Gill Disease in Barramundi (*Lates calcarifer*)

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This thesis is presented for the degree of

Master of Veterinary Science (with training)

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Declaration

I declare that this is my own account of my research, except where duly acknowledged and contains work that has not been submitted for a degree at any tertiary institution.

Neil R Griffiths
PREFACE

A veterinary degree is the most versatile of the biological sciences and much is expected by the general public from a Veterinarian.

I had a successful single man small animal practice in Adelaide, until one fateful day when driving my motor vehicle, with my trusted canine friend who kept a watchful eye; watched me suffer a Grand mal seizure and then hit a tree. My dog suffered concussion and I a month in hospital, multiple operations and multiple specialists, 2 months unable to walk and unable to practice with substantial pain and seeing the world differently than before, having time to think without the rigors of veterinary practice. I have no regrets.

For I always wished to use my veterinary degree for food production and with God saving my life and that of Caleb, I decided to investigate aquaculture, realising I knew little and needed training, which initially came from a course work masters from a non veterinary university. I started to discover a new world under my microscope from typical cytology I used in my practice every day, particularly for avian species which I did mainly for interest. I met Barramundi re-circulating aquaculture farmers at a meeting of the Inland Aquaculture Association of South Australia, asking for help with disease issues. I helped a little, but realising they needed more than what I had to offer, finding organisms or cells I did not know what they were and to my surprise others also.

So I went home to Perth, back to Murdoch University Veterinary School and asked for help. There I met Shane Raidal who taught me a decade before with an avian medicine workshop and had developed a passion for aquaculture and kindly offered me a Veterinary Master’s in Barramundi Gill Disease, Thank you Shane.

The skills developed from this Masters Degree with training, however long the road was from 2003 to 2008 have allowed me to fulfil what I wished to do in my second year of veterinary school, feed people rich or poor by developing an array of skills that any veterinarian can. Small animal practice served me well and I hope I served my clients and their pets well for nearly one and half decades, but the transition into intensive animal husbandry has put me on the frontier of where agriculture must go and I hope to contribute just a little and I thank my veterinary degrees and those that suffered me, Thank you Shane and my family, particularly my wife Leanne, who one day had the same husband, but with a different outlook.

Neil R Griffiths
ABSTRACT

Disease is a major impediment to world aquaculture, amplified by the increase of the intensity of aquaculture relieving pressure from over depleted wild stocks, but with intensity brings disease and particularly disease of the fragile gill organ, exposed directly to the water environment. There is little literature on barramundi biology and the various forms of culture impacting on health, particularly the gill and much research is required in gaining a further understanding of this popular eating fish.

The light microscope is a pivotal tool with cytology and histology mandatory in assessing gill health. The gill biopsy should be considered part of a clinical examination as the water medium surrounding the gill and on the gill contains often fragile organisms that would otherwise be lost in fixation for histology alone, but easily viewed with cytology. Barramundi are easily anaesthetised and recovered like many terrestrials and gill re-growth is rapid, healing within days. Biopsies should be viewed unstained with and without phase contrast and then stained and reviewed, recognizing some ectoparasites maybe lost with anaesthetic agents and stains. The sacrificing of the fish after a live gill biopsy is necessary with histology and microbiology our major tools for diagnostics, with no other non invasive methods readily available as for terrestrials. Every year many new water organisms related to aquaculture are described in the literature and the finding of novel and new organisms makes the veterinary examination of the live fish exciting yet imperative.

A major concern is the gill pathogens found in wild barramundi were similar to those found in culture. For example the prevalence of the parasite Henneguya a Myxosporidean was 90% in sea cages 60 km offshore from Darwin in the Bathurst Island river system and 66% for ponded fish with water drawn from the Darwin Elizabeth river, compared to 33% infected in the wild habitat of the Mary river system close to Darwin by road. However the bacterial disease Epitheliocystis had a prevalence of 66% in the sea cages and 18% of similarly sized fish in the Mary river system, yet nil found in the pond farm, but in this case sample numbers were restricted. Consequently the surveillance for new fish pathogens and monitoring for existing pathogens in the wild ecosystems and aquaculture facilities is necessary and must include the macro and micro flora and fauna surrounding such facilities as they are potentially affected from aquaculture waste streams. The sustainability of aquaculture in open water culture must be considered with great concern for many reasons, but disease by its nature could overwhelm a species and other aquatic life quickly disseminated in a dynamic water medium.

Freshwater culture of barramundi has problems with off flavour and disease, particularly re-circulating aquaculture systems due to undercapitalization and possibly at this stage with existing type farms not suited for the culture of barramundi with one farm having all fish
sampled diagnosed with systemic bacteraemia and gill Epitheliocystis. Commonly fish sampled from freshwater culture had suffered pathological changes to the gill, particularly hyperplasia indicating the fish are continually affected by issues of water quality and disease.

Pond culture appeared to control gill disease issues by affording lower stocking rates, high water exchanges from a river within metres, fallow and the flavour of the fish similar to wild catch or sea cage culture, when purged in brackish water. The decreased environmental and ecosystem risks, coupled with the pond farmer reporting good profits with a simple form of culture, also suitable for intensification is a success story for barramundi production for today and the future.
AKNOWLEDGEMENTS

This project was predominantly funded by the insurance company that kindly upon medical advice offered me a re-training package, of which funded the purchase of laboratory grade microscopes and a digital camera interface taking all of the photos for this thesis and most of the travelling expenses completing the survey.

I would like to thank Murdoch University for crediting the course work component from Deakin University, which gave me the necessary background, particularly in water quality, nutrition and the overall world stage of Aquaculture in performing this research project. Additionally thanking Murdoch Universities Fish Health Unit and Shane Raidal for supplying funding and facilities, for histology, TEM, SEM and staff facilitating a graduate from the 1980’s with the necessary diagnostic tools and support to explore a new frontier for myself and a fledgling Australian aquaculture industry.

A Thankyou to Western Australian Fisheries Pathology Department headed by Brian Jones, who taught and encouraged many veterinarians with his thirty years of service in histopathology in aquatic science and encouraged me to keep looking at the unusual cells discussed and shown in the thesis beginning in 2003, when I asked him what are they? I remember him saying ‘I have seen them before, but have not the time to investigate them.’ Hopefully a little light has been shed for more research into these unusual cells found on gills of a few Australian major finfish species, particularly the icon Barramundi.

Mentoring is part of teaching and I thank Shane Raidal again for patiently leading me in the right directions of this thesis and through the skills and confidence gained not only bridging the gap into aquaculture, but also intensive animal husbandry of meat rabbits, both requiring an interface by a veterinarian at the farm level, to pathologists, laboratories and tertiary institutions.
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LIST OF ABBREVIATIONS

DE Digitally enhanced
EGC Eosinophilic granular cell
EUS Epizootic Ulcerative Syndrome
FAO Food and Agriculture Organisation of the United Nations
MFHU Murdoch Fish Health Unit
MM Melanomacrophage
MRC Mitochondrial rich cells (also known as chloride cells)
NT Northern Territory
PAAS Periodic Acid Ammoniacal Silver
RAS Recirculating Aquaculture System
SA South Australia
SEM Scanning Electron Microscopy
TEM Transmission Electron Microscopy
TAN Total Ammonia Nitrogen
WA Western Australia
Chapter 1.  LITERATURE REVIEW

1.1.  INTRODUCTION

Barramundi or *Lates calcarifer* is an Australian icon fish sought after by sports fisherman and particularly consumers now on four continents as a table fish for its premium flesh quality. Wild caught fishing has been at unsustainable levels for approximately 15 years, despite aquaculture steadily increasing in the contribution to world demand from 3.9% of total production in 1970 to 31.7% in 2003 and rising to approximately 42% today (Haliwell, 2008), with the major constraint being sustainability to meet world demand for fish and protein products.

Developmental trends in aquaculture have seen the shift from small scale subsistence fish farming and low yielding semi-intensive production for local consumption, to higher yielding semi-intensive to intensive practices from increases in market demand and trends forged from an increase in knowledge, technology, finance and a global economy.  Trends have been focused upon lowering costs of production allowing aquaculture products to be competitive against wild catch, yet shifting to higher value fish as the market grows in affluence as seen in Japan since the 1970’s and more recently China, the largest consumer of fish products in the world consuming 67% of world supply.  The Food and Agricultural Organisation of the United Nations (FAO) have major concerns of the pace and direction of fisheries research and supporting information systems that are falling behind the need to understanding the relationships between the environment, wild fisheries management and aquaculture development. Included within these concerns are aquatic animal health issues; a major
constraint for future aquaculture development with trends driving towards intensification, thereby amplifying disease issues.

It is the purpose of this thesis to discuss the causes and pathology of Barramundi gill disease by performing a gill disease survey on different farms using different culture methods, including a wild catch within Australia of this euryhaline fish.

1.2. OVERVIEW OF ANATOMY, MORPHOLOGY, BIOLOGY, HISTOLOGY AND PATHOLOGY OF THE GILL OF TELEOST FISH

1.2.1. Gross anatomy and morphology

The gill is a thin delicate organ allowing water, gas, ion and biological waste transport. Each arch of teleosts comprise of two rows of lamellae, allowing interdigititation of the secondary lamellae for rapid oxygen absorption as is shown by the diagram from Evans, Piermarini and Choe (2005). Primary and secondary lamellae are now termed filaments and lamellae respectively by many recent authors since 2000.
Figure 1.1 Schematic of a simplified gill functional morphology of water flow (Evans et al., 2005)

Figure 1.2 Basic histology of a teleost gill showing secondary lamellae coming off each side of a primary lamella National Conservation Training Centre (www.training.fws.gov./BART/fish/histol1.html)
1.2.2. **Histology and specific cell function**

The cellular changes seen with disease can only be understood with familiarity of normal gill tissue and cell types. Figure 1.2 and 1.3 show Haematoxylin and Eosin (H&E) stained sections of the gills from teleost Rainbow trout; a freshwater fish commonly cultured in Australia showing the major epithelial cells.

![Image of histology](image.png)

**Figure 1.3 Magnified view of the base of secondary lamellae a normal teleost**

(www.training.fws.gov./BART/fish/histol1.html)

The gill filament is made up of epithelium, endothelium, fibrous and cartilaginous support and specialised cells including mucous and chloride cells. The predominant lamella cell is the internal pillar cell; a modified endothelial cell forming the blood spaces unique to fish gill. Creating a counter current flow of blood versus water particularly for oxygen uptake and probably other gaseous & ion/water molecules by passive exchange through the pavement cell, but this is unclear and more research is required (Evans, Piermarini and Choe, 2005).
The gill epithelium is 90% pavement cells of squamous to cuboidal in shape having microridges on the surface projecting into the water medium; commonly referred to as respiratory cells. The microridges increase the functional surface area of the epithelium and possibly helping mucous adherence. There is evidence that the tight junctions and pavement cell function varies between teleosts requiring further research (Evans, et al. 2005).

The chloride cells, now termed mitochondrial rich cells (MRCs), are typically found at the base of the lamellae on the afferent or trailing edge of the filament and are considered as the primary site of active physiological activity (Evans, et al. 2005; Wilson and Laurent, 2002). In teleosts the form and function of MRCs appear to vary with marine fish having clusters occurring with accessory cells. Accessory cells are similar in function to MRCs or developing MRCs. However freshwater MRCs clusters and accessory cells are uncommon, with MRCs usually occurring singularly with multi-stranded intercellular junctions with surrounding pavement cells or other MRCs forming an impermeable barrier to ions. The evidence for freshwater MRCs involved in chlorine changes is indirect or lacking at all (Wilson and Laurent, 2002). The few freshwater and euryhaline species studied including Atlantic salmon and Brown trout, demonstrated two subtypes of MRCs being α and β cells between the distal filaments called the interlamellar space or septa. α is associated with the arterioarterial circulation and β with the arteriovenous circulation. In brown trout the α MRC proliferate into the typical seawater teleost MRC and the β degenerate upon transfer to seawater, however in the Killifish also a euryhaline species upon transfer from seawater to freshwater, MRCs first morphologically and immunochemically converted to freshwater MRCs and then gradually were replaced by newly generated freshwater MRCs. How these and other
euryhaline fish and particularly Barramundi, can swim daily from saltwater to brackish water and probably to freshwater and vice versa is unknown. Evidence suggests the molecular or biochemical processes accompanying acute salinity transfers, may be species specific with commonalities between species, allowing changes in plasma osmolarity to elicit both rapid sodium and chloride extrusion by the gill epithelium in Killifish and probably other functions yet to be shown (Evans, et al. 2005).

Goblet type mucus cells are large ovoid cells composed of mucus secretory granules and are found in descending numbers in the efferent edge, afferent edge, interlamellar septa and base of the filament and there is great variability between species. There are larger numbers of mucus cells in freshwater fish than saltwater for reasons unknown and can be differentiated by either acidic or neutral mucins present in their granules (Wilson and Laurent, 2002). The primary function of mucus is protecting the gill surface particularly from micro-organisms by entrapping and continuously sloughing to the water medium (Roberts, 2001).

Eosinophilic granular cells (EGCs) are the most common immune based cell present in gill tissue, which may be homologous to mammalian mast cell (Wilson & Laurent, 2002) and the Rodlet cell first thought to be parasitic, but probably inflammatory in function with a similar role as eosinophilic granular cells are infrequent in comparison (Manera and Dezfuli, 2004). *Aeromonas salmonicida* (Ellis, 1991) and the parasitic copepod *Ergasilus* (Dezfuli et al. 2003) produced increased numbers and degranulation of EGCs in the gill, possibly having an antibacterial and antiparasitic effect respectively. Similarly high Rodlet cell numbers were also present with the copepod infestation, but their presence and function of both cells requires further elucidation (Dezfuli et al. 2003).
Fixed macrophages often with melanosomes within lysosomes are found in the gill epithelium engulfing microbes and carbon particles, particularly bacteria. Melanin probably plays a bactericidal role for reasons not yet known (Roberts, 2001). Occasionally melano-macrophages centres develop in association with chronic inflammatory lesions in the gill and are commonly found elsewhere particularly internal organs, as complex discrete centres containing lymphocytes and macrophages; possibly analogous to the mammalian lymph node. Melano-macrophages major function appears to be the capture and storage of iron in haemolytic disease, with minor functions of antigen trapping and presenting antigen to lymphocytes. Sequestrations of degraded cellular products including melanins and catabolic by-products have been suggested. They also increase in size or frequency with environmental stress and potentially could be used as bio-markers for water quality from internal organs (Agius, Roberts, 2003).

Lymphocytes were identifiable from an isolated suspension of Atlantic salmon gill cells with their typical large nucleus and little cytoplasm (Lin, Davidson, Secombes, Ellis, 1998) and probably increase in number during certain pathological or physiological circumstances in particular infection or irritation (Roberts, 2001).

Additionally (Lin, et al. 1998) suspended neutrophils from gills and they appear to have similar morphological and histochemical staining properties to mammalian neutrophils (Roberts, 2001) and are phagocytic, chemotactic and bactericidal, but usually are few in number compared to macrophages.
Much research is required in the area of gill immunity in all finfish species as the literature is not only scant, but extrapolates cellular function and interrelationships from their apparent common or similar role with terrestrial species, for example the EGC and mast cell and are struggling to define the function of other cells such as the Rodlet cell first identified in 1892 (Manera and Dezfuli, 2004). Greater knowledge of immunity not only elucidates pathogen impact on the host, but treatment development and a measurable response from fish.

1.2.3. Disease Responses of the Gill

Situated within the environment the relatively unprotected gills are vulnerable to chemical and physical trauma from dissolved and suspended material within the water column. Additionally the gill environment favours external parasite infestations from nutrient availability and suitable binding sites and habitat (Roberts, 2001). It has been established that bacteria, viruses and soluble agents can enter through the gill (Roberts, 2001) causing disease such as the bacteria *Edwardsiella ictaluri*, the causative agent of enteric septicemia in Channel Catfish (Nausbaum, Morrison, 2002), and the skin & gill viral disease of many finfish species Lymphocystis (Roberts, 2001). Primary gill disease such as Bacterial Gill Disease caused by *Flavobacterium branchiophila* affecting salmonids and *Flavobacterium columnare* causing Columnaris Disease in freshwater species both have the ability to bind to the gill epithelium causing hyperaemia, oedema and gill pathology. Fish gills respond rapidly to irritants and their response can enhance secondary bacterial and fungal infection risks from within the water medium (Noga, 2000; Decostere, Haesebrouck, Van Driessche, Charlier, Ducatelle, 1999).
With few components making up the gill, the pathological responses to pathogens are few and are evident histologically. The most common change resulting from mild irritants are changes in membrane permeability of cells and tissues, resulting in epithelial cell and sub-epithelial oedema, often with an increase in mucus also occurring on the gill surface (Roberts, 2001) and these changes and effects can be reversed with the irritant removed. However severe gill filament insults showing histological changes such as oedema, necrosis, hyperplasia, fusion and clubbing (Sanchez, Speare, Johnson, 1997; Clark, Nowak, Handler, Munday, Percival, 1997), are often non-reversible and potentially lethal if widespread.

Gill oedema often results from chemical insults particularly in freshwater culture, such as pollution from certain heavy metals, algae overgrowths, pesticides and chemical treatments including commonly used formalin and hydrogen peroxide. The use of calcium commonly in the form of finely ground limestone not only buffers the water with carbonates against acid production from freshwater fish culture, but calcium ion can potentially reduce the number of binding sites from the gills large charged surface area, particularly heavy metals, thus reducing the toxic effects & pathology in the gill (Roberts, 2001; Tao, Long, Xu & Dawson, 2002).

Necrosis is the death of cells or a portion of tissue or organ in the living body. Mild necrosis includes epithelial lifting, epithelial cell swelling and nuclear ghosting viewed as large very pale nuclei with clumped chromatin. Severe necrosis can be described by nuclei changes, including pyknosis, leaving the nucleus shrunken and very dark; karyorhexis, the rupturing of the nuclear membrane and fragmentation of the nuclear chromatin; and karyolysis, the
nucleic acids losing their basophilia and the tissue becoming shiny pink in colour. After severe necrotic changes, outlines of individual cells are indistinct, with affected cells possibly merging. Autolytic post mortem changes are readily confused with pre-mortem necrotic changes, except the changes are more generalised throughout the body, but sacrificing live fish provides a clearer picture of disease with gill autolysis very rapid, requiring immediate formalin fixation for accurate histology (Roberts, 2001; Clark, Nowak, Handlinger, Munday, Percival, 1997).

Filament and lamellae hyperplasia is a long term response, usually from a low level insult. Hyperplastic gills from an inflammatory process are excessively covered in mucus and thickened from cellular infiltration, changing consistency of normal gill anatomy and where extensive, impairing respiration and acting as a substrate for bacterial colonisation (Roberts, 2001).

The mucus cell is commonly found in increasing numbers after an initial decrease at the base of the lamellae from an insult and is recognised as being important in the gill defence against fish pathogens particularly bacteria and parasites, yet few studies have been performed as to the composition of fish mucus. Fish mucus between species of similar environments can differ in pH and viscosity within varying salinities and with disease states for example the euryhaline Atlantic salmon, Rainbow trout and Brown trout infected with Amoebic Gill Disease (AGD). Gill mucus cells vary in the number and ratio of acid and neutral mucus cells with a general trend in a shift from neutral in freshwater to acid in saltwater for all three species, with Atlantic salmon producing a more acidic mucus than the other two species for reasons unknown. The glucose and protein content between these species also varies within
the two types of mucus cells (Roberts and Powell, 2005). Mucus thickens in presence of
disease for reasons unknown and much research is required to better understand the first line
of defence of the gill, skin and gastrointestinal systems including immunoglobulin
involvement (Roberts, 2001).

Likewise MRC numbers increase from changes in salinity for fish including euryhaline
species or in response to osmoregulatory distress, but is difficult determining the cause being
pathological from poor water quality or adaptive (Haaparanta, Valtonen, Hoffmann, 1997).
Increasing numbers can be found in the filament, but also moving distally on the lamellae.
For example acidification of the water supply and soil can increase aluminium solubility,
increasing MRC numbers extending on to the filament and the lamellae; probably a direct
toxic response possibly demonstrating multiple functions of MRCs (Roberts, 2001).

Immune cell infiltration is known to occur particularly including mononuclear inflammatory
cells (Khoo, Leard, Waterstrat, Jack, Camp, 1998) mononuclear lymphoid cells (Clark,
Nowak, Handlinger, Munday, Percival, 1997), macrophages and eosinophils (Bennett &
Bennett, 2001) but the descriptions in disease responses within the literature are few and
generally a broad description of hyperplasia is given. This may reflect the few articles written
on gill diseases in finfish.

Lamellae fusion often results from lamellae and filament hyperplasia with associated mucus
changes also causing lamellae to adhere focally. This produces a cribriform three-
dimensional lamellar complex and can be a frequent response to a number of stimuli
including many protozoan ectoparasites, particularly *Ichthyobodo necator* (Roberts, 2001).
Filament clubbing is seen with shortening, expanding and blunting of a filament or filaments, often becoming fused with a hyperplastic infiltrate of epithelioid cells. This can be seen with infectious and non infectious gill disease, for example Clubbing and gill necrosis syndrome and pantothenic acid deficiency in Rainbow trout (Clark, Nowak, Handlinger, Munday, Percival, 1997).

Additionally, injuries resulting from culture due to grading, handling, disease, parasites and chemical pollution to the delicate vascularity of the fine lamellae can be seen with pillar channel thrombosis caused from the rupturing of the pillar cells resulting in telangiectasis. Respiration will be compromised only with extensive telangiectasis (Roberts, 2001; Noga, 2000).

1.3. BARRAMUNDI CULTURE

1.3.1. Natural Habitat

*L. calcarifer* is a tropical catadromous (living in freshwater but reproducing in saltwater) teleost finfish species, found from the northern Indian and tropical western Pacific Oceans including the countries Iran, China, Taiwan and Papua New Guinea and the northern third of Australia (22 degree 30'S in Western Australia to 26 degree 30'S in Queensland). They spawn and go through egg and early larval stages in saltwater, but juveniles prefer to live in fresh or nearly fresh water with the protection of mangroves or river system hides until sexual maturity, then returning to saltwater (Tucker, et al. 2002)
Rimmer and Russell (1997/98) found releasing tagged Barramundi juveniles into Northern Australian waterways 62% of the fish, particularly juveniles were recaptured within 3 km of their release location, and 37% made intra-riverine movements of between 3 and 37 km. Five fish made inter-riverine or coastal movements indicating that the species often cover substantial distances in their natural environment, but do not migrate substantial distances possibly accounting for the different varieties in different regions, including Australia (Keenan, 1994).

1.3.2. Barramundi production worldwide

*L. calcarifer* is known by other names including Asian seabass, seabass, giant seabass, white seabass, twofin seabass, blind seabass, twofinned seabass, giant perch, silver sea perch, palmer, giant palmer, nairfish, kokop putih, bekti, apahap, plakapong, nokogirihata, and silver barramundi (Tucker, et al. 2002). In Asian countries such as Taiwan and Thailand production has decreased in the 1990’s (Tucker et al. 2002) attributed to low market prices. Yet since 2000 establishment of the Marine Harvest pilot sea cage grow out facility in Northern Territory and in 2005 fingerling exports to the USA & Europe from Australia began using tank culture. Australian Barramundi production has risen steadily from 1250 tonnes produced in 2001/02 to approximately 2,500 tonnes for 2006 (ABARE Economics, 2004, 2007). However with the closure of the Lake Argyle and Marine Harvest pilot facilities, production in Australia due principally to economics will have a short term reduction.
1.3.3. **Barramundi culture in Australia**

Barramundi are spawned and fingerlings grown in seawater in tropical Australia and then grown out to market size in fresh, brackish and seawater water in various regions of Australia. Feeding and growth are best at 27-30°C and drop sharply below 25°C with feeding ceasing at 20°C and death at 14°C, consequently climate controlled tank systems are used in temperate regions (Katersky, 2005).

Barramundi are grown predominantly in sea cages and ponds in Australia and Asia alike for market before they reach sexual maturity from 0.6 kg to 3 kg for the whole fish per plate and fillet markets taking 8-24 months respectively.

Tank systems require sophisticated filtration, disinfection and production methods increasing production costs, but when production approaches 100 tonnes per year, it becomes economically viable as many smaller farms have folded in recent times.

Stocking densities vary from extensive levels in ponds at less than 1kg/m³, 40kg/m³ for cages, to 45-100 kg/m³ for intensive tank culture, with food supplied commonly in Australia by a pellet based on a salmonid diet made by specialized agricultural feed companies (Kailasam, Chandra, Muralidhar, Thirunavukkarasu, 2001; Chou, Lee, 1997; Tucker, et al. 2002; Williams, Barlow, Rodgers, Hockings, Agecopra, Ruscoe, I, 2003).
1.3.4. **Gill pathogens and Gill Disease in Cultured Barramundi**

1.3.4.1. **Introduction**

In Satul Thailand 1988, the Barramundi or Asian Sea Bass workshop on disease discussed causes of mortality in South East Asia. Diseases were viral Lymphocystis; bacterial Aeromonads, Vibriosis, Columnaris disease; the parasitic protozoa *Cryptocaryon*, Trichodina, Henneguya, Epistylia; parasitic helminths, Monogenetic Trematodes, Digenetic Trematodes, nematodes, Acanthocephalan; the parasitic copepods Caligus, Lernanthropus; and parasitic isopods (Kungvanki, et al. 1986). Likewise in Australia diseases discussed at Queensland workshops (Philips, 1998) and from government Federally and State websites report of the common pathogens causing deaths in culture, as being VNN, Lymphocystis; *Cryptocaryon*, Vibriosis, Columnaris, *S. iniae* and general bacterial diseases including opportunistic Aeromonads and Vibrios.

Only diseases that are reported in the literature affecting the Barramundi gill will be discussed.

1.3.4.2. **Viral**

Lymphocystis caused by an iridovirus is a chronic, self limiting disease of many fresh and saltwater finfish species affecting gill and skin, but only specific isolates affect the same fish family or genus. It predominantly affects juvenile Barramundi with high stocking rates increasing cannibalism and/or frequent grading aimed at reducing cannibalism with both being stressful for the fish. Transmission is probably from the rupture of lesions and most likely many fish carry the infection sub-clinically, but when stressed clinical lesions of various sizes appear from pinpoint to coalesced grape size in white to pink colour masses.
Histologically hypertrophied fibroblasts with basophilic intracytoplasmic inclusions are seen (Noga, 2000). Diagnosis is often made clinically, but should be differentiated by histology and cytology from epitheliocystis, which mainly affects gills and mild infestations of *Ichthyophthirius* affecting gills and skin. Treatment is usually not necessary if no secondary infection requiring antibiotics. Reducing causative stressors if possible and minimising skin trauma when grading or handling is advisable. It is likely that affected fish are contagious and should be quarantined for at least 1 month (Noga, 2000), but further studies are required for definite quarantine periods.

1.3.4.3. **Bacterial**

The predominantly opportunistic bacteria such as *Vibrio* and Aeromonads generally enter via the skin and gastrointestinal system causing systemic disease, and there is no literature suggesting gill entry occurs. Columnaris however, a freshwater bacterial disease, can enter the skin or gills of which *Flavobacterium columnare* is most common, affecting most finfish species (Kunttu, Suomalainen, Jokinen, Valtonen, 2009). It was previously named *Flexibacter columnaris* (Thomas-Jinu & Goodwin, 2004). It is similar to saltwater *Vibrio* strains, where virulence of strains is the most important factor determining severity of disease. Other factors increasing bacterial pathogenicity in fish generally, include water hardness greater than 70 mg/L measured by total calcium, high organic loadings promoting bacterial growth, low oxygen, high nitrite and a drop in temperature below 25°C for warm water species and above 20 °C for cold water fish. Virulence of strains varies between fish species of both warm and cold freshwater varieties, with some strains killing cold water species with no apparent pathology at 15 °C (Thomas-Jinu and Goodwin, 2004). Clinically gill lesions are less common but more serious than skin lesions, which are confirmed by
Chapter 1. Literature Review

histology seeing erosions leading to necrosis beginning at the tip of the filament, moving towards the arch. Wet preparations quickly show long, thin rods with no flexing or gliding motion as seen on other species of Flavobacteria, such as Flavobacterium johnsoniae and Flavobacterium psychrophilum and if left for a while all aggregate, forming colonies (Kunttu, Suomalainen, Jokinen, Valtonen, 2009). Commonly diagnosis is made by wet mounts with no histology (Noga, 2000). Culture and antibiotic sensitivities should be performed if antibiotics are required, growing on Cytophaga agar or Anacker & Ordal agar (Cepeda, García-Márquez, Santos, 2004), but not on standard media. Antibiotics should be used if gill lesions are present or greater than 5% of body surface area is affected by skin lesions or systemic signs such as anorexia, piping or lethargy are present, otherwise potassium permanganate will often suffice used as an external bath.

It is currently thought that Epitheliocystis is a Gram negative intracellular microbe of bacterial origin belonging to the order Chlamydiales derived by molecular methods in Barramundi and other fish species (Nowak and LaPatra, 2006). Additionally the results suggested that Lymphocystis cysts known to be caused by iridovirus infection could be coinfected with the epitheliocystis agent, aiding its spread and potentially to other organisms including ectoparasites. Epitheliocystis is usually benign in low numbers in freshwater or saltwater Barramundi culture, but for any fish species high numbers can result when stressed in culture, particularly fingerlings which may show respiratory distress, excessive mucus on the gills, failure to thrive and occasionally death, possibly related to secondary opportunistic pathogens. Microscopically either by cytology or histology the enlargement of infected epithelial cells can be readily seen, although in other species of fish other gill cells can become infected such as MRCs and goblet cells. The gills are the most common site of
infection, but lesions can be found in the integument and less commonly the orobranchial cavity, nostrils, the mucosa of oesophagus and pseudobranch of many fish species.

Histology diagnoses the pathogen showing the circumscribed spherical cysts with an eosinophilic hyaline capsule, containing coccoid or coccobacillary organisms that complete a pleomorphic developmental cycle. *Epitheliocystis* can cause a significant proliferative reaction in some species and little reaction in others, yet can have a variable reaction in the one fish. The cellular response can vary from little reaction with a thin layer of squamous epithelial cells walling the lesion, to varying degrees of lamellar fusion, inflammation, hyperplasia, increasing mucus cell numbers, epithelial lifting and necrosis.

As the organism has yet to be cultured, histology is mandatory for diagnosis.

It has been suggested that *Epitheliocystis* has two differing developmental cycles and the specific morphological characteristics depend on the stage of intracellular development for both cycles. Currently results from research papers suggest high diversity and host species-specificity of the *Epitheliocystis* agents and transmission between species is limited (Nowak and LaPatra, 2006).

There is no known effective treatment, but removal of known stressors and infected fish with quarantining of the batch of fish is recommended. Possibly a reduction in infection rates may occur in cultured fish if contained in re-circulating tanks or raceways by the use of ultra violet sterilizers and/or ozonation, otherwise increasing water flow and decreasing stocking rates in open or closed systems may help.
1.3.4.4. **Parasitic**

Parasitic diseases are a common problem for gills and skin in culture and are often difficult to classify with detail as disputes surround the taxonomy of many, but are typically classified into the Kingdoms of Protozoa and Metazoa. Protozoa comprises many phyla that are single celled eukaryotes and the multicellular Metazoa includes monogeneans, digeneans, crustaceans, larval molluscs, leeches and lampreys (Roberts, 2001).

The protozoa are the most common parasites experienced in the culture of teleosts (Noga, 2000) and with knowledge increasing rapidly; a greater understanding will unfold of the vast array of protozoal organisms. Damage is generally caused by their feeding activity, causing hyperplasia histologically with excessive mucus production on the gills and skin, usually resulting from increased numbers with deteriorating water quality.

*Cryptocaryon* or Marine Ich is a common tropical marine ectoparasite causing marine white spot and typically seen as small white lesions smaller than 1mm, but often interconnect forming larger masses on gills, skin and occasionally the eyes. It has a similar lifecycle as the freshwater white spot, *Ichthyophthirius multifilis*, but temperatures are higher inhabiting warm marine environments of 20 to 30 °C, with optimal reproduction at 30 °C, completing its lifecycle less than 6 days. It has been reported in wild stocks from Australasia, Israel, Caribbean to South America, including many Mariculture facilities in those regions, with intra-specific variants in rDNA sequences for different temperature regions, but is not teleost fish species specific, but does not affect elasmobranchs. A mature adult trophont will break out of the epithelium producing up to 200 infective theronts within a tomont encysted on a substrate usually on the bottom of the container, which are released asynchronously from 3 to
72 days by each trophont on the fish, but peaking at around 6 days for an infected fish batch (Noga, 2000; Roberts, 2001). The mechanism of host location is unknown, but probably occurs at night when many fish species are inactive and experimental results showed only up to 20% infect a fish in ideal conditions, but culture containers increase burdens significantly (Colorni & Burgess, 1997). Irritation is caused from feeding producing pruritis seen as fin tremors to hyperactivity with sudden darting movements and flashing by quick movements of the body on the side rubbing against container walls or floor (Noga, 2000). Clinically respiratory difficulty is common, seen as piping at the surface or congregating at air outlets resulting from high pathogen numbers and often leading to death. Pathology typically is epithelial hyperplasia, mucous production, and secondary bacterial and/or fungal infections with diagnosis usually via cytology, seeing continuously revolving pear-shaped ciliates within the fish epithelium in fresh gill or skin biopsies. Histologically the parasite settles at the basement membrane of the gill filament and within 30 minutes becomes enclosed by a thin layer of epithelial cells. Fusion of the lamellae and irreversible obliteration of the interlamellar septa occurs after frequent and heavy re-infections (Noga, 2000; Roberts, 2001).

Unlike *Ichthyophthirius multifilis* there is no C-shaped macro-nucleus and recent DNA comparisons have placed them in different classes, despite similar survival strategies. Treatment for barramundi as other teleosts would be 4 consecutive hyposaline baths at 8-10 g/L for 3 hours at 3 day intervals. Tomonts are intolerant to rapid salinity changes and this procedure can eradicate the disease in 7-10 days. However sea cage and pond culture would prove practically difficult. Formalin alone or with acriflavine, malachite green, methylene blue or copper sulphate has been tried with variable success in reducing burdens due to the impervious cyst wall and all but formalin are not registered or permissible in aquaculture.
Fish surviving infections have varying degrees of acquired immunity lasting up to 6 months, possibly accounting for reported treatment success, potentially driving a preventative measure through vaccine development. Possibly treatment after dark will prove more effective with the emerging trophonts, but eradication thus far has proven difficult with its complex lifecycle and lack of effective fish and environmentally friendly chemotherapeutics. Tank culture disinfection systems using ozone and/or ultraviolet, has shown effectiveness in destroying the non host stages (Colorni & Burgess, 1997).

Barramundi however being a euryhaline could possibly be treated successfully with a 3 hour exposure or prolonged exposure to freshwater for 7-10 days, including the container killing the tomonts and theronts, but no literature to this effect has been cited.

The ciliate *Trichodina* was first reported in causing gill disease in 1942 by Padnos and Nigreili and in different species of fish both cultured and wild, infesting particularly warm marine or freshwater fish with some *Trichodina* species infecting at all salinities. Generally *Trichodina* inhabit the skin and gills with many species infecting both, all with a direct lifecycle (Khan, 2004). The gill only variety are generally smaller at less than 30 micron with a smaller host range compared to the skin larger at less than 90 micron in size, whilst having a larger host range. Clinically acute mortalities can occur in younger fish with high burdens particularly with secondary bacterial infections, but often only causing morbidity issues such as anorexia and ill thrift. Chronic infections can have mortality rates at 1% per week and often another condition underlies a *Trichinosis* problem. An unstained gill clip quickly demonstrates their characteristic scooting; spinning top type motion but their presence is lost during formalin fixation for histology. Moribund fish may have more than 1 per 100 ×
magnification on cytology, but interpretation causing primary disease is unusual. Treatment is similar as for many external ciliates with formalin the most common (Noga, 2000; Khan, 2004).

Metazoa reported infesting Barramundi gills are the Monogenean trematode flukes *Gyrodactylidae* and *Dactylogyroidea*; Myxozoan *Henneguya*; Copepods *Caligus*, *Lernanthropus*; and parasitic isopods (Kungvankij, et al. 1986).

Monogenetic trematodes are a group of flat worms completing their life cycle on a single host, with each species having a narrow host range restricted to one species, genus or family; however this is often lost in aquaculture. A major identifying characteristic of these helminths is their organ of attachment, the haptor. The single haptor or monopisthocotylea is more common, particularly the Gyrodactylid and Dactylogyrid in Barramundi and other fish species, but the Gyrodactylid is exotic to Australia infesting marine or freshwater and due to their viviparous live young on the fish, numbers can quickly over whelm fish skin and gills. The less pathogenic Dactylogyrids are freshwater egg laying parasites found in Australian finfish primarily infesting the gill and distinguished by 2 pairs of eyespots which are absent in Gyrodactylids (WA Fisheries, 2006; Noga, 2000).

Lesions observed histologically are primarily haemorrhage and necrosis of epithelial tissue of the gill and skin with a typical hyperplastic response, but diagnosis is made by gill biopsy, easily demonstrating the large parasites caterpillar-like motion. Treatment may include bath applications of formalin or potassium permanganate, with the later also treating mild superficial secondary infections. High burden treatment of choice is organophosphates, but
environmental issues prohibit use for open water ways, but Praziqantel has been successful on some species of monogeneans (Noga, 2000).

*Henneguya* is the largest genus of the Myxozoa phyla comprising about 150 species that are obligate parasites of fish, found in differing salinities from freshwater to seawater affecting the gills and skin of fish, including commercial fish species such as catfish (Noga, 2000) and salmonids (Kallert, Eszterbauer, El-Matbouli, Erseus, Haas, 2005). Like all Myxozoa many species of *Henneguya* present little effect in the health of many fish, unlike the exotic *Myxobolus cerebralis* causative agent of Whirling Disease. However significant economic loss has occurred for example in salmonids and carp from destructive flesh pseudocysts (Noga, 2000) and soft tissue inflammation respectively (Liyanage, Yokoyama, Wakabayashi, 2003) from Myxozoan infections. Little is known about Myxozoan biology and pathogenesis, with the intermediate host stage of their lifecycle proving difficult to elucidate apart from four species infecting tubifex worms (Kallert, Eszterbauer, El-Matbouli, Erseus, Haas, 2005). *Theohanellus horvakai* producing tissue inflammation can enter carp through the gill and gut; therefore other species including *Henneguya* may have similar routes, but ingestion is probably the most common route (Noga 2000).

*Henneguya lateolabracis* recently identified by Yokoyama, Kawakami, Yasuda, Tanaka, (2003), caused Cardiac Henneguyosis in Chinese Sea Bass, Lateolabrax species, a euryhaline species similar to Barramundi. Clinically the gills were anaemic and at post mortem the bulbus arteriosis of the heart was enlarged. Histologically irregular plasmodia released large numbers of mature spores into the lumen from the bulbus arteriosis, which were found occluding and congesting gill capillaries, with filament hypertrophy and degeneration of gill
epithelium present, resulting in clinical gill disease. Typically diagnosis is made by identifying the multicellular spores within tissue plasmodia containing developing spores with polar capsules. The spores are very resistant except for heat and unslaked lime disinfection and surviving fish may shed spores for long periods of time, but little is known apart from *M. cerebralis* of this phylum, therefore disease prevention is necessary for control of cysts if in large numbers affecting fish health. Prevention is possible by removing the intermediate host by the fine filtration of incoming water using chlorine based products or fine micron sand filters or by reducing their numbers by the lining of ponds. Other management strategies could include control of the intermediate hosts’ habitat, stocking larger fish into infected waters, not stocking infected fish, and stocking less susceptible species.

Copepods are crustaceans and comprise typically the sea lice, fish maggots and anchor worms varying in size from barely visible to the naked eye to 10mm. The lifecycles are predominantly free-living, with some species having intermediate stages parasitising other fish species, before the adult parasite finds its final fish host for egg production. *Argulus* the sea louse, a common general culture problem, attaches to the skin only, but the less common *Lernanthropus* anchor worm females attaches by a filament to the gill filament near the arch producing irritation and localised hyperplasia, with a risk of secondary bacterial and fungal infections. The males once mated die and high female numbers would be required to cause impairment to gill function (Noga, 2000; Roberts, 2001). The literature is scant concerning Copepod infestations outside of the Caligiform sea lice and treatment using organophosphates for Lice is reported effective for Lernaeids.
1.3.4.5. **Fungal**

The most common fungal infections in fish are the ubiquitous water molds mainly in freshwater, but the class Oomycetes can also occur in estuarine conditions, but not in marine. Barramundi are susceptible when water temperatures drop below 20°C (WA Fisheries, 2006), probably due to a compromised immune system at the lower end of barramundi’s physiological range and many Oomycetes are more active at cooler temperatures. The Saprolegnia family is the most common opportunistic invader of this class feeding saprophytically on dead organic matter in both fresh and brackish waters. Primary pathogenicity does occur in some species, for example *Saprolegnia parasitica* and *P. diclina* in salmonids, but little work has been performed in other species (Noga, 2000). Transmission often occurs by motile zoospores furthering dissemination, but most infections probably occur from dead organic loadings within an aquaculture system (Noga, 2000). Clinically fish can be seen with cottony growths on skin and gills unless out of water appearing as a slimy matted mass, which will spread if left untreated and under optimum conditions this can be within 24hrs. Fresh lesions are white, but become coloured from sediment and organic loadings from algae and debris (Bruno & Wood, 1994).

Morbidity and mortality rates depend on how much of the fish is affected losing electrolytes from its superficial lesions created from feeding enzymes. Diagnosis is confirmed on live fish with cytological wet mounts, demonstrating broad aseptate fungal hyphae (hyphae lacking cross walls) which can be seen histologically demonstrating shallow lesions with loss of epithelium with little inflammation using H&E stain. Identifying the water mould as oomycete requires observation of asexual sporangia for genus, which is not often seen on wet mounts and some species will not produce sexual stages in culture (Noga, 2000, Roberts...
2001). Treatment regimes at this stage are general for fungal infections, therefore it becomes
dademic for definitive identification and primary causes of disease should be investigated
when water moulds are diagnosed. Fish with mild coverage of skin or organs usually recover
with prolonged saltwater immersion, whilst reducing osmotic stress from infection and
antibiotics may be necessary for opportunistic bacteria. Malachite green is very effective, but
is prohibited in food fish (Bruno & Wood, 1994).

Epizootic Ulcerative Syndrome (EUS) is a reportable disease occurring in Barramundi
(Herfort, Rawlin, 1999), causing mass mortalities from deep skin ulcers and secondary
bacterial disease, but does not affect the gill directly.

1.4. SUMMARY

Barramundi gill anatomy, morphology, histology, physiology and disease are often
extrapolated from other commercial cultured species, also lacking much detail particularly in
gill disease resistance and immunity and consequently may only be partially accurate.
Furthermore differing forms of Barramundi culture possibly influences pathogens and
disease, therefore much research is required for Barramundi biology, disease and culture
techniques for reducing gill disease. This thesis documents for the first time gill disease in
Barramundi with different culture methods and hopefully elucidates future areas of research
for this popular sporting and table fish.
Chapter 2. Methodologies and Descriptions

2.1. FISH EXAMINATION ON EACH FARM OR HATCHERY

At all facilities 6 fish were randomly selected from tank, pond or cage for each batch per facility by net, or rod. Live fish were sampled, the number was the minimum accepted by government departments and the financial and time constraints of a predominantly self-funded project. Many species of ectoparasites leave a host immediately at death and non-obligate or opportunistic ectoparasites may increase in number and histological changes occur rapidly at post mortem in fish. Samples of gill were fixed within 3 minutes of death (Speare & Ferguson, 1989) avoiding post mortem changes.

Fish are anaesthetised using clove oil at 6ppm, measured in length and examined with the aid of a dissecting microscope for fingerlings under 80mm. There are better anaesthetic agents registered for fish requiring recovery, including the clove oil derived Aqui-S and the limited registration benzocaine, in particular. However the fish sampled were to be sacrificed and many aquaculture operators use clove oil, for it does not require a veterinary script to purchase. It was deemed appropriate to see the impact if any, of this widely used anaesthetic and sedation drug on the health and effect if any on gill pathology and pathogen biology (Yuzo, Woody, Shoji & Ueda, 2008).

Gill and skin biopsies were performed and viewed with a compound microscope from low power through to oil using a cover slip. Contrast improved by microscope diaphragm adjustments for gill and skin parasite identification/counts up to 400 × magnification and at
oil with and without phase contrast. Repeat cytology using 20% Diff Quik number 2 solution, diluted with distilled water highlighting cells of fish and non fish origin.

All skin biopsies performed with a scalpel scrape or the use of a glass coverslip and gill biopsies using curved iris scissors for 35-150 mm long fish and straight dissecting scissors for greater than 150mm. Gill biopsies less than 50 mm fish performed with magnification.

2.1.1. Internal examination at post mortem

Fish were sacrificed by severing the spinal column behind the operculum with sharp scissors or scalpel up to 80 mm long or a filleting knife for 60-150 mm, or via sharp pruning shears for fish larger than 150 mm.

Figure 2.1 45 mm barramundi fingerling cut behind operculum dorsally killing the fish quickly with little blood loss
Post mortem examination begins with the fish in lateral recumbency removing the operculum and excising gill arches 1-4 less than 50 mm, 1-2 above 50 mm and very large fish greater than 300 mm excise one arch for adequate primary lamellae numbers for histology and ease of storage in 50mL sterile containers with 90% of volume filled with buffered 10% formalin and remaining 10% with tissue.

The body cavity is opened via a stab incision in ventral recumbency just cranial to the vent, extending cranially to the gill chamber. The subopercle bone severed with scissors or garden shears depending on size to reflect the lateral wall exposing the body cavity. Gross examinations of internal organs are made in situ, excluding the kidney obscured by the swim bladder. The heart, liver and gastrointestinal system including the vent are removed by sharp dissection taking care to avoid gut spillage. Reflect the swim bladder exposing the kidney ventral to the vertebral column by careful blunt or sharp dissection. Aseptically excising up to 50% of the kidney is possible by blunt and sharp dissection. The swim bladder is then opened inspecting for signs of pathology.

The nervous and sensory systems are not examined by histology for this project as all batches are screened for Noda virus before leaving a hatchery, unless clinical signs of these systems were evident.

Culture and sensitivity can be performed without contamination by placing the kidney sample on a sterile dish and excised further. This student has performed greater than 20 kidney cultures with no gut, skin or other contamination for bacteria for disease investigation outside of this project. No bacterial cultures will be performed in the survey due to cost constraints.
Histological samples of gills, kidneys, livers and spleens were prepared and potted in standard 10% buffered formalin and stored until July 2004, for processing at Murdoch University Pathology and Histology Departments for a more holistic pathological assessment of gill disease. Samples of gills and kidneys of fish from South Australia, Northern Territory and Northern Western Australia were collected and stored in 5% buffered glutaraldehyde for transmission and scanning electronmicroscopy, discussed in Chapter 4.

2.2. FARM DESIGN AND DISEASE HISTORY

On site visits were conducted in the years 2003 to 2004, gaining an understanding of gill disease and production methods from spawning to grow out of Barramundi culture in Australia.

Each farm or hatchery was visited to gain a good understanding of facility design, production methods, management and disease issues for the last 12 months of production, by interview and available records.

Questions asked included were:

- In the last 12 months what disease issues has there been on the farm/facility?
- Is routine sampling for fish disease performed, how often and what methods are used, for example clinical examination, microscopy evaluation including gills?
- Do you use a Veterinarian and commercial pathology laboratory for routine sampling and disease investigations?
- Has a Veterinarian visited the farm in the last 12 months and under what circumstances?
• What treatments are routine and during disease episodes have been used and who oversees such treatments?
• Method of record keeping.
• Staff qualifications and experience.
• What disease or diseases concerns you?

2.2.1.  

**South Australian Re-circulation Aquaculture (RAS)**

2.2.1.1.  

**RAS farm 1**

Farm 1 was located south of Adelaide in the foot hills and is made up of four sheds with water sourced from a central bore, which is aerated before supplied to each shed removing potentially harmful free iron and manganese ions settling out as oxides at the bottom of the common water storage tank. Each shed is made up of 2 grow out containers of 120kl each. Each container has four tanks using a single common filtration system. One shed has a separate nursery/quarantine in a dedicated nursery room for incoming fish with its own filtration system; however this supplies only 2 of the sheds with replacement stock once at 50-70 mm long and are then placed in small square cages within grow out tanks as seen below in figures 2.3 and 2.4. The remaining sheds place all fingerlings directly into the main grow out tanks within square floating cages reducing cannibalism for this occurs until 100 – 120 mm in length.

Aeration and supplemented oxygen via air stones kept oxygen levels above 5 mg/L post feeding and 7 mg/L otherwise, with low oxygen areas present down to 4 mg/L oxygen measured by an electronic anode/cathode oxygen meter.
Filtration begins with primary solid waste removal using 62 micron meshed screens. Secondary filtration reduces the highly toxic chemical waste product ammonia from un-eaten fish food, faeces and other decaying organic debris by in tank submerged bio-filtration. Bacteria adhere to plastic media submerged in a container below the primary meshed screen, converting ammonia by nitrifying bacteria to the less toxic nitrate, which is then reduced by a 10% water exchange rate per day. The water after mechanical and biological filtration is sent back to the tanks with no disinfection protocols.

Disinfection of equipment was performed by chlorine, drying and/or sunlight, but no standard filtration disinfection systems such as Ozone and Ultra-violet was used.

Salinity levels were raised for purging fasted fish in separate containers with either flow through water delivery up to 100% per hour or 50% water exchanges daily with no filtration and this was seen in all RAS farms using 10 g/L of sea salt.

2.2.1.2. RAS farm 2

Farm 2 was not part of the survey, but was included for reasons to be discussed in chapter 5. It is of the same design as farm 1 located in Northern Adelaide utilising bore water, but all purchased fingerlings were housed for 7-30 days in an a quarantine/nursery system within the one large shed. They would then be transferred to the main tanks held within small square cages until the large tank was emptied of larger fish as farm 1. Oxygen was delivered only by aeration achieving 5-6 mg/L, with many areas below 5 mg/L in each tank and post feeding levels down to 2 mg/L in areas of each tank.
Six fish were sampled for mass mortalities and a diagnosis and treatment strategies were made with cytology and culture & sensitivity, but only one was sent for histology due to abnormalities of anatomy and gills.

Figure 2.2 Schematic of South Australian RAS farm 1 & 2
Figure 2.3  SA RAS farm 2 showing drum filter* and fingerling cages within main grow out tank

Figure 2.4  SA RAS farm 2 with excessive foaming
2.2.1.3. **RAS Farm 3**

Farm 3 also in Adelaide was not part of the official survey of 2004, but was included, because it utilized a modern design of round tanks, external filtration with 150 micron primary screen and bio-filter with UV disinfection utilising carbon filtered mains water. Oxygen was by aeration only, achieving 5.5 mg/L dropping to 4 mg/L post feeding, with little variation within tanks and improved circulation of water than farms 1 and 2. Separate filtration systems for each 2 × 9000 Litre tanks for grow out and 2 × 2000 litre tanks for the nursery were used. No water was transferred between filtration systems within the same shed, affording some disease management from incoming fish and grow-out systems, but as pictured below small floating square cages was used as farms 1 and 2. Chemical disinfection for equipment was by chlorine and drying.

Figure 2.5  Schematic of SA RAS farm 3
2.2.2. Northern Territory Barramundi Production

2.2.2.1. Darwin Channel Island Barramundi Hatchery

The hatchery brood-stock was caught in local waters and then housed in a seawater closed to re-circulating tank system, with minimal water exchanges, operating at a low stocking density of 0.5 kg/1000 L, minimising disease and maximizing conditions for reproduction. When spawning is scheduled, they are transferred to a flow through spawning tank system using filtered seawater. Scheduled artificial spawnings can be performed by maintaining summer water temperatures greater than 28°C and light for 13 hours/day.

Female Barramundi are sensitive to the synthetic mammalian hormones, luteinizing hormone releasing analogue [LHRHa] or human chorionic hormone [HCG]. Fish spawn 24 to 36 hours after the injection, with each female releasing up to 3 to 6 million eggs depending on maturity. The males then immediately 'pirouette' around the females' tail releasing sperm, resulting in excess of 1.2 million fertilized eggs. Males do not require hormonal stimulation receiving visual cues from the female and pheromone release from the female adding to the attraction cannot be ruled out.
The larvae collected from the spawning tank are transferred to a closed re-circulated system and fed live food zooplankton. Feeding begins with rotifers until day 9 and then *Artemia* days 9-10, upon opening their mouths about 30-36 hours post hatch and 600,000 surviving larvae are placed into a 6,000 L semi-closed re-circulation tank. Weaning times have been reduced in recent years from 23 to 18 days using poly unsaturated fatty acid enhanced rotifers, rather than the less digestible *Artemia* reducing weaning stress, pollution and size variation. Grading once weaned can be up to daily depending on cannibalism rates.

Larvae are randomly sampled and fixed in formalin every 5 days from hatching until 25 - 30 days old, when moved to a flow through trough nursery. A timeline can be created by histopathology if disease occurs, creating a clearer pathological picture with a better understanding of causes than sampling during a disease episode.
Mortalities are often unknown and not investigated with the resulting number satisfying production needs. Weaned larvae (fingerlings) are sent the nursery and expected losses from
the nursery are less than 10% in a normal batch, when shipped to grow-out ponds or tank culture at 25-40 mm and 80 mm in length for sea-cage culture respectively.

2.2.2.2. Commercial Sea Cage Grow out facility on Bathurst Island

The Marine Harvest facility 60 km north of Darwin on Bathurst Island at the time of the survey was a commercial pilot facility, based on supply of fingerlings from the Channel Island government run hatchery based in Darwin. Only 24 hours were available at this facility due to the construction of circular polar cages seen in figure 2.10, rather than the pictured square cages for grow out in figure 2.9; consequently only six 150 mm fish and four 600 mm fish were sampled.

Figure 2.9 Marine Harvest grow out sea cage facility
Stocking densities are typical of sea cages containing carnivorous species at 40-50 kg/m³ and grading is performed until 120 mm. Cage depth was 3 metres in a water depth of 12 m at low tide with backup aeration by a diesel aerator. The below figures 2.11 to 2.13 indicates water quality issues from reduced water flow.

Figure 2.10 Marine Harvest upgrading to polar circle cage system

Figure 2.11 Sea cages with heavy anti shark netting, fish and fingerling (black) netting reducing flow within cage system
Figure 2.12 Sea cages with heavy anti shark netting, fish and fingerling (black) netting reducing flow within cage system

Figure 2.13 Organic foaming indicating poor water quality
2.2.2.3. **Pond Grow Out Facility**

11,000 fingerlings from Channel Island Hatchery are batched into a previously dried fallowed pond and allowed to grow without grading into a 50 m² pond with a centre of 2.5m deep with sloping sides. Stocking rates are thus low at 1-3 kg/m³ standing crop compared to other species farmed similarly, such as non-carnivorous species Carp, Tilapia and Channel Catfish at 20-40 kg/m³, but no grading is necessary from low densities reducing cannibalism.

Pond salinity will vary depending on the season and salt added for disease treatments particularly gill flukes, but operates between 0 mg/L and 6-8 mg/L. The salinity in the ponds at the time of sampling was 0 mg/L at the end of the wet season, with water sourced from the Elizabeth River. Aeration when needed is supplied by a series of electric paddle wheels and aerator blowers. Water quality issues are ammonia spikes solved by water exchanges,
turbidity from source water & unlined ponds, and plankton blooms when low turbidity, causing potential oxygen issues solved with aeration. Mortality rates are generally less than 2%, which is less than the standard 10% for intensive tank aquaculture.
2.2.2.4. *Wild Catch Mary River System*

A permit was obtained in April 2004 with the help of Northern Territory Fisheries to net or hook up to 200 Barramundi from the River Mary System. Fish were collected on the seawater side of the final water barrage by hook in a section of the river heading out to open sea and sampled alive. Freshwater predominated in both sections with 0 salinity recorded using a refractometer. Fish were typically blacker in colour and not the silver colour seen in a saltwater ecosystem.

![Figure 2.16 Natural habitat of Barramundi fingerlings and adults looking for food](image)

![Figure 2.17 Wild caught 600 mm long Barramundi caught in zero salinity](image)
2.2.3. Western Australia

2.2.3.1. Lake Argyle

In Northern Western Australia at Lake Argyle cage culture system was based near the dam wall was being assessed as to its viability by Veterinarian Dr Steve Percival. The great expanse of calm water allowed a simple farm construction of a series of square cages 2 metres deep, interlocked within a main frame allowing husbandry management. Fingerlings were covered with shade cloth reducing freshwater algae growth and grow out was in larger circular pens and grading was performed in grading boxes.
Stocking rates were high above 100 kg/1000 m³ at times and levels of such can be farmed successfully in large volume lakes with non-carnivorous carp species to 150 kg/1000 m³, with
high water flow through cages at 1 cage volume every 30-60 seconds (Schmittou, 1991).
Unfortunately flow rates were close to 0 L/minute when observed, with no ancillary oxygen
or air delivery noted. *S. iniae* and production issues particularly product quality of freshwater
barramundi has since closed the facility.

2.2.3.2. *Broome TAFE Hatchery*

The hatchery is similar in basic design and function, but on a smaller scale to the Darwin
Channel Island facility. Brood-stock is sourced from the region and is a different variety to
the Northern Territory’s, with a grey iris not tan and a lighter shade of silver in seawater of
which the photos do not highlight. Fecundity and survival rates are similar to the Channel
Island Hatchery.

![Western Australian variety of saltwater grown Barramundi approximately 100 mm in length](image)

Figure 2.21 Western Australian variety of saltwater grown Barramundi approximately 100 mm in length
2.3. MURDOCH UNIVERSITY FISH HEALTH UNIT

Fifty 150 mm fish were translocated from Re-circulation farm 1 in South Australia to the Fish Health Unit at Murdoch University July 2004 for further investigation of the unusual cell of possibly non-host origin found by the author prior and during the survey.

Two virgin tubs to house the fish were rinsed, filled for 2 days with clean water, rinsed and dried before the fish arrived. Despite this, within 24 hours one tub had 20 mortalities, possibly due to a plastic contamination problem. The fish in tub 2 appeared unaffected and fish were then further subdivided into 3 tubs excluding the affected tub, reducing stocking densities. 50% water exchanges using unheated carbon filtered mains freshwater were performed daily. Daily water quality monitoring included pH, ammonia products [TAN], oxygen and temperature [28°C]. On Day 1 seven fish were sampled and the remaining fish were placed in freshwater for 7 days. Between Days 8 to 14 the salinity was increased from 0 to 10 g/L. From day 14 two fish were caught daily for gill biopsy examination which was
performed under anaesthesia and then recovered. Otherwise, other fish showing signs of
disease were assessed by cytology and histopathology after euthanasia.

Six fish were to be sacrificed on Days 1, 8 and 13 and samples were collected for cytology,
histopathology and if cytology warranted for electronmicroscopy assessments.

2.4. Clinical Pathology Specimen Methodologies

2.4.1. Gill wash cytospin method

Freshly excised gill arches were washed aseptically with distilled water in a petri dish.
Suspended cells were collected by pipette and 0.5 mL was placed in a cytospin funnel
coupled to a glass slide and cytospin cover. This was centrifuged at 500 rpm for 8 minutes
and then stained once air dried.

2.4.2. Cytological Staining Techniques

Periodic Acid Shift (PAAS) stains were done according to Appendix 4.4.2 and Wright’s
stained smears were stained according to Appendix 4.4.3.

2.4.3. Histopathology Procedures

2.4.3.1. Sample fixing and processing before staining

Using a scalpel blade to dissect samples of heart, liver, intestinal tract, spleen and kidney
were placed immediately in 50mL containers with 10% buffered formalin. A 1:10 ratio of
specimen to fixative solution for non obvious pathological specimens and 1:20 for suspected pathological specimens. Samples less than 1 cm$^3$ afforded good preservation.

After a minimum of 24 hrs preservation at room temperature samples can be sectioned unless decalcification is required for larger gill arches. 5% Nitric Acid decalcifies gill tissue and concentrated ammonia is used to precipitate calcium and giving an end point once no further precipitation occurs, this process should not exceed 60 minutes with fish tissue. 2-5% fabric softener is then used to remove fat for staining and allowing easy cutting.

The samples are then set in wax with careful placement of gills for longitudinal and transverse sections and are cut to 5 microns. Gills may require further cuts for longitudinal sections if lamellae are not present in 4-6 filaments; the minimum for an adequate picture of gill pathology.

2.4.3.2. **Haematoxylin and eosin and other stains for general histology**

After freshly dissected tissues destined for histopathology were fixed, they were sub-sampled and routinely processed for paraffin embedding and sectioned at 4-60 µm. Sections were then stained with haematoxylin and eosin as described in Appendix 4.4.4.

To demonstrate fungal infections PAAS stain was used as described in Appendix 4.4.5. Similarly suspect bacterial infections were investigated using Gram stained sections as described in Appendix 4.4.6. A modified trichrome stain was used to demonstrate Microsporidia in paraffin sections as described in Appendix 4.4.7 and Giemsa stained sections were also occasionally used as described in Appendix 4.4.8.
2.4.4. Transmission Electronmicroscopy

Samples for transmission electronmicroscopy were fixed in 5% glutaraldehyde in phosphate buffer with 1% calcium chloride (200:1) and stored for a minimum of 2 hours at room temperature. Fixed tissues were washed in Sorenson’s buffer and cover with Dalton’s Chrome Osmic Acid for 1.5 hours at 4°C and shaken at 30 min intervals to increase exposure. The samples were then washed and dehydrated with several changes of 70%, 90% and then 95% ethanol before washed in 3 changes of 100% ethanol over 15 minutes then in 2 changes of propylene oxide over 15 minutes. Samples were then placed in propylene oxide/EPON 812 (60:40) for an hour at 4°C before being embedded in capsules for 24 hours at 6°C. Resulting hardened blocks were trimmed to a rhomboid shape and cut for semi-thin or ultra-thin sections for light and EM. The semi-thin sections were cut at approximately 1 micron thickness and stained with Toluidine blue for histological assessment. Ultra-thin sections were cut at approximately 90 nm thick placed on 200 mesh copper grids then stained with Uranyl Acetate for about 5-7 minutes, washed and then with Lead Citrate for about 4 minutes with the grid floating face-down on top of the drop of Lead Citrate and a pellet of NaOH. Sections were then washed thoroughly by dipping the grid in distilled water about 40 times then allowed to dry on clean filter paper and replaced back in the grid box, if not carbon coated immediately.

2.3.4 Scanning Electronmicroscopy

Samples were fixed as for TEM, washed in buffer which was then replaced with Dalton’s Chrome OsO₄ covering the tissue well in a fume hood. Samples were then washed repeatedly
for 2-3 times using increased concentrations of alcohol 30-100% each time for 15 minutes
then washed in 3 changes of absolute alcohol over 30 minutes followed by super dry alcohol
over 1 hour then in 50:50 super dry alcohol:amylacetate over 30 minutes. They were then
placed in 2 changes of amylacetate over 1 hour, placed in a critical point dryer and then
mounted onto a specimen stub with silver or carbon paint before coated with Gold/Palladium.
Chapter 3. Results

3.1. SOUTH AUSTRALIAN

3.1.1. RAS farm 1

Farm 1 was visited and stock was sampled multiple times from December 2002 – November 2004. The initial visit was due to an accidental release of iron oxide from the bore water header and aeration storage tank causing mortalities in the fish culture systems. Iron oxide precipitates and settles after aeration if contained in bore water. The gills were grossly covered in iron oxide asphyxiating the fish. It was this incident that catalysed the thesis topic.

Since that time the main issues addressing this farm prior to November 2004 were intermittent mass mortalities due to bacterial septicaemias. Despite this, no routine sampling of fish occurred, except for the health certificate from the hatcheries covering notifiable diseases. On most occasions of mass mortalities, moribund and randomly sampled fish were sent to a government or private pathology laboratory, usually with laboratory Veterinarian involvement. The repeated opportunistic bacterial septicaemias were the main concern of management, who had no formal aquaculture training managing these farms for 10 years. Since the survey the pathogen *Streptococcus iniae* had become the major disease issue, despite greater aquaculture experience and training under new management from January 2004.

Treatments were routinely performed by the owners and one employee. Commonly salt or formalin was used for bacterial treatments at 10 g/L and 200ppm for 30-60 minutes.
respectively, in separate containers and within the fish culture systems. At one stage formalin was added to the main tanks every 5-7 days for bacterial disease control, without Veterinary advice.

Records apart from fish numbers only included water quality records for daily temperature, salinity, oxygen and TAN (total ammonia nitrogen).

In November 2004 the new owners reported fish were off feed, piping and an increase in mortalities in the last few days before visited. Clinical examination revealed 6 live fish with skin mottling, dark red gills with excessive mucus of gill and skin to the naked eye. Fish 1, 3 and 5 had haemorrhages through the gill on biopsy (Figure 3.12) probably due to them struggling with clove oil anaesthesia.

<table>
<thead>
<tr>
<th>Table 1. Cytological results for RAS farm 1 barramundi</th>
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<tbody>
<tr>
<td>External gill parasites</td>
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<tr>
<td>Bacterial</td>
</tr>
<tr>
<td>Fungal &amp; Other</td>
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Histology of samples taken in November 2004 using H&E stain showed mild hyperplasia in fish 4 & 5 with *Epitheliocystis* present in fish 5 only and haemorrhage was present in fish 3. Occasional cocci bacteria in fish 1, 5 & 6 gill lamellae were also noted.
Figure 3.1 *Ichthyobodo* with contrast DE using microscope diaphragm >1000 × magnification

Figure 3.2 *Ichthyobodo* using phase contrast (DE) highlighting flagella* and bacteria unstained
Ichthyophthirius multifilis outbreak pre-dated the survey on RAS farm 1, but occurred in shed 2 flow through purging tank at 10 g/L salinity killing 50% of the purging 600g fish. This was the only case on this farm or seen in Barramundi during the survey and it was decided to include it as a primary gill disease issue. Clinically there were thickened gills with excessive mucus loadings and no white spots on gills or skin could be seen with the naked eye, but evident on gills under the dissecting microscope. No fish from the main tank were affected. Gill cytology quickly revealed invasive adults within the epithelium, with vibrating cilia and the characteristic or pathognomonic C-shaped macro-nucleus in some free swimming trophonts, confirming the diagnosis of Ich as seen in (Figure 3.3 & 3.4).

Figure 3.3 Ichthyophthirius multifilis gill biopsy SA RAS farm 1
Figure 3.4 *Ichthyophthirius multifilis* with characteristic C shape nucleus free trophont

Figure 3.5 Water mold spore-like structures possibly *Saprolegnia* species* in gill biopsy SA RAS
Figure 3.6  SA RAS farm 1 gill biopsy using Diff Quik 2 highlighting possible *Epitheliocystis* lesion with enlarged epithelia cell warranting histological conformation

Figure 3.7  Gill biopsy of same batch of Barramundi with high numbers of bacteria stained with Diff Quik 2 justifying histology
Figure 3.8 Gill H&E histology of same fish in fig.3.4b showing cocci bacteria within gill tissue*

Figure 3.9 Gill wash of different fish same batch sampled, showing unidentified cells in gill wash stained with Dif Quik 2
Figure 3.10 Gill wash showing unidentified cell heavily stained (SA video chapter)

Figure 3.11 Kidney wet preparation using phase contrast showing spherical cells similar to gill washes (SA video chapter)
Figure 3.12 Histology (H&E) showing haemorrhage to gill filaments from a fish of the same batch

3.1.2. RAS farm 2

Farm 2 was of similar design built by the same manufacturer as farm 1, but larger and also suffering bacterial mass mortalities within the last 12 months when visited in December 2004. There was no routine sampling of fish, except the health certificate from hatcheries for fish notifiable disease and sick and dead fish were sent to a commercial pathology laboratory when disease outbreaks occurred on most occasions, but no Veterinarian had visited the farm in the last 12 months, despite *S. iniae* being a major concern. Treatments were performed by owner, including salt used within the fish culture tanks at 10 g/L and methylene blue for prolonged immersion in the juvenile nursery at 1-3 mg/L. Water quality records only included daily temperature, salinity, oxygen and TAN and management records included fish numbers. Once again there was no formal tertiary training, but the owner had 15 years experience with lay staff employed.
The farm when visited reported mass mortalities post grading of juvenile fish that had reduced cannibalistic behaviour by placing similar sized fingerlings in the same container, allowing smaller fish to obtain adequate food improving the overall batch growth rates and thus minimising size variation. Clinical examination revealed excessive gill mucus on 2 fish including one with a deformed jaw displaying variable length filaments on histology (Figure 3.15). This fish was sampled for histology because of the high numbers of unidentified cells (Figure 3.16) and blunted gills seen on gill cytology possibly related to the malformed mandible.

Table 2. Cytological results for RAS Farm 2 barramundi

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<table>
<thead>
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<tr>
<td>External gill parasites</td>
<td>Occasional <em>Ichthyobodo</em> all fish</td>
</tr>
<tr>
<td>Bacterial</td>
<td>Occasional <em>Epitheliocystis</em> all fish.</td>
</tr>
<tr>
<td></td>
<td>Motile Filamentous rods bacteria from fish with deformed lower mandible of fish 1 (Figure 3.14).</td>
</tr>
<tr>
<td></td>
<td>Gram positive cocci on kidney Gram stains of all six fish.</td>
</tr>
<tr>
<td></td>
<td>[S. iniae cultured]</td>
</tr>
<tr>
<td>Fungal &amp; Other</td>
<td>Unidentified non host cells in all fish sampled.</td>
</tr>
</tbody>
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Histology with H&E of gills generally showed mild hyperplasia with occasional epitheliocystis lesion (Figure 3.17) on all fish and one fish with an unidentified cell trapped in an area of moderate hyperplasia within the filament (Figure 3.18). Gram staining revealed Gram negative cells containing cocci like material (Figure 3.19 & Figure 3.20), similar to the PAAS positive cells found in many histological sections (Figure 3.21, 3.22, 3.23, 3.24, 3.25 & 3.26).
Chapter 3. Results

Figure 3.13  SA RAS farm 2 malformed mandible

Figure 3.14  Gill biopsy of malformed mandible fingerling showing very high numbers of motile curved bacteria stained with Dif Quik2
Figure 3.15 Histology (H&E) of gill arch from deformed jaw

Figure 3.16 Cytology of gill biopsy revealing large unidentified cell (see SA video chapter)
Figure 3.17  Histology (H&E) revealing an *Epithelioystis* lesion with mild hyperplasia.

Figure 3.18  Showing a hyperplastic filament tip with a cystic type structure, with basophilic cocci structures within and similar to bacteria in size*. 

*Note: Image captions and explanations are based on the visible content of the document, as no additional context or references were provided.*
Figure 3.19  Histological Gram stain revealing structures encased with Gram negative cocci type structures

Figure 3.20  Magnified view of these structures
Figure 3.21  PAAS histological staining of these filaments (low magnification)

Figure 3.22  Magnification of one filament with these PAAS positive structures
Figure 3.23 1000 × magnification of filament edges

Figure 3.24 1000 × magnification Gill filament with small cocci structures* present in some of the PAAS positive cells
Chapter 3. Results

Figure 3.25  1000 × magnification of filament tip

Figure 3.26  Magnified by computer beyond 1000 × magnification of gill lamellae tip
3.1.3. **RAS farm 3**

Farm 3 was owned and operated by one man, who had no previous experience of aquaculture, but had some limited training by the designer and manufacturer of the fish farm. I was called to this farm in the previous 12 months of 2003 with a tentative diagnosis of skin Lymphocystis (Appendix 1.1 Lymphocystis lesions from SA RAS farm 1 2003) due to lack of grading of juveniles with high stocking densities greater than 80 kg/m³. No histology was performed due to costs and clinically resolved by grading within 7 days as the fingerlings were obviously uneven in size distribution. The fish presented with raised coalescing skin lesions and there were no respiratory signs and cytology of the gill appeared normal.

The farm had been operating since 1999 and bacterial disease had been a previous issue on one occasion, but the owner was not concerned about disease generally. There was no routine sampling of fish by owner or by a laboratory. Water quality tests were daily pH, temperature, oxygen & TAN and records of the number of fish only. Treatments performed were salt at 10-15 g/L in treatment tanks and not within the main system.

In May 2003 I visited the farm because of the occasional mortality increasing to 3-5 mortalities per day in last 7 days in 200 mm long fish. Clinically 2 of 6 randomly selected fish had petechial haemorrhages to ventral abdomen and bacterial tail rot and were confirmed with a skin biopsy & Gram staining of kidney revealing Gram negative rod bacteria and the remaining fish were unremarkable.
Table 3 Cytological results for RAS farm 3 barramundi

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>External gill parasites</td>
<td>Occasional <em>Ichthyobodo</em> all fish.</td>
</tr>
<tr>
<td>Bacterial</td>
<td>Occasional <em>Epitheliocystis</em> all fish and fish 2 gill filaments appeared thickened and increased mucus production [Occasional Gram negative rod on skin of clinically sick fish].</td>
</tr>
<tr>
<td>Fungal &amp; Other</td>
<td>Unidentified non host cells in all fish sampled. Water mold spores</td>
</tr>
<tr>
<td>Viral</td>
<td>Nil.</td>
</tr>
</tbody>
</table>

Histologically all fish had mild hyperplasia to few filaments with fish 3 being a representable sample, with cocci bacteria found within the filaments (Figure 3.28) and a possibly a dislodged MRC (Figure 3.115) from processing. Clinically non affected fish were purged in saltwater at 10 g/L for 7 days and sent to market as they were of marketable size rather than a further workup and treatment plan with bacterial culture and sensitivities for antibiotic treatment. Clinically sick fish were culled.
Figure 3.27 SA RAS farm 3 H&E histology low magnification

Figure 3.28 Occasional filament showing necrosis and bacterial infection
3.2. NORTHERN TERRITORY

3.2.1. *Darwin Channel Island Barramundi Hatchery*

In the last 12 months the disease issues had been of a routine nature with gill and skin flukes of brood-stock and the occasional mortality of spawned fish due to bacterial disease. Disease issues that concern the production team are Noda virus, nutritional and unexplained mortalities. Sampling of all fish growth stages is routine beginning with all brood-stock quarantined for 42 days when caught with gills and skin biopsies performed. Eggs from spawnings have bacterial and fungal assessments by culture. Larval clinical examinations for nutrition, then examined by post mortem using random sampling, which included culture for bacterial and fungal elements when found using Gram staining or if deemed necessary. Noda virus screening is performed by polymerase chain reaction DNA testing of fingerlings before shipment at the minimum age 21 days old. Fingerlings are examined clinically and if necessary by post mortem after grading for cannibalistic lesions, such as bacterial tail and fin rot and all are removed from culture if clinical signs are seen immediately. Additionally larvae are time lined weekly by formalin fixation for each spawning and kept for 12 weeks, allowing a trace back if disease or other issues arise from a batch shipped to farms. All post mortems are carried out by trained fish pathologists at a government laboratory and on site clinical examinations are conducted by a tertiary qualified with a Research Masters Degree aquaculturist and an aquatic fish veterinarian who routinely visits the facility. All lay staff undergoes training and a minimum of a diploma in aquaculture is encouraged for all team leaders.
Treatments that were administered included fallowing by dry out and chlorine disinfection after each batch in spawning, larval and nursery systems; praziquantel medication for flukes in brood-stock; ozone treatment of eggs at 0.4mg for 2 minutes for Nodavirus; and antibiotics following culture and sensitivity for bacterial diseases via Veterinary prescriptions. Records consisted of hand written and computer entries for all stages of production including husbandry, production and disease episodes on a daily basis.

All 45 mm and 80 mm fingerlings appeared healthy with normal conformation, except fish 6 (45mm) with tail rot (Figure 3.29), probably due to a secondary bacterial infection from tail bites. Gills of this fish were fragile upon cytology (Figure 3.30 & 3.31), with melanomacrophage centres evident on cytology (Figure 3.32).

<table>
<thead>
<tr>
<th>Table 4. Cytological results for Darwin hatchery barramundi</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>External gill parasites</strong></td>
</tr>
<tr>
<td><strong>Bacterial</strong></td>
</tr>
<tr>
<td><strong>Fungal &amp; Other</strong></td>
</tr>
<tr>
<td><strong>Viral</strong></td>
</tr>
</tbody>
</table>

Histologically most fish had occasional telangiectasis of the filaments, but otherwise normal, except for occasional mild hyperplasia of lamellae and fish 6 of 45 mm batch had occasional cocci bacteria in the filament (Figure 3.34) and a Gram stain of kidney was not performed.
Figure 3.29  45 mm long Fingerling cannibalistic bite wound

Figure 3.30  Gill biopsy of using Diff Quik stain 2 highlighting fragile filaments
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Figure 3.31  Melanomacrophage infiltration evident with necrosis at margins of filament

Figure 3.32  400 × magnification of melanomacrophage centres
Figure 3.33  Cytology with DQ2 stain revealing unidentified cells in gill wash*

Figure 3.34  Histological section of filament tip showing possible bacteria in parenchyma
3.2.2. **Tidal River Sea Cage Grow Out Facility**

This multi national company commercial pilot was well equipped with a Veterinarian managing the site remotely with on site visits and a government aquatic veterinarian visiting from Darwin regularly. Key on site staff members either held an aquaculture degree or a TAFE diploma and it was their responsibility in keeping hand written and computer records for all stages of production, including on a daily basis water quality monitoring of oxygen and ammonia levels and weekly production and husbandry records. Husbandry records included food conversion ratios of fish, clinical inspections of stock, but no microscopy. Any disease episodes had live sick fish flown to a Darwin fish pathologist and the disease issues of concern were bacterial and parasitic pathogens, which were ongoing in an open water culture system where chemical or antibiotic treatments were prohibited. All six 150 and four 400 mm fish sampled appeared healthy and with normal conformation.
Table 5  Cytological results for Tidal River sea cage grow out barramundi

<table>
<thead>
<tr>
<th>External gill parasites</th>
<th>Occasional <em>Trichodina</em> ciliate (Figures 3.38 &amp; 3.39) 3 of 6 150mm. 1 of 4 400mm. Moderate numbers of encysted Metazoan species with <em>Henneguya</em> spores for all 150 mm &amp; low numbers for one 600 mm (Figures 3.36 and 3.37).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial</td>
<td>Nil.</td>
</tr>
<tr>
<td>Fungal &amp; Other</td>
<td>Unidentified cells of non host origin on all fish (Figure 3.41). Fungal spores of water mould 600 mm Fish 1 (Figure 3.40).</td>
</tr>
<tr>
<td>Viral</td>
<td>Nil.</td>
</tr>
</tbody>
</table>

The histology of 150 mm fish gills showed a general picture of mild hyperplasia of filaments with epitheliocystis (4 of 6) (Figure 3.42) and an incidental finding of a Chondroma was noted (Figure 3.45).

The 600 mm fish had too few lamellae to give an accurate picture, but mild hyperplasia was evident around metazoan plasmodia (Figure 3.43).

Figure 3.36  Sea cage culture cytology of gill filament showing large numbers of encysted non host structures
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Figure 3.37  400 × magnification of Fig. 3.36 identifying Myxosporidea *Henneguya* polar capsules* of spores in plasmodium

Figure 3.38  DQ2 stained gill ciliate ectoparasite *Trichodina* lateral view showing ‘bowler hat’ shape from sea cage culture
Figure 3.39  Unstained *Trichodina* in motion ventral view with cilia visible

Figure 3.40  Gill wash from biopsy showing probable fungal spores stained with DQ2 from sea cage
Figure 3.41 Unidentified stained cells in gill wash from sea cage
Figure 3.42 Multiple *Epitheliocystis* lesions on H&E histology in 150 mm sea caged fish
Figure 3.43  600 mm sea caged fish with Myxosporidea plasmodium within lamellae

Figure 3.44  Plasmodium revealing *Henneguya* polar capsules* at 1000 × magnification
Figure 3.45 Incidental finding of a filament Chondroma in a 150 mm sea caged fish
3.2.3. Pond Farm Grow Out Facility

The manager of the facility was also a major partner in the business with an agriculture degree and the day to day operations manager had an aquaculture TAFE diploma and remaining staff were trained by the operations manager. In the last 12 months bacterial disease was the main issue and the government aquaculture veterinarian had visited the farm accordingly, which remained a concern for the farm. Other concerns included parasitic diseases brought in by the unfiltered river source water. Water quality measurements were daily ammonia levels and an oxygen probe in the largest pond which sounded an audible alarm when working. No treatments had been given recently, but would be administered by the operations manager under veterinary supervision.

Only 6 300 mm fish sampled (constraints placed upon the survey at the facility) and fish sampled had no abnormalities upon physical examination, except for haemorrhage of gills in one fish from thrashing when exposed to diluted clove oil (Figure 3.50).

Table 6 Cytological results for TAFE pond farm grow out system barramundi

<table>
<thead>
<tr>
<th>External gill parasites</th>
<th>Encysted Trematode fluke in low numbers on fish 3 (Figures 3.46 &amp; 3.47) Occasional Metazoan plasmodium encysted on 4 fish (Figures 3.48 &amp; 3.49).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial</td>
<td>Nil.</td>
</tr>
<tr>
<td>Fungal &amp; Other</td>
<td>Unidentified cells of non host origin on all fish.</td>
</tr>
<tr>
<td>Viral</td>
<td>Nil.</td>
</tr>
</tbody>
</table>

Histology did not reveal pathology apart from the occasional telangiectasis (Figure 3.51) on a few lamellae of 4 fish with one sample having too few lamellae to comment on. Repeat
histological sections were performed, but there were not enough filaments with good lamellae to comment for an overall health picture of that fish, possibly due to the lack of gill arch from biopsies of larger fish.

Figure 3.46  Ponded fish with unusual trematode fluke eggs encysted within filament
Figure 3.47  Higher magnification of possible trematode fluke of crocodile origin and not fish

Figure 3.48  Myxosporidea plasmodium from ponded fish taking water from river system unfiltered
Figure 3.49  Higher magnification not revealing spores capsules
Figure 3.50  Haemorrhage in filaments from thrashing when sedated with clove oil

Figure 3.51  Mild Telangiectasis probably unassociated with clove oil irritation
3.2.4. *Wild Catch Mary River System*

No abnormality was detected upon clinical examination of 300 and 150 mm wild caught fish.

Table 7  Cytological results for Mary River System Barramundi

<table>
<thead>
<tr>
<th>External gill parasites</th>
<th>Occasional Metazoan <em>Henneguya</em> plasmodium encysted on one 300 mm and three 150 mm with spores in wash (Figures 3.52 &amp; 3.53).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial</td>
<td>Nil</td>
</tr>
<tr>
<td>Fungal &amp; Other</td>
<td>Unidentified cells of non host origin on all fish (Figure 3.54).</td>
</tr>
<tr>
<td>Viral</td>
<td>Nil.</td>
</tr>
</tbody>
</table>

Too few good histological sections of lamellae to comment on for an overall health picture, except occasional telangiectasis and hyperplasia of lamellae for both batches, but cytology revealed healthy gills with the said pathology. However histopathology found in 150 mm an *Epitheliocystis* lesion (Figures 3.59 & 3.60) on fish 1, an encysted fluke on fish 2 (Figure 3.61) on the edge of a filament and fish 3 encysted *Henneguya* plasmodia (Figures 3.56, 3.57 & 3.58) were noted. Of the 300 mm Fish 4 fish had a encysted fluke within a filament 4 (Figure 3.62) with associated hyperplasia and fish 6 displayed mild to moderate hyperplasia with lamellae absent in a small area on a filament.
Figure 3.52  Myxosporidea plasmodium on filament from wild caught fish in freshwater

Figure 3.53  Typical *Henneguya* bipolar capsule* in gill wash of same fish stained with DQ2
Figure 3.54  Stained unidentified cells *(yellow) versus pavement cells (red) in gill wash

Figure 3.55  Phase contrast used with DQ2 highlighting similar cells found behind pectoral fin of freshwater wild caught fish in fast flowing section of river
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Figure 3.56 Myxosporidea plasmodium between filaments of 150 mm wild caught Barramundi

Figure 3.57 1000 × magnification able to determine *Henneguya* polar capsules* within plasmodium
Figure 3.58  Immature plasmodium between lamellae

Figure 3.59  Occasional epitheliocystis seen in wild caught on histology
Figure 3.60  1000 × magnification showing lamellae processing artefact of epithelial lifting
Figure 3.61 Developing Dactylogyrid fluke within its egg loosely attached to filament
Figure 3.62  Walled off developing fluke causing a hyperplastic response within a filament
3.3. WESTERN AUSTRALIA

3.3.1. *Lake Argyle freshwater Grow out Cage Culture*

For the last 3 weeks before arrival an aquatic veterinarian was employed to identify if the bacterial disease *Streptococcus iniae* was causing mortalities and other production issues due to lake culture and could be controlled and better managed for profit. Previously help from the Western Australian Department of Fisheries had been extensive with live fish shipped for assessment, particularly for *S. iniae*; however this farm had a number of local owners with no aquaculture experience and employed labour was untrained at the time of the survey. Records were hand written for water quality including ammonia and oxygen daily readings and at the time treatments were salt blocks at the bottom of the cages allowing barramundi to swim in
and out of higher salinities and formalin & malachite green baths in attempt to control external parasites particularly on 70 mm fish.

Clinically apart from Argulus lice on all tails there were no abnormalities found on gross examination of all 70, 250 and 600 mm fish, but 70 mm had been treated with formalin/malachite green 7 days prior to examination.

Table 8 Cytological results for Lake Argyle Barramundi

<table>
<thead>
<tr>
<th>External gill parasites</th>
<th>Argulus were grossly evident on tail fin (Appendix 1.2 &amp; 1.3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low numbers of <em>Trichodina</em> on gills of 250 mm fish 1.</td>
</tr>
<tr>
<td></td>
<td>Occasional Dactylogyrid fluke on 5 of 6 fish and 600 mm fish (Figures 3.64, 3.65 &amp; 3.67).</td>
</tr>
<tr>
<td>Bacterial</td>
<td>Nil.</td>
</tr>
<tr>
<td>Fungal &amp; Other</td>
<td>Unidentified cells of non host origin on all fish.</td>
</tr>
<tr>
<td>Viral</td>
<td>Nil.</td>
</tr>
</tbody>
</table>

Histology revealed mild to moderate hyperplasia of 250 mm and 600 mm fish gills with one fluke in one 250 mm fish (Figure 3.67 & 3.68). 70 mm fish had no abnormalities apart from occasional shortening and fusion of lamellae. One 700 mm long fish of the 600 mm batch fish had fusion at the base of the filaments and moderate hyperplasia (Figures 3.69 & 3.70) with 2 fish having the occasional fluke, but often sections were too deeply cut for assessment of lamellae for this batch.
Figure 3.64  Freshwater Dactylogyrid adult flukes embedded into a filament at Lake Argyle

Figure 3.65  Typical 2 pairs of eyespots* of Dactylogyrid gill fluke
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Figure 3.66 Hooks attaching fluke on to filament

Figure 3.67 Hyperplasia of gill with attached fluke*
Figure 3.68  Fluke embedded between hyperplastic lamellae

Figure 3.69  Filament fusion at base of filaments from hyperplasia*
3.3.2. *Broome TAFE Hatchery*

The remote location only affords a government veterinarian visit if a disease outbreak occurs such as the recent Noda virus outbreak; otherwise fish are examined by a tertiary qualified aquaculturist and if necessary shipped live to Perth for specialist pathologist assessment from WA Fisheries.

Routine sampling occurs at various production stages including all brood-stock are screened for parasites with skin and gill biopsies when caught and treated if necessary. Each batch of eggs if they fail to hatch are cultured for bacterial & fungal diseases and Noda virus is
prevented by the recent introduction of iodine washing of eggs controlling vertical transmission of the disease.

Larvae are examined clinically for nutritional deficiencies looking for deformities and disease and if found or suspected samples are shipped to WA Fisheries. Noda virus screening is performed similarly by PCR test as Darwin Hatchery and fingerlings are assessed at grading for cannibalism lesions and removed if necessary. All treatments are given by the operations manager, a tertiary qualified aquaculturist with veterinary prescriptions from Western Australian Fisheries. Assistant staff is TAFE trained.

At the time of the visit only 80 mm fish were available and the 6 fish examined had no abnormalities upon physical examination.

<table>
<thead>
<tr>
<th></th>
<th>Broome TAFE Hatchery barramundi</th>
</tr>
</thead>
<tbody>
<tr>
<td>External gill parasites</td>
<td>Nil.</td>
</tr>
<tr>
<td>Bacterial</td>
<td>Nil.</td>
</tr>
<tr>
<td>Fungal &amp; Other</td>
<td>Unidentified cells of non host origin on all fish.</td>
</tr>
<tr>
<td>Viral</td>
<td>Nil.</td>
</tr>
</tbody>
</table>

Histology only revealed the occasional Telangiectasis.
Figure 3.71  Broome TAFE saltwater hatchery: Cytology gill biopsy of 80 mm fingerling using DQ2 showing unidentified cell* on distorted filament from probable pressure of glass coverslip & handling

Figure 3.72  Phase contrast further highlighting these unidentified cells
3.4. MURDOCH UNIVERSITY FISH HEALTH UNIT

Most fish arrived from the South Australian RAS farm 1 stressed with mottled skin colour indicating transport stress in plastic bags filled with 1/3 water and 2/3 oxygen. Fish would not feed possibly from transport stress and/or virgin tank problem. The history of this facility and the fish was discussed in 3.1.1.

<table>
<thead>
<tr>
<th>Table 10. Cytological results for Murdoch University FHU barramundi</th>
</tr>
</thead>
<tbody>
<tr>
<td>External gill parasites</td>
</tr>
<tr>
<td>Bacterial</td>
</tr>
<tr>
<td>Fungal &amp; Other</td>
</tr>
</tbody>
</table>

3.4.1. *Identified and Unidentified Non Host Cells*

Unidentified non host cells found on gill biopsies were also found in histology (Figures 3.76 & 3.77) with the occasional *Epitheliocystis* (Figure 3.73) and some fish showing mild (Figure 3.76) to moderate hyperplasia (Figure 3.77) upon arrival July 7 2004. Additionally one fish demonstrated some filament necrosis with cocci bacteria destroying the parenchyma (Figures 3.78 & 3.79). TEM demonstrated *Epitheliocystis* (Figure 3.74) with the infective elements within the lesion.
Figure 3.73  Upon arrival from South Australia 150 mm Barramundi fingerlings had occasional *Epitheliocystis* lesions as seen on this H&E histological section.

Figure 3.74  Electronmicrograph showing epitheliocystis containing infective elementary bodies.
Figure 3.75  Day 1 of Barramundi gill study at Murdoch University showing unidentified cells* in gill wash using DQ2 stain

Figure 3.76  Day 1 gill filament showing unidentified cell* in parenchyma with mild hyperplasia
Figure 3.77  Day 1 gill filament showing unidentified cell* in parenchyma of another fish with moderate hyperplasia

Figure 3.78  Gill arch showing necrosis of some filaments yet no respiratory stress was evident
Figure 3.79 1000 × magnification demonstrating severe filament necrosis and a cocci bacterial infection

Days 1 to 7 fish were eating little and their skin colour remained mottled, indicating stress. On day 5 the ammonia nitrogen levels rose above 7 mg/L with a pH of 8 at 28°C or 1-2 mg/L of unionized ammonia, due to uneaten food and poor water quality despite daily water exchanges. Clinically the fish had excess mucus to skin and gills confirmed by cytology. Fish became clinically ill with sluggish and erratic swimming behaviour and 6 became moribund on day 5 which were post mortemed. All had increased gill and skin mucus with moderate numbers on the skin (Figure 3.80) and low to moderate numbers on the gills of these unidentified cells. Similar findings were found on moribund fish sampled days 6 and 7 and all fish sampled were post mortemed and samples taken for histology and electronmicroscopy.
Despite water exchanges ammonia levels remained above 7 mg/L until day 8 when they dropped below 2 mg/L.

Figure 3.80  Skin biopsy post ammonia spike stained with DQ2 showing unidentified cells

Histology did reveal oedema and necrosis too many gills as shown in (Figures 3.81 & 3.82) demonstrating the pathology of ammonia toxicity. Cytospins on day 7 revealed with Wrights and PAAS stains unidentified cells from gill washes (Figures 3.84 &.85).
Figure 3.81  Day 5 Acute ammonia spike associated with gill necrosis histologically

Figure 3.82  Gill filament necrosis and oedematous with an *Epitheliocystis* lesion between two lamellae
Figure 3.83 Normal gill filaments day 13 with some filaments cut too deep*
Figure 3.84  Cytospin of gill wash showing unidentified cell appearing differentiated using wrights stain

Figure 3.85  PAAS stain of cytospin from gill wash showing unidentified cell structures
The increase of salinity on day 8 improved fish health and increased gill mucus, trapping various unidentified cell types, including cells with what appeared to be motile internal structures in 2 of the 5 fish sampled cytologically, but nothing revealed histologically. Days 9 to 14 fish had significantly improved health and normal swimming behaviour and gill biopsies showed improving gill health each day cytologically, with normally behaving fish by the end of the study. Figure 3.83 demonstrates normal gill histology of the 6 fish sacrificed day 13. There were however a few filaments with small areas of hyperplasia and some mild epithelial lifting at the lamellae base on fish 3.

Day 13 revealed by cytospins PAAS positive cells (Figures 3.87 & 3.88) and a comparison with Wright’s stain (Figure 3.86).
Figure 3.86  Day 13 Cytospin using Wrights stain highlighting* an unidentified cell next to red blood cells

Figure 3.87  Cytospin using PAAS stain highlighting* unidentified cell
The remaining fish were sacrificed day 14 with a good example of a large multinucleated non host cell approximating a gill filament with what appears to be motile structures within, stained with 20% Diff Quik on fish 5 (Figures 89 & 3.90 & MFHU video chapter) and an example from a skin biopsy of a larger unidentified cell (Figure 3.91).
Figure 3.89  Day 14 cytology of gill biopsy showing a DQ2 stained differentiated cell, possibly multinucleated (see video)

Figure 3.90  Day 14 cytology of gill biopsy showing a DQ2 stained differentiated cell, possibly multinucleated (1000x)
SEM revealed numerous unidentified cells from spherical cells around 10 micron in diameter (Figures 3.95, 3.96 & 3.97) to small 1 micron spore (Figure 3.94) like structures, particularly in a debris mucus field most likely. On one gill filament these small spherical structures are visible (Figure 3.98). Figure 3.99 reveals a smooth raised area on the gill epithelium which is possibly a ruptured pavement cell.
Figure 3.92  SEM showing mucus debris* at base of gill filaments

Figure 3.93  Small structures upon surface of filament under debris field
Figure 3.94  High magnification of the smallest structures on a filament

Figure 3.95  Unidentified spherical body under mucus debris
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Figure 3.96 Unidentified spherical cell

Figure 3.97 Ruptured degraded spherical cell
Figure 3.98 Surface of gill filament tip showing multiple spherical cells of various sizes

Figure 3.99 Possible pavement cell rupture on a filament
3.4.2. *Fish Gill Cells Identified*

Gill cytology showed epithelial cells, which often washed off the gill, particularly with necrosis and inflammation. With deteriorating water quality the epithelial cells became easily recognised particularly with background staining with Diff Quik 2 on and of the gill as seen in Figures 3.100 &.101.

![Figure 3.100 Epithelial cells (pavement cells) on gill from RAS farm 1 (400x)](image-url)
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Figure 3.101  Heavy staining highlighting epithelial or pavement cells (1000x)

SEM of the gill also highlighted the epithelial (pavement cell) with their microridges as seen in figure 3.99 and the filament’s rounded anatomy below in Figure 102.

Figure 3.102  Gill arch showing rounded nature of filaments with lamellae folded down
EGC’s were seen cytologically within the gill particularly along the central vein and if magnified using a digital camera and video (SA video chapter) appear to vibrate or move in a wave like manner.

Histology clarified the anatomy of the gill structure and cells, particularly differentiating MRCs (chloride) and mucus cells, which will be discussed in chapter 4.

Figure 3.103 Hyperplastic gill from Murdoch after ammonia toxicity showing Eosinophilic Granular cells (EGC’s) * (400x)
Figure 3.104  EGC* on TEM at base of lamellae with ovoid granules
Figure 3.105  EGC within lamellae with both round and ovoid granules
Figure 3.106 70 mm freshwater fingerling from lake Argyle with melanomacrophage* infiltration

Figure 3.107 45 mm saltwater fingerling from Darwin Hatchery with higher numbers of melanomacrophages
3.4.3. *Histology Sectioning Artefacts*

Figure 3.108 Variable thickness cuts of filaments and lamellae

Figure 3.109 Oblique filament cuts giving impression to lack of lamellae and degree of hyperplasia
Figure 3.110 Filament cuts too deep

Figure 3.111 Filament cuts deeply on longitudinal section revealing what could appear as excessive mucus glands
Figure 3.112  Central vein appears enlarged & lamellae oedematous, but is a processing artefact most likely.

Figure 3.113  Similar to previous but only a few lamellae artefacts and only one epithelial wall lifted of those affected.
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Figure 3.114 From RAS farm 3 a private laboratory with over staining makes EGC infiltration and MRC (chloride cell) identification difficult. The cell highlighted* could possibly be a mucus cell, but further staining would be required.

Figure 3.115 Highlighted* is possibly a dislodged MRC from processing from RAS farm 3
3.4.4. **Kidney Crystals**

Kidney cytology commonly displayed what appeared to be crystals that did not take up stains and were not in found on gill cytology. Similar crystal structures were found in fish from the Darwin Hatchery (all fish sampled); barramundi in sea cages (n=2 of 6 fish measuring 150 mm); ponded fish (n=2 of 6) as well as wild fish (n=1 of 6 measuring 300 mm and n=2 of 6 fish measuring 150 mm). Barramundi in Lake Argyle measuring 70 mm also had crystals (n=2 of 6 sampled) as well as fish at Broome TAFE (n=1 of 6). Very small numbers of crystals were often found in South Australian fish. The highest in numbers of crystals were hatchery fingerlings grown in seawater and the lowest in freshwater culture.

![Image](image-url)

Figure 3.116  SA RAS farm 1 occasional chisel shaped crystals seen on kidney squash Gram stain preparation with Gram positive cocci (DE)
Figure 3.117 High numbers of crystals in saltwater culture of similar size and shape as South Australia from Darwin hatchery under phase contrast and then higher magnification below
Figure 3.118  * Higher magnification of figure 3.117

Figure 3.119  High numbers of kidney crystals from Broome hatchery under normal light microscopy
Figure 3.120  High numbers of kidney crystals from Broome hatchery under phase contrast and higher magnification

Figure 3.121  Lower numbers of kidney crystals in wild caught Barramundi in freshwater
Figure 3.122  RAS farm 2 Gram stain of kidney wet preparation showing crystals (yellow*), Gram positive cocci bacteria (pink)*, and melanin granules*.
Chapter 4. DISCUSSION OF RESULTS

4.1. INTRODUCTION

The gill was chosen for a master’s thesis being an external organ that is delicate and fragile and possibly a portal for infectious and non-infectious agents in causing pathology and disease. For aquaculture to be profitable stocking rates are variably high with varying husbandry intensity and technology within a species farmed, amplifying the risks of disease, particularly gill disease which has not been studied extensively or reported in the literature. This thesis will bring together a literature search of texts and journal articles of finfish gill disease and practical application of knowledge gained in the field, improving a clinical work-up of disease investigation and monitoring of the Barramundi gill. It is the hope that this thesis will provide a tool for students of aquaculture and veterinary science alike, in dealing with Barramundi and other teleost finfish species in the challenge of modern aquaculture. Furthermore to equip a qualified veterinarian with some aquarium hobbyist experience, but nil in commercial aquaculture, with the necessary skill base to interface between farm and specialist pathologist services. Disease is a major impediment to the growth of aquaculture [recently discussed at the OIE (World Organization for Animal Health) global conference on aquatic animal health October 2006] and consequently veterinarians have a major role to play in this developing industry, reducing pressure from our depleting wild fish populations by improving sustainability.
Chapter 4. Discussion

The species Barramundi was chosen for this thesis due to its biological catadromous, euryhaline and disease hardy reputation, yet was suffering from what appeared to be an acute mortality rate increase, due to systemic bacterial infections early 2003 in South Australia RAS farms. The subsequent investigation highlighted the potential disease issues associated with intensive culture, which will be elucidated within the body of the discussion. The literature is scant for barramundi diseases and information is often extrapolated from salmonids, carp, catfish, tilapia and ornamental species.

4.2. GENERAL SAMPLING METHODS AND TECHNIQUES

USEFULNESS AND LIMITATIONS FOR AQUACULTURE

4.2.1. History

History for any disease outbreak investigation of any animal farmed is paramount and it is quoted anecdotally that 80% of the problem is solved from a good history. This is true for terrestrial small animal practice, if the history is detailed, but I believe with animal husbandry and particularly with intensification in tank culture, husbandry issues bear the largest source of problems and disease issues, yet may not be realized in the history unless structured, detailed and then thoroughly investigated. This is a bold statement, but barramundi culture as for all aquatic species farmed, have brood-stock from the wild and water sourced often from above ground supplies, bringing in potential pathogens and many are difficult to eradicate, such as Epitheliocystis. Australian aquaculture often operates with intensification, potentially amplifying the disease process. The benefit of Australia is our isolation and clean environments to date.
A good history and farm investigation often highlights constraints in record keeping, 
husbandry practices, system design flaws and basic biological principles that are either not 
followed due to time, costs, or lack of knowledge by the producer or body of science.

A good understanding of the farming system, husbandry practices and health of fish are 
required for management of all fish health issues. A revised history sheet adapted from the 
textbook Fish Disease, Diagnosis and Treatment by Edward Noga (Noga, 2000) was 
developed and is presented in Appendix 4. After initial surveys I realised my history 
questions were inadequate, hence I sought an improved version and used Edward Noga’s 
detailed history from his invaluable textbook, but adjusted it suiting my observations from the 
survey experiences, knowing all grow-out farms will have pathogens, but are they or will 
they, be of significance today or potentially in the future, preventing the veterinarian from 
latching immediately on to the pathogens found as the cause of the problem. In Appendix 4 I 
added more detail to indicate producer knowledge and management before stock 
examination, finding many farms operating with non tertiary trained staff, a major constraint 
and prior knowledge can alleviate public relations problems when on farm. This history form 
can be faxed or emailed once a farmer has called for assistance for the first time. The 
practitioner is then better informed when on farm looking at the problem or problems, if the 
history is studied beforehand particularly in system design, which for RAS farms can be 
complicated to many.
From the history, diseases of the gill are usually highlighted by a history of fish seeking more oxygen by either:

- Piping at the surface, screens, or inactive at more oxygen rich areas of their container.
- Operculum respiratory rate increased.
- Easily stressed.
- Lower food conversion ratios.
- Flared gills at post mortem.

It may be obvious by an experienced and well-trained aquaculturist to note in the history:

- The colour of gill tissue.
- Excess mucus.
- High numbers of flukes and other large parasites greater than 200 microns and thus visible to the trained naked eye and able to identify smaller ectoparasites by microscopy. (a hand held good quality magnifying glass or head magnifying loop with a white light source is also a good clinical tool)
- Shortening of the filaments and gross defects such as atrophy.

4.2.2. **History Report**

The history must record farm name, address and date, species, number farmed and stocking density \([\text{kg/m}^3]\), along with a system design sketch and materials used, including quarantine areas, disinfection systems and protocols. The duration of farm operations and whether fallowing management is regularly used, is important information as to potential disease. Regular measurement and recording of water quality parameters for each biofiltration unit
used in RAS systems, include yearly concentrations for iron, manganese and zinc if bore water is used, salinity levels (seasonal if non bore water and daily to weekly if it fluctuates from source or changed for treatments); daily for oxygen, carbon dioxide, ammonia, nitrite, nitrates, or total ammonia nitrogen (TAN) in mg/L, turbidity, temperature, and pH; weekly for hardness, alkalinity, nitrogen (bore water); pollutants and pre-water treatments effectiveness (e.g. filtration, aeration and settling containers), should be measured or assessed depending on risk periods and intensity of farming. Both hatcheries used spectrophotometers and oxygen & temperature probes measuring the above, except for the bore water components, unlike the RAS growout farms where oxygen, temperature and TAN were commonly only measured (Lucas & Southgate, 2003).

Ponded fish water temperature and oxygen at different water depths should be measured and recorded through the day in the tropics particularly, often hourly at different risk periods, such as warm still days, determining if improved water circulation and aeration or increased water exchanges are required, reducing any oxygen deficit below the warmer surface water reducing the risk of fish kills. Ponded and sea cage culture both measured ammonia levels daily particularly post feeding, indicating if stocking and feeding rates are adequate. If a pond farm is intensified with mechanical and microbial biofiltration then RAS measurements should be included.

Oxygen concentrations should be measured prior to feeding; 30-60mins; and 2 hours post feeding, measured at 100 mm below the surface; in the middle of the water column & the bottom and this should be repeated in at least 2 other areas of the water body. The appearance of the water including colour, cloudiness or anything unusual should be recorded, along with
the water exchange rates and frequency of replacement such as daily or weekly. Suspended solids which is often a major component of turbidity, is preferably measured by a secchi disc for non tank culture and quantitatively (Australian Water Quality Centre) for tank culture if afforded or a secchi disc as a minimum.

If a delay in analysis occurs, then water samples could be stored for up to one week at 4°C for further laboratory studies in the face of a disease outbreak (Lucas & Southgate, 2003).

In the analysis of an outbreak of disease, stock movements for up to 6 weeks prior to the problem should be analysed, allowing for incubation periods for most pathogens, but this is a conservative guideline from the FAO manual ‘Procedures for the quarantine of live aquatic animals’ (Arthur, Bondad-Reantaso, Subasinghe, 2008) depending on species of fish and pathogens suspected. Including stock moved within farm and new stock imported to farm for all tanks or containers, allows an investigation not only to determine if a pathogen was introduced, but if internal farm practices are aiding pathogen dissemination. The case commencement date and progression of disease facilitated by accurate daily mortality rate data, date started, date ended and total numbers dead from the beginning of the problem, can add valuable information as to what type of disease process could be occurring or has occurred. The feeding rates, appetite of fish and food conversion ratios prior and since the problem began, along with information on the size, sinking rate and break up rates of pellets, are also helpful in disease analysis (Noga, 2000).

Physical examination and note taking by the farmer could include the position of healthy and sick fish in a container; the behaviour of sick fish paying particular attention to the colour of
the fish, skin health, piping for air, flared gills, fast respiratory rates, erratic swimming and flashing seen by rapid side to side movement.

The greater the intensity of farming, the more important this monitoring becomes including both fish and water. The minimum that is required is highlighted in the history appendix 4 and it is common for records to be minimal, as was found particularly where farmers and farm staff have no appropriate tertiary training.

4.2.3. Physical examination

Physical examination of fish is not usually as detailed as per terrestrial animals, but the gill examination should be performed with the addition of the gill biopsy and considered as part of the physical examination, because of the water medium where disease transmission rates can be amplified compared to air and the microscopic nature of most pathogens.

A physical examination should include a water quality examination as a clinician, including some basic water quality parameters that may not have been measured by the farmer or incorrectly recorded or doubts exist as to accuracy. Many smaller aquaculture farms use chemical colour analysis kits, which can be prone to misinterpretation. Handheld electronic equipment are available to measure oxygen, pH, salinity & temperature and a portable spectrophotometer is able to measure ammonia, chlorine, copper, nitrate, nitrite, dissolved oxygen, pH and phosphate as individual tests and can be performed quickly, on site and relatively inexpensive when compared to commercial water laboratories.
Blood diagnostics in fish is in the research development stage for most aquatic species and no work has been formally done on Barramundi and would not be part of a diagnostic work up at this point in time.

4.2.3.1. Cytology

Gill cytology is a rapid inexpensive method of diagnosing and monitoring a number of problems, for example gill parasites and response to treatment which can be performed on a regular basis with the same fish with little risk, given gill tissue can begin to regenerate within 4 days post acute toxicity with malathion (Dutta, et al. 1996), probably depending on species, water quality and age of fish. A brief study preceding the survey with 6 juvenile 150 mm or smaller barramundi was conducted, where I repeatedly took gill biopsies and regeneration took approximately 7 days, for the gills to appear grossly normal with magnification by taking the first third of a filament only.

Cytology views cells that are easily detached from the gill surface of host and non host origin and some of the superficial gill structure, including lamellae can be seen clearly for fish up to 50mm. Furthermore gill pathology, such as increased mucus production and gill thickening not always seen by the naked eye, can be identified in many cases, justifying the need for histology, a more expensive and longer time line for interpretation, but giving a more accurate picture of gill health and disease. Live gill biopsies contains a layer of water from the water medium, often containing non adhering ectoparasites such as Trichodina.

Establishing monitoring or treatment regimes qualitatively and quantitatively, requires accuracy and standardization of the biopsy procedure, needing to be established by the operator for the species, including which gill arch or arches to sample, how many filaments to
sample per arch and how deep to biopsy without causing harm to the fish, if recovery is required.

Consequently the addition of cytology in fish health cases, provides a more thorough assessment of gill health than using histology alone, but requires assessment either on site or the shipment of live fish, but transport stress can cause fish gills to become increased in mucus, depending on how much water to oxygen ratio and time in transit. Fish sent to Murdoch arrived highly stressed, due to low water levels and possibly the lengthy transport time. Sedation can be used, but this may change the gill biopsy and possibly skin biopsy results from reducing parasite loadings. Few studies have been published on transporting fish and none on Barramundi, but not only water to oxygen ratio needs to be clarified, but a stable optimal temperature and the minimisation of water quality deterioration needs to be investigated. A trial using plastic bags, fasted fish and the varying weight of fish per litre of water, affected fish health and mortalities in Silver catfish fingerlings over different time periods (Golombieski et al. 2003).

Anaesthetics have been reported reducing parasite numbers, such as Tricaine (MS-222), causing rapid detachment and mortality of *Ichthyobodo necator* from the gills of Striped bass and can narcotize *Trichodina* at high concentrations (Callahan & Noga, 2002). This should be considered if counts are to be performed for monitoring purposes of external parasites. This was evident in Barramundi with Clove oil with the freshwater protozoa *Trichodina*, where numbers were reduced due to nil or reduced cilia movement, but sacrificing the animal without drugs, or careful choice of sedation or anaesthetic method if drug and organism interaction are known, can reduce misinterpretation.
Stains used to identify living cells can also reduce external parasitic counts particularly *Trichodina*. This was noted using diff Quik 2 containing Thiazine, causing mortalities of some ectoparasites at different rates, such as *Trichodina* appearing to die more quickly than *Ichthyobodo*. Consequently non stained biopsies should be performed before staining, allowing a more rapid parasite count, using motion detection for many protozoa and some bacteria. Phase contrast with colourless organisms often affords good visibility, such as the unidentified cells seen in figure 3.54 and possibly better identification of the anatomy of some parasites such as the presence of flagella in *Ichthyobodo*.

Different sized fish incurred different problems performing gill cytology. Gill biopsy for Barramundi less than 50 mm can prove difficult without a dissecting microscope and experience. Consequently the entire arch often requires dissection, but filaments can float or be difficult to view with the arch present with and without a cover slip, however the lamellae were often easily viewed affording a good in situ type examination. Juvenile fish require a thorough assessment in culture for gill pathogens, often overwhelming them with typical high stocking rates and greater individual susceptibilities to pathogens due to age (Noga, 2000). The removal of the gill arch and then the dissection using a sharp scalpel under the dissecting microscope of the filaments produced the best results, eliminating focusing problems, yet preserving the floating and separation of filaments and lamellae in diagnostic numbers for accurate cytological evaluation.

Conversely, cytology of large Barramundi gills over 400 mm were too thick to be accurate, but gill scrapes or washes with gill biopsy, affords ectoparasite identification and some quantification of parasite numbers. This was initially performed using distilled water with...
either a 3ml plastic pipette or wash bottle in a petri dish, then examining a few drops of wash under the microscope. But by viewing a small amount of filament half the length of a 40-50 mm coverslip placed on a slide with 2-3 drops of distilled water from a 3ml pipette, parasites would readily leave the gill for a count that is repeatable and possibly quantifiable. An unstained biopsy should be scanned with contrast at 50-400 × magnification, looking for various external parasite motions, such as the fast random motion of the small Red Blood Cell size *Ichthyobodo* and the bowler hat shaped spinning swimming action of *Trichodina*. Stain could then be added using the 20% Diff Quik solution which killed the ectoparasites slowly, giving either a contrasted background highlighting cells or the cells taking up the stain highlighting them directly, such as *Ichthyobodo* and *Trichodina* respectively. Stain was very useful if 1000 × magnification was required to identify small cells and organisms.

Squashed preparations were too difficult to interpret unlike other denser organs, such as the kidney for host and non host cell identification, but the common ectoparasites could be identified, but their motion is restricted within debris fields not allowing quantification.

Bacteria, fungi and parasites are not always clearly visible within gill tissue cytologically, but often in clinically sick fish, stains can reveal for example, high bacterial loadings in gill washes possibly indicating disease. Histology needs to be performed including systemic bacterial Gram stains of kidney tissue and cultures in these cases, determining if the bacteria in the wash are an incidental finding, secondary opportunists to a clinically ill fish, or part of a primary disease process. Histology can not always confirm fungi and parasite pathogens, such as flukes and metazoans that are trapped or have their lifecycle within gill tissue, because of a small sample of tissue often viewed. However cytology can evaluate rapidly,
larger amounts of gill tissue particularly in small fish. 4 of 6 fish from the pond farm demonstrated immature Metazoan plasmodia and none histologically. 1 of 6 150 mm wild caught fish revealed *Henneguya* plasmodia on histology, yet 3 of 5 on cytology. 2 of the 6 600 mm fish from Lake Argyle showed flukes on histology, but 5 out of 6 on cytology revealed the occasional fluke. RAS farm 1 showed gill epithelial cell enlargement on all fish sampled indicative of *Epitheliocystis*, but only one fish histologically.

Cytology was fundamental in discovering the unidentified cells found in chapter 3, with the use of Diff Quik number 2 stain and phase contrast, but host cell knowledge is required in determining non host cells on and in the gill. For example under 1000 × magnification on fish under 150 mm, EGC’s will heat up with the microscope lamp, producing a barely detectable wave like motion of their granules, but using a head up display to a monitor via a digital camera further magnifying the image, this motion becomes remarkable as demonstrated in SA video chapter. Initially thought to be non host cells, however a pattern of location occurred with further investigation. The numbers appeared to increase with fish cultured in recirculating tanks particularly with poor water quality. They did not wash off the gill, therefore an internal gill cell and appeared to be in a predictable pattern along the central vein in high numbers. Then seen in increasing numbers within the parenchyma of the gill with diversity of shape, but lamellae were often overlying the gill, possibly confusing their location in fish larger than 50 mm unlike histology revealing gill structure clearly. EGC’s were also found in TEM migrating up lamellae in diseased gills. The literature search did not reveal all of this information and once again requires further investigation.
Another cell readily identified was the immune cell the melanomacrophage, as seen in the fish with tail rot and bacterial gill disease from Darwin Hatchery. These fragile gills were indicated by the filaments irregular shape and the edges taking up much stain.

The melanin granules contained within melanomacrophages are of similar size and shape to cocci bacteria. If the melanomacrophage is ruptured, the contained granules can be found scattered within tissue and can then be confused with similar sized cocci bacteria at 400 × or with at 1000 ×, particularly in cytology with no staining. The granules take up little or no stain with commonly used stains, such as gram and Diff Quik and with melanin colour can be confused with Gram positive cocci. The granules however will show their brown colour while focusing at 1000 × magnification, but due to their small size further magnification via computer or video screen enlargement can be necessary for the untrained eye. An example is of a kidney Gram stain squashed preparation from a fish of a South Australian RAS farm with a S. iniae septicemia, also demonstrating kidney crystals.

Gill washes of fish from recirculating systems and poor water quality produced in the wash, epithelial cells probably pavement cells and seen on the gill in Figure 3.100 being highlighted from the same fish batch using DQ2 as a background stain. Detailed information of the cytology of the gill of teleosts was not found in the literature, highlighting the need for further study of the cytology, histology and the immune system of cultured teleosts.

4.2.4.  *Histology*

Histology is mandatory for gill health assessments, revealing a greater understanding than clinical and gill cytological assessments alone, particularly when a commercial batch of fish
can be from 20,000 or greater and the ability of fish to show little clinical signs until moribund or dead.

Histology offers a variety of further staining techniques that may further elucidate many disease processes and pathogens, but generally standard H & E stain shows normal gill histology very well. Specialised staining that were of benefit was Gram stain staining for further classification of bacteria and Periodic Acid Ammoniacal Silver for suspected fungal water molds, staining carbohydrates brown.

A problem encountered from sampling was gill biopsies used for histology from large fish without the arch present, did not allow easy orientation of the filaments and lamellae and should be avoided unless a post mortem is not permissible. At post mortem a fret saw for very large fish for example 3kg or above, can be used to remove part of the arch small enough to be potted in standard formalin 50mL to 100mL containers, after the entire arch is removed by pruning shears reducing filament damage. Filament orientation with the arch present also caused positioning problems with individual filaments, commonly missing lamellae and filament anatomy, from oblique cuts of an entire arch, individual filaments obliquely orientated within the arch section, filament tips having deeper cuts than the proximal sections and cuts too deep on small filaments. The gill is a rounded organ with fine and delicate anatomy and careful orientation by the laboratory technician is required. Artefacts from processing were commonly, delamination of the epithelium particularly of the fine lamellae and dilation of the central vein and should not be confused with oedema usually associated with necrosis and this was discussed in the literature (Roberts, 2001).
Dr Brian Jones of Western Australian Fisheries, a fish pathologist for 30 years, suggests a minimum of 4-6 filaments with lamellae are necessary to form any opinion on gill health. Re-cuts were performed on many samples, despite the experience of Murdoch University Pathology Department to view filaments and lamellae adequately and on occasion this was not possible on all sizes of fish sampled. Further cuts are also helpful in the identification of key cells, viewing different levels per cut reducing potential errors of interpretation, particularly in larger fish, for example Figure 3.111 does not reveal excessive mucous glands necessarily. Sectioning of gill in longitudinal and transverse planes can reveal good detail and the make up of cell types & numbers within the gill and an improved interpretation may be given, but the longitudinal sections are those typically presented in the literature and in this thesis.

Figure 3.114 is an example of a laboratory that does not normally perform histological sections of fish and the over staining using H&E can cause some confusion of cell identification, particularly with the EGC and others that may hypertrophy, such as the mucous cell, possibly seen in the section. Additionally Figure 3.115 from the same sample batch, gives the impression of parenchyma disruption with an isolated MRC, but is more than likely a processing artefact.

Despite the most experienced technician some cells such as the mucous cells in Figure 3.111 can take up various amounts of stains. This may be due to the two different types of mucous cells found in teleosts, but no work has been done on Barramundi. Further clarification by the use of the mucin stains such as Alcian for acid pH mucus and Periodic Acid Schiff (PAS) for
base pH mucus, would help clarify the distribution of these mucous cells and other cells that may appear to be mucous cells in some sections (Wilson and Laurent, 2002).

There is no literature for barramundi mucous cells as to their ability to combat pathogens and likewise for potentially the different MRCs, enabling euryhaline barramundi to freely travel from saltwater to freshwater and visa versa, much research is required in gill physiology.

The histology of the Darwin Hatchery fish with tail rot showed gill necrosis evident with pale nuclei and oedema seen by vacuolation with epithelial lifting at the base of the lamellae. Melanomacrophages not only increased with clinically chronic disease seen in this fish, but appeared to also be common in small clinically healthy fingerling gills of both freshwater and salt water culture. However the freshwater Lake Argyle fingerlings showing melanomacrophages were being treated with malachite green and formalin at the time, possibly increasing melanomacrophage numbers from the *Trichodina* infestations and/or treatment. Additionally the bases of the filaments appear more basophilic indicative of cellular infiltration possibly causing hyperplasia, indicating a chronic disease response, yet clinically the fish appeared normal. Therefore a study of gill cell types should be investigated with various Barramundi size classifications in different culture methods and salinities, giving a clearer picture of what are normal histological cellular pictures for the culture of this species. When this study is performed gill sections of various depths are required with longitudinal and transverse sections, giving a complete histological anatomical profile of this delicate rounded organ.
4.2.5. *Electronmicroscopy*

Electronmicroscopy has two disciplines Transmission Electronmicroscopy (TEM) and Scanning Electronmicroscopy (SEM). TEM was useful in identifying host cells, in particular the EGC morphology with pseudopodia, seen commonly along the central vein and in hyperplastic gills found near or within lamellae (Figures 3.104 & 3.105). EGC granule numbers and morphology appeared to vary, possibly a maturing feature or a variation with subtypes of EGC’s in Barramundi, or simply artefact from varied cross sectional orientation on the fine TEM sections. Little is known about eosinophilic granular cell in the barramundi and much research is required.

SEM highlighted the anatomy and possibly non host cells caught within lamellae or adhered to the surface, but there are limitations with expense in money and time for both disciplines; therefore reserved predominantly for research and not a general disease investigation tool, but viewing pictures of fish gills with these techniques gave a clearer understanding of the functional morphology of the gill.

4.2.6. *Microbiology*

Microbiology was used with Gram stains in cytology and histology, but due to cost no bacterial and fungal cultures were performed in this study. They are very useful diagnostic tools and performed often for systemic disease investigations in all species, particularly if the microscopic disciplines reveal organisms requiring identification. The identification of bacterial gill disease would normally be followed by bacterial culture, samples obtained from kidney or spleen tissue swab at the time of sampling and the laboratory being informed if
hard to grow organisms are suspected and potentially zoonotic bacteria, such as the skin bacterium Mycobacteria (Noga, 2000). This bacterium often causes skin ulcers in the fish particularly in tank culture, but can be overlooked with fungal overgrowth commonly associated with an ulcer. Therefore deep scrapings should be taken, preferably accompanied with a skin biopsy of the same ulcer, including the normal tissue ulcer interface if the fish was post mortemed for histology.

Microbiological water quality testing is a new discipline, probably becoming common place within a decade as intensive aquaculture increases worldwide. It is not routinely performed unless diagnosis of disease warrants monitoring, such as S. iniae which is known to grow within the environment, including the walls of tanks as was determined by Aquaculture SA in 2005 by Dr Colin Johnston in a tank based Barramundi disease outbreak.

The measuring of coliform bacteria and total bacterial numbers could prove useful in warning aquaculture producers of impending disease, which has been used by an oyster hatchery as advice from WA Fisheries, but bacterial sampling needs to be frequent and total bacterial plate counts are time consuming and costly. However a microbiological marker probably of fish bacterial origin, such as gastrointestinal lactobacilli or an opportunistic pathogen, could be used monitoring their increase in deteriorating water, before true pathogens or more pathogenic organisms increase in number. If the test is rapid, relatively simple, specific, quantifiable and cost effective, early husbandry and treatment protocols could be utilised preferably before subclinical or clinical disease episodes occurs. This possible future trend was discussed with water quality expert and author Claude Boyd at the World Aquaculture
conference in Bali in 2005 with myself and his comment was “this is the next step in water quality management.”

4.3. GILL PATHOLOGY AND PATHOLOGICAL AGENTS FOUND

4.3.1. Hyperplasia

Hyperplasia of varying degrees was evident in many histological specimens, particularly where poor water quality or high stocking rates or parasites were evident, occasionally trapping pathogens and the unidentified cells; indicating a chronic response and a similar response to different stimuli. Severe hyperplasia was seen in older fish, where filament fusion can occur, beginning at the base and then becoming usually less severe if the hyperplasia occurs more distally. Removing the causative agents may allow gill tissue to resolve to normal architecture, probably with varying degrees depending on age and severity if the fish is not clinically affected, otherwise death would normally result. However a study on barramundi and other cultured species including ornamentals, as to the time period and ability of gill tissue to resolve varying degrees of hyperplasia should be done with toxic and pathogen agents. This would afford the producer a choice to sell his stock prematurely if food fish, if the increased time line and his method of culture increases the risk of further disease and potential losses.

4.3.2. Necrosis and Oedema

The evidence of oedema seen as vacuolation within the gill filament and lamellae and gill necrosis indicated by epithelial lifting and epithelial cell destruction was evident with acute ammonia toxicity. Other organs remained unaffected histologically, indicates the delicate
nature of the gill. An ammonia spike greater than 1 mg/L of unionized ammonia was lethal in 1-4 days for freshwater finfish, due to the necrosis and oedema of gill tissue developing in 2 to 3 days (Dutta, et al, 1996). There is little difference between fresh and brackish water in unionized ammonia levels, but full strength seawater can have up to 20% less unionized ammonia with otherwise similar water quality parameters (Noga, 2000).

The biopsy of the live fish gills produced mucus, protecting the gill from infection and regeneration of gill tissue cells can begin as early as 92 hours after a toxic insult in catfish (Dutta, et al, 1996) and therefore possibly earlier from a sharp cut from a good biopsy technique. Generally there is little information on the regenerative capacity of teleost fish gill from pathological changes. Studies demonstrating using ammonia toxicity as a model, and/or the removal of healthy filaments surgically, in an aim to determine the functional filament reserve of popular finfish species, such as barramundi under various water quality parameters, may be helpful information during acute disease episodes. This information would be of importance for treatment, recovery and prognosis, particularly for acute gill pathology and determine management decisions to harvest, cull clinically affected, treat or just remove the dead and moribund after removing the causative agent. This study could also establish regenerative times for healthy barramundi gill tissue.

4.3.3. Telangiectasis and Haematoma

Telangiectasis was commonly found but in numbers that did not affect gill performance and only one incidence of a haematoma between lamellae on a wild caught fish was seen. Telangiectasis possibly resulting from mild trauma either from high stocking rates as seen in the Darwin hatchery, or suspended solids in pond culture and in the wild that was probably
due to in part, to the wet season producing muddy water in the rivers, but disease issues cannot be ruled out. The ponded fish where there had been a history of bacterial disease issues previously had 4 fish affected, but was in low numbers on each fish, but poor histological sections despite repeat sectioning, did not reveal any more information. No information of how much telangiectasis can cause clinical signs or the gills recovery rate from telangiectasis was found in the literature. It is possible that the fast striking predatory ambushing of prey, may increase the presence of telangiectasis and possibly the more severe form of a haematoma occasionally, when suspended solids or high stocking rates are present causing filament trauma.

4.3.4 Parasitic

4.3.4.1 Henneguya Myxosporidea

*Henneguya* was diagnosed by cytology, finding the diagnostic bi-polar spores with paired filaments in the surrounding wash of a gill biopsy and within the gill identifying the myxozoan plasmodia in the Northern Territory only. Cases found were in low and occasionally moderate numbers on filaments causing pathology, but not appearing to affect the health of the fish. The encased spores could not always be identified and if no free spores were seen in the plasmodia, a squashed wet preparation stained with Diff Quik 2 was useful revealing the spore morphology if in mature plasmodia. The prevalence of *Henneguya* in the region is 90% (n=10) for cage culture, 66% (n=6) for pond culture and 33% (n=12) for wild fish, with a biased sample size not including all wild barramundi size variations. The Hatchery had no cases found and all case reports reviewed at the hatchery and at Marine Harvest, showed none were positive for this Myxosporidea upon the arrival of fingerlings.
There was no evidence of plasmodia in other tissues sampled, but the survey did not include muscle and nervous tissues systems.

This parasite appeared to be seasonal with the smaller 150 mm fish having significant numbers from both wild and Bathurst Island ecosystems, but the sample size was small and only performed once, therefore further research is required. The histology showed the *Henneguya* plasmodia residing between lamellae and within lamellae, but not the intercellular spaces of the parenchyma of the filament, as described in the literature (Noga, 2000), but few histological sections were found with plasmodia. Prevention as the literature indicates is performed by filtering the source water and 35 micron sand filtration is the cheapest, yet reliable as the Darwin Hatchery utilises, probably removing the intermediate host. Additionally if the seasonal intermediate host cannot be defined, the risk period maybe defined by a epidemiological study and source water could be taken from another source if commercially viable, such as a well for the pond farm during the risk period, acting as a filter near the river bank.

### 4.3.4.2 Flukes

Two trematode eggs possibly with a crocodile being the definitive host was found as an incidental finding on one fish on the pond farm, drawing water from crocodile infested waters, this probably is of no clinical consequence and an uncommon occurrence, but highlights the need for some form of biosecurity for water intake from wild ecosystems.

Additionally a wild caught fish had what appears to be either an egg containing a fluke, or less likely a walled off fluke dislodged during histology processing and if a fluke egg; a Dactylogyrid is most likely as these fish were caught in a freshwater mangrove river habitat, at the end of the wet season. Interestingly none were found on cytology of all wild caught.
The prevalence of flukes found on histology only was 16% (n=2), a low sensitivity when compared to cytology counts viewing more gill tissue.

Lake Argyle fish gills also had Dactylogyrids present, at a prevalence of 36% (n=18) probably lower than expected, due to treatments occurring at the time and the low numbers found posed no gill health issues clinically, but how much contributed to the hyperplasia found particularly of the larger fish is unknown. Cytology was more sensitive as expected in fluke detection, but a 250 mm fish histological section showed a fluke, yet cytology did not. The limitations of using histology for quantitative counts, is possibly due to some of the flukes being lost in processing, for example many of the filaments were cut too deep, losing lamellae and filament parenchyma. Additionally cytology is a superior monitoring tool for flukes, as recovery of the fish is possible and a response to treatments or the necessity for treatments could be assessed rapidly.

4.3.4.3. Protozoa

*Ichthyobodo* was found in all South Australian farms with a likelihood of 100% being infected in low numbers, based on a sample size of 100,000 fish for RAS farms 1 & 2 and 20,000 in RAS farm 3, sourcing fingerlings from South Australia or Queensland, where the freshwater Argyle farm sourced fingerlings from Western Australia and occasionally Northern Territory, where none were found. It is possible for some species of *Ichthyobodo* not to be limited by salinity (Callahan, Litaker and Noga, 2005), but this is unknown with the specie or species found. The numbers found and the lack of pruritis usually demonstrated by flashing, indicated parasite loadings caused little or no production problems. This parasite was not discussed in the literature concerning Barramundi, but mortalities often occur in juveniles with poor water quality and it is suggested that >2 per high powered field in a wet
preparation, is significant and may cause clinical signs in other finfish species (Noga, 2000). *Ichthyobodo* reproduces on the fish by binary fission and numbers can quickly increase, but chemical treatments such as formalin were commonly used on affected farms, possibly controlling population numbers and this cannot be ruled out for Lake Argyle culture, however unlikely, as RAS farms also used high treatment regimes. Significant damage to the gill epithelium from high burdens, when they attach intermittently can possibly result in respiratory distress, particularly if secondary infection from bacteria and/or fungi occurs. Worldwide it is considered a parasite of importance and recent molecular studies indicate that some *Ichthyobodo* species can infect multiple fish species posing an environmental translocation risk (Callahan, Litaker and Noga, 2005).

Diagnosis is the characteristic flickering motion at low power and should not be confused with ectocommensal flagellates often encountered. It is the smallest obligate ectoparasites that infest fish, measuring similar size to a red blood cell measuring at approximately 10-15 micron in length, excluding its flagella. Histopathology often demonstrates attached parasites, but this was not evident in the survey probably due to low numbers. Cytology would be used to monitor this flagellate similarly for all gill parasites.

*Ichthyophthirius multifilis* outbreak on the re-circulation farm 1 occurred with acute clinical signs of respiratory distress evident by piping for air then death. Commonly outbreaks occur in a temperature range of 15-25°C as in this case, where fish were transferred to a flow through trough at 24-25°C from a 28°C tank, adding to the stress of the fish. This primary gill pathogen did not occur in any of the other 3 sheds, therefore may possibly of been introduced unwittingly from contamination from another freshwater aquaria. This Barramundi only farm
had fingerlings acclimatized in freshwater before shipment by the hatchery. Freshwater Ich lifecycle cannot be completed with salt at levels greater than 1 mg/L, which proved a good control measure in this case, including quarantine of shared equipment and fish to other sheds was initiated where possible. Histopathology was not performed, because it pre-dated the survey and a diagnosis is definitive by the pathognomonic cytological findings. To my knowledge no further outbreaks occurred up until 2005, where contact with the farm ceased.

*Trichodina* was found in low numbers in both freshwater and seawater culture and were of similar size greater than 30 micron, causing no apparent clinical problems in the sea cages at a prevalence of 40% (n=10) or at Lake Argyle at 17% prevalence (n=18), but their were multiple problems affecting both facilities. Lake Argyle fish had suffered *S. iniae* mortalities in 2003/2004 (Creeper & Buller, 2006) and the fish were under constant veterinary monitoring and treatment during my visit in 2004, possibly kept *Trichodina* numbers low. Marine Harvest government records, noted commonly *Trichodina* in their veterinary reports, but deemed of no clinical significance and no direct treatments were given for its presence. Both sourced fingerlings from hatcheries, even though hatchery fish are often sourced from the wild and kept in re-circulating systems. The rearing methods including filtered water flow through systems and disinfection of eggs and that there was no history of the parasite found in both hatcheries, indicating that *Trichodina* was probably introduced from wild fish populations in the open water culture systems.

Clove oil should be used with caution when assessing *Trichodina* and other anaesthetic agents should be explored, such as benzocaine which is registered for use in fish. It is possible a lighter dosing with clove oil, may avoid *Trichodina* counts to drop from sedation,
but the use of Aqui-s partially derived from clove oil and registered for use in fish has not been explored in this thesis.

4.3.5 Bacterial

It was uncommon to find bacteria with histology within the gill during the survey, except for RAS farms.

Cocci bacteria were found in gills histologically as seen in the results for the Darwin Hatchery fish, suffering from tail rot. The systemic nature of the disease due to the deep ulcerative skin lesion, with motile slender Gram negative rod bacteria found by a cotton swab sample, indicates the few gill cocci bacteria is probably not the primary pathogen, but opportunistic or part of a mixed infection. The primary tail skin lesion was probably a consequence from a tail bite, commonly occurring during the cannibalistic fingerling stage, which occurs up until approximately 120 mm in length generally (Tucker, 2001). This fish for a more thorough workup would require systemic bacterial culture from an uncontaminated kidney tissue sample.

The hatchery grades at least weekly reducing the problem and culls the affected fish at grading. If numbers rise beyond the occasional fish, culture and sensitivities are performed and the remaining fish are treated with antibiotics and a disinfection bath using Benzalkonium chloride. Prevention using freshwater to brackish water baths were routinely used for all fingerlings prior to shipment. I could not establish a prevalence rate, due to the data from the hatchery not being available for this disease issue.
Cocci bacteria found in South Australian recirculation farms probably entered the fish via the gut with no skin lesions present. Gill entry however cannot be excluded when hyperplasia and necrosis was common. *S. iniae* was known to be present in South Australian recirculating farms at the time and studies to date indicate the fish gut and nares are the primary routes of infection, but can enter via the gill (McNulty, 2003). Gram stained sections of the gill were histologically unremarkable, but the kidney cytological Gram stain revealed Gram positive cocci and the farms was known by PIRSA to have had *S. iniae*. The farms currently operating are controlling *S. iniae* by an autogenous vaccine at the writing of this thesis, therefore the gill cocci seen on H&E staining were likely this organism. Likewise on RAS farm 3 the gill necrosis caused by cocci bacteria were possibly *S. iniae*, but Gram negative rod bacteria were found from a kidney wet preparation and bacterial culture would be required to clarify if *S. iniae* was present, but this was not performed because of economics and harvest of clinically unaffected fish, which were at marketable size was carried out upon diagnosis.

However RAS farm 2 despite all 6 fish kidney swabs revealing Gram positive cocci and culturing *S. iniae* from those kidney samples from a batch greater than 100,000 (100% prevalence n=6), histological Gram stains of the gill of one fish only showed Gram negative cocci type structures of similar size to *S. iniae*, generally encased within a thin walled structure and positive for PAAS, ruling out more than likely bacterial origin for the gill specimens.

Fish sent to Murdoch Fish Health Unit July 7th 2004 were from SA RAS farm 1 and one fish sampled on the day of arrival had some filaments with gill necrosis caused by cocci bacteria
with a prevalence rate of 16% (n=6). Histology of the liver, spleen and kidney did not reveal a bacterial infection, but no Gram stains or cultures of the kidney or spleen were performed. This additional information could have possibly confirmed the gill infection was localised or present systemically. Bacterial gill disease appears to be more common with high intensity farming with re-circulating water system designs. None of these farms had state of the art ozone and ultra violet disinfection systems or foam fractionation, reducing the suspended solid levels. This possibly increased \textit{S. iniae} numbers, including the tank walls and the RAS system generally, potentially increases the loadings on the gill, nares or olfactory orifice and gastrointestinal system.

\textit{Epitheliocystis} was commonly found in low numbers but at high prevalence’s in SA RAS farms. All fish sampled had gill lesions cytologically, yet not found as commonly histologically, except where numbers were high cytologically in RAS farm 2, where all were positive histologically, with varying lesion numbers per section. The interpretation of enlarged epithelial cells forming a diagnosis cytologically would have a low sensitivity and specificity, in comparison with histology being very specific in the interpretation and diagnosis of \textit{Epitheliocystis}. Additionally cytological evaluation for \textit{Epitheliocystis} in large gill filaments was difficult and this appeared to be fish over 200 mm in length. The RAS farms were supplied with fingerlings from similar hatcheries, all applying good hygiene and production practices, including the disinfection of eggs reducing transmissibility.

The sea cages in the 150 mm size had a prevalence of 66%, but poor histological sections precluded a count for the larger fish for \textit{Epitheliocystis}. The wild caught fish total prevalence rate was 8% overall and 18% for 150 mm size. The hatcheries, pond farm and Lake Argyle
had no *Epitheliocystis* lesions. The pond farm however only 6 fish were sampled and too few filaments were examinable histologically, therefore determining zero prevalence is probably erroneous, with low stocking rates possibly contributing in reducing lesion numbers for *Epitheliocystis*. Also no wild fish are able to enter the ponds, with a primary filter screen in place for incoming water using meshed wire, potentially reducing or removing the wild fish infections compared to the sea cage farm.

The *Epitheliocystis* lesions found in sea cage culture and in the wild catch in 150mm sized fish, suggests a possible seasonal life cycle or as the literature reports a possible water temperature variation (Nowak & LaPatra 2006) and other co-factors increase the spread of this Chlamydial type pathogen. All lesions appeared not to cause any clinical or production problems, consequently no facility treated *Epitheliocystis* with great concern.

The lake facility had 18 fish samples of which revealed adequate filament structure. The fact that all were free of lesions is curious, as the farm had suffered serious stressors from *S. iniae* mass mortalities in late December 2003 and early 2004, including silt and organic matter runoff from tropical rains, increasing water turbidity and organic loadings at this time (Creeper, Buller, 2006). Commonly *Epitheliocystis* lesions would be expected to increase over the ensuing months and many of the gills histologically had moderate levels of hyperplasia, indicating gill health was compromised similarly for RAS farms.
4.4. UNIDENTIFIED CELLS POSSIBLY OF NON HOST ORIGIN

4.4.1. Methods Used to Identify Non Host Cells at Murdoch Fish Health Unit

Cytology was the initial tool in finding these unidentified cells, firstly in gill washes then on the gill using normal light microscopy with high contrast and a video head up display, digitally magnifying these structures beyond 1000 ×. Due to the difficulty in seeing these structures despite good magnification, staining was initially tried and Diff Quik was chosen for its ability to stain cells well in terrestrials.

Diff Quik number 1 solution does not stain bacteria or fungi readily, but number 2 with the basophilic dye Thiazine penetrated these cells well, but often resulted in over staining. Dilution to a 20% solution with distilled water highlighted fish and non fish cells adequately and in the case of the larger unidentified cells, did not appear to stop the apparent movement of some of these cells. The samples were easily prepared using plastic pipettes to pre-drop 2-3 drops of diluted stain on a slide, then place the gill biopsy on the slide and tease the filaments apart using iris forceps and scissors. A dissecting microscope for accuracy of filament manipulation from fingerlings smaller than 80 mm was used with success in a lead up to this study. Placing a 50 mm coverslip to disperse coverslip pressure on the gill sample for fish up to 150 mm and a 60 mm for slightly larger fish worked well in reducing filament pressure artefact and debris. If the gill biopsy was occupying less than 20% of coverslip surface area, these cells could be readily viewed on the gill and in the gill wash for most specimens. Phase contrast at 1000 × was used to further highlight these cells and act as a rapid screening test in the gill and kidney wet preparations with and without staining.
Cytospins were useful in determining more information of these cells, particularly with the specialised PAAS and Wright’s stains. The drawback of the cytospins is the splattering effect, but some information of physiology could be inferred.

Histology trapped basophilic staining cells occasionally, with varying structural makeup within hyperplastic gill tissue in H&E and PAAS stains. All of the cells seen in histology were from the same farm tank design, with the highest suspended solids and organic loadings of any fish system sampled. The fish with the highest numbers found cytologically were clinically sick, moribund or in one fish with a deformed mandible delivering the highest numbers as seen on the PAAS stained sections.

Scanning electronmicroscopy found cells trapped within mucus at the base of a section of filaments from fish held in brackish water, except for the spherical cells on the filament seen in Figure 3.98 and the single cell in Figure 3.96. None of these unidentified cells were found in TEM scans.

4.4.2 Initial Characterisation

Cytologically the cells appear loosely attached to the gill surface and there appears to be 3 basic varieties. SA video chapter particularly showed the largest more complex cells sliding off the gill and in to the gill wash. These non spherical cells on the gill appear fragile in nature and possibly multinucleated as seen on the video; light tan in colour and difficult to see on the gill, but were identifiable unstained in some gill washes and easily identifiable by staining. Surface cells appeared to be lost with formalin or glutaraldehyde fixation from the
gill surface and on dead fish. These larger more complex cells appear to range in diameter from 20-65micron approximately, as measured by a light microscope eyepiece ruler.

This fragile multinucleated form is possibly gelatinous in nature, allowing it to conform to the gill surface. This would explain squashed preparations, rough handling of the coverslip or specimen, or too small amount of water under the coverslip, probably rupturing these cells and overlooked as debris.

Heat generated from the light microscope readily confuses micron sized organism motion and Brownian artefact motion, due to light microscope magnification limitations and our knowledge of micro water biology and biological/non biological Brownian motion processes. But a pattern of motion was noticed when captured on video at 1000 × magnification on and off the gill, with non spherical unidentified cells changing shape in SA and NT video chapters, including moving along the gill and sliding off the gill with presumably coverslip pressure particularly in the SA chapter.

The rupture of a single multinucleated cell in the SA video chapter, possibly from coverslip pressure could indicate non Brownian motion. The larger more differentiated cells appear to sequestrate and bud off smaller cells containing varying cellular structures, including the micron sized vibrating structures, howbeit seen 2 dimensionally within a debris field in the SA chapter, shadowing accurate interpretation. Smaller spherical cells containing moving or vibrating micron sized particles appeared evident, with specimens from the Darwin hatchery and the wild catch seen in the NT video chapter.
The gill washes commonly displayed two spherical cell types: the larger more differentiated (approximately 20 – 30 micron) and smaller (5-15 micron) undifferentiated cells as seen with the light microscope eyepiece ruler, both also appearing colourless to tan depending on size, organelle or structural development and made easily identifiable by phase contrast at 1000 × magnification. Some small spherical cells contained small micron sized elements that appeared to move or vibrate within the cell and another cell type with no internal movement, with varying amounts of undifferentiated intracellular material. Spherical cell types appear to adhere weakly to the gill when compared to the larger non spherical cells, possibly due to their shape decreasing surface contact on the gill.

The smaller spherical cells were found in kidney squashed wet preparations also stained with DQ2 and using phase contrast, indicating their robust spherical nature. Both gill and kidney spherical cells appeared to float within the wash unless trapped in debris. Therefore possibly buoyant when compared to other cells such as the gill epithelial and red blood cells, commonly found in gill wet preparations seen in video chapters from SA, NT & MHFU. The smaller spherical form with little internal structures containing non differentiated cellular material often appeared clumped in groups in cytological gill washes in aquaculture and in wild Barramundi samples. SEM Figure 3. showed what appeared to be surface impressions and often a similar pattern was seen with similar sized cytological stained cells, with non differentiated cellular material appearing within the cell.

The larger spherical cells appeared to become more differentiated and the larger possibly multi-nucleated cells, such as those seen in the SA video chapter and the video of Figure 3., contained what appeared to be motile smaller spore like structures. These small possibly
motile structures appear similar to a variety contained within the smaller spherical cells, but without other cellular material or nucleus.

The appearance of similar spherical cells in kidney wet preparations, suggest these cells may enter the fish in a smaller state or are part of the host cells, yet to be identified formally in barramundi. A rupturing cell within the kidney of a fish from Sea cage culture released micron sized particles, appearing to follow each other in the NT video chapter, but its brief time frame, possible micro currents and Brownian motion cannot be ruled out causing such an effect. Histology of kidney tissue for each fish specimen did not show any of the spherical unidentified cells, suggesting they are possibly of non host origin if excreted in the urine, or either host or non host origin being destroyed during processing.

Additionally out of curiosity a skin scrape was taken from a wild caught fish under the pectoral fin finding spherical cells, despite swimming in fast water flow. These small spherical cells with little cellular development, similar to those found in gill washes, indicate possibly a sticky property. This was performed by lightly scraping a glass coverslip over a small section of skin and placing the material on a slide, with 20% DQ staining solution, using the collection coverslip to view the specimen under phase contrast and normal light microscopy.

Similar spherical cells were found cytologically in other species of finfish gill, including Black Bream from MFHU (Appendix 1.4) and Murray Cod from Deakin University (Appendix 1.5).
Initially when the experimental fish arrived from SA RAS farm 1 at MFHU, H&E histological sections of gills showed mild to moderate hyperplasia revealing 2 unidentified basophilic staining cells. These cells may be similar to, or a variant of, or another type of cell to the cells in gill cytology during the survey. These cells have not been documented histologically in barramundi or finfish or other aquatic animal gills in the literature.

Typically the unidentified spherical cells found on the gill and gill wash in freshwater and brackish water, were in increasing numbers with deteriorating water quality, measured by increasing ammonia levels and suspended solids which were visually discerned, despite water exchanges of 50% per day during the experiment at MFHU. The fish showed typical signs of stress from transport, new container syndrome and husbandry stress from constant water exchanges and container floor vacuuming, with mottling of the skin and inappetence for the 14 day duration. The new container syndrome may have been from contamination or a variation in plastic, despite rigorous cleaning and drying in preparation for the fish. I have successfully used the same brand of plastic container many times before in South Australia with Barramundi.

The spherical cells proliferated more quickly when salt concentrations were raised from 0 to 10 g/L at a constant 28°C, but were found in all salinities at all farms, as per the results of the survey.

Cytospins highlighted the smallest spherical cells containing micron sized cellular material, possibly the same cellular material having individual movement seen on cytology, staining positive with PAAS and eosinophilic with Wright’s stains. The intracellular staining density
possibly increased by the centrifugal forces, flattening the cells and making them appear more heavily stained. These small non nucleated cells contain energy sources of carbohydrate and possibly lipids from the respective stains.

Cytospins showed a larger potentially more differentiated spherical cell with Wright’s stain in Figure 3.84, containing eosinophilic structures possibly indicating proteins with a weak basophilic cytoplasm. Figure 3.75 may be the same cell type in the gill wash cytology showing differentiation and possibly nucleated. However the PAAS positive cell Figure 3.88, demonstrates various carbohydrate containing organelles and/or stores or materials of differing size and densities, from small spherical structures with strong PAAS uptake, to diffuse weakly positive material.

The SEMs highlight spherical cells ranging from 3-20 micron in diameter, possibly the undifferentiated more robust smaller spherical cells. With the aid of deteriorating water quality and a container hindering swimming at the end of the Murdoch study, more complex unidentified cells from skin biopsies were seen.

The histology from SA farm 2 in December 2004 performed at MFHU demonstrates in a hyperplastic gill filament in Figures 3.18 a cell containing small cocci elements. These elements appear to stain PAAS positive in Figure 3.23 and Figure 3.24 with similar micron sized cocci shaped material, but clumped to the edges of the walled structure in the H & E section, possibly a variation of cell type, or from histological processing or sectioning, or is a different unidentified cell. The Gram stained histological slides demonstrate similar structures and possibly similar to the cytospins of the smaller spherical type, but not as dense
and may not be true Gram negative, but taking up the neutral red counter stain of the gram process, possibly indicating lipids and probably the same cell type seen in Figure 3.18. These cells appear to be morphologically different than from RAS farm 1 seen on day 1 histological sections. This may be sectional variation from the histological cuts, or more than likely different cells.

To summarise the cytology and histology results, the larger spherical cells appear to become differentiated potentially containing proteins, carbohydrates & lipids and appear to be nucleated and may contain moving micron sized elements as they develop. The smaller cells may also contain moving micron sized particles, containing carbohydrates and possibly proteins. They may be two separate cell types of host origin or non host origin and if non host, potentially part of a lifecycle of an unidentified organism or two separate organisms. The non spherical largest cells appear related to the larger differentiated spherical cells and potentially the smaller spherical cells.

4.4.3. Further Characterisation

Further investigation is required to characterise these cells, particularly those that are nucleated on the gill and similarly appear in the kidney. A biopsy of gill and kidney tissue of a fish can be gently flushed with distilled water and the nucleated spherical cells are identified, using a water immersion phase contrast lens, without a coverslip and extracted using mechanically manipulated micropipettes at 1000 × magnification for DNA typing. Furthermore the larger gelatinous cells could similarly be extracted and if they rupture, DNA material could be collected comparing the samples.
The cells if proven to be of non fish origin could be grown in nutrient rich fish broth from extracted intact nucleated cells and growing the cells at fish chemical water quality parameters in 10 g/L brackish water at 28°C. Gelatinising and sectioning the broth, could begin the lifecycle identification process of these fragile and possibly non fragile cells. Additionally the neutrally buoyant spherical cells could also be extracted by air lift from fish gills, to a fine micron screen for broth culture for possible identification and determine if they can further develop and if so, are they part of the larger cells lifecycle? Conversely if these cells are of fish origin, what are they and what is their role in fish biology?

Determining pathogenicity or if they play a role in aiding pathogens, is performed on fish that are free of the characterised cells. Water quality can then be deteriorated with a controlled study, by altering various parameters including temperature, salinity, hardness and organic loadings. Irrespective of primary disease results, the introduction of a known bacterial pathogen such as *S. iniae* or various opportunistic bacteria could be included in a pathogenicity study, determining if these cells aid known pathogens and/or increase microbial loadings by their apparent adherent nature.

If these cells prove to be an organism, geographical distribution and what fish and non fish species harbour the organism should be determined. Furthermore what circumstances if at all they affect our wild marine and freshwater ecosystems should be studied, particularly if aquaculture contributes to its growth and potential dissemination. Management and quarantine strategies maybe required if this shows to be an organism in Australia only.
I postulate the micron sized spore like elements are sequestered from larger spherical and non-spherical differentiated cells to a common area near the surface of the cell, which are budded off with and without other cellular material. The water column and currents then possibly move away the budded cell from the gill, which ruptures disseminating the spore like elements. The spore like elements possibly form the small spherical cells with little or no cellular material, which are neutrally buoyant become sticky in nature as they begin to develop and attaching to the gill and potentially other surfaces of many aquatic species. The urinary and gastrointestinal systems of aquatic animals, could potentially also void the spherical cells or spores, having found similar cells in kidney wet preparations and have additionally documented similar differentiated cells in the gut of barramundi fingerlings at Broome TAFE. Also these organisms have been found in eels direct from Tasmanian rivers, Victorian Murray cod, Western Australian Black Bream and an oyster gill from Darwin harbour, all documented with photos or video evidence, with the eel having the least number possibly due to large natural mucus loadings. If they are an organism potentially of fungal origin they are ubiquitous with differing species at the very least.

My theory is they are natural and may only cause a direct problem in high numbers if they do feed by enzymes on living organic tissue. Their sticky gelatinous nature when mature, potentially amplifies other microbial loadings, such as bacteria and parasites, possibly feeding on these unidentified cells and the aquatic species being cultured. In culture they increased in number on diseased fish and in deteriorating water quality and preferred brackish water, yet were found in all salinities of similar morphology in barramundi in three Australian states.
CHAPTER 5 GENERAL DISCUSSION, LIMITATIONS AND FUTURE DIRECTIONS

Population size should be considered when establishing the number to sample for surveillance, determining what pathogens are there as per the aim of this survey. Monitoring establishes any changes in prevalence or geographical distribution of known pathogens, giving a health profile for a batch of fish. The absence of standardised blood workup and specific gill pathogen tests necessitates the sacrificing of fish for pathological testing for detection and monitoring, which is costly and time consuming. Consequently budget constraints precluded high sample numbers for this survey for Barramundi gill disease.

Known gill pathogens are easily defined with cytology and histology, possibly excluding bacterial, due to the water medium and the need for bacterial culture for verification. Viruses generally affect other organs and were not considered relevant for the survey with our current knowledge in Barramundi gill diseases, excluding Lymphocystis which can be tentatively diagnosed clinically and histologically with tumour like lesions (Noga, 2000), but not carriers of the virus (Cano, Alonso, et al. 2006).

State and Federal government departments accept 4-6 fish sampled per batch in establishing the need for prescription treatments for over 100,000 fish to be treated. 6 fish from the wild per batch size would be inadequate for an accurate comparison for pathogens and disease between wild and cultured barramundi, particularly in a 3 day sampling period. This sample size could possibly not paint a true pattern for wild barramundi gill disease, for example
Myxosporidea (Noga, 2000) and Epitheliocystis (Nowak, LaPatra 2006) often having a seasonal occurrence in infectivity and severity respectively, but there was commonality in pathogens found despite this constraint. More than 200 fish are required per batch size for wild fish, for a particular habitat necessary to give a 95% confidence, if tests used to detect are 95% specific and 90% sensitive, with a disease prevalence of 10% determining a regional health profile using AUSVET \(^1\) sample size estimator. Additionally various sampling techniques to include netting at random locations in the habitat would decrease the bias of fish hooking alone healthier fish, potentially establishing a broader health profile of the wild habitat. A total of 106 fish were sampled including 30 from the MFHU study and 12 from the wild.

An intensive farm having a confined population of 20,000 to 100,000 per batch looking for pathogens excluding bacteria and viruses, will have a high sensitivity and specificity using cytology and histology as the predominant diagnostic tools. If we assume them to be collectively 0.9 sensitive in determining a pathogen and 0.99 confident in identifying known fish pathogens and there is a 10% prevalence in culture; which is the expected mortality rates per batch of fish for most grow out facilities, there is a 1% likelihood of false negatives in a tested individual occurring using AUSVET Epi Tool probability of infection program. However the aim of this survey was to document what pathogens are infecting the gill and what disease issues results from the pathogen or pathogens in various forms of aquaculture of barramundi. Consequently sampling 6 fish assuming 10% of the batch will be positive, gives

\(^{1}\)www.ausvet.com.au
a likelihood of greater than 90% using AUSVET probability of infection in a tested individual program, that the pathogen if there will be found.

General Veterinary skills and disciplines are a foundation for fish disease investigation as was outlined in chapter 4, particularly of the gill. A systematic approach used in terrestrial herd health investigations apply, however the environment is as important in an aquatic disease investigation as the animal. Water and water organisms increase and accelerate pathogen dissemination within an aquaculture facility and when aquaculture water is part of a wild ecosystem, then surveillance and monitoring of the larger flora and fauna of that ecosystem must be performed for sustainability. In a recent study of Mediterranean Sea cages at water depths between 16-28m culturing Sea bream and Sea Bass farms, both had impacts from nutrient waste on sea grass destruction extending 100m beyond penned fish. Sediment phosphorous loadings and benthic species monitoring is necessary to manage and predict aquaculture farm impacts on natural ecosystems, but protocols have yet to be developed worldwide (Diaz-Almela et al. 2008). Filter feeders don’t appear to have the same impact with feed sources derived from the open ecosystem in farms today (Diaz-Almela et al. 2008), but finfish culture impacts our open ecosystems and potentially making it unsustainable with some current practices.

Wild Barramundi prefer open water systems, including freshwater river systems and ocean with it anecdotally said; their survival in billabongs demonstrates their hardiness for intensive land culture. Wild dark skinned fingerlings use the river systems to hide and catch prey until sexual maturity and many including mature fish, maybe caught in billabongs at the end of the wet season, dieing from deteriorating water quality. The barramundi prefer to hide and strike,
accelerating rapidly catching its prey of fish and crustaceans according to the fish base organisation\(^2\) and Barramundi anglers.

Various culture methods shown during the survey have been established for over 20 years for various finfish species and improvements, such as container design and husbandry equipment and practices (Tucker, 2001). Even though disease issues have been cited in the literature since the 1980’s, many land fish farm designs are engineering based, with price driven guidelines for small to medium facilities in Australia, not biologically based for the farmed species, despite an increase in knowledge in all areas of aquaculture. Higher valued carnivorous species, such as barramundi are often grown in systems designed for maximum efficiency of land and infrastructure, despite designs available today that maybe better suited for the species in clearing of fish wastes and improving swimming behaviour with good water circulation.

Barramundi appear to be hardy, but studies in their biology for Australian culture was based on the lifecycle being closed with hormonal induced ovulation, very high fecundity and has been farmed with intensity in Asia in brackish to seawater salt concentrations. However freshwater culture particularly in RAS and lake culture appears to impact on the quality of the flesh. All of the freshwater tanked fish had a muddy flavour, including fish from Lake Argyle, yet fish farmed in low stocking freshwater ponds purged in 6 g/L brackish water or higher in salt concentrations, tasted similar to wild fish caught in the Mary river system or the ocean, which are silver in colour and not dark skinned, indicating a non freshwater habitat.

\(^2\) [www.fishbase.org](http://www.fishbase.org)
Taste appears to be related to fat levels (Percival, Drabsch & Glencross, 2008) and high stocking rates with high fat diets promoting fatty fish. All fish surveyed excluding wild caught had fatty livers histologically fed a modified salmon diet, probably too high in fats for Barramundi.

Historically and today in Australia, intensive aquaculture facilities using the square type containers such as RAS farms in South Australia, Lake Argyle and the sea cage farm design used at the time of the survey, can produce poor water quality. These designs were based on largest containers, cages or pens per hectare, growing high value fish in seawater and lower valued omnivorous fish species in freshwater from Asia and Europe, which for the latter can apparently achieve high yields due to their hardier biology (Schmittou, H. 1991). The designs in part forced the closure of the caged farms, due primarily to disease and financial problems at Lake Argyle and Bathurst Island, with a cyclone speeding up the announced closure of the latter.

Inadequate circulation, poor oxygen concentrations in areas of the containers, increased suspended solids and organic loadings promote microbial growth using the square interlocking design. The design probably increases fish stress from lack of fast swimming, bacterial & parasitic numbers and consequently mortality and morbidity rates.

Finfish sea cage culture in recent years have been established in open and deeper water, but the Marine Harvest Bathurst Island pilot facility was positioned near the mouth of a tidal seawater and freshwater river system, giving the farm some protection from expected cyclones and tropical storms. The bacteria causing mortalities were opportunistic in nature,
such as *Vibrio* species which were verified by an independent laboratory upon viewing their reports. This could be attributed to high stocking rates, poor water flow rates through the fingerling pens particularly increased by shark, fish and fingerling netting, increasing the organic loadings, which is further increased in shallow water at low tide with polluted sediment from culture. However the benefits of square module pens are the use of boardwalks reducing the reliance on machinery and reducing labour costs.

At the time of the survey polar cages were being constructed replacing this recognised design flaw, allowing the natural schooling or swimming behaviour of barramundi, improving water circulation particularly over the gills and if required air blowers could be used increasing centrifugal dispersal of wastes and increase oxygen levels, with no corners impeding circulation as for the previous design. The Polar pens design are stable as an individual unit, designed for open sea culture of Atlantic Salmon in Tasmania, affording greater distances between pens and therefore improved dispersal of waste streams from a farm, potentially reducing environmental impact and pathogen transmissibility than the previous design.

Pond farming in tropical Australia has realised good economic returns according to the owner of the pond farm, who intended doubling output of large 3kg Barramundi for the fillet market.

The pond farm utilised paddle wheel and blower aeration systems which appeared to work well at low stocking rates, but if stocking rates were to be increased, water exchange rates, aeration and possibly biofiltration systems need to be increased or added, removing additional fish waste products. The water circulation created by paddle wheels in opposite
corners of each pond, does not always improve circulation and oxygen levels at the bottom of
the pond, depending on pond depth. The use of air aspirators on the bottom in conjunction
with paddlewheels, produces higher levels and improved distribution of dissolved oxygen,
while increases the natural conversion of ammonia to nitrate by nitrogenous bacteria from
improved circulation within the entire pond (Samocha, et al. 2005). Additional biofiltration
could include the circulation of water through primary screens, removing uneaten food and
faeces through a central drain in the middle of a concave pond, with appropriate water
circulation and pumps. The addition of biofiltration artificial media increases the number of
nitrogenous bacteria, allowing higher ammonia levels with increased stocking rates, to be
converted to the less toxic nitrate end product of the bio-filtration process. This could
increase production without the use of more land and using existing infrastructure, without
compromising fish health.

Pond depths greater than 1m rarely can have an oxygen stratification problem, with carbon
dioxide increasing and oxygen levels decreasing during the day on the bottom, while oxygen
levels at the surface to a depth of 500 mm increase from phytoplankton oxygen production.
Then at night the surface water cools and phytoplankton consume oxygen, the gas layers can
invert, often killing fish due to the low oxygen and high carbon dioxide levels\(^3\). This was not
a problem for the pond farm with high clay based suspended solids, reducing phytoplankton
growth, but hindered stock observation and possibly increasing telangiectasis at a prevalence
of 88% (n=5), but not enough adequate gill histological sections were viewed, therefore the
true prevalence and number per fish could differ. The pond farm according to other sources

\(^3\) Deakin training Unit Modules, 2002
had suffered with bacterial disease earlier that year, but as to its nature, Veterinarian confidentiality prevented full disclosure. However telangiectasis trauma to the gill caused from the fish accelerating through the water while feeding, may give bacteria an easy portal of entry, potentially causing disease issues particularly bacterial gill disease and should be addressed if stocking rates were increased.

Stratification had been a problem at Lake Argyle and they measured oxygen on high risk days at the bottom of the cages. Particularly hot still days increasing stratification risks and oxygen was appropriately on hand, for the highly stocked fingerling cages being most susceptible due to age, stocking rates and waste production from high feed intake per kilogram of fish.

Oxygen levels should be in excess of 5 mg/L for production of most tropical finfish species (Morris and Selock, 1994), but oxygen levels when measured in RAS 1 & 2 designed farms post feeding, were 2 mg/L in areas of the tank corners and never exceeded 5.5 mg/L unless oxygen supplementation occurred as in RAS farm 1, which raised levels to 8 mg/L pre-feeding and dropped to 5 mg/L post feeding. RAS farm 2 exchanged larger volumes of water as the ground source water temperature was 28°C at 0 salinity and increased aeration during feeding, achieving 5 mg/L, but I was unable to measure for low oxygen levels, relying on farm data for RAS farm 2. RAS farm 3 never dropped below 5 mg/L using round conical floored tanks and an oxygen concentrator system, improving circulation and oxygenation with the aid of rotating spray bars. The improved circulation removed uneaten food and faeces before break-up and were further removed by passive mechanical filtration, reducing organic loadings within the system, therefore the water was only tea colour from the tannins released from pelleted feed, but remaining clear enough to visually inspect the fish within the
container. I noted in Victoria in 2004 with a new incomplete RAS facility not appearing in the survey, that the freshwater barramundi held with high ambient light levels and clear water, cowered near standing pipes as a group and were silver in colour not dark skinned, possibly indicating stress. Barramundi are not usually found in clear water habitats, except if they can freely swim such as the marine environment. This has not been noted in the literature, but farmers anecdotally report barramundi prefer low light and dark water, measured by feeding and growth rates and this is supported in part that cannibalism is reduced in low light levels with hides (Qin, Mittiga and I’Olengh, 2004).

RAS farms 1 & 2 have positive economic returns with lay skilled operators stocking at maximum for the design at 40-50kg/M^3, however high treatment levels and water exchanges are required to keep pathogens controlled and this was not a continual issue in RAS farm 3. Chemical treatments were effective, particularly for bacterial diseases in fingerling to 150 mm sized fish, which is the higher risk period of production; however treatments were not always legal, such as the use of methylene blue at one farm for food fish, which is only permissible in ornamental fish. One RAS farmer had directly added formalin for disease reduction to levels of 200ppm, the recommended dosing for external parasites and bacterial diseases to his main tanks. However the formalin levels were not measurable in 24 hours by the use of a handheld meter, probably due to formalin absorption in to the high suspended and non suspended organics and potentially hindering the biofilter, by killing bacteria indiscriminately similarly to methylene blue. RAS farms 1 & 2 produced more fish per kilogram per year, versus total running costs and start up costs than RAS farm 3, but disease issues were greater. RAS farm design 1 & 2 were the first design introduced into Australia in the late 1980’s and those entering the industry at the time were on tight budgets and the
design was not proven to successfully culture barramundi, but proved costly for many in
disease control, as fallow was not afforded with cost constraints and design. It is this under
capitalization that often precludes the use of veterinary surveillance, monitoring and advice
regularly and possibly the growing of another species may improve profitability in these
systems. The typical RAS farmer had no formal training as the results stated and a definite
lack of aquarium knowledge, fish biology and intensification, forcing many to close or be
sold to a more educated second generation of investors, with formally trained aquaculturists
beginning to be employed on RAS farm 1. Despite this improvement, due to the high running
costs of RAS farms and the poor farm gate prices of freshwater grown barramundi at the
time, no fallow is afforded and the water becomes a microbial soup as seen in the RAS farm
2. Ultraviolet sterilisation used in RAS farms with high suspended solids is rendered
ineffective in reducing microbial counts by the lack of penetration, but are effective in clear
water, highlights a lack of conceptual principles of intensive aquaculture. A lack of intensive
animal husbandry knowledge of the millennia biological based principle of fallow by the
original RAS farms 1 and 2 designer, builder and initial technical support person, who was an
engineer, is evident. Fallow is still used today controlling many terrestrial transmissible
diseases, particularly those involving microbes and parasites. As aquaculture intensification
increases for many aquatic species worldwide, veterinary scientists and other science
disciplines must be involved not only in the design process of aquaculture systems,
procedures and husbandry practices, but also the effectiveness of new innovations occurring
with this rapid technological expansion.

RAS farms are used for other species around the world, particularly good tasting Tilapia an
omnivorous African fish, with Nile Tilapia the most common in all forms of aquaculture
second to carp species. They are able to tolerate high stocking rates and appear a better
species type for RAS culture (Deakin workshop, 2004), with substantial stocking rates possible up to 80-100kg/m³ of water in well designed and managed systems. This is achieved at Northern State Carolina University, using a fallow system by dry out and/or chemical disinfection between batches and UV/Ozone disinfection during the batch grow out, often incorporating foam fractionating and protein skimmers as does the Darwin Hatchery, excluding ozone for their brood-stock culture.

*Epitheliocystis* had the highest prevalence, probably due to poor water quality and high stocking rates for this predatory species. Potentially a pathogen like *Epitheliocystis* could be used as an indicator of the effectiveness of a RAS system design in disease management or any other intensive fish farming method, which can be quantified by histology for barramundi.

The lack of fast swimming in the containers with high stocking rates and poor design, prevent the natural flushing of the gills, potentially allowing a build up of microbes that would normally be kept at lower numbers in the wild. This could explain the mucus build up and hyperplasia on gills found in all RAS culture during the survey, including the MFHU experiment.

Additionally the common finding of water with low flow rates, high stocking rates, high suspended organics and/or suspended solids and gill pathology was strongly evident with this study.
This lack of veterinary surveillance and monitoring at non commercial and commercial sized farms is a major constraint in South Australian RAS aquaculture when compared to other facilities, excluding Lake Argyle. Commercial returns for RAS is deemed 100 tonne of fish produced per year, affording a return for investment as per the Inland Aquaculture Association of South Australia, established from their members farming Barramundi. Only RAS farm 2 at the time was making profit beyond owner’s wages, producing in excess of 100 tonne per year, but the fish appeared pushed to their biological limit with all kidney Gram stains showing systemic disease in fish surveyed.

A veterinary health check was only performed at the government aquaculture laboratory, when more than 10% of stock mortalities occur as a provision for their South Australian aquaculture licence. Fish sent were often not selected randomly, being moribund or dead and until the *S. iniae* diagnosis in 2004, most reports were attributed to poor water quality and husbandry issues. This highlights the lack of formal training in intensive RAS husbandry and barramundi biology knowledge by state authorities, as there are few veterinarians with aquaculture knowledge employed or used regularly by regulatory authorities in South Australia. Currently at the time of writing of this thesis, two interstate Aquatic Veterinarians visit the largest RAS farms in Adelaide periodically for *S. iniae* management.

Veterinarians are well placed upon graduation with their general knowledge in the disciplines of epidemiology, population medicine, general pathology, microbiology, parasitology, pharmacology and our training in working within budget constraints, to consider further training in aquaculture. It was general veterinary ability with the light microscope that identified kidney crystals using cytology.
The kidney crystals were found in all Barramundi, but the highest numbers appear to be in seawater culture, potentially indicating that this euryhaline species osmoregulates not only through the gill, but the kidney also. These crystals are either calcium oxalate or magnesium ammonium phosphate (struvite), as determined by Philip Clark a clinical pathologist at Murdoch University (2004), with their chisel crystalline shape. The crystals were only found by the use of cytological wet preparations and would normally be excreted and lost in the histological processing. This highlights the need for research not only gill physiology, but general physiology of this popular eating fish with input from veterinarians.

There appeared a commonality of wild stock diseases and aquaculture of barramundi in the same region. A published study for potentially new and existing pathogens should be conducted through at least a calendar year periodically, for wild stock barramundi and the findings used for surveillance and monitoring programs at and near aquaculture facilities that expose fish wastes to the natural ecosystem. This could inform and alert regulatory authorities accountable for our wild stocks and ecosystems, to potential disease or pathogen increases in aquaculture areas. This should be mandatory for governments worldwide for non land based aquaculture, minimising potential ecological problems and not left in the hands of aquaculture companies, avoiding a conflict of interest.

The finding of Henneguya in another ecosystem at the Marine harvest facility and in the Mary river system separated by at least 60 km of ocean highlights that many pathogens are endemic potentially in all of tropical Australia. Henneguya did not appear to be of a concern clinically, but in time without further research in to barramundi and other species living in the
said ecosystems, open water aquaculture could increase burdens, proving costly for all concerned by *Henneguya*.

However the additional finding of *Epitheliocystis* and Dactylogyrid flukes in wild stocks and in aquaculture grow out facilities, potentially increases the exposure for disease with multifactorial causes not only for gill disease, but other organ systems by other pathogens including viruses to our wild stocks. This increases the need for surveillance and monitoring with a systematic systemic approach to aquatic animal health, however costly, but making good research for post graduate degrees in science, possibly reducing costs and should be prioritized by governments and regional boards, rather than industry driven research. Barramundi from hatcheries appears to transmit *Epitheliocystis*, despite safe guards such as disinfection of eggs to closed ecosystem RAS farms and potentially increasing barramundi wild stock burdens from local fish swimming near cage culture, or effluent flowing into their ecosystem from aquaculture facilities.

If saltwater aquaculture can be performed on land, such as the pond farm and all waste water treated and monitored before returned to the wild ecosystem, this could reduce wild stock disease threats and help preserve wild ecosystems. Otherwise open deep ocean culture appears to be the most likely the potential trend as these technologies increase. However remotely caged fish in deep ocean water will have considerable cost and practical difficulties, including fish environmental factors without direct human monitoring in remote offshore locations (Celikkol, Langan 2007) and be limited to a few species and a few companies.
Chapter 5. Future Directions

The thesis has highlighted the need for a thorough pathological workup for a batch of fish in sufficient numbers to monitor existing pathogens, but also in open culture in sufficient numbers for the surveillance of potentially new pathogens. To do this with definite outcomes, a veterinarian must know their limitations and have a sound knowledge of what are normal anatomy, physiology and histology of the species or type of fish they are dealing with. This thesis would have been completed earlier if the student had a clearer picture of normal cells and tissues in a variety of diagnostic disciplines, but text books and literature on barramundi were lacking.

The cytology identification of the gill epithelial cells was identified by ruling out pathogens and studying the video showing no movement of these cells externally or internally, once water currents had subsided. The epithelial cells probably washed off gills, that could have showed pathology histologically such as hyperplasia, but these photos pre-dated the official survey. However no epithelial cells were generally found cytologically with healthy gills of fingerlings verified histologically, unless rough handling of the fragile gill specimen occurred.

It was tempting to label unknown cells as non host, but as the survey results were reviewed, it became apparent with the use of TEM, SEM, cytology and histology that the various host cells could be identified repeatedly. The use of high magnification demonstrating ECG granules vibrating initially brought confusion, until TEM and histology results were correlated with all information, including video evidence.

It was unfortunate the time at Murdoch University was heavily devoted during the 2 week trial, describing the non host cells found on Barramundi gills. Consequently more cytological
photos were not taken of gills suffering from disease, such as the ammonia toxicity and associated gill biopsies then correlated with histological findings. This should be done, possibly giving a shorter time line for treatment or correction of husbandry issues, if epithelial cells are found in gill washes from gill biopsies, potentially indicating a diseased fragile gill requiring further investigation and treatment.

The lack of bacterial cultures hindered identification of bacterial gill colonies and their potential relationship in systemic infections in this thesis, highlighted by the multifactorial nature of disease for many conditions, such as tail bites from cannibalistic behaviour. The pond farm and sea cage culture, only allowed few fish sampled and limited time respectively and the methods of culture exposed to the open ecosystem, may have delivered more information of gill pathogens, gill trauma and consequently gill pathology, potentially affecting wild stocks if more time and access was available.

The finding of the unidentified cells particularly with high organic loadings within gill tissue has posed more questions than answered. But if they are indeed a microbial organism, why have they gone unnoticed in the literature and by scientists involved in aquatic animal health and biology? It was the examination of live fish, particularly using the rudimentary veterinary skill of cytology and post mortem, with the necessary field equipment that led to the investigation of these cells. But a university laboratory, skilled research professionals for histological, SEM, TEM and specialised cytological processing, familiar with aquatic samples, including Brian Jones of Western Australian Fisheries, aided a field veterinarian to illuminate at a rudimentary level these cells.
Chapter 5. Future Directions

The questions posed in chapter 4 of the unidentified cells need to be answered, given they were found in the wild and with similar looking cells found in other finfish species from different ecosystems within Australia and as intensity of culture increased so did these cells.

The lack of knowledge in Barramundi biology including gill physiology and health in various salinities and culture methods, highlighted by these unusual cells and kidney crystals, makes one realise that there is much research required for sustainability and profitability for barramundi culture. Additionally should freshwater culture continue, given not only disease of the gill appears to intensify, but the quality of the fish flesh is inferior to brackish or seawater culture in non RAS systems? However RAS for the biosecurity of brood-stock at very low stocking densities in seawater appears to be very successful with suitably well trained staff, veterinary support and less economic constraints than a commercial venture alone.

The research of teleost aquaculture appears to be nutritionally biased and not biological, with much of the biology of any species deficient in many areas, as highlighted during the literature search. If the lifecycle can be closed and an artificial diet established, the next step in aquaculture and our knowledge of a fish species, should be fish biology with a system based approach including its response to disease, which has historically worked well for terrestrial livestock. But it may be prudent to further research the biology of current species farmed successfully around the world, particularly those that are suitable for the rigors of high stocking densities and affording easier fish meal replacement, such as the omnivorous species farmed in Europe and Asia. Co-currently a sound knowledge of microbial water quality in all forms of aquaculture and improved water re-cycling technology within RAS and
pond facilities, is essential for ecosystem biosecurity, water conservation and sustainability on land.

Additionally the science community must concentrate on disease agents, a major impediment to aquaculture. But often disease issues as highlighted in this discussion, maybe understanding the limitations of a species being cultured, with a particular culture method and establishing the best method of culture, for that species as our knowledge base builds. Suitably trained Veterinarians should have inputs in species to be farmed, conceptual facility designs and management of aquaculture systems, allowing us to push past the need to further deplete and reduce disease risks to our wild fish stocks and ecosystems. Aquaculture the most efficient producer of protein for human consumption and multiple benefits to human health, can be performed without the need for ecological disasters, if farmed on land or with greater knowledge minimising the risk to open water ecosystems. Indeed aquaculture will become our main source of protein worldwide in years to come, as it continues to grow steadily, with the lowest food conversion ratio and less disease risks to humans, than chickens and other terrestrials currently farmed intensively.
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Appendices

Appendix 1. FIGURES OF OTHER BARRAMUNDI PATHOGENS FOUND

Appendix 1.1 Lymphocystis lesions from SA RAS farm 1 2003
Appendix 1.2 Argulus from tail easily seen by naked eye Lake Agyle
Appendix 1.3 Ventral surface of Argulus identifying suckers* & feeding tube
Appendix 1.4 Black Bream gill wash from Murdoch Fish Health Unit

Appendix 1.5 Murray cod gill wash from Deakin University
Appendix 2. Cytological Staining techniques

4.4.2. *Periodic Acid Ammoniacal Silver – Microwave Technique [PAAS]*

Solutions used:

- 1% periodic acid.
- 5% Sodium thiosulphate.
- 0.2% gold chloride.
- 10% Silver Nitrate in distilled water.
- Light Green solution containing 0.2g light green SF, 0.2 mL Glacial Acetic acid and 500 mL of distilled water.
- Saturated Picric acid.
- Ammonia hydroxide.
- 5 mL of working silver solution of 10% silver nitrate. (Add ammonia hydroxide drop by drop until solution is clear. Make to 50 mL with distilled water.

Method:

1. Heat fix slide.
2. Treat with periodic acid for 20 mins.
3. Wash in distilled water.
4. Microwave 45 mL dilute Ammoniacal silver Nitrate solution for 30 sec. without slides.
5. Place slides in heated solution, and microwave for 30 more secs.
6. Tone in 0.2% Gold Chloride for 30 secs.
7. Rinse in distilled water.
8. Fix in 5% Sodium Thiosulphate for 30 secs.
9. Wash thoroughly in water.
10. Counterstain briefly with equal volumes of light green solution and saturated picric acid.
11. Dehydrate in ethanol, clear and mount under D.P.X [synthetic resin]

Results:

Fungi sharply outlined in black with green background. Red blood cells stain yellow.
4.4.3. *Modified Wright’s Stain [Diff Quik] Automated.*

Fixative [Triarylmethane dye in methyl alcohol].

Solution 1 [Xanthene die in methyl alcohol]

Solution 2 [Azure A & Methylene blue]

Results: cells blue through to red.
Appendix 3. Histological staining techniques

4.4.4. *Haematoxylin & Eosin*

Paraffin embedded sections cut at 4-6.0 µm were de-waxed in Xylene using 2 changes each of 5-10 minutes duration, rinsed in absolute alcohol for 2-5 minutes and then passed through descending grades [e.g. 90%, 70%, 50%] of alcohol avoiding section removal by diffusion currents. The sections were then rinsed in water. Stained with Harris' Haematoxylin for 5 minutes. Washed in running tap water for 2 minutes and then treated with bluing solution. Cellular differentiation was checked microscopically before being washed in running tap water for 2-3 minutes. It was then transferred to 95% ethanol, stained with 0.5% alcoholic eosin for 30 seconds. Sections were differentiated in 95% ethanol twice for 15 seconds each time and dehydrated in 100% ethanol twice for 15 seconds each time and cleared in Xylene. Stained sections were then coverslipped using synthetic resin [DPX].

4.4.5. *Periodic Acid Ammoniacal Silver (Microwave Technique)*

Solutions used were as for cytology preparation (above).

**Method:**

- Take paraffin sections to water.
- Treat with periodic acid for 20 mins.
- Wash in distilled water.
- Tone in 0.2% Gold Chloride for 30 seconds.
- Rinse in distilled water.
- Fix in 5% Sodium Thiosulphate for 30 seconds.
- Wash thoroughly in water.
- Counterstain briefly with equal volumes of light green solution and saturated picric acid.
- Dehydrate in ethanol, clear and mount under D.P.X.

**Results:** Fungi stained sharply outlined in black against a green background and erythrocytes yellow.
4.4.6. *Gram stain*

**Method:**
- De-wax sections, rinse in alcohol, rinse in water.
- Stain with Lillie's crystal violet - 1 minute. (Filter stain onto slide.)
- Wash off with tap water.
- Treat with Gram's iodine - 2 minutes.
- Wash in tap water.
- Differentiate in acetone 1-2 seconds.
- Wash in tap water and check the staining using a microscope. The background should not be purple.
- Rinse in distilled water.
- Counterstain with 1% neutral red for 3-5 minutes. (Filter the stain onto the slide.)
- Wash in distilled water.
- Dehydrate, clear and mount. Prolong the alcohol step until the red stops coming out and mount in D.P.X.

**Results:** Gram +ve organisms - purple. Gram -ve organisms - red. Nuclei - red

4.4.7. *Modified Trichrome for Microsporidia in Paraffin Sections*

**Reagents:**
- Modified Trichrome 6.0 g of chromotrope 2R, 0.15g of fast green, and 0.7g are mixed. These are added to 3 mL of glacial acetic acid and allowed to stand for 30 minutes, then dissolved in 100 mL of distilled water.
- Acid Alcohol 0.45 mL of acetic acid is made up in 100 mL of 90% ethanol.

**Method:**
- Remove from paraffin and rehydrate sections.
- Rinse with distilled water stain in Trichrome for 90 minutes in coplin jar.
- Rinse in acid alcohol for 10 seconds.
- Rinse briefly in 95% ethanol.
- Dehydrate in 95% ethanol for 5 minutes.
- 100% alcohol for 10 minutes.
- Take through xylene and mount in DPX.

**Results:** Microsporidia stain pinkish-red, bacteria stain faint green.

Reference: NEJM Volume 326:161-166
4.4.8. *Giemsa stain*

**Method:**

- De-wax and bring sections to tap water.
- Stain with Giemsa solution diluted 1:25 with distilled water for 24 hours.
- Rinse rapidly with distilled water.
- Place in 0.5% Acetic acid until the section is pink.
- Rinse in distilled water and then blot sections with filter paper.
- Dehydrate rapidly in absolute alcohol.
- Clean in xylene.
- Mount in a synthetic resin medium.

The stain is used for the demonstration of metacercarial cysts.
Appendix 4. Questionnaire for Farmers

1. Farm name, address and date.
2. Species, number farmed and stocking density [kg/M³].
3. System design sketch and materials used [including quarantine, disinfection systems and protocols in writing], how long has it been operating, do you fallow by dry out and when was it last performed?
4. Water quality parameters for each biofilter [if not known or not used leave blank and how often are they measured?]
   a. Source water including levels of:
      i. Iron, Manganese, Zinc (bore water)
      ii. Yearly salinity levels (seasonal if non bore water)
      iii. Oxygen
      iv. Carbon dioxide and Nitrogen (bore water)
      v. Turbidity
      vi. Hardness
      vii. Alkalinity
      viii. Pollutants
   b. Pre-water treatments (e.g. filtration, aeration and settling containers)
   c. Salinity
   d. Hardness
   e. Alkalinity
   f. Temperature
   g. pH
   h. Oxygen prior to feed, 30-60mins and 2 hours post feed, measured at 100mm below surface, middle of water column & bottom and repeated in 2 other areas of the container.
   i. Water appearance [colour, cloudy and anything unusual]
   j. Nitrogen products [ammonia, nitrite, nitrates, or total nitrogen in mg/L.]
   k. Water exchange rate and frequency such as daily or weekly.
   l. Suspended solids measured by a secchi disc for non tank culture and quantitative value for tank culture (Australian Water Quality Centre).
   m. Store for a week a water sample in at 4°C for potential further laboratory studies if a disease outbreak
5. History of routine maintenance since problem began [including cleaning, repairs to pumps, pipe work, etc.]
6. Stock movements for up to 6 weeks prior to problem. [Including stock moved within farm and new stock imported to farm for all tanks/containers. Can use a diagram.]
7. When did the problem start and how did the problem progress [e.g. off feed, then lethargic and began dying]?
8. Morbidity daily rate and date started and ended.
9. Mortality daily rate, date started, date ended and total numbers dead from the beginning of the problem. (State regulations will require notification over a certain percentage.)
10. Feeding rates, appetite of fish and food conversion ratio prior and since problem began. Feed used including size and sinking rate.
11. Physical examination by farmer:
    a. The position of healthy and sick fish in tank.
    b. Behaviour of sick fish [for e.g. piping for air, flared gills, faster respiratory rate when compared with normal fish, erratic swimming, or none before death.]
    c. Colour of skin and describe any lesions. [Photograph if possible to including sides, top, bottom and gills by pulling the operculum or gill covers back of 6 affected fish.]
12. Other clinical signs or comments: