a small percentage of fish *(TABLE 4-17)*, no significant pathogens like *Streptococcus iniae* and *S. agalactiae , Yersinia ruckeri*, or any *Edwardsiella* spp. were cultured.

Aeromonas spp. (Motile *Aeromonas* Septicaemia), was to be expected as it is a ubiquitous bacterium within the aquatic environment and all freshwater fish are considered susceptible. Any stressor could potentially compromise fish immunity and predispose to secondary *Aeromonas* septicaemia (Noga,2010.b). *Aeromonas* also often presents with chronic low-grade mortalities which was a common thread through almost 90% of farms assessed.

Pathology or clinical signs, typical of Streptococcal diseases like, petechiation and haemorrhage of trunk and viscera, or areas of focal necrosis, and erratic swimming or high mortalities, were not seen. However, meningoencephalitis *(PLATE 18A)* which is a common additional, but non-pathognomonic, pathological finding (Noga, 2010.b), was evident in 17.9% of fish. Pericarditis (seen in 28% of fish), *(PLATE 18D and E)* can also reflect *Streptococcal* spp. septicaemia (Soto, 2015). Chronic manifestation of disease tends to be associated with cooler water temperatures

and acute disease with warmer temperatures (The Fish Site, 2006). With 47% of farms assessed, presenting with temperatures below 25 ^{o}C, this may be playing a role in suppressing clinical evidence of this disease in these systems. Low prevalence of ammonia toxicity, as a predisposing factor (Huchzermeyer, 1993), may also play a role. *Lactococcus garviae*, as part of the *Streptococcal* group, is a globally distributed aquatic pathogen, and already recognized in South African waters (Meyburgh *et al.*, 2017). It is associated often with poor water quality and underlying poor husbandry practice. The system where this pathogen was encountered (Farm ND), was characterized with multiple stressor factors including sub-25 ^{o}C water temperature, low DO, high CO_2, high ammonia and nitrite, in the presence of hard water with a climbing CH:TA ratio.

Further research into sub-clinical prevalence and impact, of this group of pathogens in South Africa would be valuable.

Considering the bacterial isolations from the study:

Table 5-5 Categorization of bacteria isolated:

Opportunistic bacteria (facultative pathogen)	**Obligate pathogen**	**Potential emerging fish pathogens?**	**Contaminants**
Aeromonas hydrophila ***(AH)***	*Lactococcus garviae* **(LG)**	*Myroides (flavobacterium) odoratum* **(MO)**	*Staphylococcus pseudointermedius* **(StP)**
Shewenella putrifaciens **(SP)**		*Aeromonas schubertii* **(AS)**	*Acinetobacter* **(A)**
Brevimundus vescicularis **(BV)**			
*Bacillu*s spp. **(B)**			
Staphylococcus epidermidis **(StE)**			

The *Aeromonas spp.* group are widely known as common opportunistic fish pathogens, ubiquitous in the environment, but targeting immune-compromised fish with damage to the skin barrier or primary parasitic lesions, and causing septicaemic disease and mortalities (Noga, 2010.b). In fact, all farms where *Aeromonas* spp., were cultured, had extremely high levels of *Trichodina* and/or *Ichthyobodo necator.* Correlation with higher water temperatures (Ibrahem *et al.,* 2008) was not seen, with positive cultures associated with water temperatures of both 29.2 and 21.1^{o}C. Although clinically apparent on farm ND *(Fig. 4-27)*, it is highly likely that this is more prevalent as a subclinical infection in systems, and contributing to low grade chronic mortalities. *A. schubertii*, however, seems generally to be not considered a fish pathogen, and there is only one record of it being isolated as a causative agent of disease, in an epizootic outbreak in Snakehead fish in China (Liu and Li*,* 2012). It is of interest, however, that it was

cultured in three of the 11 cultures performed, and may need monitoring as a potential emerging pathogen. It was associated with macroscopic evidence of thinning of the body wall, as well as peritonitis in two of the three positive cultures, however the remaining positive culture showed no evidence of macroscopic lesions or pathology. *Brevimundus vesicularis* is regarded as an opportunistic bacterium, belonging to the pathogenic *Pseudomonas* group (Henton,2018). As with *A. schubertii,* it also associated on necropsy with thinning of the body wall. However, both fish displaying this pathological change, were tested in the same group- it may well be that other factors like viral disease or nutrition, could have been the causative agent of this pathological change.

Bacillus spp. are common contaminants, used as fish probiotics, but rare pathogens (Henton, 2018), yet, have been implicated in reports of fish disease (Ferguson *et al.*, 2001; Oladosu *et al.*, 1994), and *Staphylococcus epidermidis*, although also generally considered a contaminant rather than a common fish pathogen (Henton, 2018), was the cause of a severe ulcerative disease outbreak in sea bream (Kubilay and Ulukoy, 2004) and mass mortalities in tilapia (Huang *et al.*, 1999). All cases are suggestive of a pathogen with an opportunistic approach. The *Bacillus* spp.(fish GF5) cultured, showed clinical association with suspected subcutaneous haemorrhage.

Shewenella putrifaciens has only been described once (Austin *et* a*l.*, 2007), as a fish pathogen in Saeed *et al.*'s, 1987, account of high mortalities in rabbitfish.

Myroides odoratum, belonging to the *Flavobacterium* group, commonly aggregate as part of the normal fish biofilm (Jacobs and Chenia, 2009), but considering the number of pathogenic

members of the *Flavobacterium* group, as well as the heavy anterior kidney culture seen- it would be worth watching closely for future signs of emerging pathogenicity or opportunism.

Acinetobacter is regarded as a contaminant with low pathogenicity, (Henton, 2018), but has been associated with haemorrhagic disease in Atlantic Salmon (Roald and Hastein, 1980). *Staphylococcus pseudointermedius* is a known mammal pathogen, not a fish pathogen (Henton, 2018).

General correlations between septicaemic disease and external findings, or macroscopic pathology on necropsy were poor, with only 66% of fish with confirmed septicaemic disease showing external lesions: 27% exophthalmos, 18% thinning of body wall, and 9% suspected subcutaneous haemorrhage. Only 45% of septicaemic fish showed possible correlations with macroscopic pathology: 9% with retrobulbar cellulitis, 18% with peritonitis, 9% with a steatitis, and 9% (1 fish) with a live suspected nematode in the pericardial cavity. With 27% of positive septicaemias confirmed in the absence of any associated macro-pathological or external lesions, it can be inferred that these diagnostic modalities are not adequate enough to confirm suspicion or presence of septicaemic bacterial disease.

RISK ASSESSMENT OF FARMS WITH RESPECT TO SEPTICAEMIC BACTERIAL DISEASE

(With High risk = presence of any obligate or emerging pathogens on appropriate organ or blood culture, Moderate risk =presence of opportunistic bacterial on anterior kidney or blood culture, Low risk = absence of these)

LOW RISK: Farms GA, GB, GC, GDa, GFb, GH, NA, NB, NC, NE, NF, NJ, LB

MOD RISK: Farms GE, LC

HIGH RISK: Farms GDb, GFa, GG, ND

5.2.3 Growth

Growth of fish populations has been extensively discussed through all relevant sections. It is clear, with only 15% of systems measuring above potential fish weight under optimum water temperatures, and 69% under 75% of their potential weight, that the cumulative effects of the large number of independent variables assessed, has one of its greatest negative impacts upon growth of fish *(Table 4-8)*. Taking into account temperature as a factor, farms still compare in a similar manner, indicating the multifactorial causes involved. Key factors playing a role in this study are water DO, temperature (particularly low), high nitrite, high stocking densities, high CO_2, poor quality and quantity of feed, and potentially high parasitic burdens. Although not assessed, chronic subclinical infections and chronic exposure to potential toxins may be playing a significant role.

One must bear in mind that growth was assessed at one point in time in this study. For thorough assessment of this parameter, population weights would need to be assessed over a period of time with multiple readings. With rapid growth of fish with good FCR being the primary objective of an aquaculture facility, further research would be valuable.

With consideration of the importance of good levels of growth within a system as a measure of good productivity, farms were assessed in the following way:

RISK ASSESSMENT OF FARMS WITH RESPECT TO GROWTH *(See Table 4-8)*

(With High risk = Extremely poor growth < 70% temperature appropriate growth , Moderate risk = Poor growth ≥ 70% < 85% temperature appropriate growth, Low risk = Moderate growth

≥ 85% <100% temperature appropriate growth , Zero Risk= Adequate growth ≥ 100% temperature appropriate growth)

ZERO RISK: Farms GB, NJ

LOW RISK: Farms GDb

MOD RISK: Farm NE

HIGH RISK: Farms GA, GC, GDa, GE, GFa, GFb, GG, GH, NA, LB

(Farms NB, NC, ND, NF, and LC could not be assessed due to mixed age and size populations)

5.3 A HEALTH ASSESSMENT MODEL FOR SOUTH AFRICAN TILAPIA RAS SYSTEMS

This study has highlighted some of the most important factors to assess when evaluating overall health of an aquaculture unit. It is understood that these factors, in a culture system, act additively or synergistically to form complexes with effect upon fish health (Shepherd, 1978) , as well as exerting significant individual impact. By taking into account and scoring key independent and dependant variables within each system as no risk: 0, low risk: 1, moderate risk: 2, and high risk: 3, an overall health score could then be matched against an ideal as a measure of overall system health.

This model *(TABLES 5-6 and 5-7)* summarizes and highlights the key independent variables in this study impacting upon fish health as CO_2, nutrition and NO_2^-, with greatest impact seen upon liver lipid and lipofuscin levels, and fish growth. Individual farm scores were used to offer feedback to farmers on key areas requiring attention and key health issues at play.

Table 5-6 Overview of scoring of independent variables through farms assessed, with highest risk variables highlighted red, moderate risk orange, and low risk green

Farm	DO	T	CO_2	NH_3-UIA	NO_2^-	CH:TA	SD	pH	N	TOTAL/27	FARM RISK
GA	2	1	3	0	3	2	1	0	3	15	MOD
GB	1	0	3	0	2	1	2	0	3	12	MOD
GC	0	1	3	1	1	2	1	0	3	12	MOD
GD(a)	1	0	1	0	2	1	1	0	2	8	LOW
GD(b)	2	0	2	1	3	1	1	0	3	13	MOD
GE	2	0	2	1	3	1	1	0	3	13	MOD
GF(a)	1	0	2	0	2	3	1	1	3	13	MOD
GF(b)	3	1	3	0	3	3	3	0	3	19	HIGH
GG	2	0	2	0	3	2	1	1	3	14	MOD
GH	0	0	3	1	3	1	0	1	2	11	MOD
NA	0	2	3	0	1	1	2	0	3	12	MOD
NB	0	2	2	0	0	1	1	1	3	10	MOD
NC	2	2	3	2	0	1	3	1	3	17	MOD
ND	3	2	3	1	3	2	2	0	2	18	HIGH
NE	3	2	3	0	3	1	2	0	2	16	MOD
NF	0	2	3	0	1	1	0	1	3	11	MOD
NJ	2	2	2	1	2	1	0	0	2	12	MOD
LB	1	3	1	0	0	1	3	1	2	12	MOD
LC	0	3	1	0	1	1	1	0	3	10	MOD
AVE.	1.3	1.2	2.4	0.4	1.9	1.4	1.4	0.4	2.7		

(DO= dissolved oxygen, T= temperature, CO_2= carbon dioxide, NH_3-UIA= un-ionized ammonia, NO_2^- = nitrite, CH:TA = complete hardness to total alkalinity ratio, SD= stocking density, pH= pH, N= nutrition)

ZERO FARM RISK =0 score

LOW FARM RISK= Score >0 <9

MOD FARM RISK= Score ≥9 <18

HIGH FARM RISK= Score ≥18

Table 5-7 Overview of scoring of dependant variables through farms assessed, with highest risk variables highlighted red, moderate risk- orange, and low risk green

Farm	T	G	D	I	A	EH	GH	EGC	LL	LF	LN	G	GR	S	TOTAL/42	RISK
GA	0	0	0	0	0	1	0	2	3	2	3	2	3	0	16	MOD
GB	1	0	1	0	0	2	1	2	3	2	3	1	0	0	16	MOD
GC	0	1	1	0	0	1	0	2	3	3	1	1	3	0	16	MOD
GD(a)	2	1	1	0	0	1	0	2	3	1	1	1	3	0	16	MOD
GD(b)	2	0	0	0	0	0	1	2	2	2	3	1	1	2	16	MOD
GE	1	0	1	0	0	1	1	1	3	3	0	2	3	1	17	MOD
GF(a)	1	1	0	0	0	3	1	2	1	3	2	2	3	1	20	MOD
GF(b)	1	0	0	0	0	1	0	1	2	3	2	1	3	0	14	MOD
GG	1	1	1	0	0	1	1	2	3	2	0	1	3	1	17	MOD
GH	0	0	0	3	0	1	2	2	2	2	1	2	3	0	18	MOD
NA	1	1	1	1	0	1	1	2	2	3	2	1	3	0	19	MOD
NB	1	0	1	1	0	1	1	2	3	3	3	1		0	20	MOD
NC	0	0	0	0	0	1	0	1	2	3	0	1		0	11	LOW
ND	0	1	0	1	0	2	1	2	2	2	3	1		2	20	MOD
NE	1	0	0	0	1	2	1	2	1	2	2	1	2	0	15	MOD
NF	1	1	1	0	0	2	1	1	3	2	2	2		0	17	MOD
NJ	0	0	1	0	0	1	0	2	2	2	3	1	0	0	12	LOW
LB	2	1	1	0	1	2	2	2	2	1	3	1	3	0	21	MOD
LC	1	1	1	0	0	2	2	2	2	1	1	3		1	20	MOD
AVE.	0.8	0.5	0.6	0.3	0.1	1.4	0.8	1.8	2.3	2.2	1.8	1.4	2.4	0.4		

WHERE ZERO FARM RISK =0 Score

LOW FARM RISK= Score >0 <14

MOD FARM RISK= Score ≥14 <28

HIGH FARM RISK= Score ≥28

CHAPTER 6 - CONCLUSION

The health of the South African RAS tilapia fish population is sub-optimal and shows concerning evidence of unhealthy, cachectic, and chronically stressed fish.

This poor health is manifesting primarily as very poor growth, evidence of gill pathology, low hepatocyte lipid, extremely high lipofuscin levels through liver and spleen particularly, evidence of gastritis, secondary parasitic and bacterial infections, and evidence of chronic low-grade mortalities.

Key underlying variables responsible for this severe impact upon health have been identified as high water CO_2, low DO, high NO_2^-, low water temperatures, and to a lesser degree, abnormal CH:TA ratios. Husbandry factors like poor matching of system filtration capacity to stocking densities, poor breeding management, questionable feed storage protocol, tunnel disrepair and poor vector management feature prominently. Extremely poor-quality feed and evidence of underfeeding, whether from inadequate supplied or anorexic impact of poor health, are playing an important role.

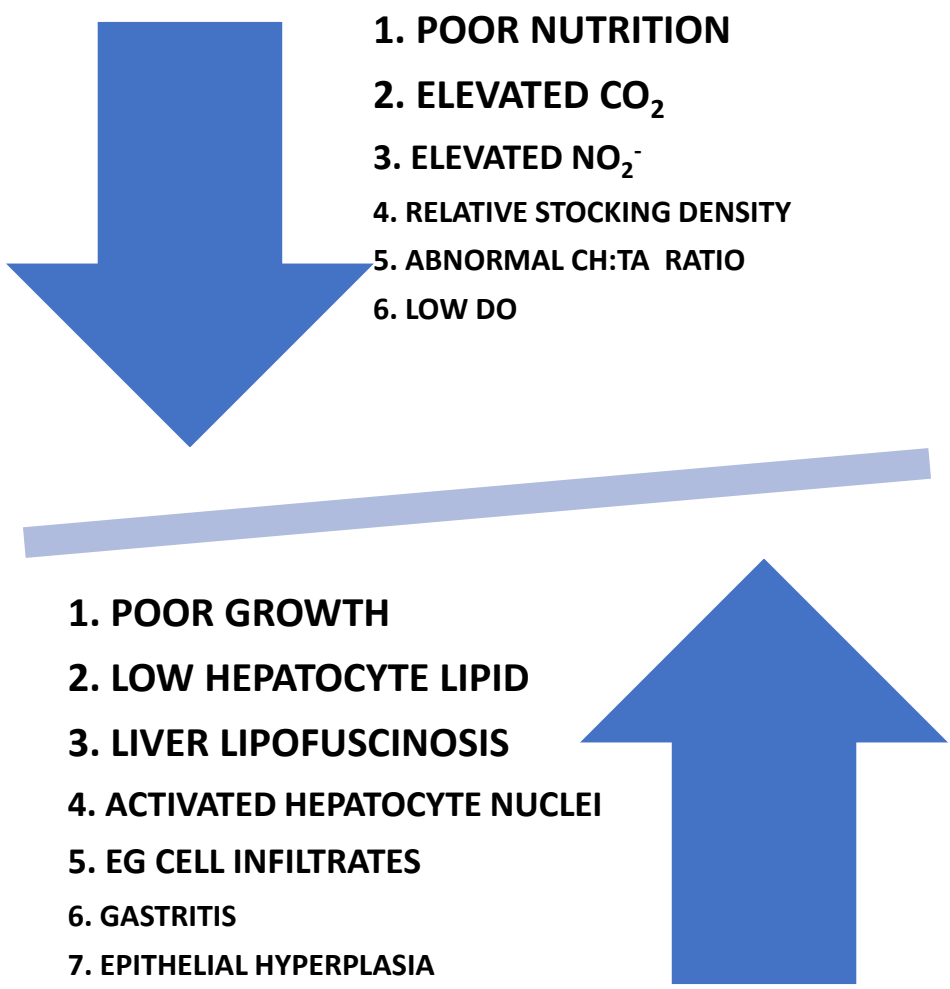


Fig. 6-1 Schematic representation of the key identified stressors and their impact upon fish health

Feed quantity and quality was highlighted as the most important independent variable in the study. With many factors suggestive of suboptimal feed quality and composition, further studies focusing on feed analysis, particularly with respect to carbohydrate/ protein ratios, source of protein, fatty acid composition, amino-acid composition, and levels of protectant antioxidants, would be of interest and value.

Evidence of inadequate intake of feed was obvious through high prevalence of low hepatocyte lipid, poor levels of growth, high lipofuscin levels, poor pancreatic activity, increased number and size of MMC's, increased hepatocyte nuclear activity, gastritis and evidence of apoptotic gastric mucosal cells, poor body condition scores, and low visceral fat levels. With so many potential stressors identified within systems, it is highly likely that this may be more a reflection of anorexia in stressed fish rather than inadequate feed offered by farmers and may be a good measure of overall fish stress levels. All stressors within systems, with low DO in particular, could potentially suppress feeding behaviour and nutrient absorption. The high ecto-parasite burdens, particularly *Trichodina* spp. will be exerting additional negative impact on feed intake through stress effect upon fish. Increased attention, on the part of the farmer, does need to be given to matching feed volume to tank biomass, and taking into account age, season and system DO levels. Inaccurate assessment of tank stocking densities may also play a role and there is a need for more accurate assessment by farmers.

Poor palatability of feed, due to rancidity, is a very likely contributory factor to low intake as well. Evidence of intake of oxidized feed was supported through the high levels of lipofuscinosis, and increased MMC size and numbers through multiple parenchymatous organs. This reflected a systemic oxidative impact rather than focal. With its known permanent effect upon hepatic function and fish health, this cannot be underestimated in its significance upon fish immune function and fish growth. Protection against fatty-acid oxidation at production level also needs to be addressed, with addition of increased anti-oxidants like Vit E, C and Selenium.

High CO_2 manifested as a poorly monitored and under-estimated water parameter, with elevated levels exerting effect upon fish health in terms of chronic stress, secondary infection, predisposition to *Ichthyobodo necator* complex infestation, likely hypercapnia- and physiological hypoxia- induced tissue damage with resulting high cellular debris and lipofuscinosis, and renal mineralization, yet, on a positive note, countering potential NH_3 toxicity. Consistent high readings through all assessed systems was reflective of influence of overstocking and possible high CO_2 levels in borehole water. Lack of use of water holding tanks and degassing techniques were predisposing to elevated levels.

High nitrite (NO_2^-) levels through many systems were exerting significant chronic stress upon the fish, probably through their impact upon osmoregulatory function. High levels reflected as increased EGC's at the base of the primary lamellae. The elevated NO_2^- levels were most likely related to organic waste build up due the uneaten feed with anorexic fish, overstocking, and compromised second stage *Nitrobacter* group nitrification, most likely due to low DO and alkalinity levels. Warmer summer months and poorly matched biofiltration to the fish stocking densities were key husbandry factors most likely to predispose to elevated NO_2^-.

Inappropriate stocking density (poorly matched to the system and filtration capacity) acted as another important chronic stressor upon fish. Systems assessed reflected high stress levels with little flexibility, where, on average, fish stocked >30kg/m^3 reflected poor growth and those <25kg/m^3 good growth, while potential under optimal management conditions exists to increase these stocking densities to much higher levels without a negative impact upon fish *(Table 5-3)*. This reflects the impacting factor of inadequate biofiltration through inappropriate

system design or poor biofilter health where bacterial biofilms have been negatively affected or destroyed by the low DO, low water temperatures below 28^0C, low water alkalinity levels or excessive water flow rates. This emphasizes the need for farmers to supplement adequate aeration or oxygenation, not only to optimize fish health but also biofilter health. Higher stocking densities predisposed to higher ecto-parasite burdens, particularly *Trichodina* spp. and *Ambiphrya* spp. Additional research into the correlation between stocking density and social stress in tilapia would be of value. High stocking densities were frequently associated with high numbers of female fish in mixed populations, reflective of underlying poor breeding management.

CH:TA ratios <1 or >5 featured as an important factor in their potential impact upon fish chronic stress, reduced feed conversion rate and resultant poor growth. This was most likely due to increased osmotic stress, and predisposition to system pH fluctuation. Renal mineralization was a concerning possible correlation.

Low DO was a significant factor, in terms of its stressor effect of upon fish through hypoxia, anaerobic impact with increased free radical production and oxidative damage, acidosis, and resulting anorexia and poor utilization of nutrients in feed and all associated pathological changes as listed above. Negative impact upon biofilter bacterial health would also have been high, contributing to the prevalent high NO_2^- levels. This was reflective of overstocked systems, inadequate aeration, and possible influence of borehole water.

Low water temperatures did not feature as a variable of great significance, largely because of variations seen over a long sampling period that encompassed both hot summer months and

cold winter months. However, low temperatures were playing a potentially significant role in many systems through slower metabolic processing, lower antioxidant enzyme function, and nitrification processes, reduced feeding (and associated pathological impact) and growth, as well as possible sub-clinical suppression of bacterial disease like *Streptococcus* spp. Together with the high CO_2, it was probably countering the potential NH_3 accumulation in systems from increased organic waste associated with anorexic fish, by facilitating conversion to NH_4^+. Poor tunnel management and cost of heating water featured as key underlying husbandry factors.

Gill pathology showed poor association with water parameters, but better association with ecto-parasites, particularly *Ambiphrya* spp. with its obvious triggering of the fish immune response and association with goblet cell hyperplasia. *Trichodina* spp. ,with its main pathological impact being epithelial hyperplasia, appeared more physically irritant than immunostimulatory in its relationship to fish. Gill pathology also served as a good reflection of increased stress and immune system activation upon fish, and generally reflected multiple inter-related pathological changes including goblet cell hyperplasia, infiltration of EGC's at base of the primary lamellae, rodlet cell infiltration and presence of Epitheliocystis. *Trichodina* spp. and *Ambiphrya* spp. showed predilection for better quality water (low CO_2 and low NH_3) and cohabiting together, while *Ichthyobodo necator* complex preferred the opposite (high CO_2 and high NH_3). Infiltration of EGCs at the base of the primary lamellae served as a potential warning of elevated water stressors like high NO_2^-, high CO_2 or low DO, with potential osmoregulatory challenge or in response to physiological hypercapnia or hypoxia in fish. With a "triad-association" with goblet and epithelial hyperplasia, activation of the immune response secondary to pathogen trigger is another likely connection. Potential super-infection of ecto-

parasites is a concern, with the common use of high-intensity RAS, poor movement control of fish, and lack of quarantine practice increasing the risk of introduction. With *O. niloticus* and hybrids showing higher infestation rates of Dactylogyridae and *Ichthyobodo necator* complex, further research into *Oreochromis* species susceptibility to ecto-parasite infestation would be of value.

Low evidence of serious pathogenic bacterial disease attests to the inherent resilience of the species in high density aqua-cultured scenarios. Despite widespread uncontrolled movement of fish locally, it appears that the national population may have been reasonably protected from disease introduction. However, the evidence of a chronically stressed population, with opportunistic infections, presence of primary pathogens like *Trichodina* spp. and *Ichthyobodo necator* complex, histopathological evidence of exposure to pathogens, and chronic low-grade mortalities may reflect a simmering time-bomb where subclinical disease becomes clinical in the face of increased stress upon fish or environmental conditions more suitable to specific diseases. The high levels of suspected parasitic cysts encountered reflected poor vector management. Their potential negative impact upon growth of fish and possible zoonotic implications needs further investigation.

Further evidence of a severely stressed population with activation of the immune system was seen in the high prevalence of gastritis, which could be used as a fairly accurate measure of level of stress in a fish, with emphasis on starvation or low feed intake. Other associations with possible toxin exposure, high endo-parasite burdens, low water DO, potential subclinical

bacterial or viral disease, and social stress were noted. Further investigation into the pathomechanism/s of gastritis in tilapia is required.

The potential impact of toxins like heavy metals or pesticides in water or feed remains unassessed. Pathological findings that potentially indicated these factors to play a role included: karyomegaly of the hepatocyte nuclei, gill pathology like telangiectasis, low liver lipid, high lipofuscin levels, cytoplasmic laking within the hepatocytes, gastric mucosal cell apoptosis, hyaline deposits in posterior kidney tissue, rupture of the MMCs with scattering of the melanin granules *(PLATE 18F)* and chronic low-grade mortalities. With a number of potentiating factors like low water temperature or high pH predisposing, further investigation would be of value.

Key farm husbandry and management factors were highlighted as predisposing to suboptimal independent variables leading to poor fish health. Attention to the following would be of value:

- ✓ Additional weekly monitoring of DO and CO_2 levels of water supply at source (borehole/ river/ municipal supply) prior to introduction into system
- ✓ Regular monitoring and recording of water parameters

  The following is suggested:

  Twice daily: DO, CO_2, temperature, pH

  Once daily: NH_3, NO_2^-

  Once weekly: Total alkalinity (TA), NO_3^-,

  Once monthly: Complete hardness (CH)
- ✓ Training on the ideal water parameter ranges, impact of sub-optimal water parameters, and corrective measures

- ✓ Implementation of pre-system water holding tanks
- ✓ Application of regular use of NaCl to improve temperature tolerance and manage any elevated NO_2^- issues
- ✓ Improved management and skills training for green water systems
- ✓ Increased use of CO_2 gas exchange techniques
- ✓ Attention to water temperature control: improved heating techniques for winter, cooling for summer, deeper tanks to afford fish accessibility to cooler water in hot summer months
- ✓ Improved breeding management with implementation of better sex-reversal techniques
- ✓ Better management of feed with use within expiry, cool and dry storage
- ✓ The need for better assessment of tank biomass, and matching with appropriate system mechanical and biofiltration, aeration and feeding regimes
- ✓ Improved manipulation of alkalinity to match complete hardness levels
- ✓ Improved vector management
- ✓ Increased aeration/ oxygenation of systems
- ✓ Optimizing biofilter health
- ✓ Improved biosecurity practices and use of quarantine facilities for new introductions
- ✓ Increased use of veterinary skills and ongoing health surveillance practice
- ✓ Regular farm-level morphometric assessment of fish to evaluate health, growth and productivity

Practical observations relevant to veterinary assessment of fish health included the following:

- ✓ Body condition score correlates well to assessment of poor nutrient intake, whether from lack of supply or anorexic impact as a result of poor health
- ✓ Macroscopic observation has little value in terms of accurately assessing fish health
- ✓ Level of skin or gill mucus was not a reliable indicator of parasite burden
- ✓ Performing all three wet mount assessments of ecto-parasites was necessary to accurately assess parasitic burdens
- ✓ Gill scrapes from the base of the primary lamellae proved a very useful tool to assess monogenean presence
- ✓ Histopathology of gills is valuable to accurately assess *Ichthyobodo necator* complex infestations
- ✓ Ecto-parasites need to be assessed individually. Total parasite score reflects poorly on patterns of individual parasites.
- ✓ Histopathological evaluation of tissues proved an invaluable tool in assessing organ / tissue lesions and thus indirectly, fish health
- ✓ Spleen: heart ratios ≥ 4, are a useful predictor of septicaemia.
- ✓ It is sensible practice to include a section of attached muscle tissue with anterior and posterior kidney samples to avoid loss of friable tissue in histological processing
- ✓ Water low in NH_3 or CO_2 may predispose to *Trichodina* spp. and *Ambiphrya* spp. infestations
- ✓ Water high in NH_3 or CO_2 may predispose to *Ichthyobodo necator* complex

The high degree of microscopic pathology versus low macroscopic visible lesions emphasize the use of both assessment modalities in fish health evaluations. It is clear that, despite high ecto-parasite burdens, nutritional deficits, and severe microscopic organ pathology, many fish showed little external macroscopic or clinical behavioural abnormalities that one would expect to see. Thorough evaluation of system water parameters, stocking density and type and volume of feed, although critical, does also not offer enough information to accurately assess fish and system health. Inclusion of veterinary diagnostic modalities of full macroscopic and microscopic evaluations, inclusive of ecto-parasite evaluation and tissue histopathology, bacterial culture and assessment of growth are invaluable tools. Correlations drawn between independent and dependent variables are significant and it is important to consider each parameter individually to weigh it on its own merit and assess its impact. However, each abnormality has to be assessed within a holistic context in an attempt to determine its interactive role/s with other parameters. In addition, all factors should be evaluated over a period of time because of their potential dynamic fluctuation and chronic impact upon fish health. A one-off assessment only offers a measure of information and this emphasizes the value of ongoing monitoring, recording and the creation of a farm data-base set of information. Using the scoring system designed in this study or a modified one to encompass key factors encountered relevant to each situation, gives one a useful tool to comparatively assess individual parameters and measure system health.

It is clear that many of the listed concerns of the farming community are not unfounded. This study has served to highlight the key factors currently impacting upon farmed tilapia in RAS systems in South Africa, and their most significant impacts upon fish health. It is clear that key

independent variables exert not only individual effect, but also act synergistically and cumulatively. Impact upon fish health is one of networks of interlinked responses, in turn triggering further physiological and immune reactions, and often serving to compound the negative stress impact even further. It is clear that RAS systems have much lower tolerance and can be very sensitive to changes in system variables. If not managed closely, impacts of unmonitored vital parameters may result in devastating fatalities or production losses, rendering this method of tilapia culture uneconomical. Despite tilapia's innate resilience, the health of the South African RAS tilapia industry is severely challenged by the multitude of stressors the fish are subjected to, with resultant poor growth. By paying attention to these underlying factors, there is potential for significant improvement in the overall population health, growth and feed conversion rates, with reduced cost to farmer and improved productivity.

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APPENDIX 1

Karen L. Veverica BS, MAq, PhD
Director, E.W. Shell Fisheries Research Center
School of Fisheries, Aquaculture and Aquatic Sciences
Auburn University, AL USA
http://www.ag.auburn.edu/fish/
Veverkl@auburn.edu

Maryke M Henton BVSc, MMedVet [Bact]
Specialist Veterinary Bacteriologist
Vetdiagnostix – Veterinary Pathology Services
South Africa
www.vetdiagnostix.com
henton@vetdx.co.za

David Huchzermeyer BSc., BVSc., MSc. in Aquatic Veterinary Studies, PhD, MRCVS.
Aquatic Veterinary Specialist Reg. No. S14/2178
Sterkspruit Veterinary Clinic, Lydenberg
South Africa
www.sterkspruitveterinaryclinic.co.za
aquavet@telkomsa.net

Nicholas James BA geography (Hons) Rhod, Grad. C.E., MSc Ichthyology (Rhodes). Pr.Sci. Nat
Rivendell Hatchery
Grahamstown
South Africa
Nickjames@intekom.co.za

Geoff Fosgate BS, DVM, PhD, Dip ACVPM Professor: Epidemiology 0000-0002-9432-0042

Faculty of Veterinary Science

University of Pretoria

www.up.ac.za

Geoffrey.fosgate@up.ac.za

Andrew P. Shinn PhD

Senior Scientist

Fish Vet Group Asia Limited (FVGAL), England/Thailand

www.fishvetgroup.asia

andy.shinn@fishvetgroup.com

Esteban Soto, MSc, DVM, PhD, Dipl. ACVM, CertAqV

Associate Professor of Aquatic Animal Health

Department of Medicine & Epidemiology

School of Veterinary Medicine

University of California-Davis

2108 Tupper Hall

Davis, CA 95616-5270

Email: sotomartinez@ucdavis.edu

APPENDIX 2

SECTION 20 AUTHORIZATION:

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1

Dr Gillian Denise Taylor
Department Para-clinical Science
Faculty of Veterinary Science
University of Pretoria
Ondesterpoort
0110
Section Pathology
E-Mail: Gillian@aquaticvet.co.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Dear Dr Gillian Denise Taylor

Your application dated the 24 July 2017 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
3. All potentially infectious material utilised, collected or generated during the study is to be destroyed at the completion of the study. A dispensation granting permission for the storage of Tissue samples taken from fish as well as some Positive bacterial

cultures is attached. Records must be kept for five years for auditing purposes. A dispensation for the storage of samples is attached.

4. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 82);
5. No part of the study may begin until the valid ethical approval has been obtained from the relevant authority;
6. Samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National *Road Traffic Act*, 1996 (*Act* No. 93 of 1996);
7. Written permission must be obtained from the responsible State Veterinarian prior to working in his/her area and records kept for 5 years.
8. A six monthly update on the project and the collection sites must be sent to Directorate animal health for as long as the study is ongoing.

Title of research/study A survey of selected pathogens of economic concern within the principle cultivated tilapia (oreochromis spp) producing regions of South Africa over the period 2017 and 2018.
Researcher: Gillian Taylor
Institution: University of Pretoria.
Our ref Number: 12/11/1/1

Kind regards,

MKMaja.

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2017 -10- 31

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1

Dr Gillian Denise Taylor
Department Paraclinical Science
Faculty of Veterinary Science
University of Pretoria
Ondesterpoort
0110
Section Pathology
E-Mail: Gillian@aquaticvet.co.za

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: A survey of selected pathogens of economic concern within the principle cultivated tilapia (oreochromis spp) producing fegions of South Africa over the period 2017 and 2018

A dispensation is hereby granted for the Section 20 approval that was issued for the above mentioned study (attached):

- Tissue samples taken from fish to be stored at the NRF Biological Bank at ondersterpoort dept Paraclinical Sciences
- And Positive bacterial cultures to be stored at Vetdiagnostix bacteriological laboratory.
- Stored samples may not be outsourced without prior written approval from the Director: Animal Health.
- Should samples be used for further research, written approval from the Director: Animal Health must be obtained prior to start of project

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2017 -10- 31

APPENDIX 3

3.1: LETTER OF INFORMED CONSENT

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

ANIMAL ETHICS COMMITTEE

INFORMED CONSENT FORM

We, the undersigned, hereby agree that the animal(s), as specified below, may be used by the researcher(s), as specified below, in the procedures as explained below:

1. To be completed by the researcher(s)

- **NAME OF THE RESEARCHER(S):**

Dr. Gillian Taylor

Dr. Johan Steyl

- **NAME OF RESEARCH PROJECT:**

Diseases of economic concern in captive tilapia spp. In South Africa.

- **PURPOSE OF RESEARCH PROJECT:**

To determine the occurrence of diseases of economic concern affecting the tilapia farming industry of South Africa.

- **DETAILED PROCEDURE(S) TO BE PERFORMED:**

Random elective euthanasia of grower tilapia (10 per site) in various captive farming systems for the purpose of detailed diagnostic sampling and examination.

- **RISK(S) INVOLVED IN SPECIFIED PROCEDURE:**

None.

- **IDENTIFICATION OF ANIMAL TO BE USED:**

Grower tilapia spp.

- **UNMISTAKEABLE DISTINGUISHING DESCRIPTION OF ANIMAL TO BE USED:**

100 to 220g of typical tilapia spp. (*O. mossambicus* or *O. niloticus* or their hybrids)

2. To be completed by the animal's owner or person duly authorized to sign on his/her behalf:

- **NAME OF OWNER:**

- **HAVE YOU RECEIVED DETAILED INFORMATION REGARDING THE PROPOSED STUDY?**

- **HAVE ALL THE RISKS INVOLVED IN THE PROCEDURE BEEN EXPLAINED TO YOU AND DO YOU FULLY UNDERSTAND THESE RISKS?**

- **DO YOU GRANT FULL CONSENT FOR THE PROCEDURE TO BE PERFORMED?**

3. The undersigned parties further agree that no compensation will be payable to the animal's owner or anybody else and that all research associated costs will be covered by the researcher(s).

4. The undersigned parties further agree that this form would serve to fully indemnify the University of Pretoria and the undersigned researcher(s) against any future claims resulting from the specified procedure by or on behalf of the animal's owner.

5. The undersigned parties further agree that no material of any kind, including data and research findings, obtained or resulting from the procedure, would be passed on to any third party or used for any purpose other than that specified in this form, except with the written consent of the undersigned owner of the animal.

_____ _____

SIGNATURE RESEARCHER(S) **SIGNATURE OWNER**

_____

SIGNATURE WITNESS

DATE: _____

3.2: HEALTH AND BIOSECURITY QUESTIONNAIRE

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Denkleiers • Leading Minds • Dikgopolo tša Dihlalefi

The Tilapia Project: Health and Biosecurity Questionnaire:

(Please note that information gained from this questionnaire will be kept confidential and purely for research purposes).

Dr Gillian Taylor BVSc CertAqV

(www.asc-aqua.org 2011, IAVBC 2017)

(A) Personal:

Name of farm: _____

Name of owner: _____

Address: _____

_____

_____

GPS coordinates: _____

Tel: (1) _____

Tel: (2) _____

Email: _____

(B) Production System:

Date Aquaculture facility started? _____

Total size of system: _____

System type: RAS: ☐ Ponds: ☐ Raceway: ☐

Please complete the following questions:

	Yes	No
Do you farm one fish species only?	☐	☐
Which species? _____		
Do you have hybrid species?	☐	☐
If more than one species farmed, please list which?		
1) _____		
2) _____		
3) _____		
4) _____		
If polyculture ,are the fish species mixed in the system?	☐	☐
Are you exclusively an aquaculture facility?	☐	☐
Do you have an aquaponic system involved?	☐	☐

Does your unit contain

1) Ova? ☐ ☐

2) Fry? ☐ ☐

3) Fingerlings? ☐ ☐

4) Grow-out? ☐ ☐

5) Broodstock? ☐ ☐

Please give approx. current stocking densities:

1) Sampled grow out stage: _____

2) Fry: _____

3) Fingerlings:_____

4) Broodstock : _____

5) Other:_____

Breeding:

	Yes	No
Are your fry hormone treated?	☐	☐
Do you use YY Supermales for breeding?	☐	☐

Breeding ratio of males to females? _____

_____

Do you inbreed? ☐ ☐

Skill:

Please describe the level of training/experience of the personnel involved in the facility:

_____

_____

_____

_____

_____

What is your current Grow out stage FCR ? _____

(C) Biosecurity:

1. Source of water for aquaculture:	Yes	No
Municipal:	☐	☐
Borehole:	☐	☐
River:	☐	☐

Other? Please specify: _____

	Yes	No
Is incoming water filtered before use?	☐	☐
Is new water treated/ disinfected before use?	☐	☐

If yes, with what? _____

	Yes	No
Is new water allowed to stand in holding tanks before entering system?	☐	☐

If yes, for how long?_____

	Yes	No
Do you monitor water parameters?	☐	☐

Which parameters are routinely checked and how often? _____

DO? _____

pH? _____

Temperature? _____

Ammonia? _____

Nitrite? _____

Nitrate? _____

Hardness? _____

Alkalinity? _____

Chloride? _____

Carbon Dioxide? _____

Other? _____

How do you assess water quality?

Probe? ☐ ☐

Test strips: ☐ ☐

Other? ☐ ☐

Please specify:_____

_____

How do you heat water? _____

_____

Is any water from aquaculture unit drained back into natural waterways?

☐ ☐

If so, does it undergo any filtration or treatment?_____

_____

_____

Please list /explain any problems you have previously had with **water quality or toxins** in the water causing morbidities or mortalities:

_____

_____

_____

_____

_____

_____

_____

Please give a general overview of your filtration protocol:

_____

_____

_____

_____

_____

_____

Have you identified any problems in the filtration system that need addressing?

_____

2. Movement of Fish:

		Yes	No
Do you purchase in:	Ova?	☐	☐
	Fry?	☐	☐
	Fingerlings?	☐	☐
	Grow out?	☐	☐

If yes, what age/weight?_____

Broodstock?	☐	☐

Please list (with as much details as you are comfortable with), where you acquire the above?

_____

_____

_____

Do you purchase from sources in other provinces?	☐	☐

If yes, please list which provinces: _____

_____

_____

Do you purchase from countries outside of SA?	☐	☐

If yes, please list which countries:_____

_____

Do your purchases come with any:

1) Veterinary Disease-free certification	☐	☐

2) Health certification ☐ ☐

3) Movement/ breeding/ farming etc permits ☐ ☐

If so, please elaborate: _____

_____

Are new purchases tested for disease before introduction? ☐ ☐

Please elaborate:_____

_____

_____

_____

Are new purchases quarantined? ☐ ☐

For how long? _____

Do you have a separate system for quarantine? ☐ ☐

Yes **No**

Are new purchases treated preventatively against disease? ☐ ☐

If so, please list medication/ chemicals used, dose and frequency of application?

_____

_____

_____

_____

Do you disinfect ova upon arrival to the farm? ☐ ☐

Are your broodstock moved/ shared between farms? ☐ ☐

If yes, please specify if locally or inter-provincially?

_____

_____

3. Husbandry

Please specify frequency of handling:

1. Fingerlings: _____
2. Growouts: _____
3. Broodstock: _____

Do you use gloves when handling fish? ☐ ☐

Is there any history of trauma/ handling stress predisposing to disease/ mortalities?

☐ ☐

Please elaborate? _____

_____

Does each tank/ system have its own set of equipment? (Nets, buckets, DO probes etc.)

☐ ☐

Is equipment (e.g. nets, buckets, clothing, aerators, vehicles, waders) cleaned and disinfected regularly? ☐ ☐

Please explain if/how tanks are cleaned, disinfected or fallowed after fish are moved or harvested? _____

_____

_____

Are ponds limed after fish are removed? ☐ ☐

Do you make use of:

1. Footbaths? _____
2. Handwash stations? _____

Do you have instructions clearly displayed for handwashes/ footdips etc?

	Yes	**No**
	☐	☐

Please list what disinfectants are used and frequency of changing? _____

_____

_____

_____

Do you share equipment with other farms? ☐ ☐

Is the property fenced and secure? ☐ ☐

Do you have problems with theft of fish? ☐ ☐

Do you have biosecurity labelling displayed? ☐ ☐

Is feed stored under cover? ☐ ☐

Is feed expiry date checked? ☐ ☐

How long do you keep feed for before using?_____

_____

Please describe brands/ types feed used:

_____

_____

_____

Please describe any suspected or diagnosed **nutrition related** diseases you have had problems with in the past?

_____

_____

_____

_____

_____

Do you grow or harvest duckweed? ☐ ☐

Yes **No**

Do you have freshwater snails in your system? ☐ ☐

Is your system open to birds? ☐ ☐

Do you control predators or wildlife? ☐ ☐

4. Disease History:

Do you monitor for sick fish? ☐ ☐

How often? _____

Are staff trained to identify sick fish? ☐ ☐

Please describe what they have been trained to look for: _____

_____

Are all sick/ dying fish removed, isolated or destroyed? ☐ ☐

Please indicate how fish are disposed of:

1) Incineration ☐ ☐

2) Burial ☐ ☐

3) Other? ☐ ☐

How often you experience fish sickness or deaths?

1) Daily ☐ ☐

2) Weekly ☐ ☐

3) Monthly ☐ ☐

4) Annually ☐ ☐

Which life stage suffers most often? _____

Do you have problems with poor growth of fish?

☐ ☐

Which life stage suffers most often? _____

Please list previous suspected/ diagnosed **infectious** diseases/ pathogens (parasites, bacteria, fungi, viruses) you have had problems with in your facility.

_____

_____

_____

_____

_____

Do you treat your system or fish preventatively against disease?

☐ ☐

If yes, please list briefly what products are used and for which stages:

_____

_____

_____

Your Disease Concerns:

Please list what top 5 diseases **you feel are most important** to your farm, listed in order of priority:

1. _____
2. _____
3. _____
4. _____
5. _____

Any other diseases of concern:

_____

Critical Risk Points that **you feel are important** where diseases could currently enter or leave your farm?

1.
2.
3.
4.
5.
6.

What would you consider are/ have been your greatest challenges in this industry?

What do you believe needs to be done to move the industry forward?

Thank you so much for your assistance and information, not only for our research, but also for its value to the development and growth of the Tilapia industry in South Africa.

APPENDIX 4 TABLE 1: Fish Parasite Grading Overview

Fish	TM	TG	TS	AT	GM	GG	GS	AG	DM	DG	DS	AD	IM	IG	IS	AI	AM	AG	AS	AA
GA1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GA2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GA3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GA4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GA5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GA6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GA7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GA8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GA9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GA10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ave farm grade				0				0				0				0				0
GB1	0	1	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GB2	1	0	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GB3	0	1	1	0.67	0	0	0	0	0	2	1	1	0	0	0	0	0	0	0	0
GB4	0	3	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GB5	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GB6	2	3	3	2.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GB7	2	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GB8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

GB9	2	2	2	2	0	0	0	0	0	0	1	0.33	0	0	0	0	0	0	0	0
GB10	2	3	3	2.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Av farm grade				1.37				0				0.13				0				0
GC1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
GC2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GC3	0	0	0	0	2	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0
GC4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GC5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GC6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GC7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GC8	0	0	0	0	0	0	0	0	0	0	1	0.33	0	0	0	0	0	0	0	0
GC9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GC10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ave farm grade				0				0.3				0.03				0				0
GDA3	2	3	3	2.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GDA4	3	2	0	1.67	0	0	0	0	0	0	1	0.33	0	0	0	0	0	0	0	0
GDA5	3	0	4	2.33	0	0	0	0	0	1	0	0.33	0	0	0	0	0	0	0	0
GDA6	3	4	0	2.33	1	1	0	0.67	0	0	0	0	0	0	0	0	0	0	0	0
Ave farm grade				2.25				0.2				0.17				0				0
GDB7	4	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GDB8	3	3	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GDB9	4	4	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GDB10	5	5	5	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ave farm grade				3.75				0				0				0				0
GE1	0	0	0	0	0	0	0	0	0	0	1	0.33	0	0	0	0	0	0	0	0
GE2	0	1	1	0.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GE3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

GE4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GE5	3	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GE6	2	1	0	1	0	0	0	0	0	0	1	0.33	0	0	0	0	0	0	0	0
GE7	4	0	0	1.33	0	0	0	0	0	1	0	0.33	0	0	0	0	0	0	0	0
GE8	1	1	2	1.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GE9	5	1	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GE10	1	0	1	0.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ave farm grade				0.9				0				0.1				0				0
GFA1	1	1	0	0.67	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	0
GFA2	2	2	3	2.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GFA3	1	4	0	1.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GFA4	1	0	1	0.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GFA5	1	3	0	1.44	0	0	1	0.33	0	0	0	0	0	0	0	0	0	0	0	0
Ave farm grade				1.4				0.27				0				0				0
GFB6	1	1	0	0.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GFB7	1	0	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GFB8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GFB9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GFB10	0	1	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ave farm grade				0.27				0				0				0				0
GG1	4	0	0	1.33	2	0	0	0.67	0	0	1	0.33	0	0	0	0	0	0	0	0
GG2	5	1	0	2	1	0	0	0.33	0	0	1	0.33	0	0	0	0	0	0	0	0
GG3	3	0	0	1	0	0	0	0	0	0	1	0.33	0	0	0	0	0	0	0	0
GG4	1	1	0	0.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GG5	4	0	0	1.33	0	0	0	0	0	2	1	1	0	0	0	0	0	0	0	0
GG6	4	0	0	1.33	0	0	0	0	0	2	0	0.67	0	0	0	0	0	0	0	0
GG7	4	1	0	1.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GG8	4	1	0	1.67	1	0	0	0.33	0	1	0	0.33	0	0	0	0	0	0	0	0
GG9	5	0	0	1.67	3	0	0	1	0	0	1	0.33	0	0	0	0	0	0	0	0
GG10	5	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ave farm grade				1.5				0.23				0.33				0				0

GH1	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	3	0	0	0	0
GH2	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	3	0	0	0	0
GH3	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	3	0	0	0	0
GH4	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	3	0	0	0	0
GH5	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	3	0	0	0	0
GH6	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	3	0	0	0	0
GH7	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	3	0	0	0	0
GH8	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	3	0	0	0	0
GH9	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	3	0	0	0	0
GH10	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	3	0	0	0	0
Ave farm grade				0				0				0				3				0
NA1	2	0	0	0.67	0	0	0	0	0	0	1	0.33	1	0	0	0.33	0	0	0	0
NA2	2	0	0	0.67	1	2	0	1	0	0	0	0	1	0	0	0.33	0	0	0	0
NA3	2	1	0	1	0	0	1	0.33	0	0	0	0	0	0	0	0	0	0	0	0
NA4	1	0	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NA5	2	0	0	0.67	1	0	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0
NA6	2	0	0	0.67	1	0	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0
NA7	2	0	0	0.67	1	0	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0
NA8	2	0	0	0.67	1	0	1	0.67	0	0	0	0	0	0	0	0	0	0	0	0
NA9	2	0	2	1.33	0	0	0	0	0	0	0	0	0	1	0	0.33	0	0	0	0
NA10	0	1	0	0.33	0	1	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0
Ave farm grade				0.7				0.33				0.03				0.1				0
NB1	2	0	0	0.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NB2	0	2	0	0.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NB3	1	0	0	0.33	0	0	0	0	0	1	1	0.67	1	0	0	0.33	0	0	0	0
NB4	1	0	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NB5	1	0	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NB6	4	1	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NB7	3	1	0	1.33	0	0	0	0	0	1	0	0.33	0	0	0	0	0	0	0	0
NB8	1	0	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NB9	3	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NB10	1	2	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Ave farm grade				0.9				0				0.1				0.03				0
NC1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NC2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NC3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NC4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NC5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NC6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NC7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NC8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NC9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NC10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ave farm grade				0				0				0				0				0
ND1	0	0	0	0	1	0	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0
ND2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ND3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ND4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ND5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ND6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ND7	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0.67	0	0	0	0
ND8	0	0	0	0	0	0	1	0.33	0	0	0	0	0	2	3	1.67	0	0	0	0
ND9	0	0	0	0	0	0	0	0	0	0	0	0	0	2	4	2	0	0	0	0
ND10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0.67	0	0	0	0
Ave score				0				0.06				0				0.5				0
NE1	0	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	1
NE2	0	2	3	1.67	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0.67
NE3	1	2	4	2.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NE4	3	1	1	1.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NE5	2	3	2	2.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NE6	2	2	4	2.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NE7	0	1	3	1.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.33
NE8	1	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0.33
NE9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NE10	2	4	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Ave farm grade				1.6				0				0				0				0.2
NF1	4	0	0	1.33	0	0	0	0	0	0	1	0.33	0	0	0	0	0	0	0	0
NF2	2	0	0	0.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NF3	0	0	2	0.67	0	0	1	0.33	0	0	0	0	0	0	0	0	0	0	0	0
NF4	2	1	0	1	0	0	0	0	0	1	0	0.33	0	0	0	0	0	0	0	0
NF5	1	1	0	0.67	0	0	0	0	0	0	1	0.33	0	0	0	0	0	0	0	0
NF6	2	2	2	2	0	0	0	0	0	1	0	0.33	0	0	0	0	0	0	0	0
NF7	0	0	0	0	0	0	0	0	0	0	1	0.33	0	0	0	0	0	0	0	0
NF8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NF9	0	0	0	0	0	1	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0
NF10	2	1	1	1.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ave farm grade				0.77				0.06				0.17				0				0
NJ1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NJ2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NJ3	0	0	0	0	0	0	0	0	0	0	1	0.33	0	0	0	0	0	0	0	0
NJ4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NJ5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NJ6	0	0	0	0	0	0	0	0	0	0	2	0.67	0	0	0	0	0	0	0	0
NJ7	0	0	0	0	0	0	0	0	0	2	0	0.67	0	0	0	0	0	0	0	0
NJ8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NJ9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NJ10	0	0	0	0	0	0	0	0	0	0	2	0.67	0	0	0	0	0	0	0	0
Ave farm grade				0				0				0.23				0				0
LB1	2	2	3	2.33	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0.67
LB2	3	2	4	3	0	0	0	0	0	2	0	0.67	0	0	0	0	0	4	0	1.33
LB3	3	2	3	2.67	0	0	0	0	0	0	1	0.33	0	0	0	0	0	2	0	0.67
LB4	3	1	2	2	0	0	0	0	0	0	0	0	0	0	0	0	1	3	4	2.67
LB5	3	3	3	3	1	0	2	1	0	0	0	0	0	0	0	0	0	2	0	0.67
LB6	3	2	3	2.67	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0.67
LB7	4	2	2	2.67	0	0	0	0	0	0	0	0	0	0	0	0	3	2	2	2.33
LB8	5	2	4	3.67	0	0	0	0	0	0	0	0	0	0	0	0	0	2	4	2
LB9	4	2	4	3.33	3	0	0	1	0	0	1	0.33	0	0	0	0	1	2	4	2.33

LB10	5	2	0	2.33	0	0	0	0	0	0	2	0.67	0	0	0	0	5	3	0	2.67
Ave farm grade				2.77				0.2				0.2				0				1.6
LC1	1	0	0	0.33	2	0	0	0.67	0	0	0	0	0	0	0	0	0	0	0	0
LC2	4	0	0	1.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC3	2	0	0	0.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC4	4	0	0	1.33	0	0	0	0	0	1	0	0.33	0	0	0	0	0	0	0	0
LC5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC6	1	1	0	0.67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC7	2	0	0	0.67	1	0	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0
LC8	1	0	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC9	4	0	0	1.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ave farm grade				0.67				0.1				0.03				0				0

Key: TM:*Trichodina*- mucous scrape; TG: *Trichodina*- gill clip; TS: *Trichodina* - gill scrape; AT: Average fish *Trichodina* grade; GM: Gyrodactylidea- mucous scrape; GG: Gyrodactylidea- gill clip; GS: Gyrodactylidea- gill scrape; AG: Average fish Gyrodactylidea grade; DM: Dactylogyridae- mucous scrape; DG: Dactylogyridae- gill clip; DS: Dactylogyridae- gill scrape; AD: Average fish Dactylogyridae grade; IM: *Ichthyobodo*- mucous scrape; IG: *Ichthyobodo*- gill clip; IS: *Ichthyobodo*- gill scrape; AI: Average fish *Ichthyobodo* grade; AM: *Ambiphrya*- mucous scrape; AG: *Ambiphrya*-gill clip; AS: *Ambiphrya*- gill scrape; AA: Average fish *Ambiphrya* grade

APPENDIX 4 TABLE 2: Necropsy data

Fish:	Sex:	Visceral fat grade:	Spleen to heart ratio:
GA1	M	1	1
GA2	M	3	1
GA3	M	3	1
GA4	M	0	1
GA5	M	1	1
GA6	M	1	1
GA7	M	1	1
GA8	M	1	1
GA9	M	2	1
GA10	M	3	1
GB1	M	0	1
GB2	M	2	1
GB3	M	3	2
GB4	M	3	2
GB5	M	1	1
GB6	M	2	1.5
GB7	M	2	1
GB8	M	4	2
GB9	M	2	1
GB10	M	3	1

GC1	M	1	1
GC2	M	3	1
GC3	M	3	1
GC4	M	3	1
GC5	M	1	1
GC6	M	2	1
GC7	M	3	1
GC8	M	3	1
GC9	M	1	1
GC10	M	1	1
GDA3	M	2	1
GDA4	M	3	1.5
GDA5	M	4	2
GDA6	M	3	2
GDB7	M	0	1
GDB8	M	1	1
GDB9	M	4	2
GDB10	M	X	X
GE1	M	2	1.5
GE2	F	2	1.5
GE3	F	2	1.5
GE4	M	1	2
GE5	M	3	2
GE6	F	1	3
GE7	M	0	3
GE8	F	1	2
GE9	M	2	2
GE10	F	2	2
GFA1	F	4	4
GFA2	M	3	4
GFA3	M	4	1.5
GFA4	M	4	3
GFA5	M	3	5
GFB6	M	3	1.5

GFB7	M	3	2
GFB8	M	X	1.5
GFB9	F	3	2
GFB10	M	3	2
GG1	M	2	1
GG2	M	2	1
GG3	M	2	1
GG4	F	2	1
GG5	F	2	1
GG6	F	2	1
GG7	M	2	3
GG8	M	2	1
GG9	M	2	2
GG10	F	X	X
GH1	F	3	3
GH2	F	2	2
GH3	F	3	3
GH4	F	3	1
GH5	F	4	0.5
GH6	F	5	2
GH7	M	4	1
GH8	F	2	2
GH9	F	3	2
GH10	M	X	0.5
NA1	M	X	1
NA2	M	X	1
NA3	M	1	0.5
NA4	F	1	1
NA5	F	0	0.5
NA6	F	0	0.5
NA7	F	2	1
NA8	F	1	1
NA9	F	0	1
NA10	M	0	X
NB1	M	2	0.7
NB2	M	1	3

NB3	M	0	1
NB4	M	2	1
NB5	M	0	0.7
NB6	M	0	1
NB7	M	0	0.5
NB8	M	0	0.5
NB9	M	0	0.7
NB10	M	0	0.5
NC1	M	1	1
NC2	F	0	0.5
NC3	H	1	1
NC4	M	1	1
NC5	M	0	1
NC6	F	0	4
NC7	M	1	5
NC8	M	2	1
NC9	F	2	1
NC10	M	0	3
ND1	M	3	3
ND2	M	5	5
ND3	F	5	1.5
ND4	F	2	5
ND5	F	4	4
ND6	F	3	2
ND7	M	4	5
ND8	M	3	2
ND9	F	4	4
ND10	F	X	5
NE1	F	5	3
NE2	M	5	1.5
NE3	M	3	1.5
NE4	M	4	1
NE5	M	4	1.5
NE6	M	5	1.5
NE7	M	3	2
NE8	M	3	2

NE9	F	4	1
NE10	M	4	1
NF1	F	0	1
NF2	F	0	1
NF3	M	0	1
NF4	M	0	0.5
NF5	F	1	1
NF6	F	1	2
NF7	M	0	1
NF8	F	1	1
NF9	F	0	1
NF10	F	1	2
NJ1	M	3	1
NJ2	M	4	1
NJ3	M	3	1.5
NJ4	M	3	1
NJ5	M	4	1.5
NJ6	M	4	1.5
NJ7	M	4	1
NJ8	M	3	1
NJ9	M	3	1
NJ10	M	4	1
LB1	M	1	1
LB2	M	2	1
LB3	M	1	1
LB4	M	1	1.5
LB5	M	2	2
LB6	M	3	1.5
LB7	M	4	2
LB8	H	3	2
LB9	M	2	2
LB10	F	3	1.5
LC1	M	4	1.5
LC2	M	4	2
LC3	M	4	1.5
LC4	M	3	1.5

LC5	M	4	1.5
LC6	M	4	1
LC7	M	4	1.5
LC8	M	2	3
LC9	M	3	1.5
LC10	M	1	2

APPENDIX 4 TABLE 3: Gill histopathology

(See material and methods grading methods: TABLES 3-8, 3-9, 3-10, 3-11)

FISH:	EH	GH	T	LE	R	E	EGC	LF
GA1	1	1	Y	N	N	N	3	0
GA2	1	1	N	N	N	N	3	0
GA3	0	1	N	N	N	N	3	0
GA4	0	1	N	N	N	N	3	1
GA5	0	0	N	N	N	N	1	0
GA6	0	0	N	N	N	N	3	1
GA7	0	0	Y	N	N	N	2	1
GA8	0	1	Y	N	N	N	2	1
GA9	0	0	N	N	N	N	1	0
GA10	1	0	N	N	N	N	3	0
Ave farm grade	0.3	0.5					2.4	
GB1	2	1	N	N	N	Y	3	2
GB2	1	1	Y	N	N	N	2	2
GB3	3	1	N	N	N	N	4	2
GB4	S	S	S	S	S	S	S	S
GB5	5	2	N	N	N	N	4	2
GB6	5	2	N	N	N	Y	4	S
GB7	4	2	N	N	N	N	3	2
GB8	2	1	N	N	N	N	3	2
GB9	2	2	N	N	N	N	2	S
GB10	3	2	N	N	N	N	4	2
Ave farm grade	3	1.6					3.2	
GC1	2	1	N	Y	N	Y	1	1
GC2	2	1	N	Y	N	N	3	0
GC3	1	1	N	Y	N	N	1	1
GC4	2	1	N	N	N	N	4	2
GC5	3	1	N	Y	N	Y	2	S
GC6	3	1	N	Y	N	N	3	2

GC7	0	0	N	Y	N	N	2	1
GC8	1	1	N	N	N	N	3	1
GC9	3	2	N	N	N	N	4	3
GC10	3	1	N	Y	N	N	5	1
Ave farm grade	2	1					2.8	
GD3	3	1	N	Y	N	N	2	2
GD4	3	1	N	N	N	Y	5	2
GD5	0	1	N	Y	N	Y	S	1
GD6	1	1	N	Y	N	N	3	0
Ave farm grade	1.75	1					3.3	
GD7	0	1	N	Y	N	N	2	0
GD8	0	2	N	Y	N	N	3	3
GD9	0	2	N	Y	N	N	4	3
GD10	0	1	N	Y	N	N	2	3
Ave farm grade	0	1.5					2.8	
GE1	4	3	N	N	N	N	2	3
GE2	3	2	N	N	Y	N	1	0
GE3	2	1	N	N	Y	N	2	0
GE4	3	4	Y	Y	Y	N	1	0
GE5	3	4	N	N	Y	N	1	1
GE6	0	0	N	N	Y	N	1	1
GE7	0	1	N	N	N	Y	1	2
GE8	1	1	N	Y	Y	N	2	2
GE9	1	3	Y	N	N	N	1	3
GE10	0	1	N	Y	N	N	1	1
Ave farm grade	1.7	2					1.3	
GFA1	4	3	N	Y	Y	N	3	1
GFA2	3	1	N	N	Y	N	2	S
GFA3	S	S	S	S	S	N	1	S
GFA4	5	2	N	N	N	Y	3	2
GFA5	5	1	N	N	Y	Y	2	1
	4.25	1.75					2.2	
GFB6	1	1	Y	N	N	N	2	0
GFB7	1	2	N	N	Y	Y	1	1
GFB8	1	1	N	N	N	Y	3	0
GFB9	0	0	N	N	N	N	1	0

GFB10	0	0	N	N	N	N	1	0
Ave farm grade	0.6	0.8					1.6	
GG1	0	2	N	N	N	N	4	1
GG2	0	1	N	Y	N	N	3	1
GG3	0	1	N	Y	N	N	4	0
GG4	0	1	N	Y	N	N	3	1
GG5	5	1	N	Y	N	N	1	1
GG6	2	3	N	Y	N	N	3	3
GG7	0	1	N	N	Y	N	3	0
GG8	1	3	N	N	Y	N	3	3
GG9	2	3	N	N	N	N	3	1
GG10	1	1	N	N	N	N	2	0
Ave farm grade	1.1	1.7					2.9	
GH1	3	1	N	N	Y	N	4	2
GH2	1	3	N	N	Y	Y	5	2
GH3	1	3	N	N	Y	N	3	1
GH4	1	2	N	N	N	N	4	1
GH5	S	S	S	S	S	S	S	S
GH6	S	S	S	S	S	S	S	S
GH7	1	3	N	N	Y	N	4	0
GH8	S	S	S	S	S	S	S	S
GH9	1	2	N	N	Y	N	2	1
GH10	1	2	N	N	Y	N	1	1
Ave farm grade	1.3	2.3					3.3	
NA1	2	1	N	N	N	N	1	0
NA2	1	1	N	N	N	N	4	0
NA3	3	2	N	N	Y	N	3	0
NA4	0	1	Y	N	N	N	4	0
NA5	3	1	N	N	N	N	2	0
NA6	S	S	S	S	S	S	S	S
NA7	0	1	N	N	N	N	2	1
NA8	0	1	N	N	N	N	1	1
NA9	0	1	N	N	N	N	1	1
NA10	0	1	N	N	N	N	1	2
Ave farm grade	1	1.1					2.1	
NB1	1	1	N	Y	N	N	2	0

NB2	2	1	N	N	N	N	3	0
NB3	3	1	N	Y	N	N	3	0
NB4	3	2	N	Y	N	N	4	0
NB5	1	1	N	Y	N	N	4	0
NB6	0	2	N	Y	N	N	1	0
NB7	1	1	N	Y	N	N	1	0
NB8	3	1	N	Y	N	N	3	0
NB9	2	1	N	N	N	N	2	0
NB10	2	1	N	Y	N	N	4	0
Ave farm grade	1.8	1.2					2.7	
NC1	0	2	N	N	N	N	2	0
NC2	0	1	N	N	N	N	3	0
NC3	0	1	N	N	N	N	2	0
NC4	3	1	Y	N	N	N	2	0
NC5	0	1	N	Y	N	N	1	0
NC6	1	0	N	N	N	N	1	0
NC7	0	1	N	N	N	N	2	0
NC8	0	1	N	N	N	N	3	1
NC9	1	1	Y	N	N	N	1	0
NC10	0	1	N	N	N	N	1	0
Ave farm grade	0.5	1					1.8	
ND1	2	1	N	N	N	Y	3	1
ND2	S	S	S	S	S	S	S	S
ND3	0	2	N	N	N	N	5	1
ND4	3	2	N	N	N	N	5	1
ND5	2	1	N	N	N	N	3	0
ND6	3	2	Y	N	N	N	4	0
ND7	2	1	N	N	N	N	5	0
ND8	2	2	N	N	N	N	5	0
ND9	3	1	N	N	N	N	2	0
ND10	3	1	N	N	N	N	3	0
Ave farm grade	2.2	1.4					3.9	
NE1	2	1	N	N	N	Y	3	1
NE2	4	3	N	N	N	N	5	2
NE3	3	1	N	N	N	N	3	0
NE4	3	1	N	N	N	N	2	0

NE5	2	1	Y	N	N	N	4	1
NE6	0	1	Y	N	N	N	3	1
NE7	2	1	N	N	N	N	4	0
NE8	2	1	N	N	N	N	5	0
NE9	3	1	N	N	N	N	4	2
NE10	3	1	N	N	N	N	5	2
Ave farm grade	2.4	1.2					3.8	
NF1	3	1	N	N	Y	N	4	2
NF2	3	2	N	N	Y	N	2	2
NF3	S	S	S	S	S	S	S	S
NF4	3	1	N	Y	N	N	2	2
NF5	3	2	N	Y	Y	N	1	0
NF6	1	1	N	N	Y	N	1	2
NF7	0	1	N	N	Y	N	1	0
NF8	2	2	N	N	Y	N	1	0
NF9	3	1	Y	Y	N	N	1	0
NF10	1	1	Y	N	N	N	1	0
Ave farm grade	2.1	1.3					1.6	
NJ1	1	1	Y	N	N	Y	4	0
NJ2	0	1	Y	N	N	N	3	0
NJ3	1	1	Y	N	N	Y	4	0
NJ4	0	1	N	N	N	Y	2	3
NJ5	1	1	Y	N	N	N	2	1
NJ6	3	1	N	N	N	Y	5	3
NJ7	1	1	N	N	N	N	S	1
NJ8	1	1	N	N	N	N	5	0
NJ9	1	1	Y	N	N	Y	5	0
NJ10	1	1	N	N	N	N	3	3
Ave farm grade	1	1					3.7	
LB1	3	2	N	Y	N	N	1	2
LB2	3	3	N	N	N	N	5	2
LB3	2	2	N	N	N	N	4	3
LB4	3	2	N	N	N	N	2	3
LB5	3	1	N	N	N	Y	1	3
LB6	5	1	N	N	N	N	3	1
LB7	2	3	N	N	N	Y	4	2

LB8	2	3	N	N	N	Y	3	1
LB9	1	2	N	N	N	Y	4	3
LB10	3	2	S	S	S	N	1	S
Ave farm grade	2.7	2.1					2.8	
LC1	3	3	N	N	N	N	4	2
LC2	3	3	N	N	Y	N	S	3
LC3	3	2	N	N	N	N	4	2
LC4	1	2	N	N	N	N	4	2
LC5	3	2	N	N	N	N	3	2
LC6	3	2	Y	Y	N	N	S	2
LC7	3	3	N	Y	N	Y	3	2
LC8	1	3	N	Y	N	Y	4	1
LC9	4	2	Y	Y	N	Y	1	2
LC10	2	1	N	N	N	N	2	0
Ave farm grade	2.6	2.3					3.1	

(EH: epithelial hyperplasia; GH: goblet cell hyperplasia; T: Telangiectasis; LE: lamellar EGC infiltration; R: lamellar rodlet cell infiltration; E: epitheliocystis; EGC: eosinophilic granular cell infiltration at base of primary lamellae; LF: secondary lamellar fusion; S: Sectioning artefact; Y: Yes; N: No)

APPENDIX 4 TABLE 4: Liver and gastric histopathology

(See material and methods grading methods: TABLES 3-12,3-13,3-14, 3-15, 3-16)

Please note that with recognition for low liver lipid being more reflective of an unhealthy system, liver grades were scored in the data sheet as: grade 1: score 5, grade 2: score 4, grade 3: score 3, grade 4: score 2, grade 5: score 1)

Fish	LL	PA	LLF	PLF	N	G
GA1	5	0	1	3	1	1
GA2	5	0	4	1	1	2
GA3	4	1	3	1	1	1
GA4	5	0	5	3	1	2
GA5	4	0	1	1	1	3
GA6	5	0	3	1	1	3
GA7	5	0	4	1	0	3
GA8	5	0	1	1	0	3
GA9	4	0	4	2	0	2
GA10	5	0	1	2	1	S
Ave farm grade	4.7		2.7		0.7	2.2
GB1	5	0	4	1	1	1
GB2	3	0	4	1	1	1
GB3	4	0	3	1	1	1
GB4	3	0	3	1	1	2
GB5	4	0	3	1	1	1
GB6	4	0	4	1	1	1
GB7	4	0	2	1	1	3
GB8	4	0	4	1	0	1
GB9	4	0	3	1	0	1
GB10	5	0	2	1	1	1
Ave farm grade	4.4		3.2		0.8	1.3
GC1	4	0	5	2	0	1
GC2	5	0	5	1	0	1

GC3	5	0	5	1	1	2
GC4	5	0	4	1	1	S
GC5	4	1	4	1	0	2
GC6	5	1	4	2	0	2
GC7	5	0	4	1	0	1
GC8	5	0	4	2	0	S
GC9	5	0	5	1	0	1
GC10	5	0	5	2	1	S
Ave farm grade	4.8		4.5		0.3	1.4
GD3	5	0	1	1	0	1
GD4	5	1	1	1	0	1
GD5	5	1	1	1	0	1
GD6	4	1	3	1	1	3
Ave farm grade	4.8		1.5		0.25	1.5
GD7	4	0	1	1	1	1
GD8	3	0	1	1	0	2
GD9	4	0	4	1	1	2
GD10	4	0	3	1	1	1
Ave farm grade	3.8		2.3		0.75	1.5
GE1	4	1	5	2	0	2
GE2	4	0	5	1	0	1
GE3	5	0	4	1	0	4
GE4	4	1	4	1	0	1
GE5	5	1	4	1	0	1
GE6	5	0	5	1	0	4
GE7	3	0	5	2	0	3
GE8	5	0	5	1	0	3
GE9	4	0	5	2	0	3
GE10	5	0	4	0	0	2
Ave farm grade	4.4		4.6		0	2.4
GF1	2	1	5	2	1	1
GF2	4	0	5	0	0	S
GF3	2	1	5	1	0	1
GF4	2	1	4	1	0	1

GF5	3	1	4	1	1	3
Ave farm grade	2.6		4.6		0.4	1.5
GF6	3	1	3	1	1	2
GF7	4	1	4	3	0	1
GF8	2	1	5	1	0	1
GF9	4	1	4	1	0	S
GF10	3	1	5	3	1	1
Ave farm grade	3.2		4.2		0.4	1.3
GG1	4	0	1	3	0	1
GG2	4	1	1	0	0	1
GG3	5	0	2	0	0	1
GG4	5	0	1	0	0	1
GG5	5	0	4	0	0	1
GG6	5	0	1	2	0	1
GG7	5	0	4	1	0	1
GG8	3	0	4	2	0	1
GG9	4	0	3	1	0	2
GG10	5	1	2	0	0	S
Ave farm grade	4.5		2.3		0	1.1
GH1	4	0	4	3	0	3
GH2	4	0	4	3	1	4
GH3	4	1	5	3	0	4
GH4	4	1	4	2	0	4
GH5	3	1	1	1	0	4
GH6	3	1	1	1	0	4
GH7	3	1	1	2	0	4
GH8	5	1	5	3	1	2
GH9	3	1	1	2	0	5
GH10	3	1	4	1	0	4
Ave farm grade	3.6		3		0.2	3.8
NA1	3	0	4	2	1	1
NA2	3	0	4	1	0	1
NA3	3	0	4	1	0	1
NA4	4	0	4	1	0	1

NA5	5	0	5	2	1	1
NA6	3	0	2	1	1	1
NA7	4	0	5	1	1	1
NA8	5	0	5	2	1	2
NA9	5	0	5	2	0	1
NA10	4	0	3	2	0	1
Ave farm grade	3.9		4.1		0.5	1.1
NB1	4	0	2	1	0	1
NB2	5	0	3	2	1	1
NB3	5	0	3	2	1	2
NB4	4	0	4	1	1	3
NB5	4	0	4	1	1	1
NB6	5	0	5	3	1	3
NB7	5	0	5	1	1	2
NB8	5	0	5	1	1	1
NB9	4	0	5	1	1	2
NB10	5	0	5	2	1	1
Ave farm grade	4.6		4.1		0.9	1.7
NC1	3	0	5	1	0	S
NC2	4	0	6	3	0	1
NC3	3	0	5	1	0	1
NC4	3	0	5	2	0	1
NC5	4	0	5	2	0	2
NC6	4	0	5	1	0	1
NC7	2	0	5	1	0	4
NC8	3	0	6	3	0	2
NC9	4	0	5	2	0	1
NC10	5	0	6	2	0	2
Ave farm grade	3.5		5.3		0	1.7
ND1	5	0	4	1	1	3
ND2	5	1	1	0	1	1
ND3	2	0	2	0	1	1
ND4	3	0	4	1	0	1
ND5	3	0	5	3	0	5

ND6	4	0	3	1	1	4
ND7	3	0	3	2	1	1
ND8	5	0	5	1	1	1
ND9	2	1	1	2	0	2
ND10	4	0	5	2	1	1
Ave farm grade	3.6		3.3		0.7	2
NE1	3	0	1	0	1	1
NE2	3	0	0	0	1	1
NE3	4	0	4	0	0	2
NE4	2	0	1	0	0	2
NE5	1	0	2	1	0	2
NE6	1	0	3	0	0	2
NE7	1	0	4	0	0	1
NE8	3	0	4	1	1	2
NE9	3	0	4	0	0	1
NE10	2	1	4	1	1	1
Ave farm grade	2.3		2.7		0.4	1.5
NF1	5	0	3	1	1	3
NF2	5	0	3	1	1	4
NF3	5	0	4	1	1	2
NF4	5	0	5	1	1	1
NF5	4	0	3	1	0	2
NF6	4	0	3	1	0	1
NF7	4	0	4	1	1	4
NF8	4	0	3	1	0	4
NF9	5	0	5	1	0	3
NF10	4	0	2	1	0	0
Ave farm grade	4.5		3.5		0.5	2.4
NJ1	4	0	3	1	1	0
NJ2	3	0	2	1	1	2
NJ3	3	0	2	1	1	3
NJ4	3	0	2	1	1	1
NJ5	4	1	3	1	1	3
NJ6	4	0	2	1	1	2

NJ7	4	1	2	2	1	1
NJ8	4	0	2	1	1	2
NJ9	4	1	2	1	1	2
NJ10	4	0	2	1	1	2
Ave farm grade	3.7		2.2		1	1.8
LB1	4	0	3	3	1	1
LB2	3	0	1	2	1	3
LB3	3	0	1	2	1	2
LB4	3	0	1	2	1	1
LB5	4	0	2	1	1	1
LB6	4	1	1	1	1	2
LB7	4	1	1	1	1	3
LB8	3	0	1	1	0	1
LB9	3	0	2	2	1	1
LB10	4	1	1	1	1	2
Ave farm grade	3.5		1.4		0.9	1.7
LC1	4	0	1	0	0	3
LC2	3	1	1	1	0	3
LC3	3	1	1	1	0	4
LC4	3	0	2	1	0	5
LC5	3	0	1	1	0	4
LC6	3	0	1	1	0	4
LC7	5	0	0	0	1	5
LC8	5	0	0	0	0	5
LC9	5	0	1	0	1	5
LC10	5	0	0	0	1	5
Ave farm grade	3.9		0.8		0.3	4.3

(LL: Hepatocyte lipid; PA: Portal adipose; LLF: Liver lipofuscin; N: Hepatocyte nuclear activity; G: Gastritis, S: Sectioning artefact)