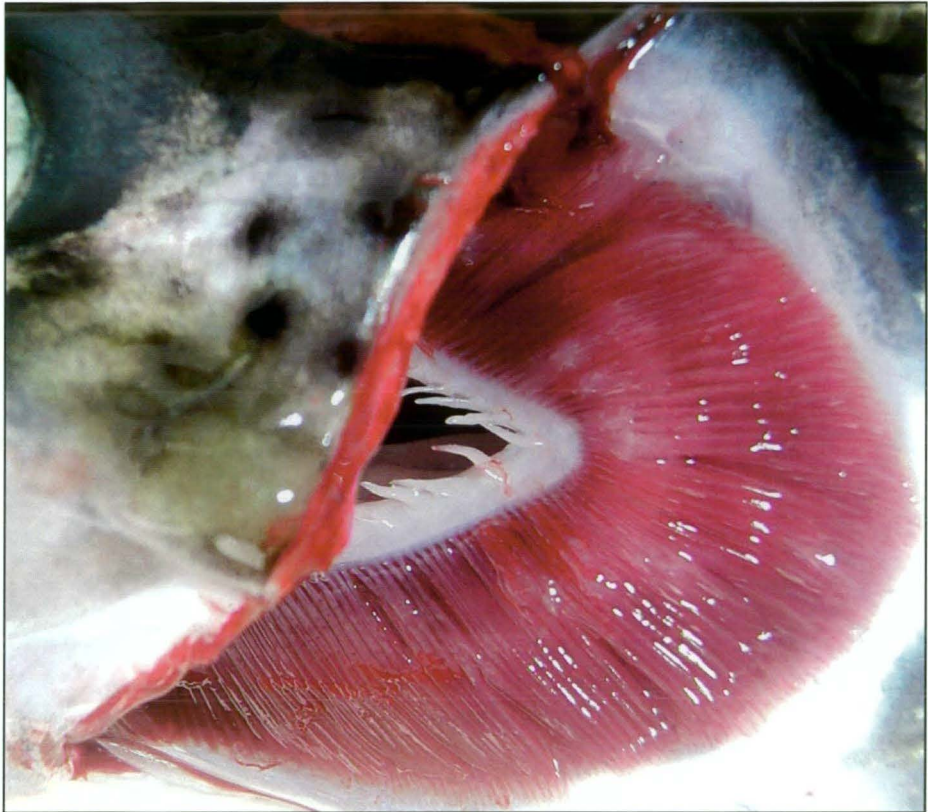


Potential risk factors of amoebic gill disease in Tasmanian Atlantic salmon.



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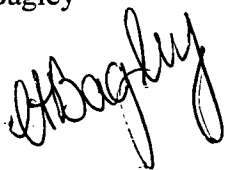
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Carley A Bagley



16.10.06

Abstract

Amoebic gill disease (AGD) is the most significant health problem affecting the production of Atlantic salmon (*Salmo salar* L.) in Tasmania, Australia. AGD affects a number of cultured fish species worldwide, however its impact is insignificant when compared to that in Tasmania. The disease was first identified in southern Tasmania in 1986 shortly after the initiation of Atlantic salmon farming. AGD is a result of an endemic parasite *Neoparamoeba* sp. attaching to the salmon's gill tissue. Research conducted over the years has resulted in a large reduction of AGD associated mortalities. However, the disease continues to place a significant financial burden on the industry with the only effective form of treatment being freshwater baths, a strategy implemented in the late 1980's.

Epidemiological studies are essential as they facilitate in identifying causal factors that may be associated with disease outbreaks. An understanding of these complex interactions is required in order to implement effective control and prevention strategies. This thesis examined a number of environmental conditions and husbandry protocols currently utilised in the Atlantic salmon industry in Tasmania.

Fallowing of lease sites as a disease management strategy for AGD was found to be unsuccessful. The mean AGD prevalence of Atlantic salmon cultured at a fallowed site ($57.5\% \pm 5.32$) was similar to fish at the control site ($52.3\% \pm 5.35$). *Neoparamoeba* sp. was isolated from environmental samples at the fallowed site despite the absence of salmon. Atlantic salmon maintained in copper based antifouling paint treated nets had a higher mean prevalence of AGD ($29.2\% \pm 6.74$) compared to salmon maintained in untreated nets ($21.3\% \pm 6.43$). The belief that copper acts as an attractant for *Neoparamoeba* sp. was not ascertained. Atlantic salmon maintained under continuous artificial lights had a similar prevalence of AGD as salmon maintained under natural light conditions for the majority of a 12 month trial. However when subjected to stressful conditions the salmon maintained under artificial lights had a significantly higher percentage of gill lesions ($43.1\% \pm 4.38$) compared to salmon held under natural light conditions ($14.11\% \pm 1.96$).

An Atlantic salmon farm located in the Tamar River in the north of Tasmania was studied as a control site, as the farm had been operating for approximately 5 years with no history of AGD. *Neoparamoeba* sp. was isolated from the benthic sediment and nylon nets, but not detected on steel nets or the salmon's gills. However approximately 12 months after the trial concluded the farm experienced its first outbreak of AGD.

The work contained in this thesis has identified a number of environmental and husbandry practices that warrant further investigation to accurately understand their influence on the occurrence of AGD in Tasmanian cultured Atlantic salmon. Future research must also concentrate on identifying and understanding the causative agent/s involved in this disease in order to develop effective treatments.

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Firstly this PhD would not have been achievable without the wonderful support and encouragement from my fantastic mum and dad, loving partner Christopher and wonderful friends Niall Stewart and Marianne Douglas.

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Chapter 1 - General Introduction



Amoebic gill disease (AGD) is the most significant health problem affecting the production of Atlantic salmon (*Salmo salar* L.) in Tasmania, Australia. The disease was first identified in Tasmania in 1986 and to this day is a significant financial burden to the industry (Munday, 1986; Munday *et al.*, 2001). AGD is believed to be a result of opportunistic *Neoparamoeba* spp. attaching to the gills of salmon (Adams and Nowak, 2004a; Dyková *et al.*, 2005). AGD mortalities have declined over the last twenty years as a result of research combined with subsequent changes in farming practices (Munday *et al.*, 1990; Douglas-Helders *et al.*, 2000). However, farms are still reliant upon the use of freshwater baths to treat the disease, a strategy first implemented in the late 1980's (Parsons *et al.*, 2001).

1.1 Tasmanian Atlantic salmon industry

Tasmania is responsible for producing only a small proportion of cultured Atlantic salmon, approximately 15 000 tonnes in 2001-02 (DPIWE, 2004). Countries such as Norway annually produce approximately 40 times more salmon than Tasmania (www.dpiwe.tas.gov.au). The majority of salmon farming in Tasmania occurs on the southeast coast at Huon Estuary, Port Esperance, Tasman Peninsula and D'Entrecasteaux Channel (Crawford, 2003). Other production areas include Macquarie Harbour on the west coast and Tamar River on the north coast (Figure 1.1). The industry provides a high level of employment in the state and has been recorded as one of the highest employers in Australian aquaculture (www.aquafincrc.com.au, DPIWE, 2004). Approximately 90% of salmon produced in Tasmania supplies the domestic market with overseas markets including Japan, Indonesia, Hong Kong and Singapore (DPIWE, 2004). The market value for 2001-02 was estimated at \$111.5 million, an eight-fold increase in production since 1989-90 (DPIWE, 2004). AGD is currently the only significant health problem the Tasmanian salmon industry is faced with. However approximately 10-20% of annual production costs are a direct result of managing this disease. At approximately \$11 million a year, it makes AGD a very costly disease to incur.

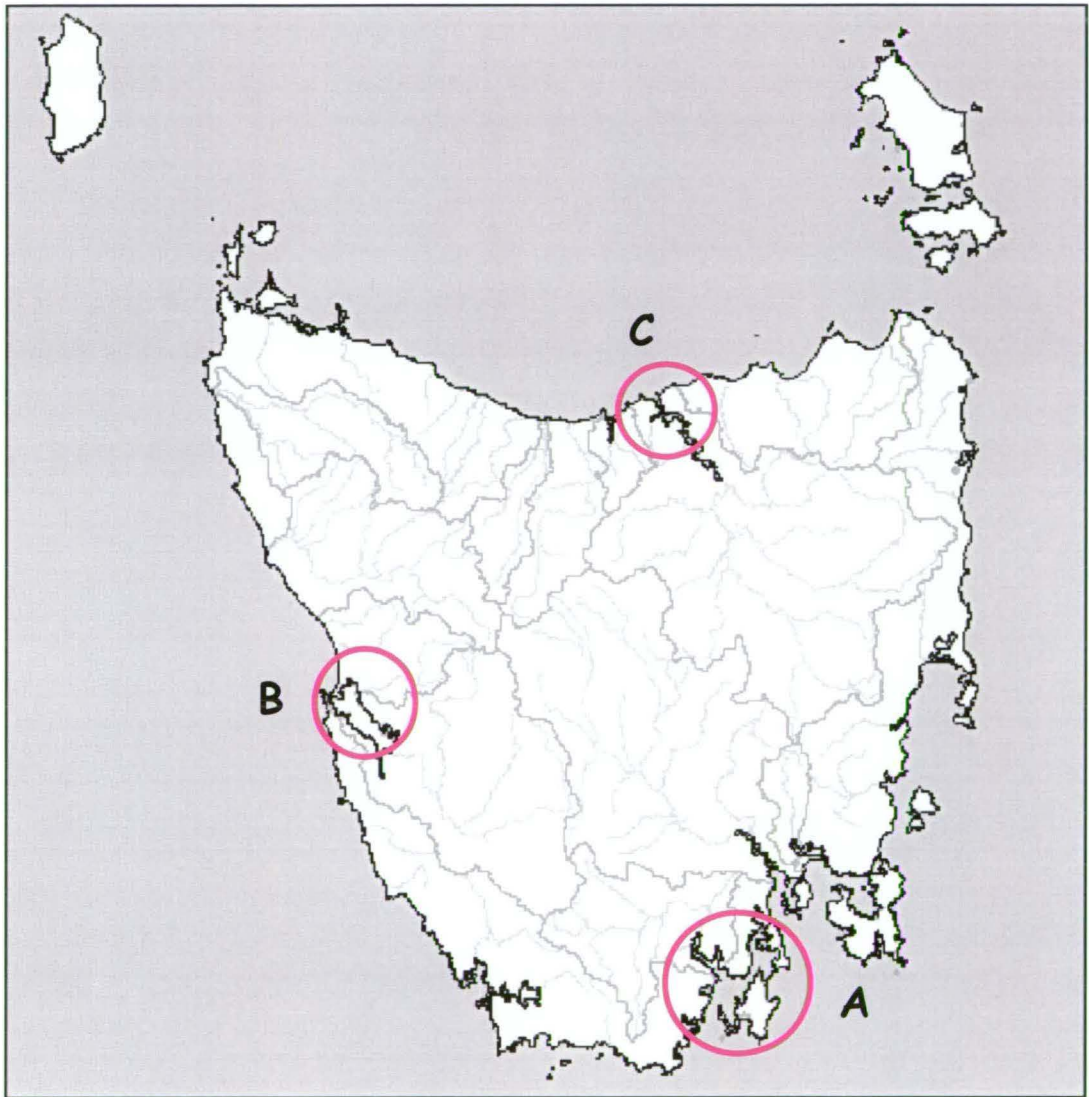


Figure 1.1: Map of Tasmania showing location of Atlantic salmon farms.
A = Huon Estuary, Port Esperance, Tasman Peninsula and D'Entrecasteaux Channel, B = Macquarie Harbour and C = Tamar River.

1.2 The causative agent

AGD was first described in Tasmanian Atlantic salmon in 1986 but the causative agent was not identified at the time (Munday *et al.*, 1986). An outbreak of AGD in cultured coho salmon *Oncorhynchus kisutch* (Walbaum), in the USA resulted in the identification of *Paramoeba pemaquidensis* as the causative agent (Kent *et al.*, 1988). Page (1987) at the same time had redescribed it as *Neoparamoeba pemaquidensis*. The genus is comprised of exclusively marine amoebae inhabiting coastal and estuarine environments in the northern and southern hemispheres (Nowak *et al.*, 2002; Wong *et al.*, 2004). *Neoparamoeba* spp. is amphizoic, capable of existing as an opportunistic pathogen and in a free-living form. Attachment to a solid surface is required for predation and asexual reproduction (Roubal and Lester, 1989; Dyková *et al.*, 2000; Wong *et al.*, 2004). *Paramoeba* can be identified by the presence of the symbiotic organism *Perkinsella amoebae*, located near the nucleus (Dyková and Novoa, 2001; Dyková *et al.*, 2003).

At present no studies have been able to fulfil Koch's postulate for AGD as the disease is unable to be reproduced in Atlantic salmon using cultured strains of *N. pemaquidensis* (Munday *et al.*, 2001; Morrison *et al.*, 2005). Current experimental infections are reliant upon cohabiting naïve salmon with 'wild' type infected salmon or using partially purified *Neoparamoeba* spp. preparations harvested from the gills of infected fish (Akhlaghi *et al.*, 1996; Zilberg *et al.*, 2001; Morrison *et al.*, 2004). A closely related species, *Neoparamoeba branchiphila*, has been isolated from the gills of AGD infected salmon, however its affiliation with the disease is unknown (Dyková *et al.*, 2005).

1.3 Geographic distribution

Neoparamoeba species associated with AGD have been isolated and identified from marine environments throughout the world (Clark and Nowak, 1999; Crosbie *et al.*, 2003; Crosbie *et al.*, 2005). AGD outbreaks have been recorded in the USA, Spain, Ireland, France, Chile and New Zealand (Kent *et al.*, 1988; Munday *et al.*, 1993; Dyková *et al.*, 1995; Rodger and McArdle, 1996; Palmer *et al.*, 1997; Findlay and Munday, 1998; Munday *et al.*, 2001; Nowak, 2001; Douglas-Helders *et al.*, 2001). Within the Tasmanian marine environment

Neoparamoeba sp. has been detected in the benthic sediment (Crosbie *et al.*, 2003), water column (Tan *et al.*, 2002; Douglas-Helders *et al.*, 2003) and on salmon nets (Tan *et al.*, 2002; Douglas-Helders *et al.*, 2003). The amoeba appears to have no preference for sediment type and has been isolated from sediments ranging from fine sand to organically rich anoxic material (Crosbie *et al.*, 2003). The presence of *Neoparamoeba* sp. at locations with no history of salmon culture suggests that it is not reliant on the fish to survive (Crosbie *et al.*, 2003).

Neoparamoeba sp. has been detected in the water column using immuno-dot blot, however to date isolation and culture has been unsuccessful (Crosbie *et al.*, 2003; Douglas-Helders *et al.*, 2003). It has also been isolated from nylon nets treated with antifouling paint, such as copper or lanolin, as well as from untreated nets (Tan *et al.*, 2002; Douglas-Helders *et al.*, 2003). A number of macro-fouling species such as the blue-lipped mussel commonly found growing on the salmon nets have also had *Neoparamoeba* sp. isolated from the shell (Tan *et al.*, 2002).

1.4 Species affected

Atlantic salmon, *Salmo salar* L., appear to be the most susceptible to AGD, however other cultured marine fish have been identified with the disease (Munday *et al.*, 2001). Salmonid species known to be affected by AGD include rainbow trout, *Oncorhynchus mykiss* (Walbaum), coho salmon, *O. kisutch* (Walbaum), and chinook salmon, *O. tshawytscha* (Walbaum) (Kent *et al.*, 1988; Munday *et al.*, 1993; Munday *et al.*, 2001). In Tasmania the production of 'ocean trout' has been significantly limited due to rainbow trout's high susceptibility to AGD (Munday *et al.*, 2001). AGD has been recorded in cultured turbot, *Scophthalmus maximus* (L.), European seabass, *Dicentrarchus labrax* (L.), and sharpsnout seabream, *Diplodus puntazzo* (Cetti) (Dyková *et al.*, 1995; Dyková and Novoa, 2001; Dyková *et al.*, 2005). On one occasion paramoeba was identified in wet preparation on the gills of immature couta, *Thyrsites atun* (Euphrasen) caught near AGD affected salmon in southern Tasmania (Foster and Percival, 1988). Field surveys have been unable to detect *Neoparamoeba* sp. on the gills of wild fish inhabiting the areas surrounding salmon cages in southern Tasmania (Dawson 1999; Munday *et al.*, 2001; Douglas-Helders *et al.*, 2002; Nowak *et al.*, 2004).

1.5 Disease characteristics

A background level of AGD can be present throughout the year in Tasmania, however outbreaks pose the greatest problem for the industry (Clark and Nowak, 1999; Adams *et al.*, 2004). Macroscopically, AGD in Atlantic salmon presents as white raised patches in conjunction with an excess of mucus. These patches are easily identified and used as a disease status by the farms during routine gill checks. As the disease intensifies so too do the number of patches and *Neoparamoeba* sp. with the lesions becoming the preferred site of attachment (Clark and Nowak, 1999; Zilberg and Munday, 2000). Eventually individual patches are unrecognisable as they meld together covering the gill arch.

Histologically the raised patches correlate with focal and multifocal hyperplasia and hypertrophy of the gill epithelium (Zilberg and Munday, 2000; Adams and Nowak, 2004a). In the advanced stages of the disease lamellar fusion is increasingly apparent resulting in the formation of cystic spaces or vesicles that can be seen with *Neoparamoeba* sp. and inflammatory infiltrates enclosed within (Munday *et al.*, 1990; Zilberg and Munday, 2000; Adams and Nowak, 2004a). As the disease progresses mucus production increases and hyperplastic epithelial tissue is sloughed off with associated amoeba (Zilberg and Munday, 2000; Adams and Nowak, 2004). The increased production of mucus cells on affected filaments has been suggested as a mechanism the fish use to control the infection and repair the damaged areas (Zilberg and Munday, 2000; Adams and Nowak, 2004a, Adams *et al.*, 2004).

Fish with AGD are reported as having sluggish behaviour, swimming close to the surface and increased opercular movement (Kent *et al.*, 1988; Munday *et al.*, 1990; Rodger and McArdle. 1996). A reduction in feed intake is commonly observed in AGD infected salmon, however this is a classic response of stressed and diseased fish (Funk *et al.*, 2004). Initially it was believed that mortalities in AGD affected fish were due to respiratory dysfunction (Munday *et al.*, 2001). Further research disputed this conclusion and the cause of death still remains unclear (Powell *et al.*, 2001)

1.6 Treatment

Currently the only form of treatment available to the industry is the use of oxygenated freshwater baths, a practice devised in the late 1980's (Parsons *et al.*, 2001). The fish are placed in freshwater (salinity less than 4 ppt), for a period of 2-6 hours based on the severity of the outbreak and environmental conditions (Parsons *et al.*, 2001). The salmon are then returned to seawater (Parsons *et al.*, 2001). Whilst *Neoparamoeba* sp. can be inactivated by this method of treatment a large proportion do remain viable and are capable of re-infecting (Howard and Carson, 1993; Clark *et al.*, 2000; Findlay *et al.*, 2000; Parsons *et al.*, 2001).

1.7 Risk factors

Epidemiological studies are essential to be able to formulate successful treatment regimes and management protocols. Identifying risk factors is an integral part of any study. Generally, risk factors are discussed in terms of host, parasite and environmental parameters. Previous research pertaining to host risk factors has identified a number of susceptible aquaculture species. Sexually mature Atlantic salmon appear to be more prone to the disease (Mitchell, 2001; Nowak, 2001). Wild fish inhabiting the surrounding area of salmon farms are not thought to be risk factors for AGD (Douglas-Helders *et al.*, 2002; Nowak *et al.*, 2004).

Information regarding the causative agent of AGD is limited. *Neoparamoeba* sp. has been isolated from sediment, netting and biofouling associated with netting (Tan *et al.*, 2002; Crosbie *et al.*, 2003), however their virulence and ability to infect salmon is unknown. It appears from experimental trials that the virulence of gill-attached *Neoparamoeba* sp. increases when sequentially passaged through naïve hosts (Findlay *et al.*, 2000). Douglas-Helders *et al.* (2000) found *Neoparamoeba* sp. can survive on dead Atlantic salmon and remain infective up to 14 days post mortem.

The majority of epidemiological studies conducted have investigated the potential of environmental parameters as risk factors. Temperature coupled with salinity has been identified as an extremely important risk factor (Clark and Nowak, 1999; Nowak, 2001). AGD outbreaks are consistently recorded throughout the warmer

months of December to March, which coincides with periods of low rainfall and increased salinity. A recent study challenged the importance of water temperature as a risk factor indicating that AGD occurs at temperatures below 10°C (Douglas-Helders *et al.*, 2001). Factors such as jellyfish, algal blooms and the presence of bacteria in the water column have been examined, however there is no conclusive evidence for their involvement in AGD outbreaks (Clark and Nowak, 1999; Nowak, 2001).

A number of recent studies have investigated husbandry protocols that may promote AGD. Atlantic salmon maintained within copper based antifouling paint treated nets had a higher prevalence of AGD when compared to fish held in untreated nets (Douglas-Helders *et al.*, 2003). Following lease sites for a period of 4 to 97 days initially resulted in a lower prevalence of AGD when salmon were re-stocked at these sites (Douglas-Helders *et al.*, 2004). The use of prophylactic freshwater baths and the use of 60 or 80 m cages did not have any significant impact upon the presence of AGD (Douglas-Helders *et al.*, 2004).

Aims

The aims of this thesis are outlined as follows;

1. To investigate the use of fallowing within the Huon Estuary in the southeast of Tasmania. Identify its impact on the prevalence of AGD and comment on its potential as a disease management protocol (Chapter 3).
2. To further investigate whether copper antifouling paint treated nets are a reservoir for *Neoparamoeba* sp. and a possible risk factor for AGD in Atlantic salmon (Chapter 4).
3. To investigate the potential of a lanolin product as an antifouling paint and its impact if any on the prevalence of AGD in Atlantic salmon (Chapter 4)
4. To investigate the effect of submerged lights used to inhibit sexual maturation in Atlantic salmon on AGD prevalence (Chapter 5).
5. To investigate an Atlantic salmon farm situated in the Tamar River on the north coast of Tasmania in an attempt to better understand why this farm has not experienced AGD in its 5 years of existence despite *Neoparamoeba* sp. being identified in the surrounding environment (Chapter 6).

Chapter 2 - General Materials and Methods



2.1 Gill scores

The gills of the salmon were examined for the presence of white patches and assigned a score in accordance with the protocols of Huon Aquaculture Company. In the field this method is used to indicate the presence of AGD. The four scores used were clear, faint spot, spot and patch with patch being the most severe (Figure 2.1).

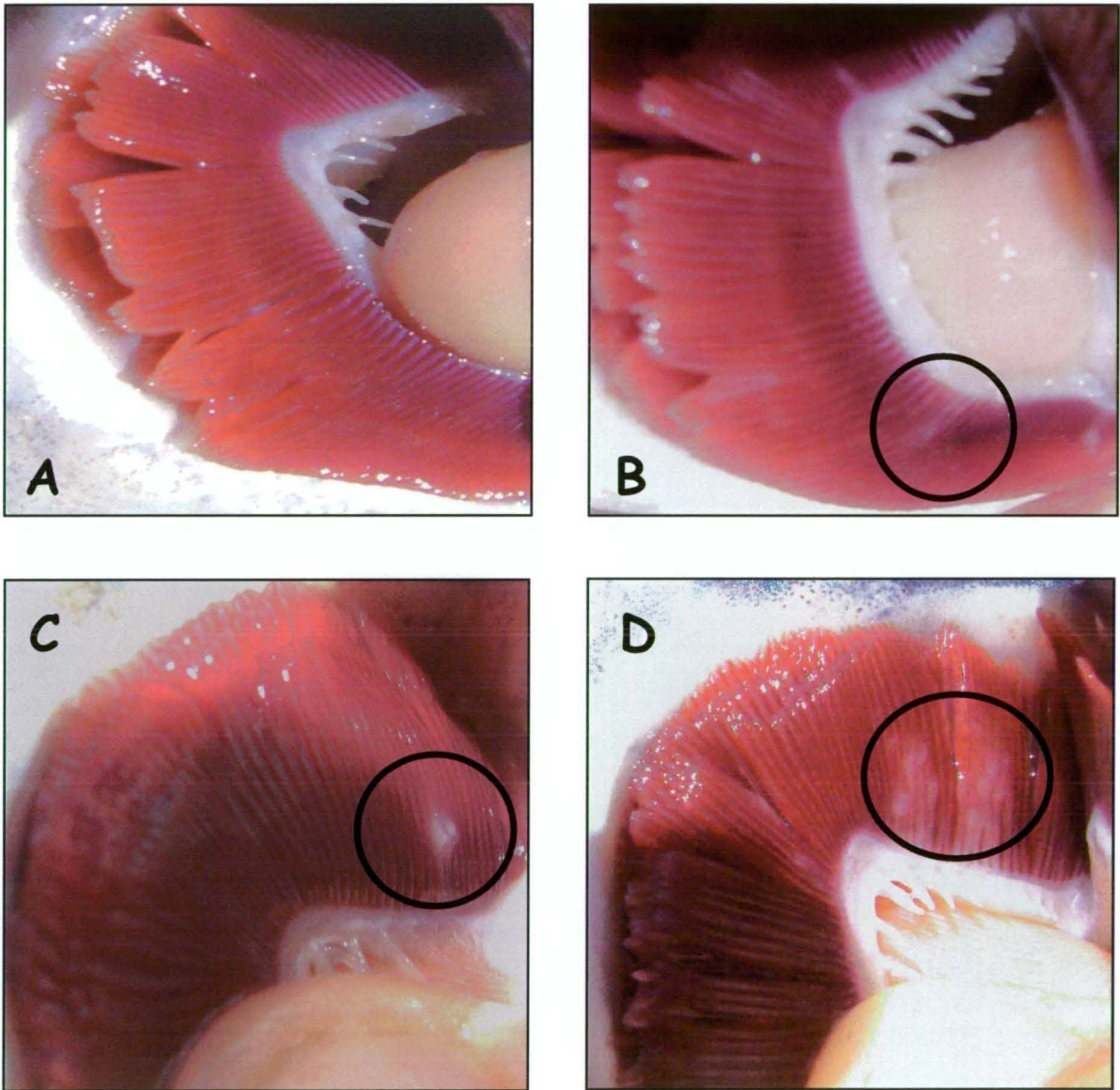


Figure 2.1: The use of gill scores as a field diagnostic tool to identify the AGD status of Atlantic salmon by the presence of white mucoid plaques. The scores were assigned in accordance with Huon Aquaculture protocols. A = clear, B = faint spot, C = spot, D = patch.

2.2 Amoebae culture

Amoebae were cultured based on methods adapted from those described by Page (1983). The malt yeast agar (MYA) agar plates (0.1g malt, 0.1 g yeast, 1 L filtered seawater) were maintained at 19-20°C in a temperature-controlled incubator for a period of 7-14 days. During this time plates were checked daily using a stereo dissecting microscope (Olympus Hamburg, Germany) to identify amoebae growth patches and estimate numbers. Growth patches were marked and the area of agar cut and removed using a sterile scalpel blade. The excised section was placed face down on a new MYA plate and approximately 1.5 ml of sterile seawater (autoclaved, 121°C for 15 minutes) was added. The new plate was then sealed and incubated for a further 7-10 days whilst being observed daily.

2.3 Cell Harvesting Protocol

Once amoebae numbers were greater than approximately 200 cells per plate harvesting occurred. Approximately 500 µl of sterile seawater was added to each plate and the surface was gently scraped for several minutes using a sterile spreader to dislodge the cells. A 200 µl aliquot was pipetted into a sterile eppendorf tube and frozen at -80°C for DNA extraction. Another 200 µl aliquot was pipetted into an eppendorf tube with 10 µl formalin (37% formaldehyde) and refrigerated for 4'6-diamidino-2-phenyli (DAPI) staining. A small drop was also placed on a glass slide and heat fixed for immunofluorescent antibody test (IFAT).

Detection of *Neoparamoeba* sp. in environmental samples

Neoparamoeba sp. in culture enriched environmental samples were detected using IFAT and a DAPI stain. Both of these tests can only identify that the amoebae belongs to the *Paramoeba* genus. A species-specific PCR was then used to determine if *N. pemaquidensis* was present. Only when PCR had been used could the organisms be defined as *N. pemaquidensis* otherwise they were referred to as *Neoparamoeba* sp.

2.4 Immunofluorescent antibody test (IFAT) protocol

The IFAT stain used followed the protocol of Howard and Carson (1993).

Slides prepared in the cell harvesting protocol were flooded with phosphate buffered saline (PBS 0.1M, pH 7.2) and incubated for 3 minutes. The PBS was discarded and the slides flooded with primary antibody (rabbit α *N.*

pemaquidensis, 1:50 dilution in PBS) and incubated at 37°C for 60 minutes in a moist chamber. The slides were then washed in PBS twice for 4 minutes. A

fluorescein labelled secondary antibody (1:60 dilution in IFAT diluent, sheep α rabbit Ig, Silenus, Melbourne, Australia) was applied and incubated at 37°C for 45 minutes in a moist chamber. The slides were then washed as above, mounted in alkaline-buffered glycerol and examined under a UV microscope (Figure 2.2).

Reference strain *N. pemaquidensis* (PA027) was used as a positive control.

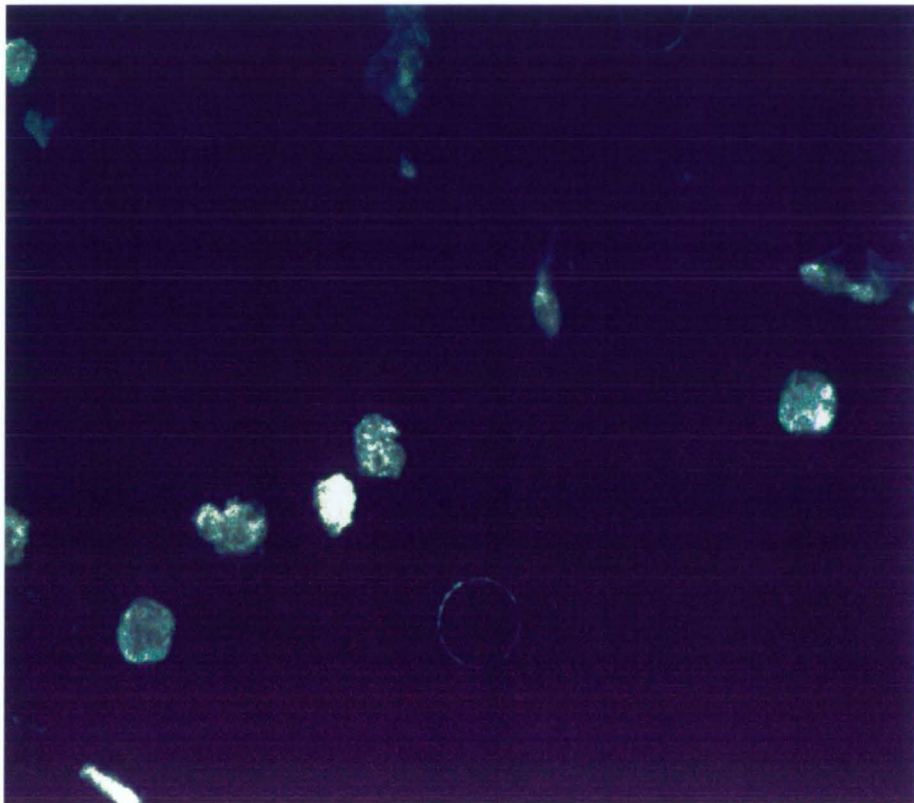


Figure 2.2: IFAT stain using a fluorescing secondary antibody to identify *Neoparamoeba* spp.

2.5 4'6-diamidino-2-phenylindole (DAPI) protocol

A 4'6-diamidino-2-phenylindole (DAPI) stain adapted from Howard (2001) was used to highlight the symbiont parasome characteristic of *Paramoeba* spp. A 1.5 ml eppendorf tube with 200 μ l of sample and 10 μ l of formalin was incubated with 10-25 μ l DAPI solution (DAPI, Sigma) in the dark for approximately 30 minutes. A wet mount was then prepared and examined using a fluorescent microscope with a filter block in the UV excitation range (Figure 2.3). Reference strain *N. pemaquidensis* (PA027) was used as a positive control.

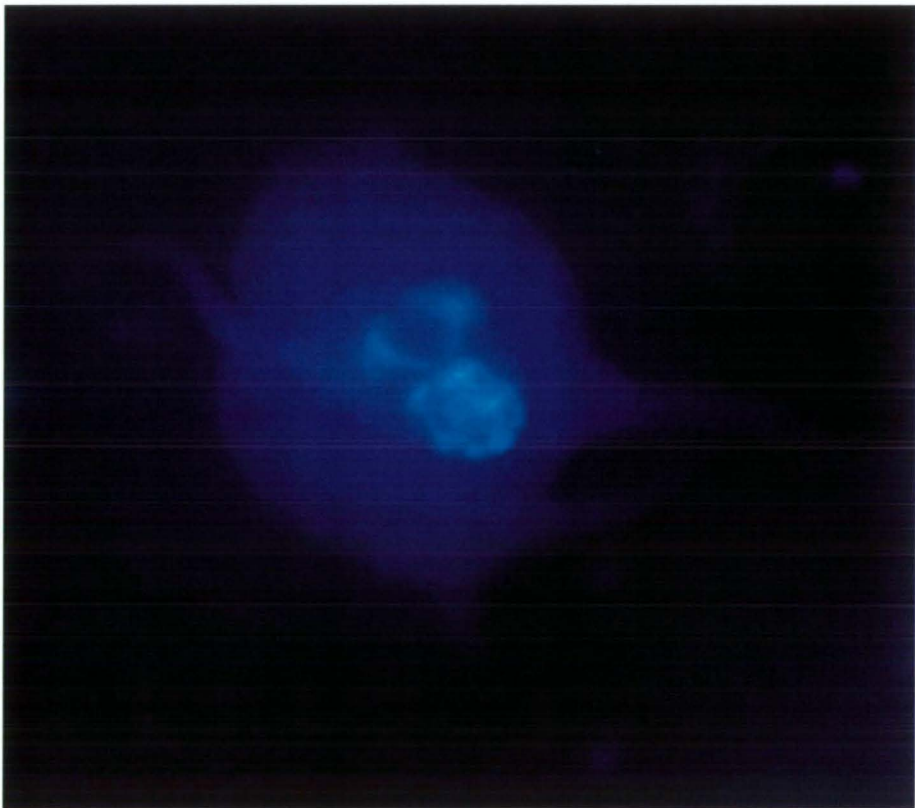


Figure 2.3: DAPI stain highlighting the nucleus and characteristic parasome used to identify *Neoparamoeba* spp.

2.6 Extraction of DNA

DNA was extracted following the methods of Wilson and Carson (2001). The method was performed using a 96 well Uni-filter plate (GF/B, Whatman, USA). Each well of the filter plate was pre-moistened with 100 μ l of reverse osmosis water. A vacuum of 5 – 10 kPa was applied for 1-2 minutes to empty the wells contents before the sample being added. A volume of 200 μ l cell suspension was placed in a 1 ml microcentrifuge tube and incubated with 500 μ l of guanidinium isothiocyanate lysis buffer (30g GuSCN to 25 ml TRIS-HCL 0.1 mol⁻¹, pH 6.4) for 50 minutes at room temperature. The lysates were then pulse centrifuged and the supernatant transferred to individual wells. The wells were washed twice with 200 μ l of lysis buffer, five times with 200 μ l of cold 70% ethanol and once with 100 μ l of acetone. A vacuum of up to 15 kPa was applied until no liquid remained in the wells. A 35 μ l volume of elution buffer (TRIS-HCL 10 mmol⁻¹, pH 8) was added to each well and incubated for 5 minutes. The bound DNA was transferred to a 96 well microtiter tray by applying a vacuum up to 15 kPa and 20 μ l of elution buffer was added to each well. A vacuum was applied until no traces of buffer could be detected. The tray was then sealed and refrigerated at 4°C overnight to allow the DNA to re-hydrate.

2.7 Polymerase Chain Reaction (PCR) protocol

A PCR protocol using species-specific primers of the 18S rDNA gene sequence was used to identify *N. pemaquidensis* in environmental samples (Elliott *et al.* 2000). Each reaction tube had total volume of 20 μ l of solution consisting of; 2 μ l 10x PCR buffer (Invitrogen Life Technologies), 1.6 μ l dNTPs (0.2 mM Epicentre Technologies), 0.8 μ l MgCl (2 mM Invitrogen Life Technologies), 0.6 μ l forward primer (fNp-Hxe23b1; 5'-GTGAGTGATGAGTAGACCTACTGG-3') and 0.6 μ l reverse primer (rNp-Hxe23b1; 5'-CACAACAAACTCGCTCTACCCG-3') (Elliott *et al.*, 2001), 0.2 μ l Platinum Taq (Invitrogen Life Technologies), 2 μ l from DNA sample, 1 μ l BSA (20 ng per reaction) and 11.2 μ l MilliQ water. PCR positive controls of purified DNA from a reference strain of *N. pemaquidensis* (PA027) and a negative control of *Pentacapsula* sp. were included.

PCR cycling conditions

PCR cycling occurred in a PTC-100 thermocycler (Bresatec).

1. 1 cycle 94°C for 3 minutes
2. 35 cycles of: 94°C for 45 seconds
 58°C for 45 seconds
 72°C for 45 seconds
3. 1 cycle of: 72°C for 4 minutes

Gel protocol

A 2% agarose gel (GibcoBRL, Life Technologies) was prepared to visualise the amplicons. A 1 kilo-base DNA ladder (Invitrogen Life Technologies) was loaded into the first well of each gel. Each well had a volume of 8 µl containing 1:5 dilution of dye and reaction solution. The gel was run at 70 V for 36 minutes. The gel was immersed in ethidium bromide staining solution (3% in distilled water) on a shaker at room temperature for 30 minutes. The gel was then viewed using a UV transilluminator and digital pictures taken (Figure 2.4).

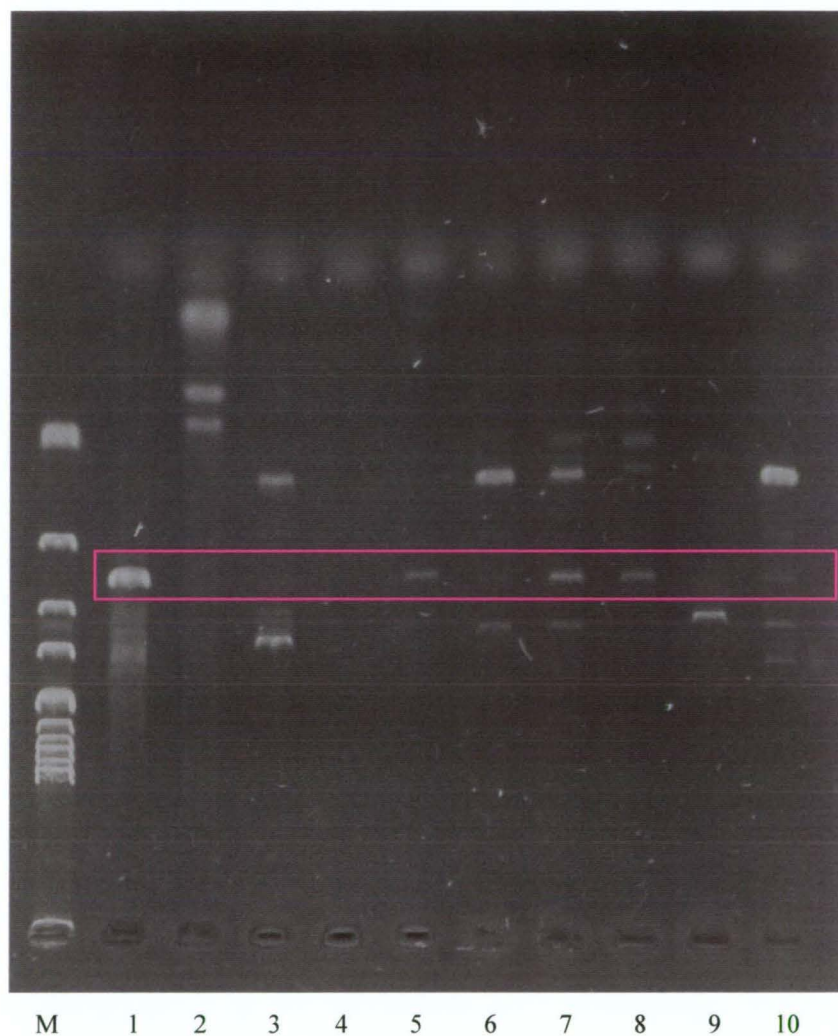


Figure 2.4: An agarose gel stained with ethidium bromide used to visualise the PCR products. Lane M = 1 Kb reference ladder, Lane 1 = positive control *N. pemaquidensis* reference strain (PA 027), Lane 2 = negative control, Lanes 3 – 10 environmental samples. The positive samples are within the pink frame.

2.8 Immuno-dot blot test

Sample preparation

The immuno-dot blot test used to detect the presence of *Neoparamoeba* sp. followed the protocol developed by Douglas-Helders et al., (2001). Gill mucus samples collected in the field were incubated with 400 µl of 1% mucolytic agent N-acetyl-L-cysteine (BDH, Melbourne) at 37°C for 1 hour. The cells were lysed by adding 40 µl of commercial bleach (0.21% v/v sodium hypochlorite and 0.045% v/v sodium hydroxide) vortexed and placed on a shaker for 8 minutes. Then 10 µl of 2N hydrochloride was added, the samples mixed by vortexing and placed on a shaker 30 minutes. The treated samples were stored at -20°C overnight. The following day samples were thawed at 37°C and centrifuged at 15 600 g for 20 seconds. Two pieces of 0.45 µm pore size Immobilon-P™ membrane (Millipore, Bedford USA) were pre-treated using the following protocol;

1. 100% ethanol for 15 seconds
2. Distilled water for 2 minutes
3. Phosphate buffered saline (PBS, 0.1M, pH 7.4) for 5 minutes

A piece of filter paper was cut to the same size as the membrane and flooded with 2-3 ml of PBS.

Assay protocol

All incubation steps in the following protocol were carried out at room temperature on a shaker. Using a 96 well vacuum dot-blotter (Millipore), 80 µl of supernatant was added to each well and incubated at room temperature for 18 minutes. A positive control consisting of 10 µl of reference strain *N. pemaquidensis* (PA027) and a negative control consisting of 80 µl digested mucus from freshwater Atlantic salmon were used. A vacuum of approximately 15mmHg was applied until all the wells appeared dry. The top membrane was then removed and washed using the following cycle:

1. Once in PBS (0.1M, pH 7.4) for 5 minutes
2. Twice in PBS-0.05% Tween 20 (PBS-T) for 5 minutes
3. Once in PBS (0.1M, pH 7.4) for 5 minutes

The membrane was incubated in casein (2.5% dilution in PBS-T, BDH) for 1 hour and washed using the above protocol. The membrane was incubated with rabbit primary antibody (1:600 dilution in PBS-T) for 25 minutes and washed as described above. The membrane was then probed with a secondary antibody (1:4000 dilution in PBS-T, Silenus, Melbourne, Australia) and incubated for 20 minutes. The membrane was then washed as follows:

1. Once in Tris buffered saline (TBS, 0.1M, pH 7.4) for 5 minutes
2. Twice in TBS-0.05% Tween 20 (TBS-T) for 5 minutes
3. Once in TBS (0.1M, pH 7.4) for 5 minutes

The membrane was then visualised using BCIP/NBT (Moss Inc., Maryland, USA) for 2-3.5 minutes ensuring that there was colour in the positive control but none in the negative. Following development, the membrane was washed twice in distilled water to stop further colour development. To achieve accurate results the membrane was read whilst still wet (Figure 2.5).

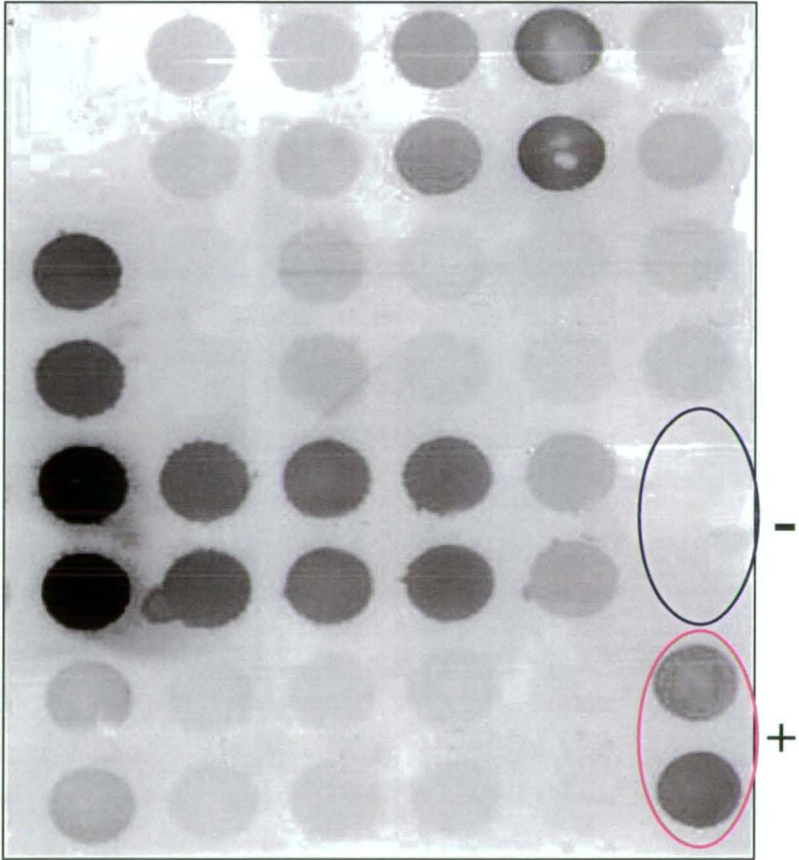


Figure 2.5: Photo of an immuno-dot blot. Positive (pink ellipsoid) and negative (blue ellipsoid) samples are indicated, as are mucus samples from Atlantic salmon.

2.9 Gill Histology

Whole gills were dissected from the fish and placed in seawater Davidson's fixative (3:3:2:1; 95 % ethanol, 0.2 μ filtered seawater, formalin, glacial acetic acid) for 48 hours before being transferred to 70% alcohol until processing. The second gill arch was excised, processed (Tissue-TEK[®] Miles Scientific, USA), embedded in paraffin, sectioned at 4 μ m and stained with haematoxylin and eosin. The number of lesions with amoebae attached was recorded for each section, as was the total number of filaments. Sections were observed by light microscope (Olympus, Hamburg, Germany) using x1 – x400 magnification. Photos were taken using a digital camera (Nikon coolpix 990).

Chapter 3 - Fallowing of farm sites: a possible disease management strategy for amoebic gill disease?



Introduction

Epidemics, whether in wild populations or in cultured stocks, are rarely the result of a single determinant. The occurrence of a disease event is a complex interaction between host, infectious agent and the environment (McVicar, 1997; Menzies *et al.*, 1998). For example, *Vibrio salmonicida* outbreaks require the presence of the pathogen, host, suitable water temperatures and other stressors such as poor nutrition or water quality (Colquhoun and Sørum, 2001; Damsgård *et al.*, 2004). Management and husbandry protocols also play a part in the complex and multifactorial nature of a disease (Menzies *et al.*, 1998). Investigating disease determinants provides information on potential risk factors, which may be the cause or source of an outbreak. An understanding of these complex interactions may enable the impact of a disease outbreak to be minimised and transmission of the causative agent to be reduced (McVicar, 1997; Menzies *et al.*, 1998; Baldock, 2002). Where the disease-causing agent is endemic to the local environment and unable to be eradicated, a certain background level of disease must be tolerated. Under these circumstances it is only when there is a rise in the number of new cases exhibiting clinical signs that a disease outbreak is identified (Baldock, 2002).

Fallowing is a common practice in terrestrial agriculture as it enhances crop production and reduces the pathogen load by rotating pastureland (Bron *et al.*, 1993). The practice of intensive fish production can result in changes to the local environment from waste feed and faeces and an increase in the abundance of pathogens (Bron *et al.*, 1993; Carroll *et al.*, 2003; Pereira *et al.*, 2004). In Tasmania the Marine Farming Planning Act 1995 states “there must be no unacceptable environmental impact 35 m outside the boundary of the marine farming lease area” (Crawford, 2003). Fallowing is mandatory upon the appearance of hydrogen sulphide bubbles and methane gasses in the sediment as these are extremely toxic to fish (McGhie *et al.*, 2000; Crawford, 2003). As a result salmon farmers in the Huon Estuary, Tasmania commonly practice fallowing to ensure lease sites do not reach this end point (McGhie *et al.*, 2000). In contrast, Scotland salmon farmers use fallowing as a disease management strategy to minimise the impact of sea lice, *Lepeophtheirus salmonis* (Bron *et al.*,

1993; Pietrak and Opitz, 2002; Bruno, 2004). When being used as a tool to break a pathogens life cycle, fallowing requires all cultured fish and infrastructure to be removed from the site (Bron *et al.*, 1993; Pereira *et al.*, 2004).

Many studies have investigated AGD, identifying the aetiological agent, susceptible hosts and the disease distribution (Kent *et al.*, 1988; Roubal and Lester, 1989; Munday *et al.*, 1990; Palmer *et al.*, 1997; Dyková *et al.*, 2000; Nowak, 2001; Dyková *et al.*, 2005). However, limited research has been conducted to identify environmental conditions and husbandry practices that may be risk factors for AGD (Clark and Nowak, 1999; Douglas-Helders *et al.*, 2000; Douglas-Helders *et al.*, 2001; Tan *et al.*, 2002; Douglas-Helders *et al.*, 2003; Douglas-Helders *et al.*, 2004; Douglas-Helders *et al.*, 2005).

Of the risk factors investigated salinity coupled with temperature has proven to be the most important (Munday *et al.*, 2001; Douglas-Helders *et al.*, 2003; Douglas-Helders *et al.*, 2005). In Tasmania, farms located in areas with salinity above 30 ppt experience a higher incidence of AGD (Akhlaghi *et al.*, 1996; Nowak, 2001; Douglas-Helders *et al.*, 2004). A recent *in vitro* study found that salinity had the greatest influence on *Neoparamoeba* sp. survival (Douglas-Helders *et al.*, 2005). Salmon farms operating in areas of low salinity such as Macquarie Harbour (17 ppt) are not affected by AGD (Clark and Nowak, 1999). Mortality rates of experimentally infected salmon were highest when salinity levels were elevated to 37- 40 ppt (Nowak, 2001). A marked decrease in AGD was observed when Atlantic salmon were maintained in experimental tanks with 27 ppt salinity in comparison to 35 ppt (M.Adams unpub. data.). AGD outbreaks in other countries have also been shown to coincide with high salinity levels (Nowak, 2001).

AGD outbreaks in Tasmania have been recorded at water temperatures ranging from 12-20°C (Akhlaghi *et al.*, 1996; Munday *et al.*, 1990; Clark and Nowak, 1999; Nowak, 2001). *Neoparamoeba* sp. has been found on salmonid gills at temperatures as low as 10°C (Clark and Nowak, 1999; Douglas-Helders *et al.*, 2001). In Ireland outbreaks in salmon have been observed between 12-21°C (Rodger and McArdle, 1996; Palmer *et al.*, 1997) and AGD has been identified in

turbot *Scophthalmus maximus* L., cultured in north-west Spain at a temperature range of 14-18°C (Dyková *et al.*, 1998). Under experimental conditions mortalities due to AGD can be minimised by maintaining water temperatures between 12-14°C (Akhlaghi *et al.* 1996; Munday *et al.*, 2001). Increasing temperatures above this range significantly increases mortality rate and control of the infection is very difficult (Munday *et al.*, 2001). AGD outbreaks in Tasmania have been consistently recorded during the summer months December to March when water temperatures are high (Clark and Nowak, 1999). Other potential environmental risk factors that have been suggested include dissolved oxygen levels (DO), water currents, dead fish, jellyfish and algal blooms (Douglas-Helders *et al.*, 2000; Munday *et al.*, 2001; Nowak, 2001; Douglas-Helders *et al.*, 2003).

The Huon Estuary, approximately 40 km in length, situated south of Hobart is an area where a significant proportion of Tasmania's Atlantic salmon is produced (Parslow, 2000; Crawford, 2003). Since 1997, the Huon Aquaculture Company has increased their production of Atlantic salmon substantially in particular at lease sites close to the mouth of the Estuary (Parslow, 2000). This study aimed to examine the effectiveness of fallowing as a husbandry protocol to reduce *Neoparamoeba* sp. in the environment and associated AGD outbreaks. Environmental conditions and the occurrence of AGD at two leases located in the Huon Estuary, Tasmania, Australia one of which had been fallowed, were monitored over a six-month period.

Materials and Methods

Experimental design

Atlantic salmon in replicated pens were located at two lease sites (Garden Island and Flathead Bay) in the Huon Estuary, Tasmania, Australia (Figure 3.1). The presence of *N. pemaquidensis* on the nets and in the sediment and the prevalence of AGD were examined over a 6 month period commencing in December 2002 and concluding in May 2003. Prior to the trial Flathead Bay was fallowed for approximately 11 months with all fish and nets removed from the site. Four trial pens with 120 m diameter nets were located at each site stocked with Atlantic salmon. Baseline values of AGD prevalence in fish and the presence of *N. pemaquidensis* were taken in November 2002 at Pillings Bay. The fish were bathed in freshwater prior to sampling.

Fish

Atlantic salmon smolts obtained from Tasmanian commercial hatcheries were transferred to Pillings Bay in the Huon Estuary, Tasmania, Australia between May and August 2002 and stocked in 80 m diameter pens (Figure 3.1). At the beginning of December the fish were transferred to 120 m diameter nets and towed to the trial sites located at Garden Island and Flathead Bay (Table 3.1).

Table 3.1: The number of Atlantic salmon stocked in each pen at the beginning of the trial.

Location	Pen	Number of fish
Garden Island (B)	1	21 382
	2	25 059
	3	51 213
	4	49 843
Flathead Bay (C)	1	21 226
	2	24 797
	3	62 569
	4	55 613

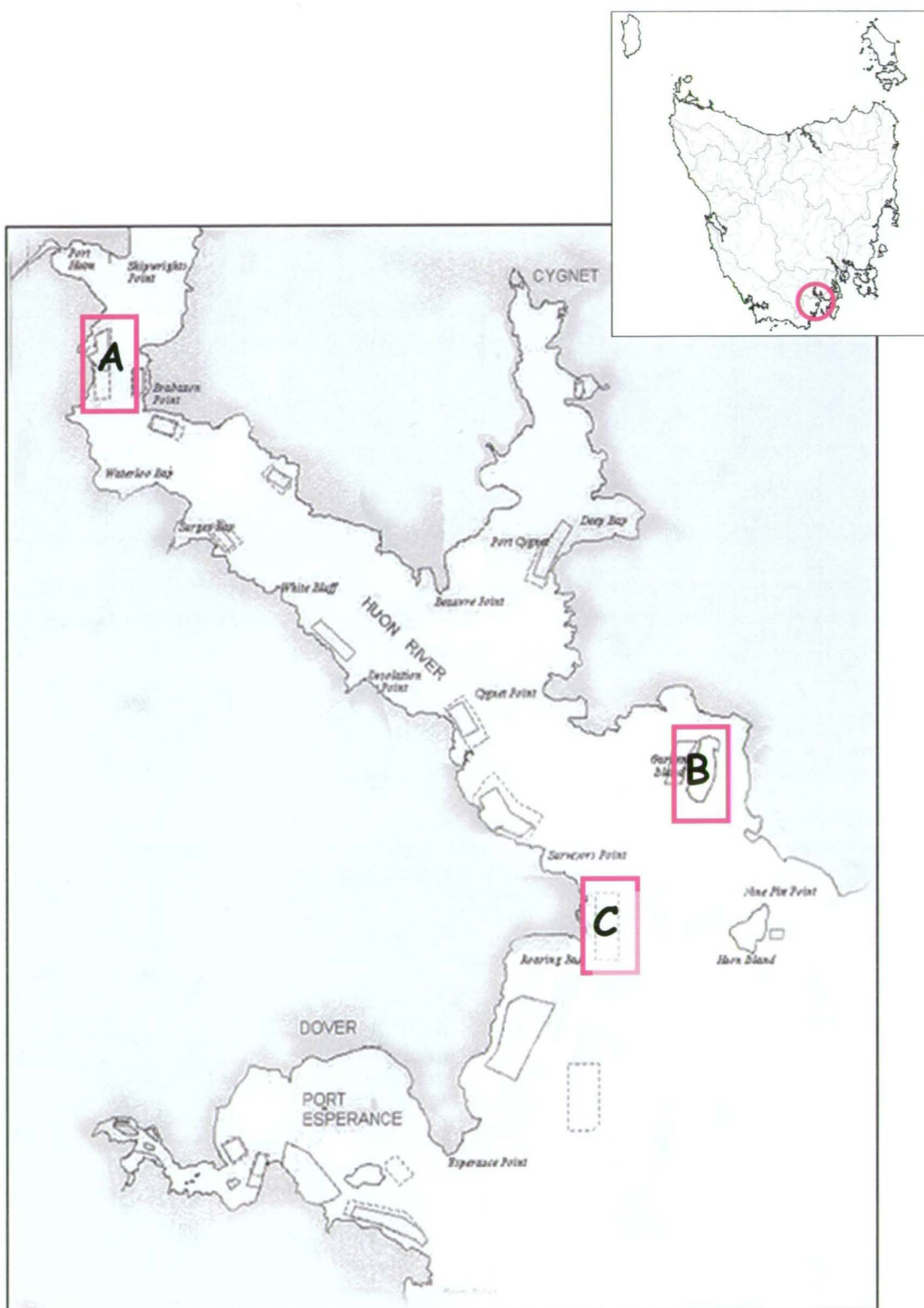


Figure 3.1: Map of Huon Estuary showing trial site locations, Pillings Bay (A), Garden Island (B) and Flathead Bay (C).

Husbandry protocol

The trial pens were fed with commercial salmon pellets (Skretting, Australia) using an Aquasmart™ automated demand feeder. The gills of 20 fish from each pen were monitored by farm staff on a weekly basis for white mucoid patches and assigned an arbitrary gill score (Refer to General Materials and Methods 2.1). Freshwater bathing was undertaken if these scores were elevated. No net changes were performed during the trial. Salinity, temperature and dissolved oxygen were recorded on a daily basis.

Sample collection

Fish gills:

Fish in the pen were crowded using a box net, removed by dip net and anaesthetised using 0.5% AquiS® (Lower Hutt, New Zealand). The gills of 20 fish were examined for the presence of white patches, assigned a gill score and a scraping of gill mucus taken to be analysed by immuno-dot blot. If white patches were present the scraping was taken from that area. The mucus samples were placed on ice until reaching the laboratory and stored at -20°C until analysis. The fish were revived and returned to the pen.

Nets:

Net segments from the different sites were examined for the presence of *N. pemaquidensis*. Segments of 0.5mm net filaments (approximately 4 x 4 cm) were cut from the 120 m diameter net at a depth of 5 m by divers and placed in sealed containers underwater. The segments were always sampled from the east side of the net within the same area. Upon reaching the shore, individual net segments were placed directly onto an MYA plate, sealed and labelled. Upon reaching the laboratory, approximately 6 hours after collection, the samples were incubated at 19-20°C for 7–14 days (n = 4 / pen).

Sediment:

One to two sediment grabs were taken from a depth of approximately 12 m on the north facing side of each cage. The sediment was placed in sealed individual 1 L containers until reaching the shore. For each cage two 5 g samples were placed on individual MYA plates, labelled and sealed. Upon returning to the laboratory these samples were incubated at 19-20°C for approximately 14 days.

Identification of *Neoparamoeba* sp.

1.1 Isolation and culture

Amoebae were isolated from net segments and cultured (Page 1983, General Materials and Methods 2.2). Cells were harvested as described (General Materials and Methods 2.3) and DNA was extracted (Wilson and Carson 2001, General Materials and Methods 2.6).

1.2 Detection of amoebae

The presence of *Neoparamoeba* sp. was confirmed by IFAT using a primary rabbit antibody prepared to *N. pemaquidensis* strain PA027 (Howard and Carson, 1993, General Materials and Methods 2.4). A DAPI stain was used to highlight the characteristic parasome and nucleus indicating that the organism belonged to family Paramoebidae (Howard, 2001, General Materials and Methods 2.5). *N. pemaquidensis* was detected on the net segments and in the sediment by PCR using specific primers of the 18S rDNA gene sequence (Elliot *et al.* 2001, General Materials and Methods 2.7). *Neoparamoeba* antigens in the gill mucus were detected using an immuno-dot blot technique (Douglas-Helders *et al.* 2002, General Materials and Methods 2.8).

AGD case definition

A fish with both positive gill score (the presence of white plaques) and positive immuno-dot blot (the presence of *Neoparamoeba* sp. antigens in gill mucus) was defined as AGD positive.

Statistics

Comparisons of AGD prevalence at the trial sites for each month were analysed by a chi-square (2x2 table) using the statistical package Epi Info™ 2002 (CDC, USA). AGD prevalence at the two sites, irrespective of time, was analysed by means test using the statistical package Epi Info™ 2002. A two-tailed student t-test was used to compare the presence of *N. pemaquidensis* on net segments and in sediment with AGD prevalence. A significance level of $p \leq 0.05$ was adopted.

Results

AGD prevalence

The mean prevalence of AGD in Atlantic salmon maintained at Flathead Bay was marginally higher than the fish held at Garden Island (Table 3.2). A comparison of the intervals between freshwater bath treatments revealed no significant difference between the two sites ($p = 0.358$).

Table 3.2: The mean AGD prevalence and average time between freshwater baths recorded at the two trial sites (n = 480 Garden Island, n = 440 Flathead Bay).

Location	Mean AGD Prevalence (% ± S.E.)	Average period between freshwater baths (days ± S.E.)
Garden Island	52.3 (5.35)	37.8 (1.44)
Flathead Bay	57.5 (5.32)	38.5 (1.97)

A comparison of AGD prevalence for salmon at each sample point showed a significant difference between the two trial sites for the months of January ($p < 0.05$) and March ($p = 0.033$; Figure 3.2). The maximum AGD prevalence was observed in February (63.8%) and January (82.5%) for salmon from Garden Island and Flathead Bay respectively.

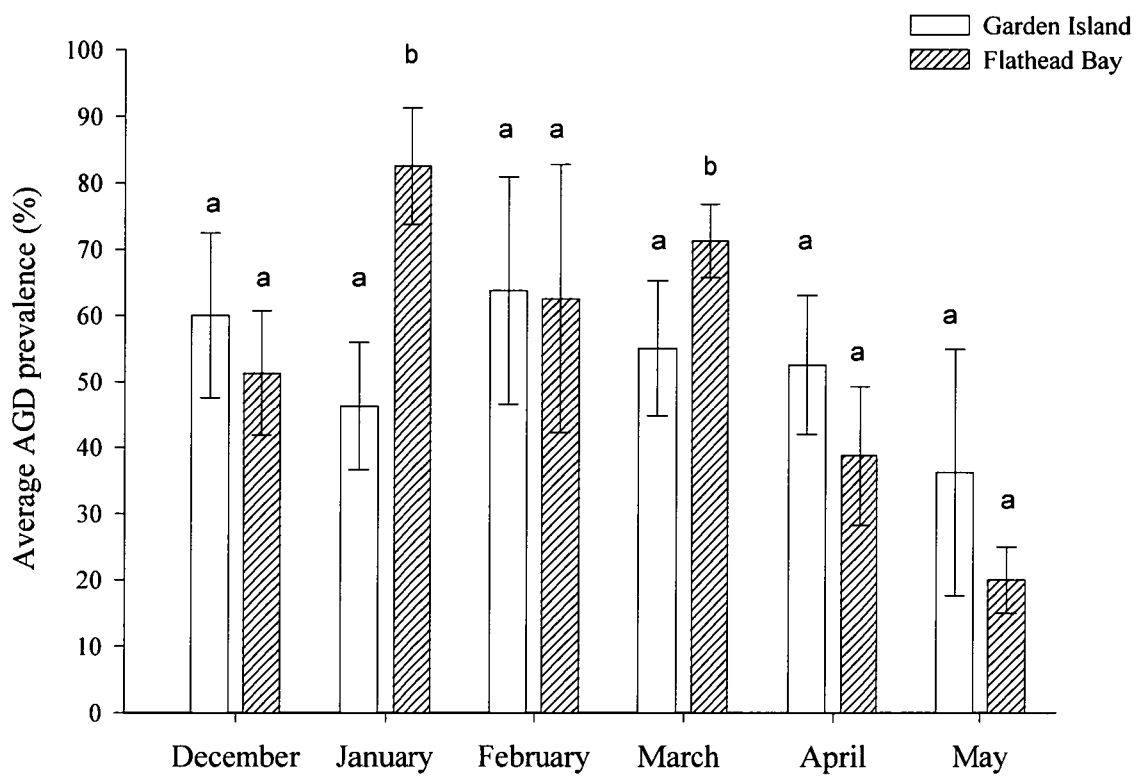


Figure 3.2: AGD prevalence in Atlantic salmon at trial sites Garden Island or Flathead Bay over a six-month period. ($n = 80$ / treatment / month, with the exception of May where $n = 40$ for Flathead Bay). Different superscripts denote significant differences within a month.

Netting samples

At both sites the highest number of net samples with *N. pemaquidensis* present was found in February (Table 3.3). Garden Island had consistently a higher number of positive net samples than Flathead Bay, with the exception of December and February. A comparison of positive net samples and AGD prevalence showed no significant relationship ($p = 0.749$).

Table 3.3: Net samples identified with *Neoparamoeba pemaquidensis* present by PCR at the two trial sites. (n = 16 / month / site).

	Number of PCR positive net samples	
	Garden Island	Flathead Bay
December	0	0
January	2	0
February	3	3
March	2	1
April	2	1
May	1	0

Sediment samples

For both sites the number of sediment samples with *N. pemaquidensis* present was low (Table 3.4). Positive samples were only recorded in December and February for Garden Island and December and March for Flathead Bay. Flathead Bay had the highest number of positive samples in March. A comparison of positive sediment samples and AGD prevalence showed no significant relationship ($p = 0.526$).

Table 3.4: Sediment samples identified with *Neoparamoeba pemaquidensis* present by PCR at the two trial sites. (n = 8 / month / site).

	Number of PCR positive sediment samples	
	Garden Island	Flathead Bay
December	1	1
January	0	0
February	1	0
March	0	2
April	0	0
May	0	0

Environmental parameters

There was no significant difference in either salinity or temperature between the two sites at a depth of 0 or 5 metres (Figures 3.3 and 3.4).

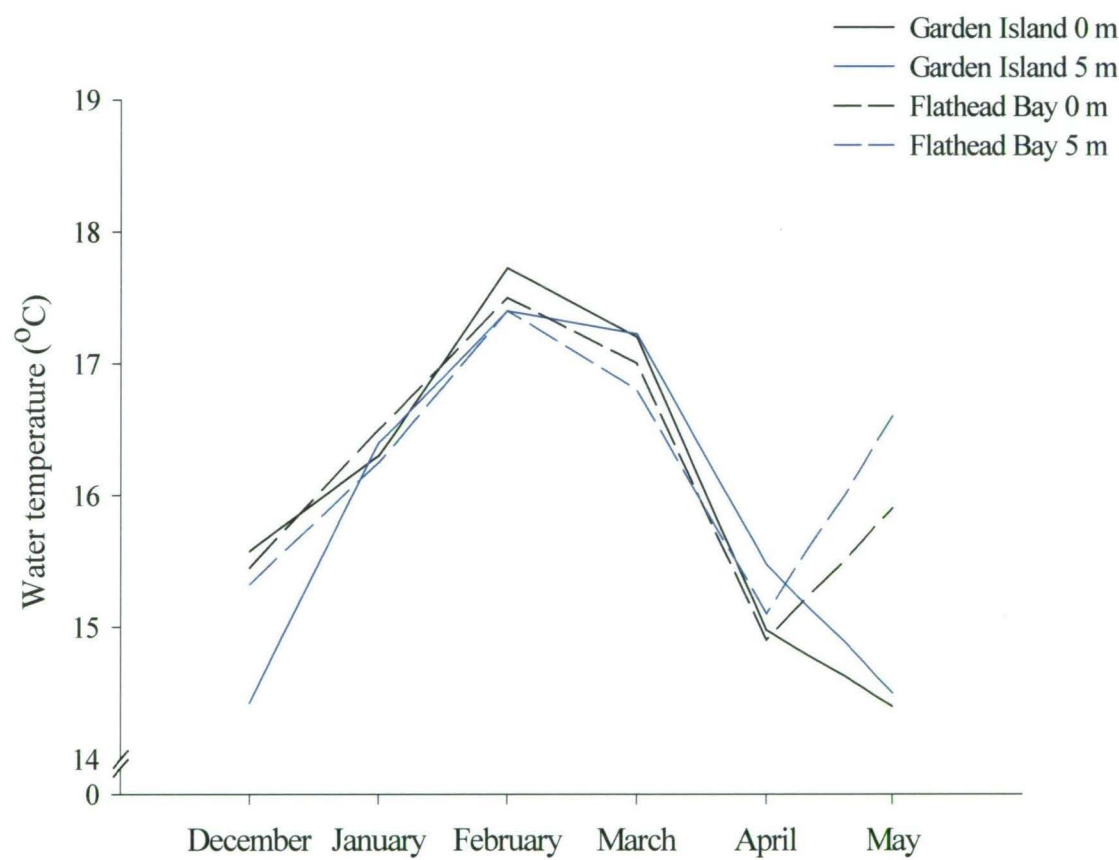


Figure 3.3: The average water temperatures recorded at the two trial sites measured at surface level and at a depth of 5 m.

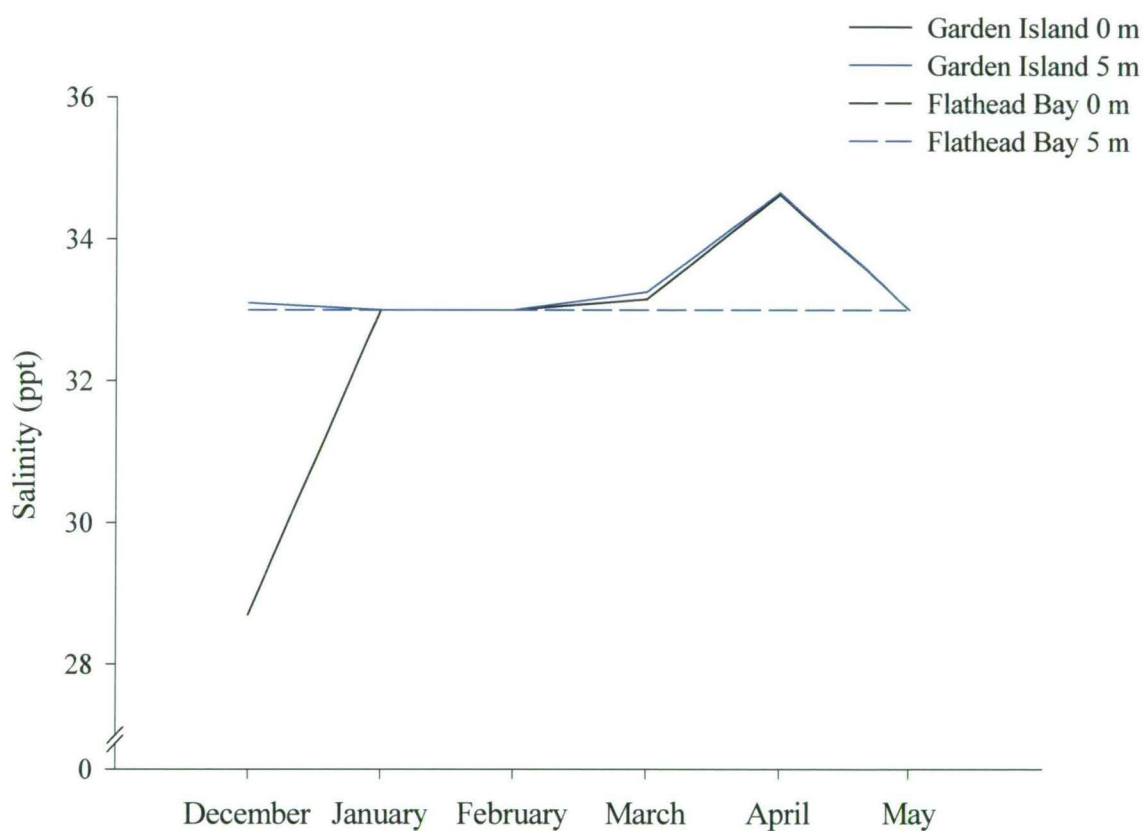


Figure 3.4: The average salinity recorded at the two trial sites measured at surface level and a depth of 5 m.

Discussion

Despite Flathead Bay having been fallowed for 11 months no significant difference was found for the prevalence of AGD in the fish or the interval between freshwater bath treatments at the trial sites. Douglas-Helders *et al.* (2004), similarly found no difference in the prevalence of AGD between sites within the Huon estuary that had been fallowed (4 to 97 days). They did however find that the interval between freshwater baths was significantly longer for cages held on fallowed sites.

Fallowing can be an effective management tool used to break a disease cycle and decrease the risk of an outbreak by reducing the pathogen population (Bruno, 2004). It has successfully been used to reduce the numbers of sea lice (*Lepeophtheirus salmonis*) on Atlantic salmon in Scotland by removing potential hosts (Bron *et al.*, 1993; Pietrak and Opitz, 2002). Sea-lice have been a consistent problem for Scotland salmon farmers for the past 25 years (Mackinnon, 1998; Denholm *et al.*, 2002). Due to perceived environmental damage and a tolerance to the organophosphorus compounds, the industry has been forced to seek alternative treatments for sea lice (Bron *et al.*, 1993). *L. salmonis* copepods can survive up to 95 days without attachment to a host and adults approximately 34 days away from a host (Bron *et al.*, 1993; Ritchie, 1997; Pietrak and Opitz, 2002). A fallowing period exceeding 95 days thus breaks the lifecycle and minimises re-infection of new smolt introduced to the site (Bron *et al.*, 1993; Pietrak and Opitz, 2002).

The effectiveness and duration of a fallow period is reliant upon a number of factors including biology of the pathogen, environmental conditions and potential reservoir hosts (Bron *et al.*, 1993; Pietrak and Opitz, 2002). *Neoparamoeba* sp. is a marine amphizoic amoeba (Page, 1983; Kent *et al.*, 1988) endemic to the Tasmanian marine environment (Crosbie *et al.*, 2003). Populations have been found in a range of sediment types including areas with no history of salmon culture (Crosbie *et al.*, 2003), on nets and on biofouling of sea cages (Tan *et al.*, 2002) and in the water column (Elliott *et al.*, 2001; Tan *et al.*, 2002; Douglas-Helders *et al.*, 2003). *Neoparamoeba* sp. trophozoites require attachment to a substrate to asexually reproduce (Martin, 1985; Dyková *et al.*, 2000). Prior to the

introduction of sea-cultured Atlantic salmon and rainbow trout to Tasmanian waters in 1984 there is no record of AGD in Australia (Munday, 1986). Wild fish existing in the surrounding areas of salmon farms had no *Neoparamoeba* sp. present on their gills (Dawson, 1999; Douglas-Helders *et al.*, 2002).

The presence of *Neoparamoeba* sp. in sediments that have no history of Atlantic salmon production, and the lack of AGD in native species, suggests that *Neoparamoeba* sp. does not require a host for survival (Crosbie *et al.*, 2003). As *Neoparamoeba* sp. are not reliant upon Atlantic salmon as a host the effectiveness of fallowing as a disease management strategy is doubtful. To obtain the maximum benefit from fallowing a sound knowledge of the organisms lifecycle and a host dependency such as with *L. salmonis* is required. Current knowledge of the *Neoparamoeba* sp. behaviour and virulence factors is limited.

Experimentally infected AGD in Atlantic salmon can only be achieved by co-habitation of naïve fish with infected fish and amoebae preparations harvested from the gills of infected salmon (Zilberg *et al.*, 2001; Morrison *et al.*, 2004). This suggests that the major mode of transmission is infected fish. This study showed that despite removing fish and nets from the Flathead Bay site for 11 months, upon the return of salmon to the site the initial prevalence of AGD was similar to the non-fallowed site. The value of fallowing is minimised if the potential for re-infection is high, which appears to have occurred in this trial due to current farming practices (Pietrak and Opitz, 2002). The salmon were maintained at Pillings Bay for approximately 6 months prior to the trial. At this company this is standard practice when transferring smolts from the hatchery. In comparison to the marine sites in the Huon Estuary, the occurrence of AGD at Pillings Bay, a brackish site, is unusual (D. O'Brien pers comm.). The baseline study at Pillings Bay detected *Neoparamoeba* sp. antigens in gill mucus from the salmon and *Neoparamoeba* sp. was isolated from the sediment below the cages. This indicates that the salmon had encountered *Neoparamoeba* sp. prior to being stocked at Flathead Bay, which may have influenced the early onset of AGD.

Even if fallowing was a possible management strategy for AGD a major problem that would affect its viability is the requirement of a co-ordinated program (Bron *et al.*, 1993; Pietrak and Opitz, 2002). The Huon Estuary region is responsible for a significant proportion of the Atlantic salmon produced in Tasmania (Crawford, 2003). Currently within the Huon Estuary there are 16 lease sites operated by two independent companies (Crawford, 2003; DPIWE, 2005). The estuary is fast flushing with times estimated to range from 2.5 days at times of high flow to 1 week during low flow (CSIRO Huon Estuary study team, 2000; Parslow, 2000). The benefits of fallowing would be negated if a co-ordinated program was not adopted. If areas surrounding a fallowed site continued to be farmed, the flow dynamics of the Huon Estuary suggest that the fallowed site would be impacted. The lack of new lease sites within the Huon Estuary, which would allow current production levels to be maintained whilst fallowing, would have significant financial ramifications for the farmers (Crawford, 2003).

Whilst the trial sites are both located within the Huon River Estuary the dynamics of the areas are quite different. Flathead Bay is situated on the western side of the estuary close to the shoreline and is classed as a marine site with limited freshwater influence (CSIRO Huon Estuary study team, 2000). Garden Island on the other hand is situated in the middle of the estuary close to Garden Island. It is classed as a marine site but has a surface freshwater layer that occurs predominantly on the northern side of the river for a significant part of the year (CSIRO Huon Estuary study team, 2000). These conditions may have influenced the AGD results at the Garden Island site due to the freshwater layer. Precipitation records indicate that a large increase was experienced in the month of March that may have altered the estuary conditions (www.bom.gov.au). Whilst farm records show no change in salinity at Garden Island, the farm did experience equipment failure for a period of the trial and as a result salinity was not always accurately recorded.

The presence of *Neoparamoeba* sp. on cage nets and in sediment beneath the salmon pens supports the findings of previous studies (Tan *et al.*, 2002, Crosbie *et al.*, 2003, Douglas-Helders *et al.*, 2003). No statistical comparison was made

between the sites due to low numbers. This is not a direct reflection upon the presence of *Neoparamoeba* sp. as isolating from environmental samples can result in false negatives as the current culture methods can experience difficulties that may render the cells non-culturable (Crosbie *et al.*, 2003). A large number of non-*Neoparamoeba* species are present in environmental samples that are capable of overgrowing the target species (P. Crosbie pers. comm.).

There was no significant relationship between the presence of *Neoparamoeba* sp. and AGD prevalence for net and sediment samples at either site. To date limited research has been conducted investigating the relationship between *Neoparamoeba* sp. in the sediment and on nets and the occurrence of AGD (Tan *et al.*, 2002; Douglas-Helders *et al.*, 2003). The virulence of these environmental strains still remains unknown and therefore it should not be assumed that they cause AGD. Dyková *et al.*, (2005) recently examined the morphological characteristics and SSU rRNA gene sequences of a number of fish gill and environmentally derived *Neoparamoeba* sp. clones. The study identified 22 strains of *Neoparamoeba pemaquidensis* and within the clade 2 distinct subdivisions were generated. Clones isolated from net samples within the Huon estuary were divided between the two subdivisions (Dyková *et al.*, 2005). Whether these strains differ in virulence is unknown. Both divisions do contain gill strains isolated from Tasmanian Atlantic salmon that have been associated with AGD (Dyková *et al.*, 2005).

This study identified a number of issues that indicate fallowing may not be an effective strategy for minimising AGD outbreaks. These included *Neoparamoeba* sp. being endemic to the Tasmanian marine environment and not reliant upon Atlantic salmon as a host. Fallowing programs would need to be a co-ordinated effort for the whole Huon Estuary, which is clearly an unrealistic requirement. Husbandry practices of initially holding smolt at a brackish site within the estuary negate the effects of fallowing. Whilst AGD is rarely recorded at this site, from this trial it is apparent that the salmon come into contact with *Neoparamoeba* sp. as indicated by positive immuno-dot blots. Fallowing is a costly exercise for the companies operating within the Huon Estuary with the benefits as a disease

management strategy perhaps not warranting such expense. Fallowing as an environmental management tool however does appear to have positive benefits such as allowing oxic conditions of surface benthic sediment to be re-established (Crawford, 2003).

Chapter 4 - The effect of copper antifouling paint treated nets
on the prevalence of amoebic gill disease.



Introduction

The optimisation of environmental conditions is an essential requirement that assists in the production of high quality Atlantic salmon. As such, biofouling is a costly problem for the Tasmanian salmon industry. The pen netting used by the salmon industry is an ideal substrate for biofouling due to its multi-filament nature, which provides protective crevices for settling organisms and a high surface area to volume ratio (Hodson *et al.*, 1997). The increased nutrient and organic load from feed wastage and fish excretion also encourages rapid fouling (Hodson *et al.*, 1997). The structural integrity of the pen is constantly under pressure due to the increased weight, necessitating frequent net changes. This is a financial burden to the farm as it is labour intensive and damage to the net can occur. This may result in a loss of stock, and growth rates may be affected due to disturbances in feeding regimes (Hodson *et al.*, 1997; Hodson *et al.*, 2000). In addition, fouled nets reduce the water exchange resulting in increased ammonia levels and reduced dissolved oxygen (Hodson *et al.*, 1997; Douglas-Helders *et al.*, 2003). As well as directly affecting the health of fish by reducing water quality, biofouling can also indirectly impact upon their health. Net changes stress the fish and may result in an increased susceptibility to diseases. Biofouling communities may act as reservoirs for disease causing organisms such as *Neoparamoeba* sp. (Alexander, 1991; Hodson *et al.*, 1997; Clark and Nowak; 1999; Nowak, 2001; Tan *et al.*, 2002).

Various agents have been used to slow the rate of biofouling. Prior to 1990 the most effective and commonly used antifouling agent was a self-polishing copolymer paint containing tributyltin (TBT) (Callow and Callow, 2002). The TBT was released as the paint surface dissolved, killing settling organisms by inhibiting energy transfer processes in respiration and photosynthesis (Callow and Callow, 2002). Whilst this type of paint was extremely effective in minimising fouling, it was found to have a detrimental effect on non-target organisms at nanogram-per-litre concentrations (Albalat *et al.*, 2002; Callow and Callow 2002; Morley *et al.*, 2003; Ohji *et al.*, 2003). Dogwhelks, *Nucella lapillus*, displayed imposex (occurrence of male sexual characteristics on the female) and disappeared from rocky shores where there was a high level of boating activity

(Callow and Callow 2002; de Mora *et al.*, 2003; Ohji *et al.*, 2003). Deformities in limbs of fiddler crabs, delay in moult, retardation of regenerative growth and impairment of egg production were other adverse effects attributed to TBT compounds (Callow and Callow 2002; Ohji *et al.*, 2003). Antifouling paints containing TBT were banned in a number of countries in the late 1980's for use on aquaculture nets and boats under 25m (Albalat *et al.*, 2002; Albanis *et al.*, 2002; Callow and Callow, 2002; Voulvoulis *et al.*, 2002; Ohji *et al.*, 2003). This led to the development of tin free products, the majority of which contain soluble cuprous oxide (Cu_2O) pigment combined with organic boosting biocides (Kiil *et al.*, 2002; Ohji *et al.*, 2003; Valkirs *et al.*, 2003).

The rate at which the Cu_2O biocide is released from the coating into the surrounding water is influenced by physical factors such as temperature, salinity and pH (Valkirs *et al.*, 2003). Whilst high concentrations of copper are toxic to certain algae, some bacteria are able to colonise the treated nets by producing a polysaccharide layer that acts as a protective barrier (Marszalek, 1979; Dempsey, 1981; Børufsen Solberg *et al.*, 2002). This initial colonisation is referred to as micro fouling as it includes bacteria, unicellular algae and cyanobacteria (Callow and Callow, 2002). The type of fouling community present is highly dependent upon the substrate, geographical location, season and competition and predation (Hodson and Burke, 1994; Callow and Callow, 2002). Under appropriate environmental conditions, micro fouling can provide a suitable substrate for larger fouling organisms by protecting the organisms from antifouling agents

Although copper treated nets may have some efficacy in reducing biofouling, previous studies have determined that such nets and associated fouling are risk factors for AGD outbreaks (Nowak, 2001; Tan *et al.*, 2002; Douglas-Helders *et al.*, 2003). A risk factor is defined as an increase in the risk of a disease outbreak due to the presence of external influences (Thrusfield, 1995; Nowak, 2001). *Neoparamoeba* sp. has been identified in association with a number of fouling organisms found on southern Tasmanian salmon nets (Tan *et al.*, 2002). These included amphipods, blue-lipped mussel *Mytilus edulis*, solitary ascidians *Ciona intestinalis*, bryozoan *Scrupocellaria bertholetti*, as well as the biofilm that

encompasses the net (Tan *et al.*, 2002). Conflicting evidence exists with regard to the effect copper treated nets have on AGD prevalence. Clark and Nowak (1999) concluded that net fouling did not have any effect on AGD prevalence based on farm net fouling scores and a multiple stepwise regression analysis incorporating other risk factors. In contrast, Douglas-Helders *et al.* (2003), found that Atlantic salmon maintained in nets treated with copper antifouling paint had a significantly higher AGD prevalence in comparison to untreated nets.

Whilst copper based paints have been proven effective in minimising fouling, environmental and consumer concerns question its continued use (Lewis, 1994; Hodson *et al.*, 2000). As a result alternative products that are more environmentally friendly are being investigated. One such product is lanolin, more commonly known as “wool grease”. It is a natural oil that forms a protective barrier on the fleece of sheep making it impervious to water. Lanolin has a number of applications ranging from corrosion control to cosmetic products. Studies conducted in Europe have found that a modified version of Penaten[®] cream, a diaper ointment with lanolin as a principle component, has promising signs as a potential antifouling paint (Magee *et al.*, 1996). Field trials conducted in the USA have shown Penaten[®] to be comparable with the commonly used copper based antifouling paint Woolsey Neptune[®] (Magee *et al.*, 1996). Anecdotal evidence from within the yachting fraternity also confirms lanolin’s potential as an antifouling product. A water ferry operator at Brisbane’s Moreton Bay currently uses a lanolin product to restrict biofouling on the hull and improve fuel efficiency (www.setsail.com). Further research is required to investigate the potential of a lanolin-based antifouling paint being used in aquaculture practices.

This study investigated the prevalence of AGD in Atlantic salmon maintained in nets treated with Hempel[®], a copper antifouling paint and Netclear[®] a lanolin antifouling paint over a six month period. The presence of *N. pemaquidensis* on the treated and untreated nets was monitored in conjunction with AGD prevalence. A laboratory trial was undertaken to further investigate the ability of *Neoparamoeba* sp. to attach to copper coated and untreated nets. The potential of

copper and lanolin nets acting as reservoirs for *Neoparamoeba* sp. and their potential as risk factors in AGD outbreaks in Atlantic salmon was examined.

Materials and Methods

FIELD TRIAL

Experimental design

Atlantic salmon maintained in replicated pens with Hempel[®] (NSW, Australia) a copper antifouling paint, Netclear[®] a lanolin based antifouling paint (Qld, Australia) or untreated nets were located in the Huon Estuary, Tasmania, Australia (Figure 4.1). AGD prevalence in fish and the presence of *N. pemaquidensis* on nets was examined over a 6 month period commencing in April, and concluding in September 2002. Samples were collected on a monthly basis. Two 120 m diameter nets were treated with Hempel[®] and two with Netclear[®] antifouling paint in accordance with the manufacturer's instructions. The nets were then suspended in the water for a minimum of two weeks, including two untreated nets, after which they were stocked with salmon. The fish were bathed in freshwater prior to sampling. No net changes were made during the trial with the exception of the lanolin treated nets. Approximately one month after the trial began the Netclear[®] treated nets had to be removed due to heavy fouling and replaced with untreated nets for the remainder of the trial. These nets are still referred to as Netclear[®] to avoid confusion with the untreated control nets.

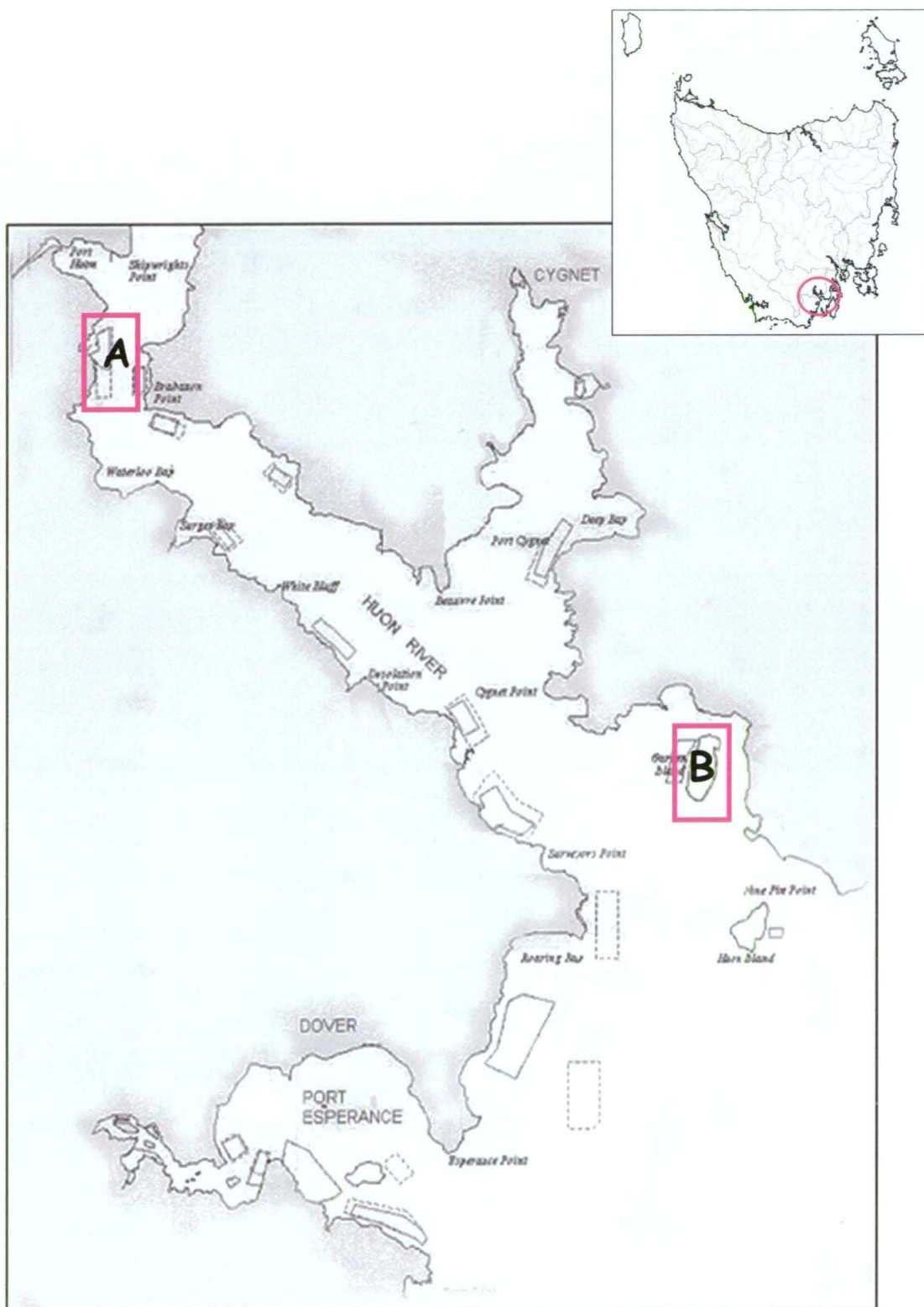


Figure 4.1: Map of Huon Estuary showing trial site locations; Pillsbury Bay (A), and Garden Island (B).

Fish

Atlantic salmon smolts were transferred from Tasmanian commercial hatcheries to Pillings Bay, in the Huon Estuary between August and September 2001 (Figure 4.1). The fish were stocked in 80 m diameter pens with untreated nets. The pens were towed to the trial site, in December 2001 and transferred to 120 m diameter pens with untreated nets until the trial commenced in April 2002 (Table 4.1).

Table 4.1: The number of Atlantic salmon stocked in each pen at the beginning of the trial.

Treatment	Number of fish stocked in pen
Control A	30 311
Control B	22 236
Hempel [®] A	37 691
Hempel [®] B	39 926
Netclear [®] A	37 132
Netclear [®] B	33 880

Husbandry protocol

Trial pens were fed with commercial salmon pellets (Skretting, Australia) using an Aquasmart™ automated demand feeder. The gills of 20 fish from each pen were monitored by farm staff on a weekly basis for white mucoid patches and assigned an arbitrary gill score (Refer to General Materials and Methods 2.1). Based on the gill scores freshwater bathing was undertaken when the farm believed necessary. No net changes were performed during the trial other than the lanolin pens as described above in ‘Experimental design’ section. Salinity, temperature and dissolved oxygen were recorded on a daily basis.

Sample collection

Fish gills:

Fish in the pen were crowded using a box net, removed by dip net and anaesthetised using 0.5% Aquis[®] (Lower Hutt, New Zealand). The gills of 20 fish were examined for the presence of white patches, assigned a gill score and a scraping of gill mucus taken to be analysed by immuno-dot blot. If white patches were present the scraping was taken from that area. The mucus samples were placed on ice until reaching the laboratory and stored at -20°C until analysis. The fish were revived and returned to the pen.

Nets:

Net segments from the different antifouling treatments were examined for the presence of *N. pemaquidensis*. Segments of 0.5mm net filaments (approximately 4 x 4 cm) were cut from the 120 m diameter net at a depth of 5 m by divers and placed in sealed containers underwater. The segments were always sampled from the east side of the net within the same area. For the initial sampling times (April and May) the nets were resuspended in sterile seawater in a 15 ml plastic tube and agitated for approximately 1 minute to dislodge the amoebae. A 20 µl sample was spread on a MYA plate using a sterile spreader, sealed and labelled. The samples were incubated at 19-20°C for 7 –14 days (n = 4 / pen). Due to a distinct lack of amoebae being isolated and the availability of improved culture methods the remaining sampling points were treated differently (I. Dyková pers. comm.) The individual net segments were placed directly onto the MYA plate, sealed and labelled. The samples were incubated at 19-20°C for 7 –14 days (n = 4 / pen).

Identification of *Neoparamoeba* sp.

1.1 Isolation and culture

Amoebae were isolated from net segments and cultured (Page 1983, General Materials and Methods 2.2). Cells were harvested as described (General Materials and Methods 2.3) and DNA was extracted (Wilson and Carson 2001, General Materials and Methods 2.6).

1.2 Detection of amoebae

The presence of *Neoparamoeba* sp. was confirmed by IFAT using a primary rabbit antibody prepared to *Neoparamoeba pemaquidensis* strain PA027 (Howard and Carson 1993, General Materials and Methods 2.4). A DAPI stain was used to highlight the characteristic parasome and nucleus indicating that the organism belonged to family Paramoebidae (Howard, 2001, General Materials and Methods 2.5). *Neoparamoeba pemaquidensis* was detected on the net segments by PCR using specific primers of the 18S rDNA gene sequence (Elliot *et al.* 2001, General Materials and Methods 2.7). *Neoparamoeba* antigens in the gill mucus were detected using an immuno-dot blot technique (Douglas-Helders *et al.* 2002, General Materials and Methods 2.8).

AGD case definition

A fish with both positive gill score (the presence of white plaques) and positive immuno-dot blot (the presence of *Neoparamoeba* sp. antigens in gill mucus) was defined as AGD positive.

LABORATORY TRIAL

In an attempt to further investigate if *Neoparamoeba* sp. are attracted to copper antifouling nets a laboratory trial was conducted.

Sampling protocol

Clean 1 cm nylon net segments were immersed in Hempel[®] antifouling paint (NSW, Australia) in 90 ml plastic petri plate for 1 hour. They were transferred to a clean plastic petri dish and placed in a 37°C oven for approximately 3 hours to dry. *Neoparamoeba* sp. were harvested and partially purified from the gills of AGD infected Atlantic salmon (Morrison *et al.*, 2004). Three 15 ml centrifuge tubes containing 1.5 ml of sterile seawater (Hempel[®] and untreated nets) or 0.2 µm filtered seawater (Hempel[®] nets) were inoculated with a 750 µl aliquot of *Neoparamoeba* sp. (245,505 cells/ml ± 15542.32). Single net segments were added to each tube and incubated for 1 hour at room temperature on a rocking platform. After 1 hour the net segments were removed and placed in individual clean 15 ml centrifuge tube containing 2.25 ml of 1% trypsin/PBS (PBS 0.1 M,

pH 7.4; Gibco, Burlington Canada) and gently agitated to dislodge the amoebae. The remaining solution in each tube was centrifuged (400 g, 5 minutes) 1 ml of the supernatant discarded replaced with 1 ml of 1% trypsin and gently agitated. The recovered amoebae were enumerated using a haemocytometer. This process was carried out on day 1 and again on day 14.

Statistics

Initially the prevalence of AGD was analysed at each time point using a stratified chi-square test. A decision was made to pool the data within each treatment due to the variation of replicate pens being greater across the sampling times (E. Seargent AUSVET, pers. comm.). The prevalence of AGD for each treatment group was analysed at each sample point by a 3x2 chi-square using the statistical package Epi Info™ 2002 (CDC, USA). No statistical analysis was completed for the nets segments due to low sample numbers. A comparison of amoebae attachment in the laboratory trial was analysed using a 3x2 chi-square. The assumption of normality and homogeneity were tested using an f-test. A significance level of $p \leq 0.05$ was adopted.

Results

FIELD TRIAL

AGD prevalence

The mean prevalence of AGD in Atlantic salmon in nets treated with Hempel[®] antifouling paint was higher than in salmon held in untreated nets (Table 4.2). Whilst the nets referred to as Netclear[®] were also untreated nets from May until the conclusion of the trial, the mean prevalence of AGD was higher in comparison to the control and Hempel[®] nets. The control nets recorded the lowest mean prevalence of AGD (21.3%) and the Netclear[®] nets the highest (32.1%).

Table 4.2: The mean AGD prevalence in Atlantic salmon maintained within antifouling paint treated and untreated nets (n = 240 / treatment).

Treatment	Mean AGD Prevalence (% ± S.E).
Control	21.3 (6.43)
Hempel [®]	29.2 (6.74)
Netclear [®]	32.1 (7.37)

A comparison of the treatment groups identified a significant difference in the prevalence of AGD within each time point (Figure 4.2). Fish maintained in Hempel® and control nets had a significantly lower prevalence than Netclear® in April ($p = 0.010$; < 0.05) and July ($p = 0.014$; < 0.05). Fish maintained in Hempel® and Netclear® treated nets had a significantly higher prevalence of AGD in comparison to the control nets in May ($p < 0.05$; < 0.05).

In June there was no significant difference in AGD prevalence between the control and Netclear® nets ($p = 0.110$). Fish held in the control nets had a higher prevalence than Hempel® treated nets however the difference was not statistically significant ($p = 0.263$). The prevalence of AGD was significantly higher in fish held in Netclear® treated nets compared to Hempel® ($p = 0.003$).

In August fish held in Hempel® and control nets had a significantly higher prevalence of AGD in comparison to Netclear® nets ($p < 0.05$; < 0.05). There was no significant difference in prevalence between the control and Hempel® pens ($p = 0.134$).

In September the prevalence of AGD for fish held in the control nets was lower than Hempel® and higher than Netclear® nets however the differences were not statistically significant ($p = 0.110$; 0.191). Fish held in Hempel® treated nets had a significantly higher prevalence of AGD in comparison to Netclear® nets ($p = 0.002$).

The highest prevalence of AGD during the trial was recorded in the fish maintained in Netclear® treated nets in July (62.5%). The lowest prevalence during the trial occurred in fish maintained in the control nets in May (0%). The lowest AGD prevalence of fish held in Netclear® treated nets (10%) was in August. The highest prevalence of AGD of fish held in control nets (52.5%) was in August. Fish held in Hempel® treated nets had the highest prevalence of AGD in September (50%) and the lowest in June (5%).

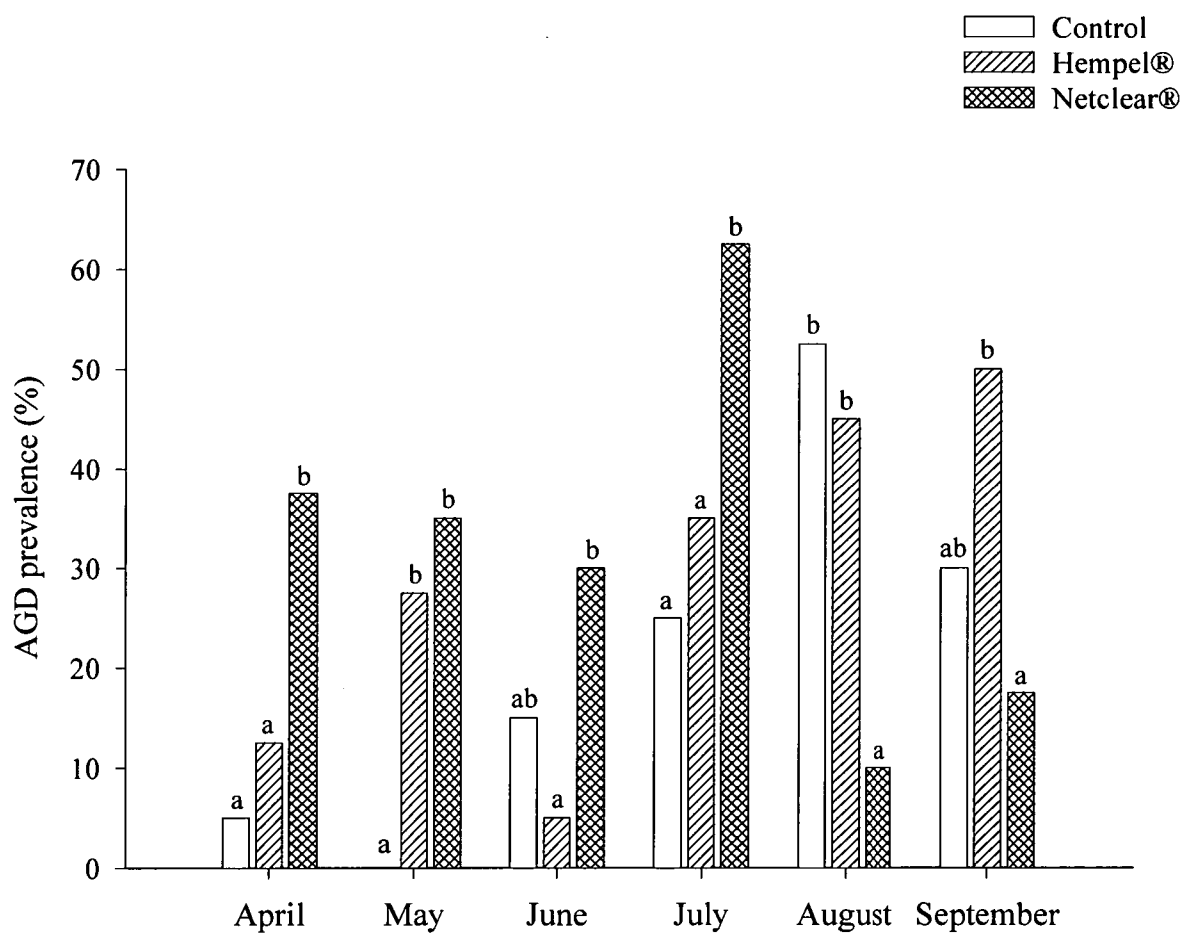


Figure 4.2: AGD prevalence in Atlantic salmon maintained within antifouling paint treated and untreated nets (n = 40 / treatment each month). Different superscripts denote significant differences between treatments within each month at $p < 0.05$.

Prior to the commencement of the trial the pens (with the exception of Hempel[®] B and Netclear[®] A), required freshwater treatment on 3 occasions to alleviate AGD. (Table 4.3) Fish in Hempel[®] pen B recorded the highest number of pre-trial baths (4) whilst the fish in Netclear[®] pen A recorded the lowest (2). Fish in Hempel[®] pen A had the shortest interval between hatchery transfer and the first freshwater bath being required (40 days), whilst fish in control pen A had the longest interval (172 days). With the exception of control pen B and Hempel[®] pen B the remaining pens required treatment with freshwater twice throughout the trial. Salmon in both control pen B and Hempel[®] pen B were treated once.

Table 4.3: The number of freshwater baths used as a treatment for AGD prior to and during the trial. The numbers within the brackets indicate the days between successive baths with the first number representing the first freshwater bath after stocking.

Treatment	Number of freshwater baths prior to trial	Number of freshwater baths during trial
Control A	3 (172, 21, 41)	2 (36, 97)
Control B	3 (160, 27, 43)	1 (36)
Hempel [®] A	3 (40, 136, 41)	2 (48, 92)
Hempel [®] B	4 (127, 29, 21, 47)	1 (43)
Netclear [®] A	2 (149, 29)	2 (47, 99)
Netclear [®] B	3 (127, 21, 48)	2 (43, 79)

Net segments

There were no PCR positive samples for the months of April and May, possibly due to the isolation method used (Table 4.4). Net segments coated with Hempel® antifouling paint had a greater number of PCR positive (5) in comparison to Netclear® (1) and control (2) nets. *N. pemaquidensis* was present on Hempel® nets in the months June, July, August and September. The maximum detection of PCR positive samples was in July (4) with Hempel® treated nets being the highest (2). Control nets were positive in June and July with only one sample for each month. Only one Netclear® treated sample was PCR positive recorded in July.

Table 4.4: The number of Hempel®, Netclear® and control net segments that were PCR positive for the presence of *Neoparamoeba pemaquidensis* (n = 8/treatment for each month).

	TREATMENT		
	Control	Hempel®	Netclear®
April	0	0	0
May	0	0	0
June	1	1	0
July	1	2	1
August	0	1	0
September	0	1	0

LABORATORY TRIAL

There was no significant difference in the number of *Neoparamoeba* sp. adhering to Hempel® paint treated and untreated net segments for days one ($p = 0.384$; Figure 4.3) and fourteen ($p = 0.285$; Figure 4.4) post inoculation. The number of amoebae attaching to the nets was variable within the treatments.

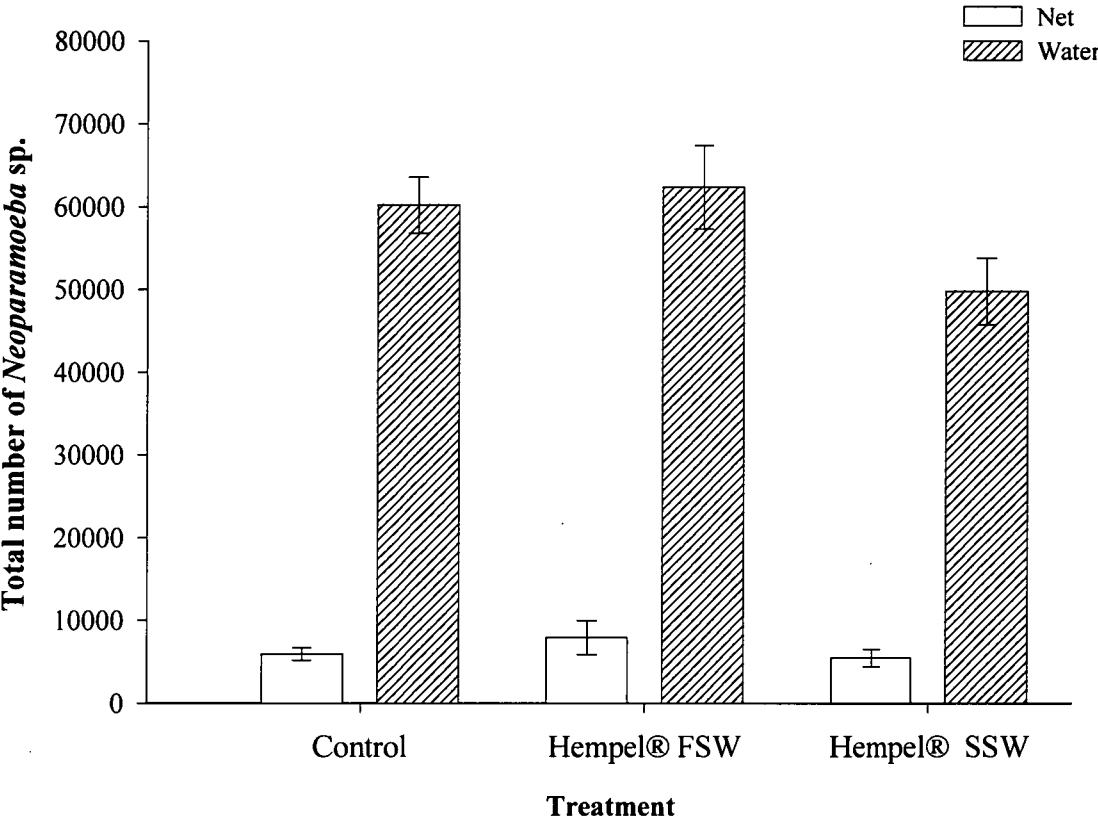


Figure 4.3: Gill derived *Neoparamoeba* sp. attached to Hempel® antifouling paint net segments in 0.2 μ m filtered seawater (FSW) and sterile seawater (SSW) and untreated net segments on day one ($n = 3$).

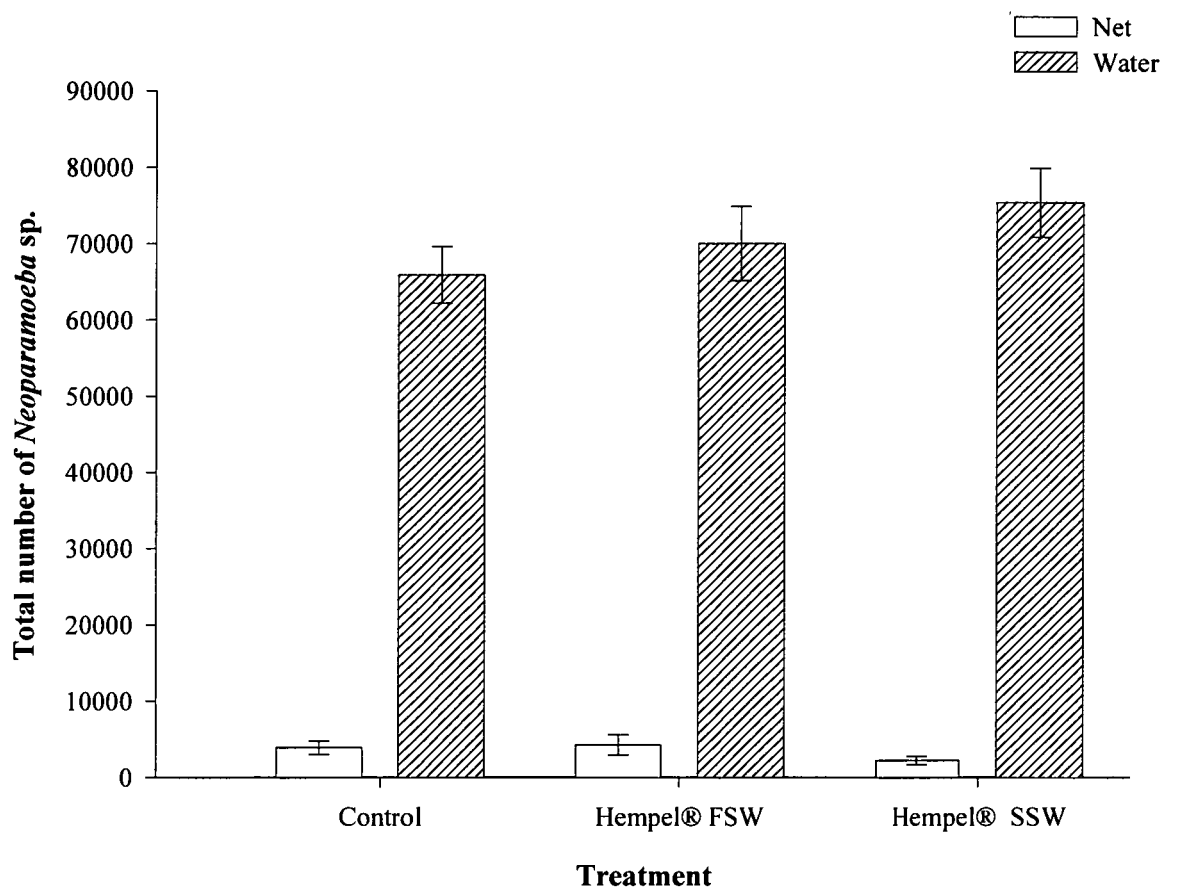


Figure 4.4: Gill derived *Neoparamoeba* sp. attached to Hempel® antifouling paint net segments in 0.2 µm filtered seawater (FSW) and sterile seawater (SSW) and untreated net segments on day fourteen (n = 3).

Discussion

Atlantic salmon maintained in nets coated with Hempel[®], a copper based antifouling paint, had a significantly higher prevalence of AGD. This supports the findings of previous work conducted at the same site in the Huon estuary (Douglas-Helders *et al.*, 2003). However, the prevalence recorded by Douglas-Helders *et al.* (2003), was considerably higher for both Hempel[®] treated nets ($59.5\% \pm 4.5$) and untreated nets ($40\% \pm 5.7$). The previous trial was only conducted over a 10 week period in contrast to this trial, which ran for a period of 6 months (Douglas-Helders *et al.*, 2003). AGD prevalence appears to fluctuate according to seasonal variation, sampling protocols, and annual environmental conditions. Initially *N. pemaquidensis* was not identified on the net samples, however this was believed to be a result of the isolation method. At the third sampling point (June) a new technique was employed (described in Materials and Methods) resulting in PCR positive results for all treatments.

The detection of *N. pemaquidensis* on net segments supports the findings of previous studies conducted in the Huon estuary (Tan *et al.*, 2002; Douglas-Helders *et al.*, 2003). Tan *et al.* (2002) also isolated and identified *N. pemaquidensis* on a number of micro and macro fouling species residing on untreated nets. Fouling can be defined by four significant stages, each a succession of biological and chemical events (Grasland *et al.*, 2003; Thouvenin *et al.*, 2003). The initial stage occurs within seconds of immersion and results in the formation of a conditioning film. The second, third and fourth stages result in the attachment of bacteria, unicellular species and eukaryotes respectively (Grasland *et al.*, 2003; Thouvenin *et al.*, 2003). A combination of chemical, biological and temporal factors in conjunction with the solid surface properties influence the organisms that adhere to the film (Grasland *et al.*, 2003).

The presence of *N. pemaquidensis* on both copper antifouling treated and untreated nets suggest that amoebae find the surface conditions of both suitable for attachment. Copper treated nets appeared to provide a more favourable environment for amoebae to attach. It is suggested that cuprous oxides ability to attract four times more bacteria provides an abundant food source that attracts

Neoparamoeba sp. (Dempsey, 1981; Douglas-Helders *et al.*, 2003). A recent study suggested that *Neoparamoeba* sp. are not primarily bacterivorous as previously believed, after *Neoparamoeba* clones were not identified with bacteria in the cytoplasm over a period of subculturing (Dyková *et al.*, 2005). Previously primary isolates of *Neoparamoeba* sp. have been cultured on malt yeast agar plates seeded with autoclaved bacteria species such as *Pseudomonas* sp. (Kent *et al.*, 1988; Munday *et al.*, 1990; Dyková *et al.*, 2000; Paniagua *et al.*, 2001; Crosbie *et al.*, 2003). During subculturing, bacteria can survive from the primary isolation (Dyková *et al.*, 2005). The observations made by Dyková *et al.* (2005) were not consistent for all subcultured samples used in the study. Previous studies have found *in vitro* *Platyamoeba* sp. and *Acanthamoeba castellanii* growth to be enhanced when certain bacteria species are present (Bottone *et al.*, 1992; Paniagua *et al.*, 2001). The presence of bacteria *Winogradskyella* sp. has been suggested to enhance the severity of AGD infections (Embar-Gopinath *et al.*, 2005). The increase in bacteria in conjunction with an increase of *Neoparamoeba* sp. may suggest that there is an association between the two with the bacteria in some form providing a food source for the amoebae.

Nets coated with copper antifouling paint may have altered surface conditions in comparison to untreated nets. Whilst all the nets used were comprised of multi-filament material that naturally provides an ideal substrate for fouling, the use of copper antifouling paint may have modified surface properties such as electrical charge that in turn influences the type of organisms able to settle on the net (Hodson *et al.*, 2000; Grasland *et al.*, 2003). High concentrations of copper are toxic to certain organisms, however a number of bacteria are able to colonise the treated surface and provide a protective barrier allowing other organisms to adhere (Dempsey, 1981; Børufsen Solberg *et al.*, 2002). The composition of the biofouling able to attach to copper treated nets may provide a preferential environment for *Neoparamoeba* sp. as a result of reduced competition. Therefore it may be the environment copper treated nets promote that attracts *Neoparamoeba* sp. not copper itself.

Whilst the results of this trial are interesting and support previous findings they must be interpreted with caution, as the trial pens were not treated consistently during the trial. Huon Aquaculture's protocol is to stock smolt from the hatchery at Pillings Bay, a brackish lease site in the Upper Huon Estuary. This practice has been shown to delay the onset of AGD when compared to smolt stocked at full salinity sites (Clark and Nowak, 1999). Despite *Neoparamoeba* sp. being in the environment and on the gills of Atlantic salmon at Pillings Bay, AGD is minimal and treatment with freshwater is rare (A. Steenholdt pers. comm.). The smolts used in this trial were maintained at Pillings Bay in untreated nets for approximately 5 months. With the exception of one pen freshwater bathing was not required until the fish were transferred to full salinity. The fish requiring freshwater bathing at Pillings Bay did so only 40 days post transfer from the hatchery and were later used to stock one of the Hempel[®] treated nets. Why this population was more susceptible to AGD is unclear. Farms identify AGD by observing salmon gills for the presence of white mucoid patches. This association can be incorrect in field applications as a number of other pathogens and toxicants can also produce this response (Adams *et al.*, 2004). If the patches were a result of AGD this suggests that these salmon had a higher sensitivity to the presence of *Neoparamoeba* sp., which may have altered the findings in this trial.

The only treatment effective in alleviating AGD outbreaks is bathing the salmon in freshwater, a practice developed in the late 1980's (Foster and Percival, 1988; Parsons *et al.*, 2001). The amoebae are dislodged from the gills due to a low tolerance to low salinity and hypersecretion of gill mucus (Munday *et al.* 1990; Clark *et al.*, 2000; Parsons *et al.*, 2001). A small proportion of the *Neoparamoeba* sp. can remain viable following treatment (Parsons *et al.*, 2001). Prior to the trial commencing the fish were treated with freshwater but not according to a consistent protocol. The majority of the pens were bathed between eight and sixteen days prior to the initial sampling point. However, for one of the Hempel[®] and one of the Lanolin pens, the fish were bathed twenty-seven and forty-one days respectively. The number of freshwater baths conducted prior to the trial commencing was also inconsistent. Three baths was common, however one of the Hempel[®] pens received four and one of the Lanolin pens only received two. The

salmon were all maintained in untreated nets prior to the trial and therefore differences cannot be attributed to the use of antifouling paint. The inconsistent bathing schedules used prior to and during the trial make it difficult to correlate differences in AGD prevalence to the use of antifouling paint on the nets.

When comparing the presence of *Neoparamoeba* sp. on the net segments against the treatment used the results must be interpreted with caution. The commercial cages used in this trial were fitted with 120 m x 12 m nets whereas the sample taken each month to culture and isolate *Neoparamoeba* sp. was 4cm x 4cm. It is quite plausible that sampling such a small area may have resulted in false negatives. The net size taken was restricted as they were being cut directly from commercial pens. Larger samples would have undermined the integrity of the net and as these were first and foremost commercial cages containing valuable stock. The farm could not risk salmon escaping or seals gaining access to the net. It is important to remember that whilst *Neoparamoeba* sp. can be detected we have no method to quantify their presence in environmental samples. Whilst untreated nets had fewer samples with *Neoparamoeba* sp. present, it cannot be assumed that there were less amoeba. All that can be said is that a greater number of copper treated net samples had *Neoparamoeba* sp. present in comparison to untreated nets. Numbers cannot be estimated and the statement “copper treated nets attract more *Neoparamoeba* sp. than untreated nets” cannot be made with conviction.

Whether copper is as an attractant for *Neoparamoeba* sp. is unclear as demonstrated by the experience of salmon farms in Macquarie Harbour. Between 1916 to 1994 Mount Lyell copper mine in Queenstown, Western Tasmania discharged tailings into King River and Macquarie Harbour, resulting in elevated dissolved and particulate copper concentrations (Teasdale *et al.*, 1996). Copper levels in Macquarie Harbour have been recorded between 6 $\mu\text{g L}^{-1}$ and 140 μm (Koehnken, 1997; Douglas-Helders *et al.*, 2005). *Neoparamoeba* sp. has been isolated from Macquarie Harbour sediment however salmon farmed in the area remains free from AGD (Crosbie *et al.*, 2003). Douglas-Helders *et al.* (2005), found that the survival of three strains of *Neoparamoeba pemaquidensis* exposed to Macquarie Harbour water was significantly reduced. The salinity of the water

however was 17 ppt and when adjusted to 36 ppt there was no effect on the amoebae's survival (Douglas-Helders *et al.*, 2005). This suggests that the salinity of Macquarie Harbour not the elevated copper concentration inhibits *Neoparamoeba* sp. survival.

The results of the laboratory trial are difficult to extrapolate to field conditions. Whilst the samples were placed on a rocking platform in an attempt to replicate water movement in the field, the simulation was not realistic especially in such a small volume of water. The use of filtered and sterile seawater may have precluded *Neoparamoeba* sp. from settling on the net segments due to the lack of a conditioning layer seen in the natural environment. Normal seawater was not used as amoebae may have been present and it would have been impossible to differentiate between these and the gill derived *Neoparamoeba* sp. The low numbers of amoebae attaching to the netting may have been a result of the plastic tube providing a more favourable attachment surface. Morrison *et al.* (2004) developed a method to partially purify *Neoparamoeba* sp. by adherence to plastic petri dishes. Despite the large variation seen even within treatments, no difference in the number of *Neoparamoeba* sp. attaching to copper coated and untreated net segments was evident. Perhaps copper antifouling paint treated nets do not act as an attractant for *Neoparamoeba* sp. as previously suggested (Tan *et al.*, 2002; Douglas-Helders *et al.*, 2003) however, further research is required to verify the outcomes of this trial.

Apart from a small laboratory trial (Tan *et al.*, 2002) no studies have investigated whether *Neoparamoeba* sp. residing on nets can cause AGD in Atlantic salmon. To date we are unsure of the movement patterns of *Neoparamoeba* sp. Do they move from the nets to the salmon or vice versa, or is their movement multi-directional? Whilst it appears that copper paint treated nets may be a reservoir for *Neoparamoeba* sp. they cannot be labelled a risk factor as there is no evidence to suggest that the amoebae on the nets cause AGD. Further research is required to investigate this relationship. As well as compare the biofouling communities present on copper treated and untreated

nets. From this study it appears that copper singularly is not an attractant for *Neoparamoeba* sp.

Chapter 5 – The effect of continuous artificial lights on the prevalence of AGD.



Introduction

The occurrence of early sexual maturation in cultured Atlantic salmon can be a costly process resulting in reduced growth and food conversion, a decline in condition and increased mortalities and susceptibility to diseases (Myers, 1984; Taranger, 2004). Generally farms must either harvest mature fish prior to the targeted size or discard them (Porter *et al.*, 2003). Harvested fish are often downgraded to a lower value product when processed as a result of soft flesh and gaping myotomal muscle (Mitchie, 2001).

The onset of early maturation is a production constraint not confined to sea cage Atlantic salmon. The production of good quality large rainbow trout is notoriously difficult as a result of early maturation and a high proportion of male flatfish and sea bass mature before reaching the required harvest size (Taranger, 2004). A number of studies have linked early maturation with several variables including environmental cues such as photoperiod and water temperature, lipid stores and nutritional status as well as genetically fixed traits (Johnston *et al.*, 2004; Taranger, 2004).

To combat early maturation Atlantic salmon farms located in the northern hemisphere are incorporating the use of artificial lights as a standard husbandry protocol (Oppedal *et al.*, 2001). Maintaining the salmon under continual artificial light until early summer has increased the efficiency of production dramatically through improved growth rates and postponed sexual maturation (Oppedal, 1997; Duncan *et al.*, 1999; Porter *et al.*, 1999; Oppedal *et al.*, 2001; Juell *et al.*, 2003). Initially surface lights were used however due to energy loss from surface reflection, an increase in hazards for boating traffic and aesthetic concerns, submerged lights have been introduced (Hevrøy *et al.*, 2003).

Both light intensity and water temperature have a strong influence over the behaviour of sea cage Atlantic salmon. The depth at which salmon swim is a result of light levels and a trade off between food availability and hunger and varies with seasonal changes (Fernö *et al.*, 1995; Oppedal *et al.*, 2001; Hevrøy *et al.*, 2003). Generally salmon swim deeper in the water column and maintain a

school structure during the day (Juell *et al.*, 2003). At dusk the salmon ascend towards the surface and typically the school structure breaks down and swimming speeds reduce (Fernö *et al.*, 1988). The mimicking of daylight conditions with continuous artificial lights results in the salmon maintaining daytime behaviour. At night the fish tend to remain deeper in the water column and maintain circular schooling and swimming speed (Oppedal *et al.*, 2001). Whilst this has been shown to improve production efficiency, limited work has been conducted on the consequences of altering the natural behaviour patterns (Juell *et al.*, 2003).

When introducing new husbandry practices it is essential to understand the flow-on effects that may result. In Scotland the initial use of artificial lights led to an increase in the number salmon infected by the sea lice *Lepeophtheirus salmonis* (Hevrøy *et al.*, 2003). *L. salmonis* is a marine parasite that relies on the presence of salmonid species such as Atlantic salmon for survival. The pelagic copepodid stage has been identified as strongly phototactic and the use of artificial lights can result in aggregates of copepodids accumulating around the light source (Bron *et al.*, 1993).

Altering the distribution and behaviour of the salmon or the amoebae as a result of a continual artificial light source may affect AGD prevalence. This study investigated the effect of continuous artificial light on the prevalence of AGD in Atlantic salmon maintained in experimental cages in the Huon Estuary, Tasmania, Australia over a 12 month period.

Materials and Methods

Experimental design

This trial was conducted in collaboration with Dr Porter from the School of Aquaculture, University of Tasmania (CRC 1A.6, FRDC 2001/246). Atlantic salmon were maintained in replicated experimental pens (5 x 5 m) located in the Huon Estuary Tasmania, Australia (Figure 5.1). Each pen was stocked with approximately 700 fish, of which approximately 250 were tagged with a passive integrated transponder (PIT) tag. Two pens had a 400 w Pisces Aquabeam™ underwater light suspended centrally at a depth of 2m. The lights were activated on the 25th April 2003 on a 24 hour regime until the 22nd December 2003 (the summer solstice). Lights were not used beyond this point. The sampling began in June 2003 and concluded in June 2004. Initially sampling occurred every 6 weeks, until November 2003 and every 9 weeks there after.

Fish

A population of female, out of season diploid Atlantic salmon smolt was sourced from Wayatinah hatchery, Tasmania, Australia. Four months prior to seawater transfer 1000 randomly selected individuals had a PIT tag placed under the skin behind the posterior dorsal fin. The posterior fin was then clipped to ensure easy visual identification. In April 2003 the fish averaging 87 g were transported from the hatchery to Hideaway Bay in the Huon Estuary, Tasmania, Australia (Figure 5.1). The fish were randomly distributed between the 4 trial pens

Husbandry protocol

The fish were fed with commercial salmon pellets (Skretting, Australia) using an Aquasmart™ automated demand feeder. The farm staff monitored the salmon for AGD on a weekly basis (Refer to General Materials and Methods 2.1), and the fish were treated with a freshwater bath approximately every 6 weeks. When a bath coincided with a sampling point it was undertaken immediately after. The trial pens were all bathed at the same time.

Sample collection

Fish were dip netted from a pen and placed into a holding container and anaesthetised with Aquis® [0.05 %] (Lower Hutt, New Zealand). The PIT tagged fish were identified and separated into a second container with a lower concentration of Aquis® (0.01%). The length and weight of the PIT tagged fish were recorded and the gills of 30 fish (identified by alcian blue marks on the abdomen) were examined for the presence of white patches and assigned a gill score (Refer to General Materials and Methods 2.1). These fish were monitored for the duration of the trial. Thirty untagged fish were lethally sampled, length and weight recorded, the gonad removed and weighed to calculate Gonadosomatic Index (GSI) and the left gill basket was removed and placed in seawater Davidson's fixative for histological processing and examination. The remaining population were transferred to a liner with oxygenated freshwater for approximately 4 hours before being returned to their original pen.

Identification of *Neoparamoeba* sp. on salmon gills

Gill sections stained with H&E were observed by light microscopy for the presence of lesions and associated *Neoparamoeba* sp. (General Materials and Methods 2.9).

AGD case definition

- 1) A fish with a positive gill score (the presence of white plaques) was defined as AGD positive (non-lethal sampling).
- 2) A fish identified by histology with gill lesions in association with amoebae containing a parasome was defined as AGD positive (lethal sampling).

GSI formula

$$\text{GSI} = \text{Gonad weight (g)} / \text{Body weight (g)} \times 100$$

Salmon were identified as maturing or mature when the GSI was greater than 1.

Statistics

The number of AGD positive fish and GSI for the artificially lit and unlit pens was examined at each time point by chi-square (2x2) using the statistical package Epi Info™ 2002 (CDC, USA). The correlation between GSI and percentage of gill lesions was analysed using Microsoft Excel. The relationship between AGD positive and negative fish and GSI was examined using a student t-test in Microsoft Excel. The total number of mortalities recorded over the trial period was analysed using a chi-square (2x2) table. A significance level of $p \leq 0.05$ was adopted (where the p value exceeds four decimal places <0.001 is used).

Results

Only 33 of the original 120 PIT tagged fish marked with alcian blue remained at the conclusion of the trial. There was no consistent pattern observed in the recorded gill scores.

Of the lethally sampled fish a total of 77 out of 343 were identified as AGD positive in the lit pens and 89 out of 355 positive in the unlit pens. Salmon maintained under artificial lights had a significantly higher prevalence of AGD in August ($p = < 0.001$; Figure 5.2). In November and February the prevalence of AGD was significantly higher in salmon held under natural light conditions ($p = 0.005$; $p = 0.002$ respectively). A comparison of AGD prevalence between the treatment groups identified no significant difference for the remaining four sample points. Salmon held under natural light conditions recorded no AGD in August and September, whilst fish in the artificially lit pens had no AGD only in September. The maximum AGD prevalence recorded (43 %) was in July in salmon maintained under artificial lights. The lowest prevalence of AGD (0 %) was observed in both treatment groups during the winter months.

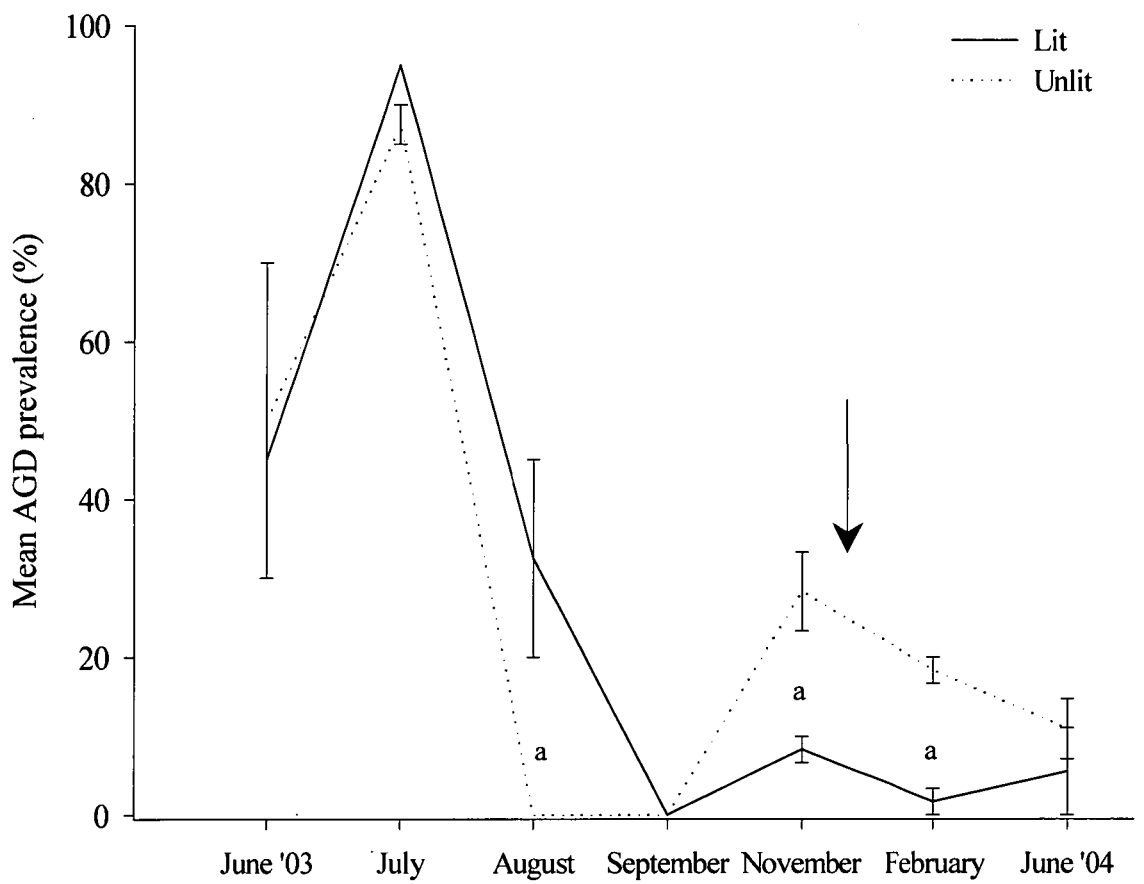


Figure 5.2: Mean AGD prevalence (\pm S.E) recorded in Atlantic salmon maintained under continuous artificial light and natural light conditions over a 12 month period. Superscripts denote a significant difference within that month. The arrow indicates when the lights were turned off.

Significant differences in the percentage of gill filaments with lesions appeared to be a reflection of AGD prevalence. In August salmon maintained under artificial lights had a significantly higher number of filaments affected with lesions ($1.49 \% \pm 0.37$) compared to the unlit fish (0% ; $p = < 0.001$; Figure 5.3). AGD prevalence was also significantly higher in the lit pens. The percentage of affected gill filaments was significantly higher in fish held under normal light conditions for November ($3.67 \% \pm 1.00$; $p = < 0.001$) and February '04 ($2.94 \% \pm 1.15$; $p = 0.02$). The prevalence of AGD was also significantly higher in the unlit pens for November and February. In July however, despite no significant difference in AGD prevalence, the fish maintained in artificially lit pens had a significantly higher percentage of gill filaments with lesions ($43.1 \% \pm 4.38$) when compared with the unlit pens ($14.11 \% \pm 1.96$; $p = < 0.001$).

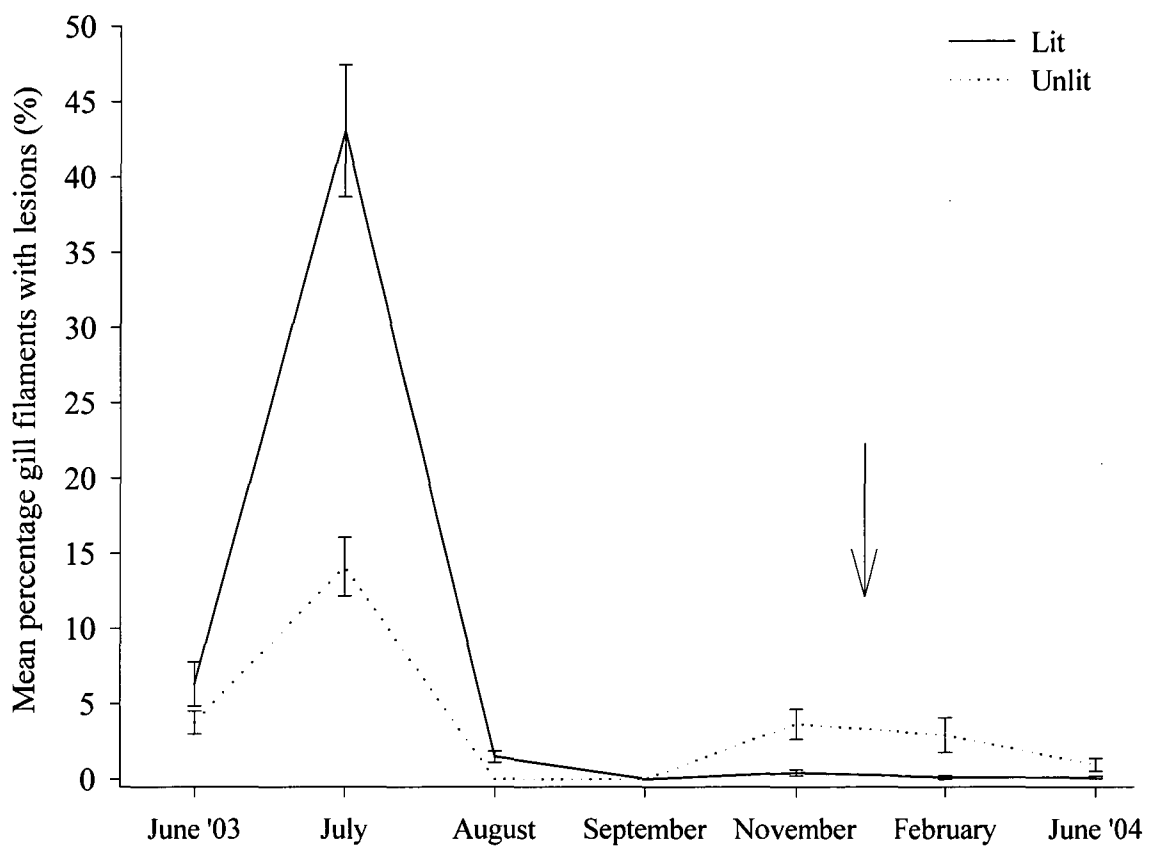


Figure 5.3: Percentage of gill filaments (\pm S.E) affected with lesions in Atlantic salmon maintained under continuous artificial light and natural light conditions over a 12 month period. Superscripts denote a significant difference within that month. The arrow indicates when the lights were turned off.



Figure 5.4: A representative gill section stained with H&E taken during the 12 month trial.

Atlantic salmon maintained under natural light conditions had significantly higher GSI for the majority of sample points. The unlit pens had a significantly higher level of mature/maturing salmon (44) in comparison to the lit pens (8) ($p = < 0.001$; Figure 5.5). The highest GSI was recorded in June '04 (30.3 %) in the unlit pens. There was no relationship between GSI and AGD particularly in February ($p = 0.436$) and June '04 ($p = 0.126$) when the maturing fish were recorded. There was no correlation between GSI and the percentage of gill filament with lesions ($r^2 = 0.001$; Figure 5.6). There was no significant difference in mortality rates between the lit and unlit pens ($p = 0.819$).

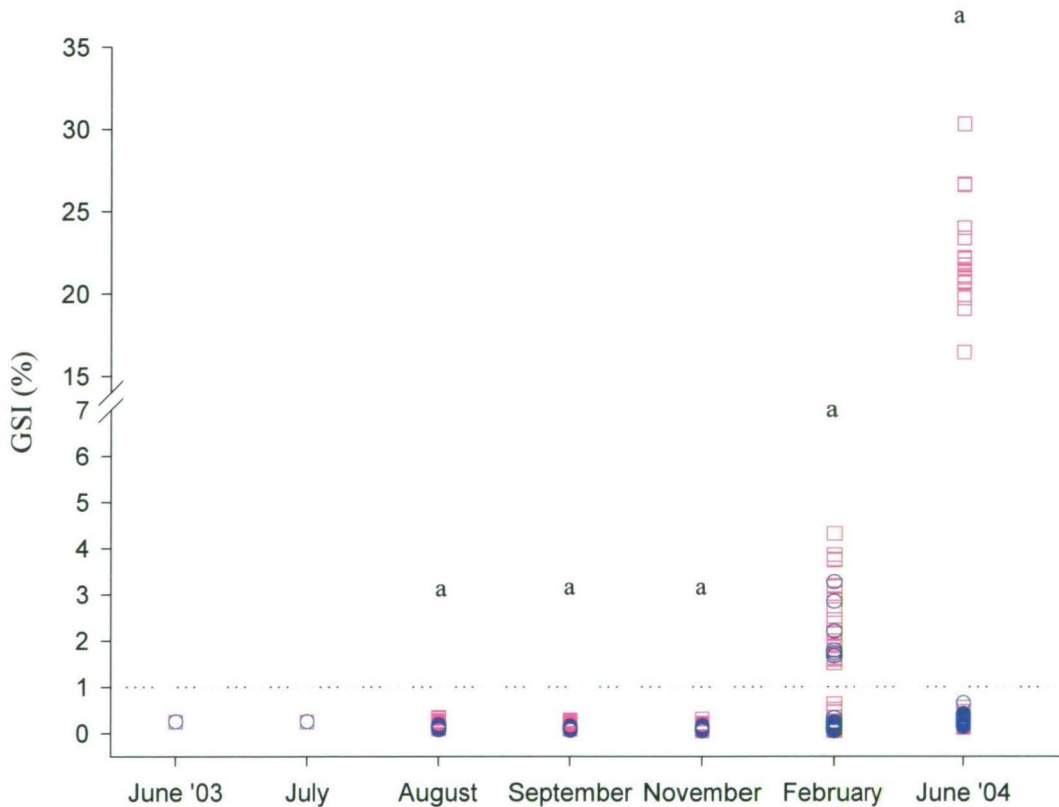


Figure 5.5: The GSI recorded in Atlantic salmon maintained under continuous artificial light (□) and natural light (○) conditions over a 12 month period. Superscripts denote a significant difference within that month. (Line indicates the point at which fish are classed as maturing)

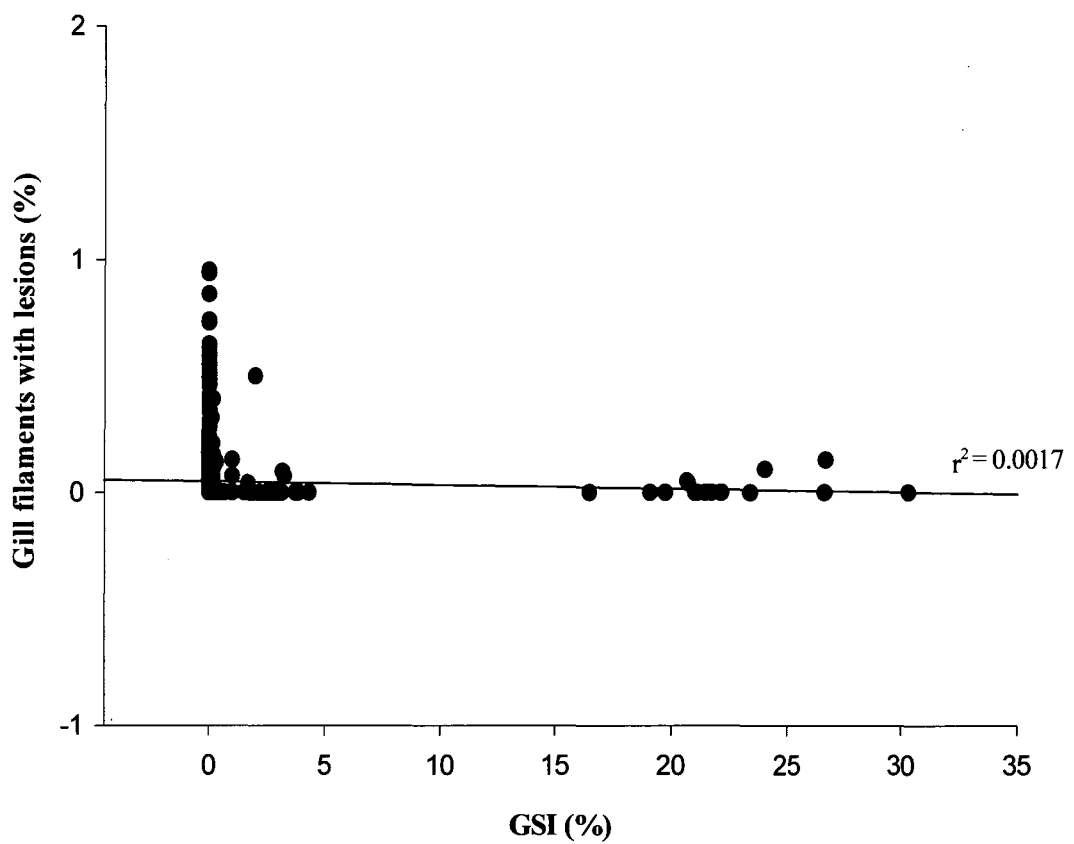


Figure 5.6: A comparison of GSI and gill filaments with lesions recorded in Atlantic salmon maintained under continuous artificial lights and natural light conditions over a 12 month period (n = 698).

Discussion

Whilst there were some significant differences observed in the prevalence of AGD for Atlantic salmon maintained under continuous artificial light and natural light conditions, the majority of the seven sampling events recorded no significant difference. The fact that all the cages were treated routinely with freshwater may have masked the impact artificial lights had on AGD prevalence and any effects on production. Generally commercial operations only use freshwater baths when required and the period between treatments can be sporadic. The percentage of gill filaments affected by lesions generally reflected the findings for AGD prevalence. Salmon reared under natural light conditions had a significantly higher number of mature or maturing fish ($GSI < 1\%$), however only a very small number of fish out of the total population were maturing or mature for both treatment groups.

Examining the prevalence of AGD as well as the percentage of gill filaments with lesions gave an indication of the severity of an outbreak. The results showed that when a significant difference in AGD prevalence was detected there was also a significant difference in the percentage of gill filaments affected. However, in July despite no significant difference in AGD prevalence, the salmon maintained under artificial lights recorded a significantly higher percentage of affected filaments. This difference is interesting as both treatment groups were subjected to stressful conditions prior to sampling. An unfortunate error in August resulted in all the salmon being bathed in water with salinity greater than 10 ppt that resulted in an AGD outbreak (A. Steenholdt pers. comm.). The use of artificial lights to suppress sexual maturation coupled with stressful culture conditions appeared to enhance the severity of the AGD outbreak.

The routine bathing of salmon in freshwater approximately every 6 weeks appeared to maintain the prevalence of AGD at an acceptable background level. When dealing with an endemic pathogen it is unrealistic to expect long periods of no disease unless an effective treatment is available. Salmon farms in Tasmania consistently record a background level of AGD in particular farms located in the southeast such as the Huon Estuary. It is only when the AGD prevalence exceeds

the background level that the term outbreak is used to describe the disease status. The use of freshwater bathing on a regular basis resulted in low levels of AGD being recorded throughout this trial even during the summer months when outbreaks are common. Whether the regular use of freshwater has future implications such as amoebae developing a resistance is unknown and needs to be investigated especially as freshwater bathing is the only effective treatment currently available to the industry.

During this trial very few fish in both treatment groups were classified as maturing ($GSI < 1\%$) or mature. Interestingly the maturing/mature fish were all observed to be AGD positive. The process of sexual maturation can be influenced by a number of factors. Oppedal *et al.* (2003) were surprised when no differences in maturity levels were detected between salmon maintained under continuous artificial light and simulated natural photoperiod. However, as with this trial the work was conducted in small research cages with lower fish densities than used in commercial conditions and may have influenced the process of sexual maturation (Oppedal *et al.*, 2003).

Since the completion of this trial a commercial scale trial has been conducted in the same area. Interestingly AGD outbreaks are regularly observed in the salmon maintained under artificial lights approximately one month in advance of the unlit pens (A. Steenholdt, pers. comm.). The use of continuous artificial lights has been shown to alter sea-cage salmon's natural behaviour (Huse and Holm, 1993; Juell *et al.*, 1994; Fernö *et al.*, 1995; Oppedal *et al.*, 2001). Instead of the typical ascent to the surface at dusk, disbanding of schooling and a reduction in swimming speed, salmon maintained under artificial lights tend to remain deep in the water column and maintain schooling and swimming speed (Oppedal *et al.*, 2001; Juell *et al.*, 2003). The implications of this modification in behaviour and AGD are unknown.

The continuation of schooling throughout the night in artificially lit pens may result in higher densities and a greater horizontal transmission of *Neoparamoeba* sp. Currently experimental infection of AGD can only be achieved by two

methods; the use of crude gill extracts containing *Neoparamoeba* sp. or the co-habiting of infected salmon with naïve fish (Zilberg et al., 2001; Morrison *et al.*, 2004). AGD infections are easily induced by the co-habitation method indicating that horizontal transmission is a major concern. The continuation of swimming speed as a result of artificial light may also hasten the onset of AGD. The greater volume of water passing through the salmon's gills may promote a greater interaction with *Neoparamoeba* sp. in the water column.

Juell *et al.* (2003) found the use of submerged lights resulted in salmon swimming at greater depths in the water column. The ramifications of this with regards to AGD are quite important. The Huon Estuary generally has a surface layer of freshwater as a result of the surrounding rivers (CSIRO Huon Estuary study team, 2000). The depth this layer penetrates is dependent upon seasonal variation but generally is down to 1 m from the surface. If submerged lights cause the salmon to remain deeper in the water column they may not be interacting with the surface freshwater layer as regularly as salmon maintained under natural light conditions and could explain the earlier onset of AGD. Whilst this surface layer will not achieve the same results as immersing fish in a freshwater bath for several hours, it may assist in extending the period between treatments. Another possibility may be that the salmon remaining deeper in the water column brings them in closer contact to amoebae within the sediment. The movement patterns and transmission method of *Neoparamoeba* sp. are not currently known and therefore it is difficult to speculate on this interaction.

In Scotland research is being conducted on the use of artificial lights and its effect on sea lice infections (Hevrøy *et al.*, 2003). Sea lice are strongly phototactic parasites that congregate near the surface during daylight hours (Kadri *et al.*, 1991; Bron *et al.*, 1993; Hevrøy *et al.*, 2003). Salmon maintained in pens that allowed a deeper vertical distribution recorded significantly lower sea lice infection as they had less contact with the parasites compared to salmon maintained in shallow pens (Hevrøy *et al.*, 2003). The positioning of artificial lights at different depths could be used as a management strategy to avoid harmful environmental conditions and parasitic infections (Juell *et al.*, 2003). Tasmanian

salmon farmers need to investigate this theory further as it may be a useful disease management strategy for AGD. Perhaps artificial lights can be positioned to draw the salmon into the surface freshwater layer more often resulting in a reduction in AGD prevalence. This would also require a sound knowledge of *Neoparamoeba* sp. in terms of phototactic response if any and position in the water column. No information was available justifying the chosen depths for positioning artificial lights in salmon pens.

It is essential that further researches undertaken to determine the impact of artificial lights on the prevalence of AGD based on the findings of this trial and the work currently being completed on a commercial scale. The very nature of sea cage aquaculture is an extremely stressful environment for Atlantic salmon. Throughout the grow-out phase they endure many stressful conditions including high water temperatures, biological stressors such as jellyfish and crowded conditions. It is important to understand how suppressing sexual maturation through the use of artificial lights impacts the immune system of Atlantic salmon and their ability to cope with an endemic pathogen such as *Neoparamoeba* sp. The introduction of a husbandry protocol that promotes the onset of AGD during stressful conditions is a costly process the industry cannot accept.

Chapter 6 – Case study of an Atlantic salmon farm located in Northern Tasmania



Introduction

Atlantic salmon farms located in the Huon Estuary, southern Tasmania, are the state's major salmon producers, however they are also the most affected by AGD. No salmon farms were located in the north of the state until 2000 when a lease in the Tamar River was granted (DPIWE, 2004). This farm is unique in Tasmania as it consists of a 'system farm' developed by Marine Construction, Norway. The system is anchored 75 m offshore and consists of eighteen 24 m x 24 m galvanised steel modular cages with zinc coated steel nets. The cages are locked together, unlike farms in southern Tasmania where individual polar circle pens with nylon nets are anchored throughout a lease site.

The site in the Tamar River is relatively small and consists of only one area, whereas other salmon companies have a number of large lease sites. Despite *Neoparamoeba* sp. having been isolated and identified in the sediment of the Tamar River (Crosbie *et al.*, 2003), this site remained AGD free until April 2005. The conditions of the Tamar River are very different to those in the Huon Estuary especially, salinity, proximity of other salmon farms and the velocity of currents (DPIWE, 2004). It has been suggested that environmental conditions have the greatest influence on AGD (Gross *et al.*, 2004). A closely related species, *Neoparamoeba branchiphila*, has been isolated from the gills of AGD infected salmon however its involvement in the disease is presently unknown (Dyková *et al.*, 2005). DNA primers were available for *N. branchiphila* therefore it was included in this study.

This trial was conducted in an attempt to further investigate the presence of *Neoparamoeba* spp. in the Tamar River and its possible presence on the salmon cage nets. Nylon and steel nets, sediment and fish were all examined over a one month period for the presence of *Neoparamoeba* spp. in an attempt to better understand why this farm was unaffected by AGD for so long.

Approximately 12 months after the conclusion of the trial the farm recorded its first outbreak of AGD. The disease began in the maturing marine pre-smolt '04 year class. According to farm records these maturing fish were laying on the

bottom of the steel nets prior to the outbreak. Initially mortalities in these fish were approximately 17 - 21 per cage per day. After one month the mortalities rose to approximately 224 per cage per day with the majority of these being mature fish. The Department of Primary Industries, Water and Environment Animal Health diagnosed AGD but detected no *Neoparamoeba* sp. using IFAT. The farm was logistically unable to treat the outbreak with freshwater and chose to cull the AGD affected, maturing and triploid fish instead. Environmental and histology sampling were undertaken to investigate this outbreak.

Materials and Methods

Experimental design

An isolated Atlantic salmon farm located in the Tamar River, northern Tasmania, Australia was investigated for the presence of AGD in fish and *N. pemaquidensis* and *N. branchiphila* in the environment (Figure 6.1). Due to a large variation in depth across the farm site (16 – 30 m) the replicated trial cages (24 m x 24 m) were assigned at opposite ends of the farm (A and B at 16 m and C and D at 30 m). The trial commenced at the beginning of March 2004 with baseline samples of sediment taken to examine the initial presence of *N. pemaquidensis* and *N. branchiphila*. The AGD status of the fish was also determined. A panel of nylon and steel net (50 cm x 50 cm) was placed side by side at a depth of 5 m within each trial cage. One month later at the beginning of April 2004, sediment and net samples were examined for the presence of *N. pemaquidensis* and *N. branchiphila* and fish for the presence of AGD.

Fish

Atlantic salmon smolt obtained from Tasmanian commercial hatcheries were transferred to the Tamar River site in 2003 and stocked at approximately 35 000 per cage. Nylon nets (1 x 1 cm) had to be hung within the steel nets until the fish were approximately 400g to prevent the smolt from escaping through the steel nets (approximately 3 months).

Husbandry protocol

The trial pens were fed with commercial salmon pellets (Skretting, Australia) using an automated feeder. Salinity, temperature and dissolved oxygen were recorded on a daily basis by farm staff.

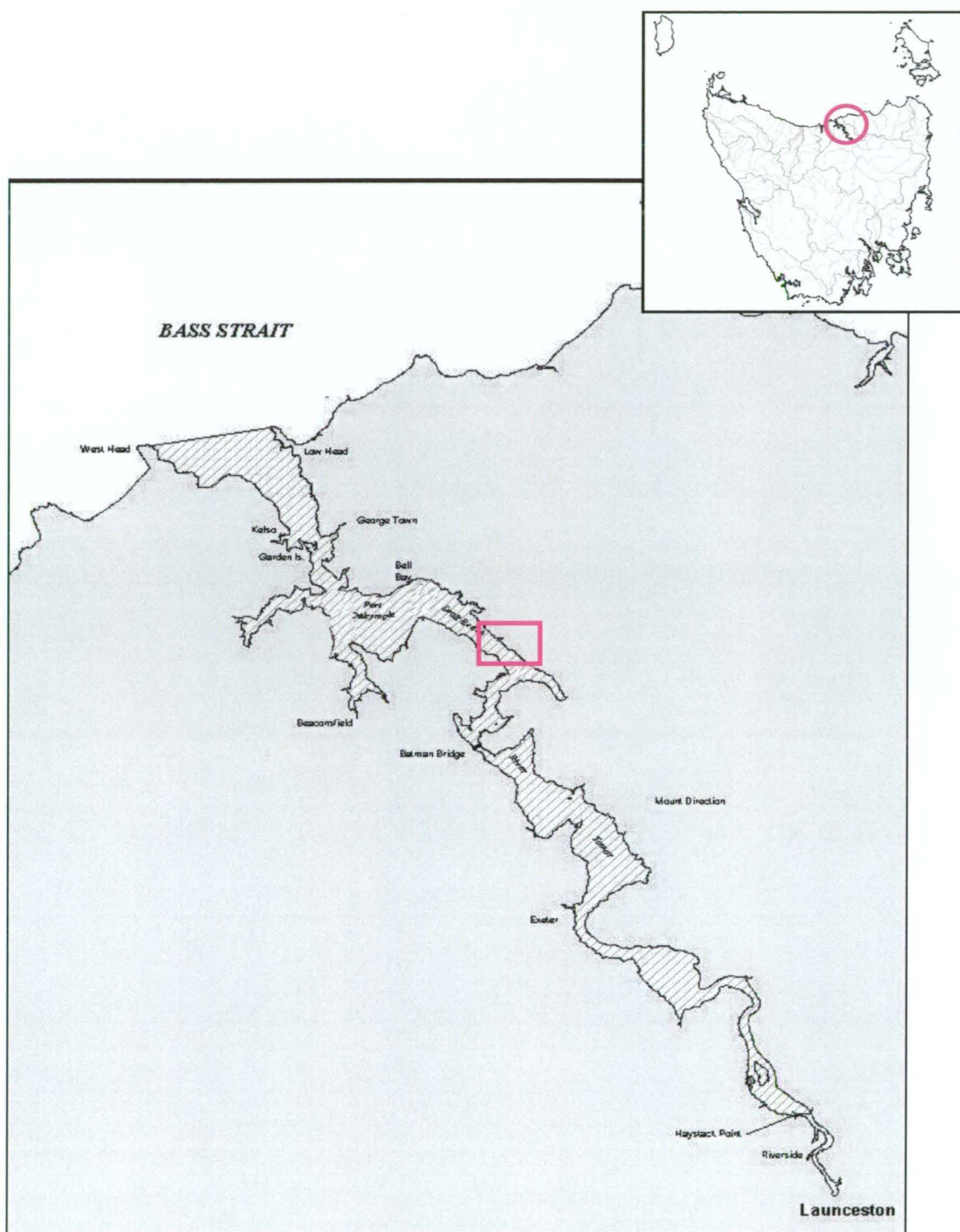


Figure 6.1: Map of Tamar River in northern Tasmania showing the location of the Atlantic salmon farm.

Sample collection

Within each cage the fish were crowded using a box net. Twenty fish were removed and anaesthetised using Aquis[®] [0.5%] (Lower Hutt, New Zealand). The gills were examined for the presence of white patches and assigned a gill score (refer to General Materials and Methods 2.1). A mucus scraping was taken from the gills and placed in sterile seawater for examination by immuno-dot blot. If white patches were observed then the scraping was taken from that area. The samples were placed on ice until reaching the laboratory and then stored at -20°C until analysis. Ten of these fish were euthanased, their left gill arch removed and placed in seawater Davidson's fixative for processing and histological examination at a later date. The remaining fish were revived and returned to the pen.

Small pieces of nylon and steel net (approximately 4cm x 4 cm) were cut from the panels suspended within each trial pen by divers and placed in a sealed container whilst underwater. Upon reaching the shore the segments were divided into four pieces and each placed on a separate MYA plate, labelled and sealed. Upon returning to the laboratory, the samples were maintained in a temperature-controlled incubator at 19-20°C for up to 14 days.

Two sediment grabs per trial cage were taken, one from either end of the cage and placed in individual 1 L containers. Five drops of sediment each being approximately 1 g, were placed onto an individual MYA plate, labelled and sealed. Upon returning to the laboratory the samples were incubated in a temperature-controlled incubator at 19-20°C for up to 14 days.

Identification of *Neoparamoeba* spp. in environmental samples

Neoparamoeba spp. were isolated and cultured from the net segments and sediment samples (Page 1983, General Materials and Methods 2.2). Cells were harvested as described (General Materials and Methods 2.3). The presence of *Neoparamoeba* spp. was confirmed by IFAT using a primary rabbit antibody prepared to *N. pemaquidensis* strain PA027 (Howard and Carson 1993, General Materials and Methods 2.4). A DAPI stain was used to highlight the characteristic

parasome and nucleus indicating that the organisms were *Paramoeba* (Howard, 2001, General Materials and Methods 2.5).

The presence of *N. pemaquidensis* and *N. branchiphila* were confirmed by PCR using species specific primers of the 18S rDNA gene sequence (Elliot *et al.*, 2001, General Materials and Methods 2.7; Dyková *et al.*, 2005)

DNA extraction protocol

N. pemaquidensis DNA was extracted from the cells harvested from the culture plates (Wilson and Carson 2001, General Materials and Methods 2.6). *N. branchiphila* DNA was extracted from the cells harvested from the culture plates using DNeasy Tissue kits (Qiagen Pty. Ltd.).

Briefly, a 200 µl cell suspension was centrifuged for 5 mins at 8 000 g. A 30 µl sample was pipetted from the pellet, placed into a sterile 1.5 ml microcentrifuge tube with 180 µl of tissue lysing buffer (ATL) and 20 µl of proteinase K, vortexed and incubated at 37°C overnight on a rocking platform with the lids taped down. The following morning the samples were mixed for 15 seconds. A 200 µl volume of buffer AL was added, mixed and incubated at 70°C for 10 minutes with the lids taped down. A 200 µl volume of 100% ethanol was added, vortexed and placed into a DNeasy mini spin column. The column was placed within a 2 ml collection tube and centrifuged at 6 000 g for 1 minute. The collection tube with its contents was discarded and the spin column placed into a new 2 ml collection tube. A 500 µl volume of wash buffer (AW2) was added and centrifuged at 20 000 g for 3 minutes. The collection tube with its contents was discarded and the spin column placed into a 1.5 ml microcentrifuge tube. A 100 µl volume of elution buffer (AE) was added and incubated for 1 min at room temperature and then centrifuged at 6 000 g for 1 minute.

PCR protocol

Each reaction tube had total volume of 25 µl of solution containing; 2.5 µl 10x PCR buffer (Invitrogen Life Technologies), 0.5 µl dNTPs (0.2 mM Epicentre Technologies), 1 µl MgCl (2 mM Invitrogen Life Technologies), 0.6 µl forward

primer *N. pemaquidensis* (5'-CATCTCCTTACTAGACTTTTCATG-3') and *N. branchiphila* (5'-GTGAGTGATRRTTAGACCTCTTGG-3') and 0.6 µl reverse primer *N. pemaquidensis* (5'-CACAACAAACTCGCTCTACCC-3') and *N. branchiphila* (5'-CACAGCAAACCTYATYYTCACAAA-3'), 0.2 µl Platinum Taq (Invitrogen Life Technologies), 2 µl from DNA sample, 1 µl BSA and 16.6 µl MilliQ water. PCR positive controls of purified DNA from a reference strain of *N. pemaquidensis* and *N. branchiphila* and a negative control of DNA were included.

PCR cycling conditions for *N. pemaquidensis*

PCR cycling conditions for *N. pemaquidensis* were as described in General Materials and Methods 2.7

PCR cycling conditions for *N. branchiphila*

PCR cycling occurred in a PTC-100 thermocycler (Bresatec).

1. 1 cycle 95°C for 10 minutes
2. 30 cycles of: 94°C for 1 minute
 58°C for 1 minute
 72°C for 1.5 minutes
3. 1 cycle of: 72°C for 10 minutes

Gel Protocol

A 1% agarose gel was used to visualise the amplicons. Each well contained 1.5 µl dye and 4 µl PCR product, with the exception of the ladder, which was 1.5 µl dye, 1 µl of 1 Kb DNA ladder (Invitrogen Life Technologies) and 3 µl of distilled water. The gel was run at 100 V for 1 hour. The gel was immersed in ethidium bromide solution (3% in distilled water) on a shaker for 30 minutes. The gel was then visualised over a U.V transilluminator and photos taken.

Identification of *Neoparamoeba* sp. on salmon gills

Neoparamoeba spp. antigens in the gill mucus were detected using an immunodot blot (Douglas-Helders *et al.* 2002, General Materials and Methods 2.8). Gill sections stained with H&E were observed by light microscopy for the presence of lesions and associated *Neoparamoeba* spp. (General Materials and Methods 2.9).

AGD case definition

- 1) A fish with both positive gill score (the presence of white plaques) and positive immuno-dot blot (the presence of *Neoparamoeba* spp. antigens in gill mucus) was defined as AGD positive (non-lethal sampling).
- 2) A fish identified by histology with gill lesions in association with amoebae containing a parasome was defined as AGD positive (lethal sampling).

Farm outbreak

Once the AGD outbreak became evident, salinity and water temperature records were obtained from the farm and analysed for the previous 5 years. AGD affected fish were examined and the gills of ten fish identified with severe lesions were sampled and processed for histology.

Statistics

The presence of *N. pemaquidensis* on net segments and in the sediment was analysed by chi-square (4x2) table using the statistical package Epi Info™ 2002 (CDC, USA). The presence of *N. branchiphila* on net segments and in the sediment was analysed by chi-square (4x2) table. A chi-square (2x2) table compared the presence of *N. pemaquidensis* and *N. branchiphila* on net segments and in the sediment. A significance level of $p \leq 0.05$ was adopted.

Results

Both *N. pemaquidensis* and *N. branchiphila* were detected in the benthic sediment and on the nylon netting. No amoebae were isolated from the steel netting. No Atlantic salmon were AGD positive. Over the sampling period the average environmental conditions were; salinity 30 ppt (± 0.264), water temperature 18.4°C (± 0.225) and dissolved oxygen 80-90%.

Sediment samples

The baseline survey of sediment found a total of eight samples (25%) with *N. pemaquidensis* present and ten samples (31.3%) with *N. branchiphila* present (Table 6.1). There was no significant difference in the number of positive samples for *N. pemaquidensis* or *N. branchiphila* as a result of the variation in depth at the site ($p = 0.661$ and 0.090). Pen D had one individual sample with both species of amoebae present.

Table 6.1: The number of sediment samples identified with *Neoparamoeba pemaquidensis* and *Neoparamoeba branchiphila* present by PCR at the baseline time point (n = 8 / site).

Baseline sediment samples PCR positive		
Site	<i>N. pemaquidensis</i>	<i>N. branchiphila</i>
A	5	1
B	0	5
C	2	1
D	1	3

Following one-month six sediment samples (18.8%) had *N. pemaquidensis* present and nine samples (28.1%) *N. branchiphila* present. There was no significant difference in the number of positive samples for *N. pemaquidensis* or *N. branchiphila* as a result of the variation in depth at the site ($p = 0.482, 0.637$ respectively: Table 6.2). No individual samples had both species of amoebae present.

A comparison of the number of sediment samples PCR positive for *N. pemaquidensis* and *N. branchiphila* found no significant difference at the baseline sampling and again one month later ($p = 0.582$ and $p = 0.380$)

Table 6.2: The number of sediment samples identified with *Neoparamoeba pemaquidensis* and *Neoparamoeba branchiphila* present by PCR following one month (n = 8 / site).

Sediment samples PCR positive after 1 month		
Site	<i>N. pemaquidensis</i>	<i>N. branchiphila</i>
A	1	1
B	1	3
C	3	3
D	1	2

Netting samples

Four nylon net samples (25%) had *N. pemaquidensis* present and ten samples (62.5%) *N. branchiphila* (Table 6.3). There was no significant difference as a result of the variation in depth at the site for both *N. pemaquidensis* and *N. branchiphila* ($p = 1.000$, $p = 0.149$ respectively). Site B and D had one individual sample with both species of amoebae present. The number of net samples with *N. branchiphila* present was significantly higher than *N. pemaquidensis* ($p = 0.035$).

Table 6.3: Nylon net samples identified with *Neoparamoeba pemaquidensis* and *Neoparamoeba branchiphila* present by PCR following one month. (n = 4 / site).

Nylon net samples PCR positive		
Site	<i>N. pemaquidensis</i>	<i>N. branchiphila</i>
A	1	1
B	1	2
C	1	3
D	1	4

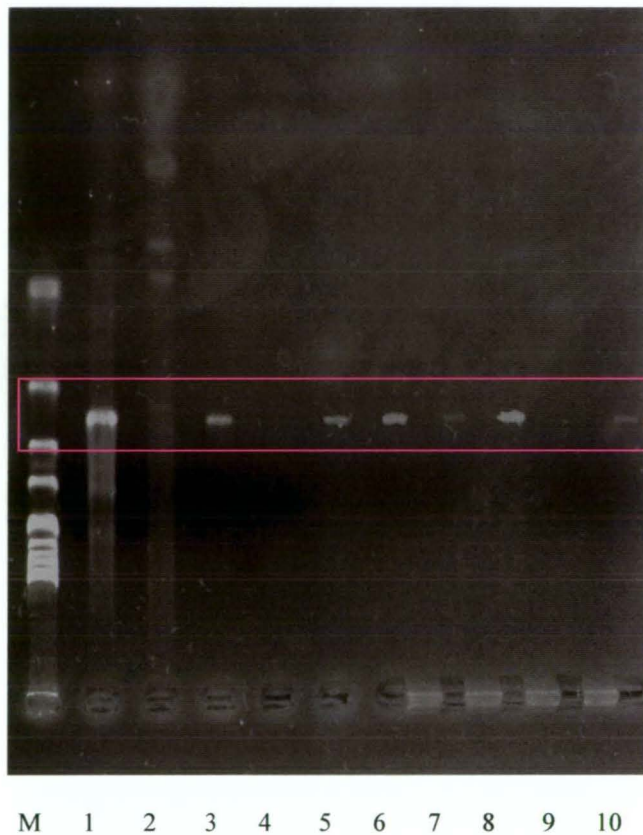


Figure 6.2: Photo of gel showing positive *Neoparamoeba branchiphila* nylon net and sediment samples. Lane M = 1 Kb DNA ladder, 1 = positive control, 2 = negative control, 3 – 6 nylon net samples, 7 - 10 sediment samples.

Gills

No white patches were found on the gills of the eighty fish examined and all immuno-dot blot results were negative indicating no *Neoparamoeba* spp. antigens being present (Figure 6.3). Routine histology found no AGD like lesions with associated *Neoparamoeba* spp. One fish did have an AGD like lesion but no amoebae were associated and therefore using the AGD case definition it was recorded as negative.



Figure 6.3: Photo of gill section stained with H&E, taken during the trial showing normal gill filaments with no amoebae or AGD present.

Farm outbreak

Salinity levels at the site were consistently in excess of 30 ppt from January to July 2005, which was unusual compared to previous years (Figure 6.4). Water temperatures were comparable to previous years with no unusual changes (Figure 6.5). In the 12 months prior to the outbreak the system farm had expanded from 10 to 18 cages, resulting in an increase in the number of fish held at the site. Maturing fish with AGD continued to be observed until August 2005 (Figure 6.6 and 6.7).

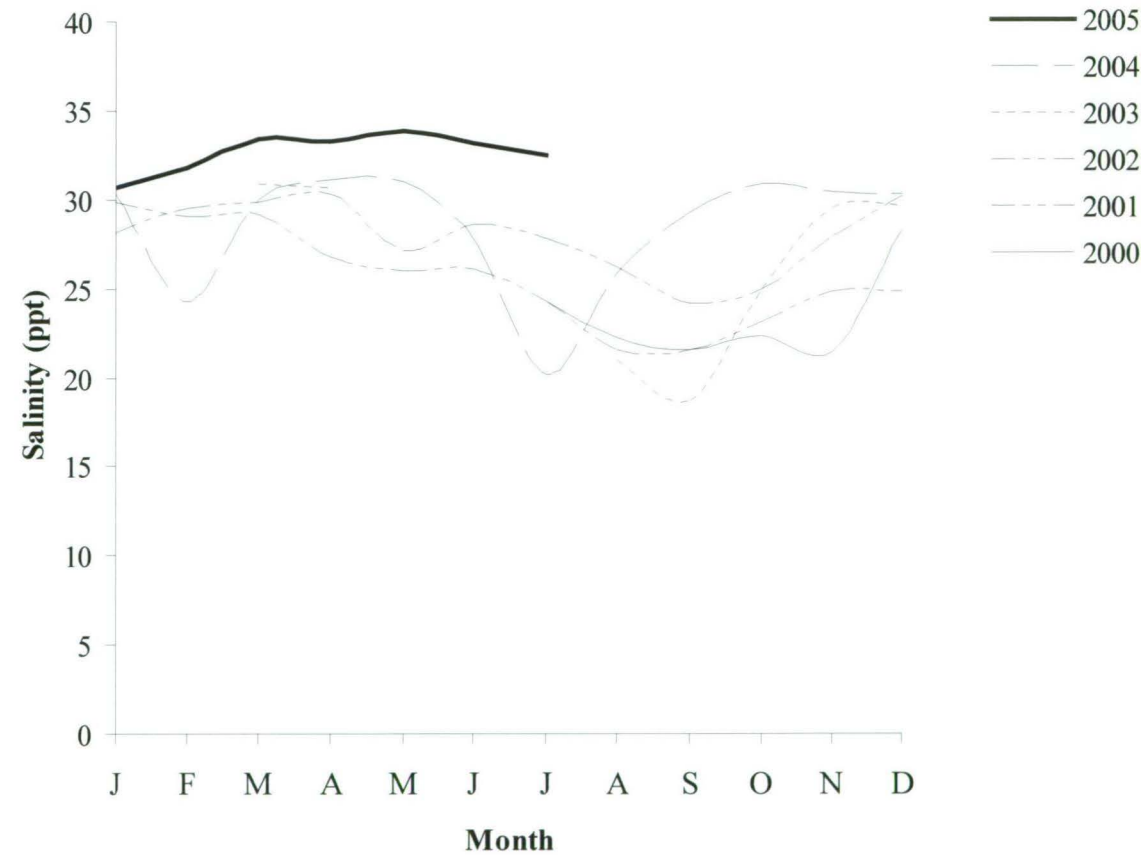


Figure 6.4: Average monthly salinity (ppt) recorded at the farm site in the Tamar River from 2000 - 2005.

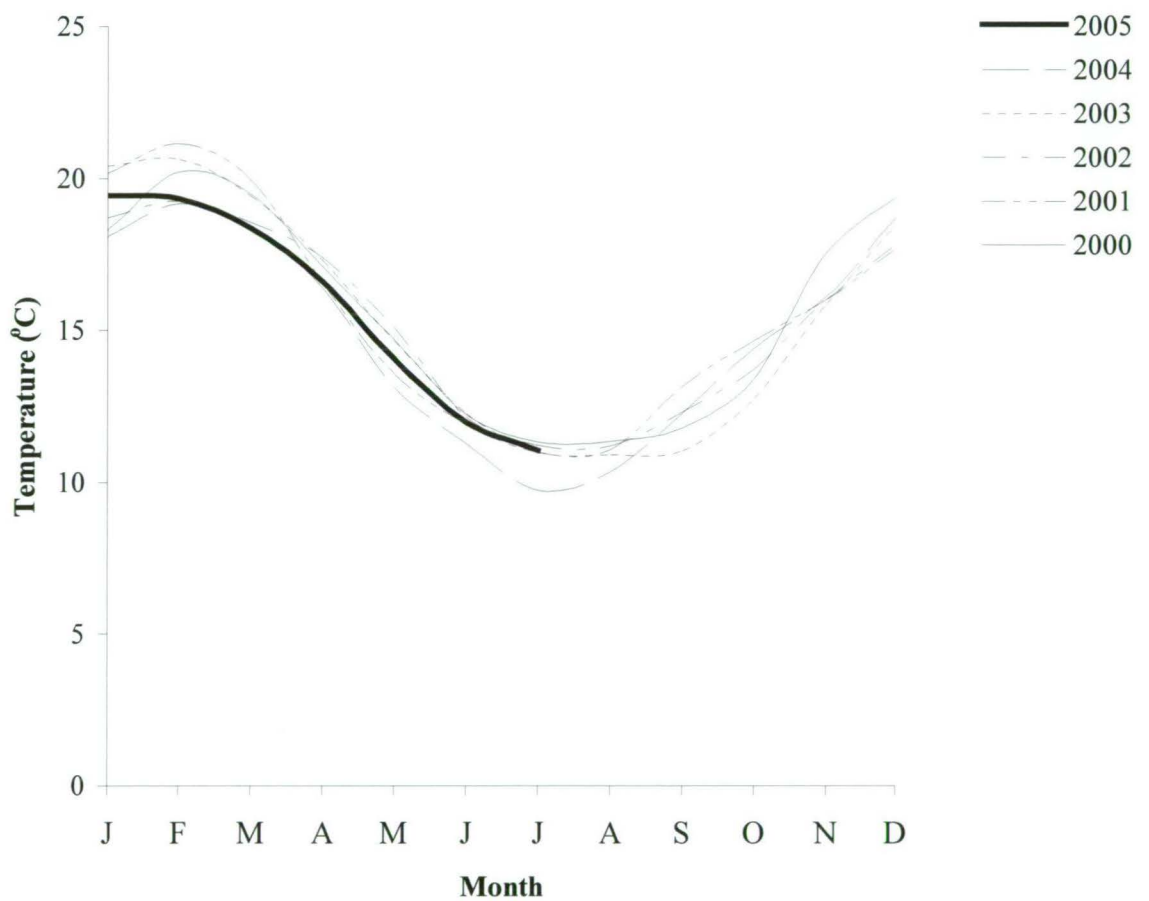


Figure 6.5: Average monthly water temperature recorded at the farm site in the Tamar River from 2000 – 2005.



Figure 6.6: An Atlantic salmon gill sampled during the AGD outbreak in March 2005 stained with H&E. This fish is in the advance stages of AGD, indicated by the lamellar fusion and the formation of interlamellar vesicles. Numerous *Neoparamoeba* spp. are present on the edge of the gill lamellae (indicated by ellipsoid).

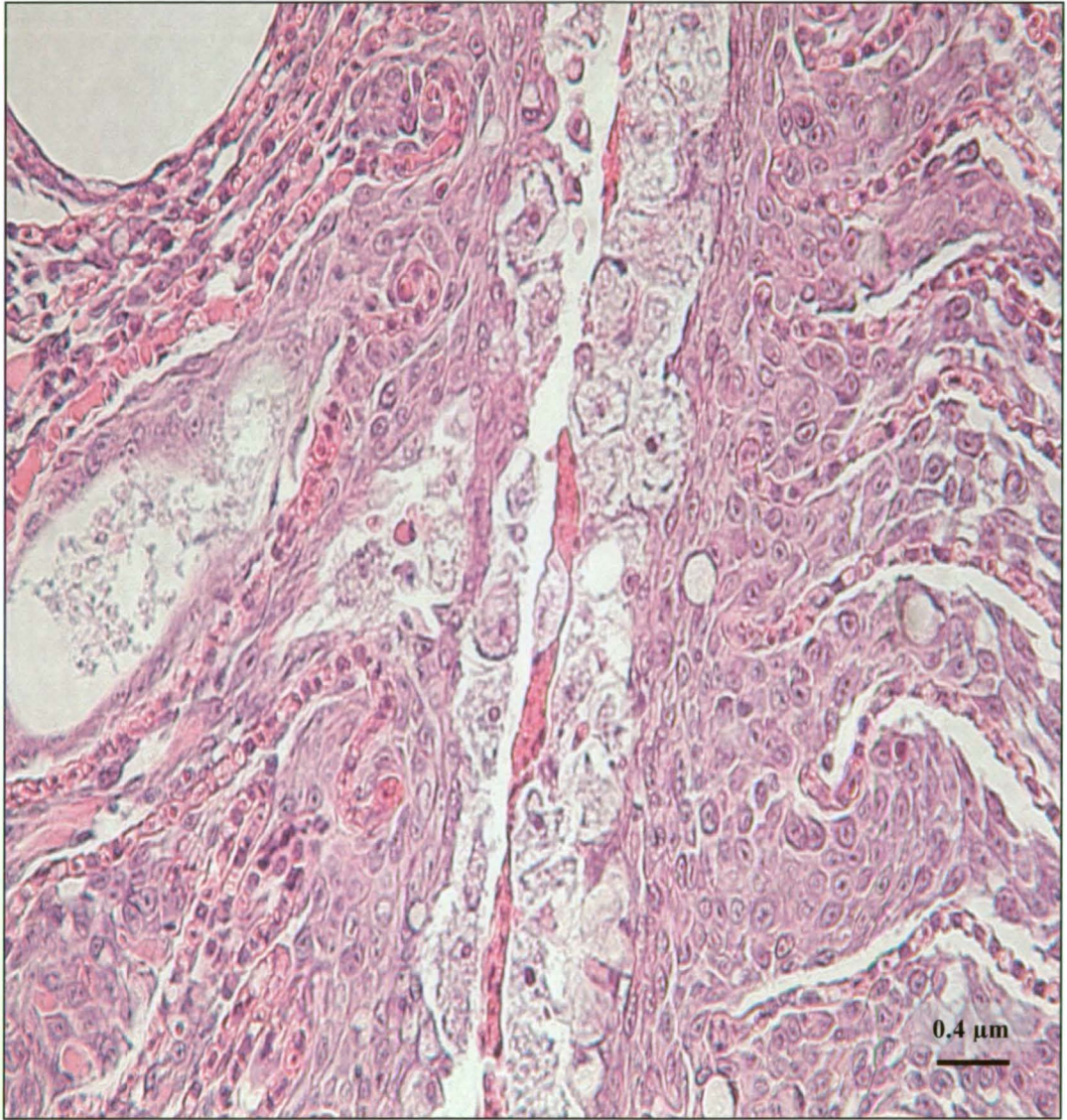


Figure 6.7: A close up view of the above H&E stained gill section with *Neoparamoeba* spp. (indicated by arrow) associated with the areas of lesions.

Discussion

Detection of *Neoparamoeba* sp. in the sediment samples is consistent with the findings of previous work conducted at the same site in the Tamar River (Crosbie *et al.*, 2003). Current knowledge on the distribution of amoebae at this site was enhanced by the isolation of *Neoparamoeba* spp. on nylon nets but not on steel nets. The AGD outbreak 12 months after the trial greatly assisted in identifying environmental conditions and husbandry practices that warrant further investigation as risk factors.

Both water temperature and salinity have been identified as risk factors for AGD (Munday *et al.*, 1990; Clark and Nowak, 1999; Douglas-Helders *et al.*, 2001 Nowak, 2001). The spike in salinity appears to be the predisposing factor for AGD at this site in the Tamar River. Water temperature not only was consistent with previous years but in the lower range. A recent study confirmed that salinity was a dominant factor influencing the survival of *Neoparamoeba* sp. (Douglas-Helders *et al.*, 2005). Cultured and gill harvested amoebae showed reduced survival when incubated in water with 17 ppt salinity (Douglas-Helders *et al.*, 2005). When the same water sample had the salinity raised above 30 ppt, as commonly seen in AGD affected areas, the survival of *Neoparamoeba* sp. increased dramatically (Douglas-Helders *et al.*, 2005). An increase in salinity from 27 to 35 ppt demonstrated a marked increase in the incidence and severity of AGD in experimentally infected salmon (M. Adams unpublished data). Farm records indicate that salinity levels above 30 ppt were infrequent however the presence of AGD in 2005 coincided with a period of high salinity in excess of 32 ppt. In the early part of 2005 rainfall in the surrounding areas was quite low, which resulted in a reduction of the surface freshwater layer usually present in the Tamar River and a spike in salinity (www.bom.gov.au). The low salinity levels in previous years may have reduced the survival of *Neoparamoeba* spp., which in turn reduced the risk of infection with AGD.

Whilst salinity appears to play a major role in AGD water temperature must not be discounted as a risk factor. The optimum water temperature for cultured Atlantic salmon is between 10 - 16°C however they can be farmed up to 20°C

(DPIWE, 2004). For the majority of the production cycle Tasmanian farms operate within this range however during the summer period water temperatures can exceed 20°C. AGD in the field has been recorded between temperatures 12 - 20°C (Munday *et al.*, 1990; Clark and Nowak, 1999) and experimental infections maintained between 16 – 20°C (Munday *et al.*, 2001; Adams and Nowak, 2004; Gross *et al.*, 2004). Findlay (2001), found that experimental AGD could be manipulated when water temperatures were between 12 -14°C however, above 16°C the disease was very pronounced with limited control (Munday *et al.*, 2001). In these experimental trials salinity was consistently maintained above 32 ppt. Salmon farmed in elevated water temperatures coupled with high salinities may be more prone to AGD as a result of increased stress levels and a compromised immune system. Further research is required to accurately determine the importance of temperature and salinity as individual risk factors.

The initial occurrence of AGD in the maturing population of Atlantic salmon supports previous findings that sexually mature fish are more susceptible to the disease (Mitchell, 2001; Munday *et al.*, 2001). The immune response of fish can be compromised by a number of events such as stress, season, diet, hormone levels and disease. The process of maturation has been shown to increase stress levels in salmonids (MacKinnon, 1998). Combined with the stress of intensive culture the salmon can become chronically stressed and more prone to disease (Mackinnon, 1998). Although cortisol levels were not measured in this trial it is well established that sexual maturation causes elevated cortisol levels and suppressed immune function (Bakke and Harris, 1998; MacKinnon, 1998). The majority of AGD affected fish in this trial were identified as mature and most likely immuno-suppressed.

The apparent lack of amoebae on the steel nets may have been a result of the low presence of biofouling. The steel and nylon nets used in this trial had never been immersed in seawater. The smooth surface of the steel and lack of crevices and niches may have resulted in the biofouling community taking longer to establish when compared with nylon nets. If the amoebae are unable to attach to the steel nets it suggests that the net itself does not act as a reservoir but in fact the

biofouling community that colonises the net. Whether *Neoparamoeba* spp. is present on steel nets once an established biofouling community is present is not known and requires further research

Prior to the outbreak it was hypothesised that the fast flowing current was a contributing factor for the lack of AGD at this farm. In comparison to the Huon Estuary (0.02 – 0.03 m/sec) the average water flow in the Tamar River (0.38 – 0.70 m/sec) is extremely fast (Aquaneal, 1999; CSIRO Huon Estuary Study Team, 2000; Woods *et al.*, 2004) and has been thought to offer a form of natural protection for the fish. One theory was that the amoebae were unable to colonise the salmon gills as the current limited attachment. From this trial it is apparent that amoebae are capable of attaching to the nylon nets. *Neoparamoeba* spp. may have preferred the nets as an attachment site due to their multi-filamented surface and numerous niches (Hodson and Burke, 1997). Another possibility is that the current trapped the amoebae in the niches of the nylon nets allowing them to continue colonising the area. In the 12 months between the trial and the outbreak another eight cages were connected to the system farm. Whether this significantly reduced the flow through the cages is not known, but seems possible. This investigation suggests that the high water flow of the Tamar River may assist in keeping AGD at bay. However environmental conditions outside the normal range coupled with immuno-compromised fish appeared to negate any protection the fast flow may have afforded the farm.

Currently the movement of *Neoparamoeba* spp. from the sediment through the water column to the salmon gills is unknown. Whether the amoebae existing in the sediment and on the nets are virulent and cause AGD is also unknown. Prior to the outbreak occurring at the site in the Tamar River, staff reported a number of maturing fish resting on the bottom of the steel nets. This may have facilitated the movement of amoebae to the fish gills however this is only speculation as information on the movement patterns is lacking. Despite no amoebae being cultured from the steel nets as previously indicated this may have been due to a lack of biofouling and therefore further investigation is necessary.

The significantly higher number of net sample with *N. branchiphila* present is a very interesting finding. The recently developed primers for *N. branchiphila* identified the amoebae in a number of previous samples taken from the sediment in Macquarie Harbour and on the gills of AGD positive salmon in experimental infection tanks at the University of Tasmania. Previously the causative agent of AGD has been cited as *N. pemaquidensis* (Adams and Nowak, 2004). With the identification of a second neoparamoeba species on the gills of AGD infected salmon the pathogen/s responsible for the disease is unclear. The inability to reproduce AGD in experimental conditions with cultured strains of *N. pemaquidensis* may be due to the disease being a mixed species infection. Without the presence of the other pathogens the disease may not develop. This is a significant area of AGD research that requires further investigation.

It cannot be assumed that the strains of *N. pemaquidensis* and *N. branchiphila* detected in the Tamar River are the same from AGD affected areas in southern Tasmania. The number of Atlantic salmon cultured in the Huon Estuary is significantly greater than the Tamar River and AGD is a continuous problem for the farms in the region. It has been suggested that *Neoparamoeba* sp. virulence increases when continually passaged through naïve hosts (Findlay *et al.*, 2000). Smolts are introduced more frequently due to a greater availability of out of seasons and triploids. Combined with the different environmental conditions found at the two areas it cannot be assumed that the amoebae are the same strains and research needs to be undertaken examining the internal transcribed spacer regions of the amoebae for possible differences.

The farms in the Huon Estuary are heavily reliant on freshwater bathing to treat AGD and maintain production levels. It has been shown that a number of amoebae can tolerate this treatment, which may result in the development of a resistant strain, a common problem with parasitic pathogens (Howard and Carson, 1993; Clark *et al.*, 2000; Findlay 2001; Parsons *et al.*, 2001). During the outbreak in the Tamar River the farm was logistically unable to treat the affected fish with freshwater and as a result all maturing, triploid and unhealthy fish were culled.

Huon estuary farms may have inadvertently selected for more pathogenic strains through their current farm practices.

Prior to the outbreak it had been suggested that the lack of salmon farms and low biomass of fish held at the site assisted in preventing AGD. At present there are no other salmon farms located in the Tamar River. The current lease has been operating for approximately 5 years and over that time has gradually increased in size. Approximately 12 months before the outbreak 8 cages were added to the system farm each stocked with approximately 35 000 salmon. This large increase in fish numbers may have provided an environment suitable for the *Neoparamoeba* spp. population to increase. Also extra cages would have increased the surface area available for attachment and reproduction. This may have resulted in the population reaching the minimum level required to induce AGD. The minimum infective dose of gill derived *Neoparamoeba* sp. currently used to induce experimental AGD infections is 10 cells L⁻¹ (Morrison *et al.*, 2005). This dose rate is similar to the number of amoebae detected in the water column of Atlantic salmon cages in southern Tasmania, and results in a gill response similar to that of AGD affected fish in the field (Douglas-Helders *et al.*, 2003). However, this dose rate should be interpreted with caution when being related to the Tamar River site as the environmental conditions such as salinity, water velocity and flush rates are quite different to those of southern Tasmania. Perhaps these factors afforded the farm some protection resulting in a higher minimum dose rate necessary for AGD to develop.

From this trial it appears that the culmination of optimal environmental conditions, the presence of maturing salmon and an increase in fish numbers at the site resulted in an AGD outbreak. It suggests that new farms should be located based on environmental conditions of the area, in particular salinity, rather than the presence of other farms. Stress appears to be a major risk factor with the holding of mature fish over suboptimal conditions resulting in the occurrence of AGD. Future monitoring must be undertaken at this site in order to gain a better understanding of this costly disease.

Chapter 7 - General Discussion



A systematic approach is essential when investigating disease outbreaks. To prevent future transmission or in the case of an endemic pathogen, minimise the occurrence of outbreaks, the cause or source of a disease must be accurately identified. Treatments based on unreliable information can be costly to the industry. Epidemiological studies are essential to gain a firm understanding of the pathogen, disease conditions, response of host and environmental conditions.

Fallowing is husbandry practice commonly used in terrestrial and aquatic intensive farming operations. This study found the practice of fallowing to be unsuccessful in minimising AGD in Atlantic salmon stocked within the Huon Estuary, southern Tasmania. The salmon restocked at a lease site fallowed for 11 months recorded a similar prevalence of AGD as a site that had no rest period. A previous study completed at the same location also found no significant difference in the prevalence of AGD in salmon restocked at sites that had been fallowed between 4 and 97 days (Douglas-Helders *et al.*, 2004). In Scotland fallowing has proven successful in reducing sea lice infections in Atlantic salmon (Bron *et al.*, 1993; Pietrak and Opitz, 2002), however a number of circumstances restrict its potential as a disease management strategy for AGD in Tasmania. *Neoparamoeba* sp., the causative agent of AGD, is endemic to the Tasmanian marine environment and its survival is not reliant on the presence of Atlantic salmon as a host. The holding of smolt at Pillings Bay, a site within the Huon Estuary, prior to the trial resulted in a pre-exposure to *Neoparamoeba* sp. This may have masked the effect restocking salmon on a fallowed site had on AGD prevalence, as the fish were not naïve. However, as the holding of smolt at Pillings Bay is common practice for the salmon farms located in the Huon Estuary this needs to be taken into consideration when assessing the potential of fallowing. The economic ramifications of a treatment program are a major factor for the salmon industry. A coordinated fallowing program would be required in the Huon Estuary and as one company is based solely in this area the financial loss would far outweigh any small benefit fallowing may provide in reducing AGD outbreaks.

Copper based antifouling paint treated nets were identified as a reservoir for *Neoparamoeba* sp. and a potential risk factor for AGD outbreaks (Tan *et al.*,

2002; Douglas-Helders *et al.*, 2003). Whilst this study also found that salmon maintained in copper antifouling paint treated nets had a statistically higher prevalence of AGD, in terms of biological significance it was negligible. Both treatment groups recorded a total AGD prevalence above 20 %, which for the industry is an unacceptable background level and would require immediate treatment with freshwater. Despite the higher number of copper treated net samples with *N. pemaquidensis* present, I am not confident in interpreting these results as copper antifouling paint acting as a risk factor or possible reservoir. Current diagnostic tools are limited to only detecting the presence of *Neoparamoeba* sp. not the quantity. Therefore, the results of the PCR can only be interpreted as presence or absence and not as a quantifiable amount. The laboratory trial conducted found no significant difference in the settlement rate of *Neoparamoeba* sp. on copper treated and untreated net segments. These findings suggest that the copper antifouling paint does not act as an attractant for the amoebae. A possibility is that the antifouling paint creates an environment, which selectively supports an organism that the amoebae utilise as a food source. At present *Neoparamoeba* sp. food preference is unknown.

Maintaining Atlantic salmon under continuous artificial lights in experimental conditions did not result in a significant difference in the prevalence of AGD for the majority of the 12 month trial. However at three of the seven sampling points a significant difference was identified. Two of these differences were a result of the fish in unlit pens having a higher AGD prevalence than the artificially lit pens. The use of freshwater bathing routinely every 6 weeks assisted in maintaining the prevalence of AGD at an acceptable level. During a stressful event it was found that despite no difference in AGD prevalence the severity of AGD recorded in the salmon maintained under artificial lights was significantly higher. Interestingly a trial currently being conducted on a commercial scale has identified AGD outbreaks occurring approximately one month earlier in salmon maintained under continuous artificial light. The behaviour of *Neoparamoeba* sp. in particular possible phototactic response, is currently unknown and requires further investigation to assist in assessing the effect of artificial lights on AGD prevalence.

A study conducted at an isolated Atlantic salmon farm located in the Tamar River northern Tasmania, found *N. pemaquidensis* and *N. branchiphila* present in the benthic sediment and on nylon net panels suspended within the salmon cages. No amoebae were able to be cultured from the steel net panels. Clinically no white patches were observed on any salmon gills sampled, a method commonly used to identify AGD presence. No *Neoparamoeba* sp. antigens were detected in the gill mucus either, indicating no previous exposure to the amoebae. This farm site was examined in an attempt to better understand the environmental parameters that may play a role in the occurrence of AGD. As no AGD had ever been recorded in the farms history of operation it was treated as a control site. Interestingly, 12 months after this study had concluded the farm suffered its first AGD outbreak. It appears that a spike in salinity, coupled with maturation and an increase in the number of fish held at the site provided optimum conditions for AGD to occur.

Over the previous 20 years our knowledge of AGD has increased greatly, however a number of significant “pieces” are still missing from the AGD puzzle. Previously *N. pemaquidensis* was cited as the sole causative agent of AGD in Tasmanian Atlantic salmon. However a recent study has cast doubt on this belief (Dyková *et al.*, 2005). A second species, *N. branchiphila* has been isolated and identified from the gills of AGD infected salmon held in experimental tanks at the University of Tasmania and from commercial farms. The role this amoeba plays in the occurrence of AGD is currently unknown and must be investigated further. At present cultured isolates of *N. pemaquidensis* and *N. branchiphila* are unable to invoke an AGD outbreak under experimental conditions (Zilberg *et al.*, 2001; Morrison *et al.*, 2005). Currently tank infections are achieved through co-habiting infected and naïve salmon or using *Neoparamoeba* sp. isolates harvested from gills of infected salmon (Zilberg *et al.*, 2001; Morrison *et al.*, 2004). Despite the claim that amoebae obtained from infected gills are “partially purified” the fact remains that unless using clonal cultures one cannot be certain of the causative agent involved. The pioneer in AGD research, the late Dr. Barry Munday suggested that further research is required to determine the differences between “wild” type and cultured *N. pemaquidensis* (Munday *et al.*, 2001). A recent study

confirmed Dr. Munday's earlier work identifying that *Neoparamoeba* sp. become non-infective when cultured (Morrison *et al.*, 2005). Villavedra *et al.*, (2005) has suggested that virulence is dependent upon host-derived factors and that the current methods used to culture *Neoparamoeba* sp. may be rendering the organisms non-virulent. Current culture conditions in no way represent the natural environment amoebae occur in particularly as they are grown on monoxenic bacteria lawns. A recent study found cultured *Neoparamoeba* sp. express a different antigen profile to freshly isolated amoebae and that the method of *in vitro* cultivation alters the antigens expressed (Villavedra *et al.*, 2005). The failure of cultured strains to elicit an AGD response restricts current research, especially as some antibodies raised against cultured parasites and used for IFAT have been shown to not recognise freshly isolated *Neoparamoeba* spp (Villavedra *et al.*, 2005).

It appears from this study that the environmental parameter salinity plays a major role in the occurrence of AGD in Tasmania. The salmon farm located in the Tamar River, northern Tasmania experienced its first outbreak in its six years of operation during a period of unusually high salinity. Several recent studies have also identified salinity as the main environmental risk factor in AGD outbreaks (Douglas-Helders *et al.*, 2005; M. Adams unpub. data, 2005). As the farm in the Tamar River is isolated from the other farms it is an invaluable study site and all attempts should be made to support a monitoring program at this site to observe the progression of AGD. If environmental conditions are the major factors influencing AGD then farmers may have to contemplate relocating farms from the Huon Estuary to sites with more suitable conditions. If this is unachievable then a study needs to investigate the effect of stocking density on the prevalence of AGD. The financial burden of AGD is mainly a result of costly freshwater baths, extra staff to undertake the bathing and increased mortalities. Whilst initially farmers will see the concept of reducing stocking densities as a loss of income, a cost/benefit analysis should be made to accurately determine the benefits as the results of this study indicate that stress is a major factor increasing the susceptibility to AGD.

The majority of the work present was undertaken as field experiments, which often limited the outcomes. Whilst the salmon industry is committed to research, they are first and foremost a commercial enterprise with production and profits being the main priorities. Unfortunately, this did result in cages not always being treated identically or the direction of the trial altering to accommodate production demands. The identification of AGD was mostly reliant upon gill scores and immuno-dot blot, both non-lethal forms of sampling. Histological examination is the preferred method for identifying AGD as gill lesions and associated *Neoparamoeba* sp. can be visualised, whereas macroscopic gill lesions are not specific to *Neoparamoeba* sp. and the primary antibody in the immuno-dot blot is not species-specific. As in excess of 1600 fish would have been killed to complete histology it was not a viable option for the farm. Field experiments are also limited as replication is often low or non-existent. Despite these constraints field experiments under commercial conditions are essential as laboratory based trials never can truly mimic the natural environment and the effects must be observed on a true scale as potential problems are magnified.

An excellent example for future AGD research is the epidemiology studies undertaken on sea lice infections in cultured Atlantic salmon in Scotland (Bron *et al.*, 1993; Costelloe *et al.*, 1999; Pike and Wadsworth, 1999; Revie *et al.*, 2002). The research conducted has identified the causative agent, its lifecycle and behaviour, host specificity, cause of mortalities and effective treatment regimes. Whilst farms in Scotland are still reliant on a chemical therapeutic to reduce lice infections, they are beginning to incorporate husbandry changes such as fallowing, based on findings from epidemiology studies (Pietrak and Opitz, 2002). In contrast, the causative agent or agents of AGD are not well defined, the cause of mortality is unclear, the parasites behaviour is unknown, the method of transmission is unknown, the virulence of different environmental samples is unknown and cultured strains of *Neoparamoeba* are unable to be used to elicit experimental AGD infections. All of these factors need to be investigated and understood before an effective treatment can be developed. The production of a vaccine is perhaps not the most appropriate path to venture along at the present time when the pathogen/s responsible for AGD remain elusive.

AGD research has achieved a lot in its 20 year history however a number of significant areas are still unknown, which affect the development of alternative treatments. Whilst AGD places an enormous financial burden on the Tasmanian Atlantic salmon industry, it is not the greatest threat the industry will face. Currently the Tasmanian farms are operating in water temperatures at the higher end of the salmon's tolerance range. If as predicted water temperatures rise beyond these tolerance limits then sea-based salmon culture in Tasmania may cease to exist rendering AGD an academic interest only.

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