Final Report

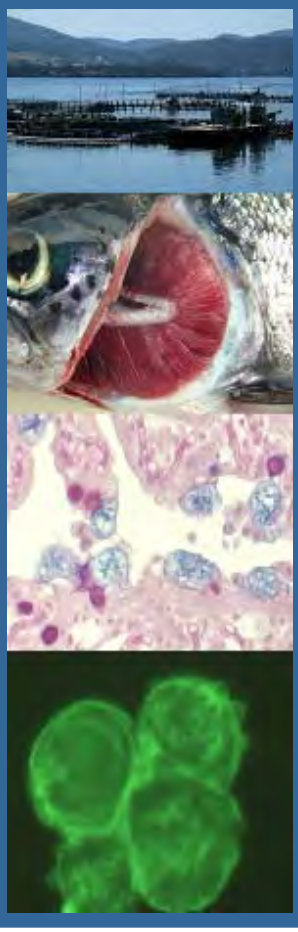
Aquafin CRC

HOST-PATHOGEN INTERACTIONS IN AMOEBIC GILL DISEASE

Barbara Nowak, Richard Morrison, Philip Crosbie, Mark Adams, Rick Butler, Andrew Bridle, Kally Gross, Benita Vincent, Sridevi Embar-Gopinath, Jeremy Carson, Robert Raison, Margarita Villavedra, Kristy McCarthy, Kevin Broady and Michael Wallach

August 2004

Aquafin CRC Project 3.4.2
(FRDC Project No.2001/244)



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2001/244 Host-pathogen interactions in Amoebic Gill Disease

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OBJECTIVES:

1. To provide knowledge base for development of novel treatments and vaccine.
2. To identify factors leading to binding of the parasite to fish gills.
3. To identify gill conditions which increase the susceptibility of the fish to AGD.
4. To develop techniques for *in vitro* work on Amoebic Gill Disease.
5. To expand *N. pemaquidensis* library of strains.
6. To implement a long term preservation for *N. pemaquidensis* based on freezing technology.
7. To develop improved culture systems based on monoxenic and axenic techniques.
8. To develop cell factory capability to produce high density cell suspensions of *N. pemaquidensis.*
9. To develop cell purification techniques to produce pure cell suspension of *N. pemaquidensis* derived from cell culture and gill associated disease.
10. To implement cell characterisation techniques for strain differentiation
11. To investigate culture strategies to develop infective strains of *in vitro* grown *N. pemaquidensis.*

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED

This project significantly increased our knowledge and understanding of Amoebic Gill Disease (AGD). We developed tools and techniques applicable to further research on AGD and other fish diseases. This project contributed to education and training in the area of fish health. Five PhD students and three postdoctoral fellows were involved in this project. Development of case definition for AGD and increased understanding of AGD outbreaks in the field were of particular interest to the salmon industry. Diagnostic services (Fish Health Unit, DPIWE) collaboratted by providing expertise and adopting this project's results.

Prior to this project, our knowledge of Amoebic Gill Disease (AGD) was fundamentally limited. An improved understanding of host-pathogen interactions was required to provide a basis from which to develop effective strategies for future control and treatment of the disease. This was achieved by the development of new techniques and adaptation of existing ones for the study of ecto-parasitic gill disease.

This project addressed the need for understanding disease development and progression. Sequential histopathology in both laboratory and field infections identified developmental stages of AGD, in terms of host response and pathogen proliferation and the disease's strong association with salinity and temperature. This work enabled the introduction of an AGD case definition, providing consistent and repeatable disease interpretation for future studies.

Several factors affecting the susceptibility of the host to infection, positively or otherwise, have been identified. A clear relationship between amoebae density and AGD severity has been demonstrated. We have shown that injection with bacterial DNA motifs can enhance survival in experimentally infected fish. However, experimental trials of commercially available oral immunostimulants have thus far been unsuccessful. Similarly, prior exposure to experimental AGD challenge did not provide subsequent protection against re-infection, even after complete recovery from initial infection. These results also suggested some degree of non-specific immune suppression associated with infection by *Neoparamoeba* sp. A preliminary experiment indicated decreased colonisation of amoebae upon physically damaged gill tissue. We also demonstrated that gill associated mucus may be necessary for amoebic attachment. Increased AGD severity has been associated with the presence of some gram negative bacteria, however the disease could be induced without exposure to any bacteria.

This project has also provided numerous improvements to existing methods and the creation of new technologies. In terms of culture techniques, the complexity of growing *Neoparamoeba* sp. had previously proved a major limitation to AGD research. We have now established a centre of expertise in

the culture of *Neoparamoeba* sp. and have standardized retrieval of relatively pure preparations of virulent, host derived *Neoparamoeba* sp. In addition, amoebae culture technologies have been adapted to provide cell factory production systems for *Neoparamoeba* sp. and enhanced cryopreservation techniques. This project has significantly expanded the library of available strains of *Neoparamoeba* sp. and led to the description of a new species. The development of a central source for this amoeba ensures uniformity of research findings and outcomes amongst the numerous research groups currently operating. The enhancement of isolation and purification techniques enabled AGD challenge methods to be fine-tuned to provide reproducible trials. This information has formed the basis for the systematic development of clearly defined challenge models for current vaccine efficacy trials. Identification and quantification methods for different cell types were developed to interpret host response to the amoebae.

Other new research tools include the development of *in vitro* methods for attachment studies of *Neoparamoeba* sp. using gill explants and cell mono-layer technologies. These methods were used in conjunction with the development and screening of monoclonal antibodies used in a protein based approach to identifying attachment factors for vaccine candidates. A large panel of monoclonal antibodies has been developed to study these attachment factors.

While we have achieved our objectives and answered many of our original questions, new questions emerged from our research. Further work is needed to fully understand the role of inflammatory responses in AGD, potential for immunomodulation, effects of challenge conditions on disease dynamics, infectivity of different strains (and species) of *Neoparamoeba* sp., association of different species of *Neoparamoeba* sp. with lesions and the relationship between amoebae numbers in the water and AGD outbreaks on the farms. There is an ongoing requirement for new methods. For example we cannot easily distinguish the different species of *Neoparamoeba* sp., measure antibody presence in the gills or quantify amoebae in the water.

In conclusion, the combination of method developments and trial results have not only significantly increased our understanding of AGD but also has enhanced our ability to instigate, support and evaluate future AGD research, ultimately leading to direct benefits for the salmon aquaculture industry.

KEYWORDS: **Amoebic Gill Disease, *Neoparamoeba* sp., Atlantic salmon, aquaculture, immunology, pathology, diagnostics**

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This study formed part of a project of Aquafin CRC, and was supported from the Australian Government's CRCs Programme, the Fisheries Research and Development Corporation and by other CRC participants. We would like to thank salmon industry for their collaboration and support, in particular Peter Bender (HAC), Dr Dom O'Brien (HAC), David Mitchell (HAC), Adrian Steenholdt (HAC), Innes Weir (formerly HAC), Dr Harry King (Saltas) and Dan Fisk (Tassal). We are grateful to Helen Statham for her help with histology. We would like to thank Mick Attard and Dr Mark Powell for providing infected fish from AGD infection tank during this project. We would like to acknowledge help from Dr Iva Dyková (Czech Academy of Sciences, Institute of Parasitology) for her advice and expertise in isolation, culture and identification of marine amoebae. Dr Dianne Elliott (USGS) contributed her expertise in histopathology. Dr Mark Crane (CSIRO) and Gemma Clark (CSIRO) have run additional cell line testing for the gill-isolated amoebae. We would like to thank Carlton United Brewery and Steve Lingard for supplying EcoActiva, Skretting for supplying MacroGard and Vistavet Ireland and Dr Marian McLoughlin for supplying Ecoboost. Prof Chris Carter's collaboration with regard to in-feed immunostimulant trials and access to his experimental system for EcoActiva and EcoBoost experiments are gratefully acknowledged. Dr Don Ward supported the EcoBoost experiment with his nutritional and technical expertise. We would like to thank Dr Chris Prideaux (CSIRO) for his interest, support, advice and discussions. Dr Stephen Pyecroft (DPIWE) and Dr Judith Handlinger (DPIWE) offered helpful comments and advice. Dr John Bowman (University of Tasmania) provided expertise in molecular microbiology. We would like to thank Dr Peter Montague (Aquafin CRC), Dr Patrick Hone (FRDC), Prof Colin Buxton (TAFI), Prof Chris Carter (TAFI), Dr Stephen Battaglene (FRDC ASAS, TAFI), Dr Jenny Cobcroft (FRDC ASAS, TAFI), Pheroze Jungalwalla (FRDC ASAS, TSGA), Erika Laws (FRDC ASAS, TAFI) for their continuing support during this project. International linkages and collaboration in this project were proudly supported by the *International Science Linkages* programme established under the Australian Government's innovation statement *Backing Australia's Ability*.

Background

This project forms a part of the Research Program of the CRC for Sustainable Aquaculture of Finfish ("Aquafin CRC"), and employed funds invested out of the CRC's Commonwealth grant and by FRDC and other Participants of the CRC.

Health is one of the major issues in aquaculture. Fish health is crucial for aquaculture to be successful. Sick fish will not only increase production costs sometimes to the point of the industry loosing its viability, but also adversely affect the industry image. For example, infectious salmon anaemia (ISA) resulted in some salmon companies in Scotland going into receivership. Additionally, diseases affecting cultured fish often result in the use of medications, which increases production cost and can negatively affect the markets.

In Australia, salmon aquaculture is relatively disease free, which gives Australian Atlantic salmon industry a marketing advantage. However, Amoebic Gill Disease costs are 9.4% of the gross value of production. The disease outbreaks seem to intensify as the industry develops and now are prevalent not only during summer months but also in winter. Outcomes of CRC Health Program such as improved treatment of AGD infected fish, AGD risk forecasting ability and vaccine production against AGD will increase the profitability and competitiveness of salmon industry in Australia.

Amoebic Gill Disease is the main disease currently affecting salmon industry in Tasmania. While it is controlled by freshwater treatment, it still causes fish mortalities and increases production costs. In the last few years, the intensity and frequency of AGD outbreaks seemed to increase.

Neoparamoeba pemaquidensis is a marine protozoan that is known to be free-living as well as epizoic, parasitizing the gills of several species of fish and causing AGD. Both free-living and parasitic form can be grown by monoxenic culture using bacteria as prey. Culture of the free-living form is reported to be readily achievable and non-exacting (Martin 1985) but culture of the parasitic form has shown that *N. pemaquidensis* isolated from fish gills are slow growing and fastidious in their culture requirements (Howard and Carson 1992; Dyková *et al*. 2000). Axenic culture of *N. pemaquidensis* has been reported to have been achieved (Kent at al. 1988) but in other studies (Howard and Carson, unpublished findings) protozoa could only be cultured monoxenically.

A source of well characterised organisms that are equivalent to naturally occurring gill associated protozoa both physiologically and in expression of virulence factors, is an essential requirement of the programme. Supply of protozoa underpins research in all the AGD programmes and is particularly important for the programme to develop a vaccine. Failure to develop culture techniques and supply sufficient quantities of *N. pemaquidensis* places in jeopardy the rest of the AGD programme. Similarly, methods to quantify *N.*

pemaquidensis numbers, and *in vitro* methods for investigation of AGD are lacking, but are crucial for development of vaccines or novel treatments.

While the previous CRC for Aquaculture provided new information about AGD, including description of pathology in experimental infection and development of direct laboratory infection using crude gill isolates (Zilberg and Munday 2000), there are still a lot of unanswered questions about host-pathogen interactions in the disease. Additionally, while no protection could be shown after vaccination with cultured *N. pemaquidensis* or crude isolates from gills of infected fish (Zilberg and Munday 2001), acquired immunity was reported after transfer of infected fish to fresh water (Findlay and Munday 1998). These results require further investigation and their confirmation would provide a basis for vaccine development.

Need

There has been some research done on potential vaccine development, but lack of information and *in vitro* methods slowed down progress. There is little knowledge about the interaction of the causative parasite *N. pemaquidensis* with the gill of the fish. Identifying factors (both host- and parasite-specific) causing the parasite attachment to the gill will provide information useful for control and treatment of the disease. While *in vitro* models have been developed for other fish parasitic conditions (for example white spot - Nielsen and Buchmann 2000), there is no *in vitro* model for investigation of Amoebic Gill Disease. This model would provide detailed information on cellular interactions between pathogen and host and could form a basis for development of novel treatment and help in vaccine development. Additionally, it could provide infective parasites, if other culture methods (see below) are not successful.

Currently all *in vitro* tests (for example investigating treatment effectiveness) rely on manual counts, which are not only slow but also could introduce operator error. There is no quantitative system developed for pathogen counts in water samples from farms or experimental tanks.

Techniques have been developed for the isolation and maintenance of *N. pemaquidensis* based on monoxenic cultures (Howard and Carson, 1991, 1992). Culture by this method is proving highly problematic for further studies because preparations of protozoa are contaminated to a greater or lesser extent with bacteria. Studies to determine protozoal cell function, protein and DNA composition have been seriously compromised by the presence of bacteria. Methodologies are required that will enable bacteria-free preparations of protozoa to be made. Culture relies on the use of agar as a solid substrate to stimulate growth of *N. pemaquidensis*. Cell propagation and harvesting by this system is time consuming and inefficient. Development of practical systems for cell factory production of *N. pemaquidensis* is required. This is particularly needed for studies to characterise *N. pemaquidensis* cell wall composition and cell function, investigations that require considerable quantities of biomass.

While there is now overwhelming evidence that *N. pemaquidensis* is the causative agent of AGD, no model of infection has been developed using protozoa derived from monoxenic or xenic cultures (Howard and Carson 1994). Failure to re-infect fish following culture represents a major limitation for several programmes of work, particularly studies where it is necessary to infect fish at will with controlled doses of a single strain of *N. pemaquidensis*. Current methods rely on the use of *N. pemaquidensis* harvested from natural infections in fish. While this strategy meets an immediate need, long-term this approach cannot be justified. Development of a method to grow *in vitro* virulent protozoa capable of infecting fish is an essential objective.

The current library of *N. pemaquidensis* isolates obtained from fish with AGD is small and consists of strains which have now been in continuous culture for

almost 10 years. There is an urgent need to re-isolate *N. pemaquidensis* from recent cases of AGD and expand the library of isolates to ensure that an adequate range of phenotypes and genotypes are available. Maintenance of *N. pemaquidensis* is based solely on continuous subculture as no adequate methods of preservation based on freezing have been identified (Howard and Carson 1992, Dr Susan Brown, Culture Collection of Algae and Protozoa, UK, personal communication). Preservation of *N. pemaquidensis* is seen as an essential requirement of the AGD programme as it will provide a means of maintaining strain integrity, a vital objective for the vaccine development programme.

Outside morphological characterisation, few markers have been identified to define strains of *N. pemaquidensis*. Recent observations (J. Carson, T. Wagner and T. Howard unpublished findings) have revealed marked differences in growth rates of strains in culture. There is also evidence that there is a difference in sensitivity to the anti-fungal agent pimaricin by free-living and epizooic forms of *N. pemaquidensis*. Clearly strains of *N. pemaquidensis* can be isolated though the significance of these is unclear but may be important in understanding host-pathogen interaction. While useful preliminary observations, further work is required to develop phenotypic markers that can be used for strain differentiation.

The complexity of growing *N. pemaquidensis* has proved a major limitation to previous studies on AGD. Establishment of a centre of expertise in the culture of *N. pemaquidensis* should overcome any difficulties associated with the supply of organism. A reference laboratory will also help ensure standardisation of cultures that are to be used by the different projects and will help to ensure uniformity of findings and research outcomes.

This project's results are directly applicable to other gill problems and may provide basis for technology useful in other fish species.

Objectives

Objective 1
To provide knowledge base for development of novel treatments and vaccine.

Objective 2
To identify factors leading to binding of the parasite to fish gills.

Objective 3
To identify gill conditions which increase the susceptibility of the fish to AGD.

Objective 4
To develop techniques for *in vitro* work on Amoebic Gill Disease.

Objective 5
To expand *N. pemaquidensis* library of strains.

Objective 6
To implement long term preservation for *N. pemaquidensis* based on freezing technology.

Objective 7
To develop improved culture systems based on monoxenic and axenic techniques.

Objective 8
To develop cell factory capability to produce high density cell suspensions of *N. pemaquidensis.*

Objective 9
To develop cell purification techniques to produce pure cell suspension of *N. pemaquidensis* derived from cell culture and gill associated disease.

Objective 10
To implement cell characterisation techniques for strain differentiation.

Objective 11
To investigate culture strategies to develop infective strains of *in vitro* grown *N. pemaquidensis.*

Methods

Objective 1
To provide knowledge base for development of novel treatments and vaccine

1. AGD infection

Relationship between numbers of amoebae and lesion severity (Morrison *et al.* 2004)

Amoebae were partially purified from the gills of AGD affected Atlantic salmon. Trophozoites were characterised by light microscopy and immunocytochemistry and designated *Neoparamoeba* sp., possibly *N. pemaquidensis*. Cells were placed into experimental infection systems ranging in concentration from 0 – 500 cells L^{-1}. AGD was detected by gross and histological examination in fish held in all systems inoculated with amoebae.

2. Immune response

Reinfection (Gross *et al*. 2004a)

Experiments were conducted to determine if previous infection of Atlantic salmon with *Neoparamoeba* sp. would provide protection against challenge and the immunological basis of any protection. Atlantic salmon were infected with *Neoparamoeba* sp. for 12 days then treated with a 4 hour freshwater bath. Fish were separated into two groups and maintained in either seawater or freshwater for 6 weeks. Fish were then transferred to 1 tank with a naïve control group and challenged with *Neoparamoeba* sp.

Immune response during AGD outbreak (Gross *et al.* 2004b)

An experiment was conducted to determine the effect of *Neoparamoeba* sp. infection on Atlantic salmon immune responses. Atlantic salmon naive to *Neoparamoeba* sp. exposure were experimentally infected and serially sampled 0, 1, 4, 6, 8 and 11 days post-exposure (dpe). Samples were taken from groups of unexposed and infected fish and at no time did the control (unexposed) fish show gross or histological signs of amoebic gill disease (AGD). Histological analysis of infected fish gill arches identified the presence of characteristic AGD lesions as early as 1 dpe with a steady increase in the number of affected gill filaments over time. Gross lesions were first observed on the gills of the exposed group 6 dpe. Immune parameters investigated were anterior kidney phagocyte function (respiratory burst, chemotaxis and phagocytosis) and total plasma protein and lysozyme.

Simple vaccine (Morrison and Nowak 2004)

AGD naïve Atlantic salmon (*Salmo salar*) (117 ± 5 g) were used for the trial. All fish (n = 22 fish per treatment) were anaesthetised using AQUI-S (according to the manufacturer's instructions), anchor tagged (Hallprint Pty Ltd., Victor Harbour, Australia) below the dorsal fin and placed into an aerated bath containing 50 L freshwater containing one of the following:

1. Placebo – bath only.
2. Wild type amoebae antigens (164900 cell equivalents L^{-1}).
3. NP251002 antigens (643889 cell equivalents L^{-1}).

Fish were bathed for 6 h at 17°C and transferred to a 3000 L recirculation system. Fish were acclimated to seawater (35‰) over a 7 d period starting at 20 d post-treatment. At 27 d post-treatment amoebae were scraped from the gills of two AGD affected fish from an experimental AGD infection tank as described by (Zilberg *et al*., 2001) without mucus digestion. The crude gill preparation was placed in the recirculation system at a concentration of 2867 amoebae L^{-1}. During the challenge, moribund fish were examined for gross signs of AGD. At the conclusion of the challenge experiment, gills of surviving fish were excised the second left gill arch placed in seawater Davidson's fixative and then processed for routine histology. The proportion of filaments affected by AGD lesions were assessed by light microscopy at 40× magnification.

CpGs (Bridle *et al.* 2003)

Atlantic salmon smolts, weighing approximately 80 g, were acclimatised to three 1000 L recirculated-seawater tanks over 14 days before treatment. Sea water was maintained at 17ºC at 37‰ salinity and the fish fed once daily to satiation with a commercial dry feed (50/14 pellets, Pivot Pty Ltd, Hobart, Australia). Groups of Atlantic salmon were anaesthetised with 50 ppm Aqui-S® and the following treatments administered by an intraperitoneal (i.p.) injection of 100 µL $fish^{-1}$: (1) untreated; (2) phosphate buffered saline (PBS); (3) non-CpG 1720 in PBS (50 µg $fish^{-1}$); (4) CpG-1668 in PBS (50 µg $fish^{-1}$). Oligodeoxynucleotides (ODNs) were purchased from Sigma Genosys (NSW, Australia) and were phosphorothioated to increase their resistance to nuclease degradation. Sequences of ODN are: CpG-ODN 1668, TCC ATG ACG TTC CTG ATG CT and non-CpG ODN 1720, TCC ATG AGC TTC CTG ATG CT. ODN sequences were selected based on their effective use in Atlantic salmon, rainbow trout, *Oncorhynchus mykiss* (Walbaum), and murine studies (Jørgensen *et al.* 2001a; Jørgensen *et al.* 2001b; Weighardt *et al.* 2000). Each replicate tank contained 7 fish from each of the 4 treatment groups. Fish were challenged 6 days post injection by the addition of gill-isolated amoebae to the recirculating water supply at a rate of 2460 amoebae L^{-1}. After inoculation with the gill isolate, fish were collected when moribund, and euthanased with 0.5% Aqui-S® (v v^{-1}). A gill smear was taken from the third left gill arch for immunocytochemical detection of *N. pemaquidensis* as previously described. The gills were then fixed in seawater Davidson's fixative, and the second left gill arch routinely processed for histology.

EcoActiva

Atlantic salmon smolt weighing approximately 150-200 g were transferred to 12 circular tanks (300 L) connected to a 3000 L holding tank and gradually acclimatised to sea water over two weeks. Sea water was maintained at 35‰ salinity, a temperature of 16 ± 0.5°C, and was U.V-irradiated and 0.2 µm filtered prior to addition to the tanks. Water quality was monitored every second day and total ammonia and nitrite were maintained at ≤ 0.5 mg L^{-1} and ≤ 0.2 mg L^{-1}, respectively. Fish were held for a further two weeks to assure they were healthy and free of gross signs of disease before starting the trial. Four treatments were assigned to the tanks with each treatment consisting of

triplicate tanks (20 fish tank^{-1}). Treatments consisted of feeding the fish one of three commercial *β*-glucan containing diets and a control diet.

The commercial *β*-glucan products, EcoActiva Paste (1%), EcoActiva Powder (0.3%), and MacroGard (1%) were incorporated into a commercial Atlantic salmon feed. The control diet had no *β*-glucan added. Briefly, an Atlantic salmon HP kernel diet supplied by Skretting (Hobart, Australia) was hammer-milled to produce a dry feed mix. The experimental 4 mm pellet diets were produced by adding fish oil, water, carboxymethyl cellulose (CMC) and the *β*-glucan product to the feed mix before pelleting. Inclusion rates of these standard ingredients were 14.3%, 8%, and 0.84% respectively, and were calculated allowing a 50% water loss after drying the pellets.

Feed intake varied over the trial and as such fish were fed once per day to satiation. As a result feed intake never exceeded 1% body weight per day. Fish were fed their respective experimental diets for 1 week before the challenge followed by 3 weeks of the control diet. This 1:3 feeding regime was maintained over the duration of the trial.

Four fish were sampled at 0 d before the experimental diets were fed to the remaining fish, followed by four fish from each treatment group at 3 and 7 d post initial feeding of the experimental diets, and anterior kidney respiratory burst activities were measured as previously described. Fish were anaesthetised with 50 ppm Aqui-S (Aqui-S NZ Ltd, Lower Hutt, New Zealand) and bled from the caudal vein before being euthanased in an overdose of Aqui-S (0.5% v v^{-1}) and the anterior kidney sampled.

Fish were challenged at the end of the first 7 days of feeding with the experimental *β*-glucan feeds by the addition of *N. pemaquidensis* to the recirculating water supply at a rate of 1.15×10^3 amoebae L^{-1}. Amoebae were harvested from the gills of fish with clinical AGD according and 384 amoebae L^{-1} d^{-1} were added to each of the 12 tanks over 3 days. Fish were collected and recorded when dead or moribund, however, moribund fish were first euthanased with 0.5% Aqui-S (v v^{-1}) before the gills were excised and fixed in seawater Davidson's fixative. The first left gill arch of each fish was routinely processed for histology (5 µm, H and E).

Data were analysed using SPSS version 10. One-way analysis of variance (ANOVA) and Student's *t*-tests (Bonferroni corrected) were used to analyse the respiratory burst data and Kaplan-Meier survival curves were compared using the log rank test. Relative percent survival (RPS) was evaluated according to Amend (1981) and calculated as: RPS = [1 – (% *β*-glucan diet mortality/ % control mortality) × 100]. Results of analyses were considered statistically significant if $P<0.05$.

<u>Ecoboost</u>

Three experimental feeds were formulated to contain 0.0%, 4.0% or 8.0% wet weight of Ecoboost. Ecoboost was added to a commercial salmon feed kernel

mash (Skretting Atlantic HP), replacing equal amounts of alpha-cellulose and bentonite (Table 1). The kernel mash was made by hammer-milling an extruded commercial kernel, which had not been oil-sprayed. All remaining dry ingredients, including Ecoboost, were added to each portion of mash separately and mixed thoroughly. Carboxymethylcellulose was included to aid in pelleting. The fish oil was added to individual batches of dry mix, and thoroughly mixed for at least 10 minutes. Immediately prior to pelleting, 100 ml distilled water kg^{-1} dry weight feed was added to the moist feed while mixing. The feeds were pelleted at room temperature with a 3.4 mm die, on a California Laboratory Pellet Mill (CL-2 laboratory pellet mill, California Pellet Mill Co., San Francisco, U.S.A.). Pelleting proceeded from the feed with the lowest concentration of Ecoboost (0.0%) to the highest (8.0%). After finishing all the experimental feeds for one concentration, the pellet mill was cleaned of any residual marked feed. The pelleted feeds were oven-dried at 40°C for over 24 h, and stored in a cold room at 2.7°C.

The experiment was conducted at the School of Aquaculture, University of Tasmania (Launceston, Tasmania, Australia). The twelve 300 L, cylindrical tanks comprising the experimental system were connected to a temperature-regulated (15.8 ± 0.76°C), 3000 L reservoir. Photoperiod was not controlled. Filtered (0.2 micron) seawater (salinity: 33‰) initially supplied to the system and replenished as required, was treated through physical and biological filters. The system supplied water to each tank at an average flow rate of 6 L min^{-1}. Water parameters (dissolved oxygen, oxygen saturation, chlorine, temperature, ammonia, nitrate and nitrite) were monitored to ensure water quality remained within limits recommended for Atlantic salmon (Wedemeyer, 1996).

Four hundred fish were acclimatised to salt water over a three week period. During this acclimation period the fish were fed to satiation twice per day (09:00 and 17:00) with the extruded commercial feed (Atlantic HP 4.0 mm), produced under commercial conditions by Skretting Australia (Cambridge, Tasmania, Australia). After being held in full-strength seawater (salinity: 33‰) for two weeks, three hundred Atlantic salmon parr were randomly allocated between each of the twelve tanks until a total of 25 fish were in each tank. Fish with obvious deformities were not included in the experiment. Four replicate tanks, one in each row determined at random, were fed one of the three treatment feeds to satiation twice per day (09:00 and 17:00) over 21 days. All feeds were stored in a cold room at 2.7°C until required.

Feeding of the treated feeds was stopped after 21 days and the fish were returned to the commercial feed (Atlantic HP 4.0 mm). The fish were challenged with gill-isolated neoparamoebae over a 4 day period (total 458 amoebae L^{-1}). Any moribund fish were removed from tanks twice daily, after feeding, and the gills from each fish were removed and stored in Davidson's seawater solution. During the challenge period no seawater was exchanged, and only several hundred litres were added to provide for the efficient running of the experimental system. The trial was terminated 35 days after the start of

the challenge. Throughout the 35 days of the challenge all the monitored water parameters remained within acceptable limits.

Inflammation in AGD

Juvenile Atlantic salmon (*Salmo salar*) weighing approximately 100 g were held in four 300 L recirculating seawater tank systems. Fish were acclimated to sea water over 10 days and the water maintained at 37‰ and 16ºC.

Two of these recirculating systems were inoculated with *Neoparamoeba* sp. (5000 amoebae L^{-1}) isolated from the gills of AGD infected Atlantic salmon as described by Zilberg *et al.* (2001). Both uninfected control and AGD infected fish were anaesthetised 8 days post inoculation and the vascular permeability of the two groups compared. Fish in the other two systems were euthanased and liver samples taken for real time reverse transcriptase PCR analysis of interleukin-1β gene expression. Vascular permeability was determined using a spectrophotometer to measure the amount of Evans blue dye bound to plasma protein and exuded into the peritoneal cavity after intravenous injection (Doherty *et al*., 1995). Liver samples were placed in RNAlater (Qiagen, Clifton Hill, Australia) and total RNA extracted using an RNeasy mini kit according to manufacturer's instructions (Qiagen, Clifton Hill, Australia). cDNA was reverse transcribed from total RNA using a High-Capacity cDNA Archive Kit (Applied Biosystems, Melbourne, Australia). Real time RT-PCR primers and MGB-TaqMan probes were designed by Applied Biosystems (Assays-by-Design Service) after submitting the Atlantic salmon interleukin-1β and β actin gene sequence data. Assays were performed on an ABI 7700 Prism Sequence Detection System (Applied Biosystems, Melbourne, Australia). Gene expression data was calculated using the comparative CT method (Applied Biosystems User Bulletin #2: Relative Quantitation of Gene Expression) and is expressed relative to the uninfected control.

3. Pathology

Primary pathogenicity of *Neoparamoeba* (Adams and Nowak 2004a).

Three groups of Atlantic salmon were exposed to either viable gill isolated amoebae, sonicated amoebae or seawater containing viable amoebae without direct contact to gill epithelia. Fish were removed 8 d post exposure and the gills assessed histologically for AGD.

Sequential pathology (Adams and Nowak 2003, 2004b)

Commercially reared Atlantic salmon were transferred from two freshwater hatcheries to Pillings Bay in the Huon Estuary, Southern Tasmania in early October 2001. Fish were initially sampled during transfer to the estuarine site (2 cages, n = 10 / cage) and were sampled identically on a weekly basis thereafter. Immediately following collection of fish at week 12 post-transfer, the cages were towed further toward the mouth of the estuary. Sampling was completed after 19 weeks immediately preceding initial freshwater bath treatment of fish for AGD. Fish were collected from each cage by box netting. Each fish was individually removed from the holding bin and terminally anaesthetized in clove oil (0.02% w/v). The gills were scored for gross signs of AGD and any abnormal observations upon the gills or upon each fish were

noted. Fish weights and lengths were recorded and the gills were excised for histology.

In 2002, sequential pathology between the first and second bathing was investigated. Commercially reared Atlantic salmon (out of season) smolts were transferred to Deep Bay in the Huon Estuary, Southern Tasmania in June 2002. Salmon populations from two 80 m pens within this site were selected and monitored approximately fortnightly for gross signs of AGD. On 5th December 2002 the trial pens were deemed by farm management as "heavy" in terms of AGD severity based on gross diagnosis. The pens were subsequently scheduled for initial freshwater bath treatment the following week. Fish were bathed for three hours in oxygenated freshwater sourced from a local dam (total hardness = 165 mg L^{-1} $CaCO_3$). Stocking densities were 3.34 kg m^{-3} and 10.72 kg m^{-3} for pens referred to hereafter as 10 and 14. The population within Pen 14 was subsequently split immediately after bathing, reducing the stocking density to 2.14 kg m^{-3}. Sampling commenced immediately before and after initial bath treatment and then on a weekly basis until a second bath was required to treat re-infection. Pen 10 was re-bathed after week 4 sampling was complete and pen 14 was re-bathed seven days later resulting in an extra week of data for this pen. Fish were collected from each pen (n = 10) by box netting. After terminal anaesthetization with 0.2% clove oil, the weight, length, gross gill score and any anomalous gross observations were recorded for each fish. The gills were then excised, rinsed gently in 0.22 µm filtered seawater and fixed for 1-2 h in seawater Davidson's fixative. Gross gill images for each fish were captured from the second left anterior hemibranch which were subsequently processed for histology.

Objective 2
To identify factors leading to binding of the parasite to fish gills

Antibody approach

In this project we have proposed a rationale for vaccine development based on the assumption that attachment of parasites to the gill epithelium is mediated by specific receptor-ligand interactions of molecules expressed at the surfaces of parasites and host cells. Three key issues must be addressed in following this approach for the development of an AGD vaccine: a) the molecular mediators of attachment of *N. pemaquidensis* to salmon gill epithelium must be identified and characterized; b) the ability of antibodies specific for the attachment factors to block the binding of the parasite must be demonstrated and c) strategies must be developed to ensure that vaccination generates specific antibodies at the gill surface. Molecules that mediate attachment and elicit antibodies that can block *N. pemaquidensis* binding to host cell *in vitro* and prevent infection in the disease model will provide defined candidate antigens for vaccines.

During the course of the project we have produced a large panel of murine monoclonal antibodies (Mab) against *N. pemaquidensis*. Initially (the first two fusions) we started producing Mab against *in vitro* cultured PAO27 (non-

infective) parasites due to the lack of wild type parasites. More recently (five fusions), after pooling many batches of wild type parasites and using a subtractive immunization regime outlined below, we have produced Mabs that are specific for wild type, infective, *N. pemaquidensis*. Initial immunisation with non-infective parasites (PA027) was followed, 24 and 48 h later, by injection with cyclophosphamide to suppress the immune response. This treatment was followed by priming with infective parasites (WTNPA) and boosting with whole WTNPA parasites (2 fusions) or deglycosylated membrane preparations of WTNPA (3 fusions).

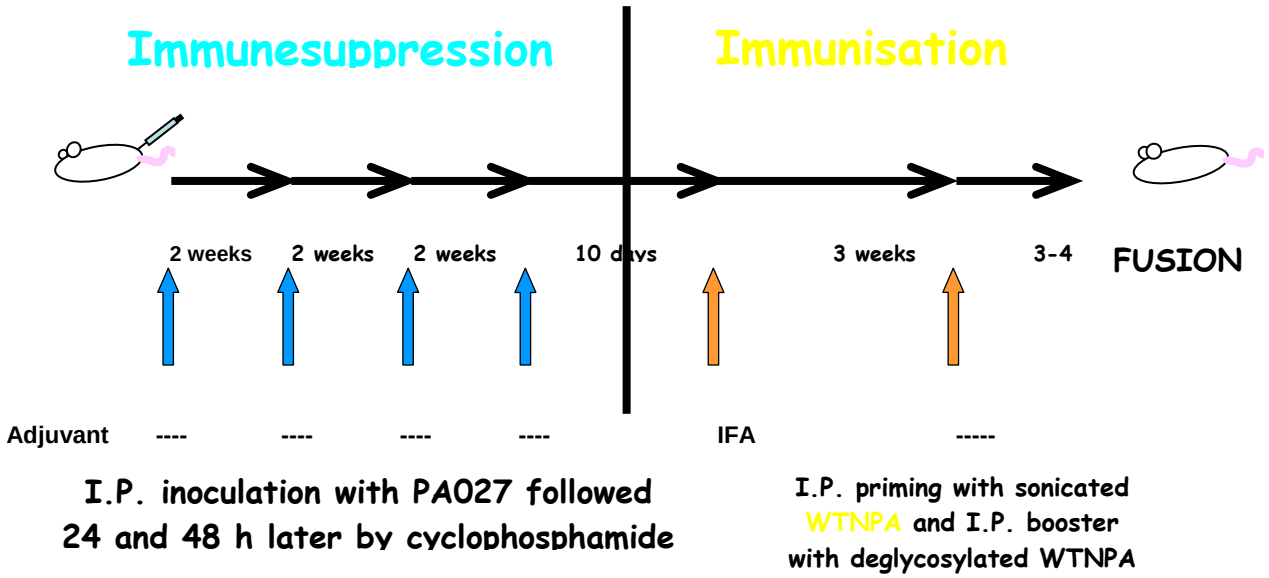


Figure 1. Subtractive immunization protocol used in fusion number 5.

Hybridomas were screened by EIA, IFAT and flow cytometry to identify Mabs reacting with cell surface antigens unique to infective parasites. The chemical nature of the antigen recognised by selected Mabs was determined: ie. peptide or carbohydrate epitopes. Hybridomas specific to the infective parasites were further tested in an *in vitro* attachment assay.

Amoeba attachment to gill explants (Butler and Nowak 2004a)

Infection tank exposure

The role of blood and mucus in the attachment of amoebae was investigated in an *in vitro* experiment. Mucus was removed using mucolytic agent and perfusion removed blood. The gill arches from duplicate fish in each of the four groups (untreated, perfused, mucolytic, perfused and mucolytic, n=16 arches per group) were separated from the gill basket and 15 cm of nylon thread tied to both afferent and efferent regions of the branchial arch. A small weight was attached to the thread at the efferent end and the afferent thread was secured to a line running across the centre of a 2000 L tank. Gills were suspended

vertically in the water column at approximately 15 cm depth and at 5 cm intervals. The tank contained recirculating seawater (35‰ ± 1‰) at ambient temperature and was used to maintain a permanent laboratory infection of AGD in Atlantic salmon. The infection at the time of this study was active and caused AGD related mortalities in naive fish following 7-9 days of exposure.

At 2, 4, 12, and 24 h after addition to the infection tank duplicate gill arches per fish were removed, rinsed in a stream of sterile seawater and then stored in sterile seawater for transport to the laboratory. Arches were individually scraped using plastic 'hockey-stick' spreaders with a diluent of 30 mL of sterile seawater. Four glass slides were placed in a plastic tray and the gill suspension was added to the surface and maintained for one hour at room temperature to allow amoebae present in the suspension to adhere. Excess gill suspension was removed and the slides washed gently in a stream of sterile seawater. Each slide was processed for immunohistochemistry and viewed x100 magnification and the number of positive immunostained cells recorded.

Assay of amoeba attachment inhibition to salmon gill explants
Various treatments were applied to amoebae to manipulate amoeba attachment. These treatments included:

1. Heat treating amoeba suspension for 30 min at 56°C.
2. Incubation of amoeba suspension with proteinase K.
3. Incubation of amoeba suspension with proteinase K and subsequent incubation in seawater for 24 h.
4. Incubation of amoeba suspension with monoclonal antibodies raised against *N. pemaquidensis.*

In addition gills prior to explantation were treated with 10 IU hyaluronidase in PBS (Sigma-Aldrich) for 30 minutes that removed the mucus layer of the tissue, to determine the involvement of mucus in the attachment process.

In each case 500 amoeba mL^{-1} were added to 12 well tissue culture plates containing a single gill explant and maintained at 18°C for 24h. Explants were then removed, rinsed in a stream of sterile seawater and the tissue scraped from the branchial arch using a plastic 'hockey stick' spreader in a diluent of 30 mL of seawater. The suspension was decanted onto the surface of four glass slides and the number of adherent amoebae after 1 hour of incubation was counted following immunohistochemical processing (Bridle *et al.* 2003).

Objective 3
To identify gill conditions which increase the susceptibility of the fish to AGD

Gill damage (Adams and Nowak 2004)

AGD infection was evaluated histologically at 12 h, 24 h and 48 h post exposure to gill-isolated amoeba (2600 cells L^{-1}) in three groups of salmon, one group being mechanically injured 12 h prior to exposure (the first and second left anterior hemibranchs abraded with a sterile cotton swab until haemorrhage was evident).

Presence of bacteria (Bowman and Nowak 2004, Embar-Gopinath *et al.* 2004)

Bacteria present on gills with and without AGD were identified as previously described (Bowman and Nowak 2004). Atlantic salmon, *Salmo salar* L., of approximately 85 g were acclimatised to sea water (35‰, 1 µm filtered) over 7 days in 6 recirculating systems each consisting of three 70 L containers and a 70 L sump.

Following acclimatisation, fish were divided into three experimental groups (n = 4 fish per container) consisting of 2 recirculating systems per group and a negative control group. Fish in group 1, were exposed to amoeba only (positive control); group 2, gram positive bacteria (*Staphylococcus* sp.) and amoeba; group 3, gram negative bacteria (*Winogradskyella* sp.) and amoeba; group 4, no treatment (negative control).

Sea water temperature was maintained at 16 $\pm$ 0.5°C, pH 8.2, dissolved oxygen 7.6 mg L^{-1}, salinity 35‰ and total ammonia-nitrogen below 0.2 mL^{-1}. Prior to inoculation, all the groups except control were bathed in sea water containing potassium permanganate ($KMnO_4$) solution (5 mg L^{-1}) for 20 minutes to remove the natural microflora on the gills. The control fish were bathed in sea water for the same time. After the bath the fish were maintained for 2 days to return to normal conditions.

Two fish were then randomly selected from all groups and were euthanased with an anaesthetic overdose (Aqui-S), and mucous samples were collected from the gills and streaked on to several bacterial media (Sheih's medium), Marine Agar (Difco), Tryptone Soya Agar (Oxoid), Todd Hewitt medium (Oxoid, Australia)) to establish baseline community structure. The remaining fish in group 2 and group 3 were exposed to sea water containing *Staphylococcus* sp. and *Winogradskyella* sp. bacteria (1×10^5 cells mL^{-1}) respectively for 1 hour. Group 1 and 4, were bathed in sea water for the same time. Again the fish were maintained for 4 days to allow the development of inoculated colonies on the gills. Later, all the groups, with the exception of group 4 were infected with *Neoparamoeba* sp. (300 cells L^{-1}).

All fish were sampled on day 8 post amoeba challenge, when white mucous patches (consistent with AGD gross pathology) were observed on the gills of all treatment groups. Fish were euthanased with an anaesthetic overdose (Aqui-S), and swabs of gill mucus and anterior kidney were taken and streaked

onto a range of bacterial media (given previously) and incubated at 20^{o}C for 48 h. After incubation the morphological characterisation of the colonies revealed that the shape, size, cell arrangement, pigmentation and staining were consistent with *Staphylococcus* sp. (group 2) and *Winogradskyella* sp. (group 3) as previously recorded (Gopinath, unpublished).

The gill basket was then removed and placed in seawater Davidson's fixative and post-fixed in 70% ethanol. Tissues was processed for routine wax histology and stained with haematoxylin and eosin. To visualise gram positive or negative bacteria on the gills and to determine their association with amoebae the group 2 and 3 sections were also stained with Brown and Brenn stain.

All sections were assessed viewed by light microscope and fish with amoebae associated with AGD typical lesions were considered AGD positive. The section where the gill was well orientated and consisted of greater than three quarters of the filament was used in disease diagnosis. The number of filaments that had a typical AGD lesion characterised by single or multifocal epithelial hyperplasia and leucocytic infiltration resulting in lamellar fusion was recorded. The percentage of affected filaments was calculated from this to give an estimation of disease severity.

Lesion size was also recorded by counting the number of hyperplastic interlamellar units within each lesion (Adams and Nowak 2001). Images of the affected tissue were taken using Leica DC300f, Wetzlar, Germany.

A one-way ANOVA using SPSS© version 11.5 was performed to test for significant difference between the experimental groups. Significant differences between groups were assessed using Tukey's HSD *post-hoc* test, homogeneity of variance was assessed using Levene's test of equality of error variances; differences were considered significant at the $P \leq 0.05$ level.

Presence of other amoebae

Field study

Total of 32 fish were sampled in the field aiming to isolate amoeba. The samples were processed as described in Dyková *et al.* (2005).

Experiment 1

Atlantic salmon (n = 27, average weight 95 g) were acclimated to seawater over a ten day period and were held at (35 ‰) approximately two weeks prior to experimentation. Acclimation was conducted in a modular tank system comprised of three interconnected circular tanks (1000 L) with a biofilter and supplied with 5 μm filtered seawater.

Following acclimation, fish were transferred and equally divided among the three modular systems each with 3 holding tanks and a reservoir (270 L per module, 1 μm filtered seawater, n = 3 fish per tank, n = 9 fish per module/treatment group). Water was exchanged at 30% per day starting at day

3. Water quality parameters for the duration of the experiment (measured daily) are outlined below in Table 1.

Table 1. Treatment group designations and water quality parameters (Exp 1)

Group	Treatment	Temp ^{o}C	pH	DO mg L^{-1}	NH_4 ppm
P1	*Platyamoeba* sp.	17.3	8.2	7.8	<2
NP1	*Neoparamoeba* sp.	17.0	8.2	7.8	<2
C1	Combined amoebae	16.8	8.2	7.8	<2

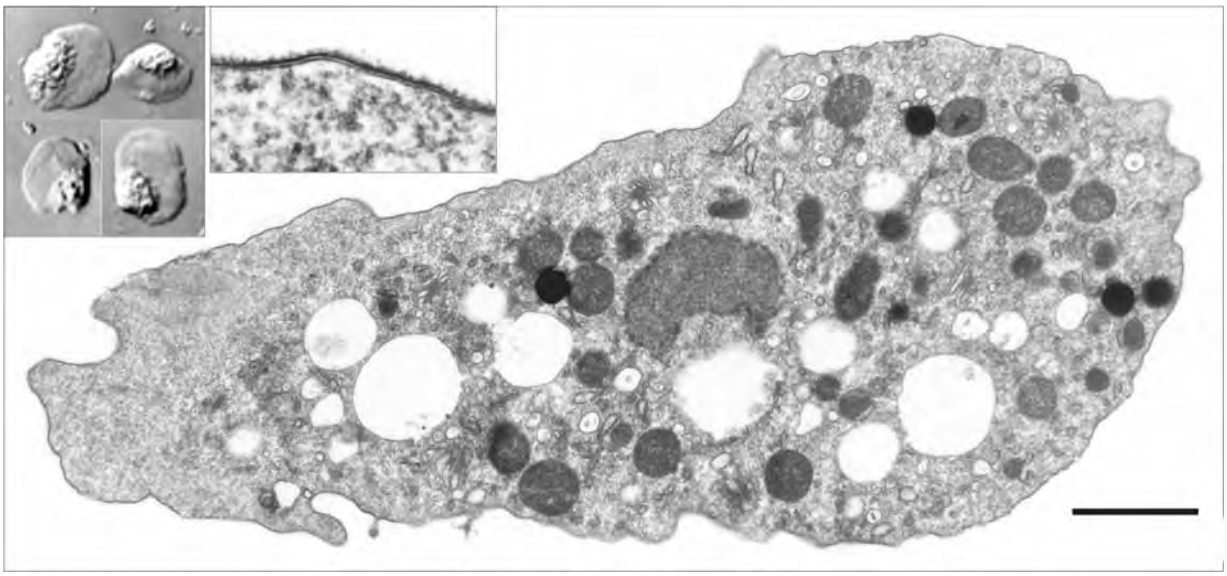


Figure 2. (Inset left) *Platyamoeba* sp. of cultured strain SS8FJ2/l viewed using Olympus Nomarski DIC system. (Inset right) TEM image of cell surface of above strain. (Foreground) TEM of whole cell of above strain.

Platyamoeba sp. (cultured strain SS8FJ2/l – Figure 2), isolated from *S. salar* farmed in Ireland (Feb 2000: primary isolation, May 2000: cryopreserved [liquid N_2 - passage no 15] thawed Oct 2000) were grown on non-nutrient agar at 18^{o}C, harvested from plates (passage no 66) and added to modules 1 (Groups P1 and C1 respectively) at approximately 800000 cells L^{-1}.

Neoparameba sp. were isolated and quantified from the gills of *S. salar*, using methods previously described by Zilberg *et al.* (2001). The source of infective *Neoparamoeba* sp. trophozoites was from an ongoing infection system located at the University of Tasmania, Launceston. Trophozoites from gill isolates of *Neoparamoeba* sp. were added to modules 2 and 3 (Groups C1 and NP1 respectively) at approximately 3900 cells L^{-1}.

During transfer of fish from the acclimation module to infection modules, four additional fish (pre-treatment control) were removed. These were terminally anesthetized (0.2% clove oil), gills excised, rinsed briefly in 0.22 μm filtered

histology. Any moribund fish were removed from their respective systems and sampled as above.

Following eight days of exposure of fish to amoebae the experiment was terminated. Following fish collection, tissue was excised from the gills for amoebae culture isolation and transmission electron microscopy as outlined by Dyková *et al.* (2000). Remaining tissues were processed for routine histology.

Experiment 2
Atlantic salmon (n = 36, mean weight 196 g, range 71 g – 297 g) were acclimated to seawater (35‰) over a ten day period. Acclimation was conducted in a 4000 L Rathburn tank with self-contained biofilter and solids separator. Following acclimation, fish were transferred and equally divided among four modular systems each with 3 holding tanks and a reservoir (270 L per module, 1 μm filtered seawater, n = 3 fish per tank, n = 9 fish per treatment group). Water quality parameters for the duration of the experiment are outlined below in table 2.

Platyamoeba sp. (details as above) were grown on non-nutrient agar at 18^{o}C, harvested from plates (passage no 80) added to modules 1 and 2 (groups P2 and C2 respectively) at approximately 50000 cells L^{-1}. *Neoparameba* sp. were isolated and quantified from the gills of *S. salar*, using improved trophozoite isolation methods described by Morrison *et al.* (2004). The source of infective *Neoparamoeba* sp. trophozoites was from an ongoing infection system located at the University of Tasmania, Launceston. Trophozoites from gill isolates of *Neoparamoeba* sp. were added to 2 and 3 (groups C2 and NP2 respectively) at approximately 500 cells L^{-1}. No trophozoites were added to the remaining group (N2) thus providing a negative control.

Any moribund fish were removed from their respective systems, recorded as a mortality and sampled for histopathology as previously described. The experiment was terminated at day 13 post-exposure (PE). Fish were terminally anesthetized (0.2% clove oil), the second and third left arches were excised, rinsed briefly in 0.22 μm filtered seawater, fixed (seawater Davidson's [12 h]) and processed for routine histology. Small samples of remaining gill tissue were removed for trophozoite isolation as described by Dyková *et al.* (2000).

Table 2. Water quality parameters* (Exp 2)

Treatment	n	Temp	Salinity	DO	pH	Cells L^{-1}
N2	9	16	37.3	7.8	8.2	0
NP2	9	16.3	37.1	7.8	8.2	500
P2	9	16.5	37.3	7.8	8.2	50000
C2	9	15.8	37.3	7.8	8.2	500 and 50000

N = Negative control

NP = Gill isolated, adherent *Neoparamoeba* sp.

P = *Platyamoeba* sp. (strain SS8FJ2/l)

C = Combined culture and gill isolates

* NH_4 maintained below 1ppm, water exchange 30%.day^{-1}

Objective 4
To develop techniques for *in vitro* work on Amoebic Gill Disease

In vitro models of fish tissues have been used extensively in the study of physiology and toxicology, but to a lesser extent in health and immunology. Simple cell monolayer models are used extensively in the isolation of viruses and in bacterial invasion studies. Similarly they have been used in the study of endoparasites and in particular intracellular parasites. They have not been used widely to work with ectoparasites because of the often physically invasive processes of parasite attachment that single cell layers cannot sustain and remain viable. This is made more complicated if the parasite is also a marine organism. The natural state of all cultured cells is low tonicity comparable to normal physiological osmolality of approximately 300 mM kg^{-1}; marine environments are typically 1000 mM kg^{-1}. In such circumstances more complex reconstructed tissue models have been used that are capable of limited osmoregulation or provide physical barriers to parasite damage and the ingress of seawater-based maintenance media.

This project adopted 3 approaches to the maintenance and study of gill amoeba with host tissues; cell monolayers; multi-layered cell cultures; and explants of host tissue.

Cell monolayer culture model

Whilst *in vitro* effects of disease-causing organisms are often recorded using non-host cells it is due to the lack of suitable cell lines from the host species rather than a deliberate choice; the preference is to study effects using appropriate tissue. There are very few gill cell lines from salmonid fish and no lines from Atlantic salmon gill.

<u>Dual-enzyme method for establishment of primary cultures from Atlantic salmon gill (Butler and Nowak 2004b)</u>

Atlantic salmon gills were perfused with 0.9% NaCl solution containing 10 IU mL^{-1} lithium heparin to remove circulating cells. The gill basket was removed and individual filaments chopped to approximately 2-3 mm^3. The tissue fragments were first incubated with a collagenase digestion medium (4mg/ml bacterial collagenase in HBSS (Sigma-Aldrich)) for 30 min. The tissue was then pelleted by centrifugation at 100 g for 10 min at 4°C, the supernatant containing isolated cells was retained and the enzyme neutralized by the addition of culture medium containing 10% v/v Foetal Calf Serum (FCS) (Sigma-Aldrich). The tissue pellet was resuspended in a trypsin digestion medium (10% v/v trypsin-EDTA solution (2.5% trypsin) in HBSS (Invitrogen)) and mixed for 30min. The tissue was centrifuged and the supernatant containing isolated cells was retained and pooled with the previously isolated cells. The tissue was then incubated with the collagenase digestion medium and this cycling of collagenase and trypsin digestion was repeated for a total of 4 cycles. The density of viable isolated cells was determined by trypan blue exclusion and the suspension was adjusted to 5 x 10^5 cells mL^{-1} and 200 µL decanted into 24 well cell culture plates. Wells were washed at 2 days post isolation to remove non-adherent cells and media replaced every 7 days (L-15

medium containing 50% v/v sterile filtered culture-conditioned media) until monolayers were confluent. Cells were passaged by the addition of trypsin-EDTA and split in the ratio 1:2 until passage 5 after which the ratio was 1:3.

In vitro interaction of gill amoeba and Atlantic salmon gill cells (Butler and Nowak 2004c)

Neoparamoebae were harvested from gills of Atlantic salmon affected by AGD maintained within the Aquaculture Centre, University of Tasmania, Launceston. Fish showing AGD pathology (white gross gill lesions) were euthanased by anaesthetic overdose (Aqui-S®), the entire gill basket removed and transported to the laboratory in sterile seawater containing antibiotic and antimycotic solution (5% v/v 5000 IU mL^{-1} penicillin and 5 mg mL^{-1} streptomycin solution (Sigma), 1% v/v 10 mg mL^{-1} gentamycin (Sigma) and 0.25 mg mL^{-1} amphotericin B (Invitrogen)). The medium was exchanged for sterile distilled water and the gills mixed for five minutes to loosen the attachment of amoeba to tissue. Gill filaments were scraped into a Petri dish using cell scrapers to remove mucus and surface epithelial cells.

The suspension was centrifuged at 400 x *g* for 10 minutes and the pellet resuspended in seawater. The suspension was further diluted with sterile seawater to approximately 50 times and 30 mL decanted into multiple Petri dishes. Amoebae were allowed to adhere to the surface of the dishes for one hour, following which the medium was decanted into fresh Petri dishes to undergo a second round of adherence. Adhered amoebae were resuspended by the addition of 3 mL trypsin-EDTA solution (0.25%) (Invitrogen) for two minutes, then pooled and diluted with sterile seawater prior to centrifugation at 400 x*g* for 10 minutes. Pelleted amoebae were resuspended in seawater and the viable population estimated using haemocytometer and trypan blue. The proportion of *N. pemaquidensis* in the population was measured by an indirect immunoperoxidase method using a primary rabbit anti-*N. pemaquidensis* (PA027) antibody using the method of Bridle *et al*. (2003).

Atlantic salmon gill cells (RGE-2 cell line) were passaged into 12 well tissue culture plates (Nunc) and allowed to reach confluency with standard culture medium (Leibovitz (L-15) medium supplemented with 10% FCS, 2 mM L-glutamine (Invitrogen), 1% v/v penicillin streptomycin solution (Sigma) and 1% v/v 30 mM NaCl). Media were removed and the monolayers washed three times with 0.01 M phosphate buffered saline (PBS) pH 7.4 to remove traces of FCS. Isolated amoebae were resuspended to a final density of 1 x 10^6 cells mL^{-1} in either serum free standard culture medium (330 mM kg^{-1}), hyperosmotic culture medium (780 mM kg^{-1}), or seawater culture medium (1000 mM kg^{-1}).

Hyperosmotic medium was prepared by the addition of 5% v/v salt solution (50% v/v 5 M $MgCl_2$, 50% v/v 2.5 M Na_2SO_4, pH 7.4) and 5% v/v sugar solution (33.3% v/v 1 M sorbitol, 33.3% v/v 1 M mannitol and 33.3% v/v 1 M xylose, pH 7.4) to serum free standard culture medium. Seawater culture medium was

prepared by the addition of 7.5% v/v salt solution and 7.5% v/v sugar solution to serum free standard culture medium.

Two hundred microlitres of amoeba suspension was added to appropriate wells of culture plates and incubated at 18°C. Control wells consisted of cells maintained without amoebae with standard, hyperosmotic or seawater culture media. Amoeba growth estimates were measured daily by counting of amoeba per field of view in five random fields in duplicate culture wells (at x200 magnification). RGE-2 cell survival was measured using a modified MTT cell viability and proliferation assay. Briefly, media were removed from four replicate wells, washed with PBS to remove amoebae and 100 µl of 5 mg mL^{-1} MTT in L-15 added and incubated at 18°C for four hours. Media were removed and 100 µl dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was then added, the contents mixed by pipetting for 10 minutes and the supernatants read at an optical density of 550nm on a Spectra Rainbow thermo-microplate reader (Tecan). A standard curve was produced by plotting haemocytometer cell counts against the corresponding OD.

Assay modifications

In addition to using RGE-2 cells the interaction of gill amoeba with cultured cells from other fish species and of different tissue origin was tested. The protocol was as above and differed only in the formulation of the standard culture medium for each cell line (Table 3).

Table 3.

Cell Line	Species Origin	Tissue Lineage	Culture Medium†	Source
RGF	Atlantic salmon	Gill - fibroblast	L-15 +10% FCS + 2 mM L-Glutamine + 1% v/v 30 mM NaCl	School of Aquaculture, Launceston Tasmania
ASE-w	Atlantic salmon	Skin - epithelial	L-15 +10% FCS + 2 mM L-Glutamine + 1% v/v 30 mM NaCl	FRS Marine Laboratory, Aberdeen, Scotland
AS-6	Atlantic salmon	Mixed tissues - fibroblast	L-15 +10% FCS + 2 mM L-Glutamine + 1% v/v 30 mM NaCl	FRS Marine Laboratory, Aberdeen, Scotland
CHSE-214	Chinook salmon	Embryo - epithelial	MEM +2mM L-Glutamine + 10% FCS	AAHL, Geelong, Australia
RTG-2	Rainbow trout	Gonad - fibroblast	MEM +2mM L-Glutamine + 10% FCS	AAHL, Geelong, Australia
BF-2	Bluegill Sunfish	Caudal trunk - fibroblast	GMEM +2mM L-Glutamine + 10% FCS	FRS Marine Laboratory, Aberdeen, Scotland
EPC	Common carp	Epidermis - epithelial	EMEM + 2mM L-Glutamine + 1% Non Essential Amino Acids + 10% FCS	FRS Marine Laboratory, Aberdeen, Scotland

†All media formulations were supplemented with 1% v/v Penicillin-streptomycin solution (Sigma-Aldrich, P0718)

Gill explant culture model

Explanted animal tissues are commonly used for the study of biological processes *in vitro*. These studies benefit in having a complete tissue with which to model the process of interest but are limited in the time they can accurately interpret the observations as being real, and not artefactual of a decaying tissue; in most cases tissue degradation is rapid. In order to study the longer-term interaction of gill amoeba and gill tissue *in vitro* we developed a method for the extended maintenance of explanted gills that was also applicable for short-term experiments for studying early pathogenesis and amoeba attachment.

Fish were anaesthetised with Aqui-S® according to manufacturer's guidelines and bled from the caudal venous sinus using vacuette tubes and 23G needles (Greiner). The heart was exposed following an incision along the ventral mid-line and a perfusate of 0.9% NaCl solution (Viaflex™) containing 10 IU mL^{-1} lithium heparin (Sigma-Aldrich) was introduced to the ventricle under gravity flow through a 25G needle. The perfusion was allowed to continue for several minutes before the caudal peduncle was severed and the perfusate and remaining blood permitted to drain. The procedure continued until either heart function stopped or until the gills were adequately perfused as indicated by their white appearance. The gills were removed and placed in ice-cold antibiotic seawater solution (5% v/v 5000 IU mL^{-1} penicillin and 5 mg mL^{-1} streptomycin solution (Sigma-Aldrich), 1% v/v 10 mg mL^{-1} gentamicin (Sigma-Aldrich) and 0.25 mg mL^{-1} amphotericin B (Invitrogen)) for transport to the laboratory.

All subsequent procedures were performed within a sterile environment. Gills were washed in a gentle stream of antibiotic seawater solution to dislodge sloughed cells and blood and submerged within an iced dish of the same solution diluted 1:5 with sterile seawater. Gill arches were removed from the gill basket and trimmed to remove approximately 1 cm from each end; the sites from where tissue degradation was observed to begin. Explants were placed into individual wells of 12 well tissue culture plates (Nunc) and maintained in ice-cold antibiotic seawater solution for 3 h after which media were replaced with sterile seawater and the plates incubated at 18°C. Tissues were fixed in 8% formal buffered saline at regular intervals between 0 h and 10 days post explantation and processed for normal wax histology for observations of tissue changes.

In vitro interactions between gill amoeba and Atlantic salmon gill explants

Explants of Atlantic salmon gill tissue were prepared as detailed above and placed in individual wells of 12 well tissue culture plates (Nunc). Gill amoeba were isolated from AGD affected fish using the previously stated method, the density adjusted to 500 viable cells mL^{-1} and 1 mL added to each explant. Controls consisted of wells incubated with seawater only, and wells of amoebae without explanted tissue. At 2, 4, 12, 24 and 48 h post incubation explants were removed and rinsed in a stream of sterile seawater and fixed in seawater Davidson's solution. Tissue was processed for normal wax histology

and observations of tissues changes, presence of adhered amoebae and in particular indications of tissue changes and interaction at the sites of amoeba attachment were made.

Application of models for *in vitro* study of gill amoeba

Assay of amoeba cytolysins

RGE-2 cell monolayers were prepared in 24 well tissue culture plates and 200 µL of 1 x 10^6 cells mL^{-1} isolated gill amoeba in hyperosmotic culture medium added to each well as described previously. Cultures were observed regularly over 5 days. Incubation of gill amoeba and RGE-2 monolayers caused complete monolayer cytolysis in 5 days. Various treatments were applied to the amoeba to manipulate this outcome which would provide evidence for the nature of the cytolysis. In each case the outcome was recorded as 'cytolysis' (the treatment had no effect on the supernatant), 'reduced cytolysis' (the treatment had some negative effect on supernatant) and 'no cytolysis' (the treatment prevented action of the supernatant). The following treatments were applied:

1. Heat treating amoeba suspension for 30 min at 56°C.
2. Incubation of amoeba suspension with proteinase K.
3. Incubation of amoeba suspension with proteinase K and subsequent incubation in seawater for 24 h.
4. Incubation of amoeba suspension with monoclonal antibodies raised against *N. pemaquidensis.*

Assay of amoeba secreted/ excreted cytolysins

RGE-2 cells were prepared in 24 well tissue culture plates and isolated gill amoeba added to each well as described previously. Following 24 h or 48 h incubation at 18°C plates were centrifuged at 200 x *g* for 5 minutes and the supernatants collected. Supernatants from each plate were pooled and dialysed overnight against 4 changes of PBS at 4°C to bring them to physiological osmolality. They were then filter sterilised (0.22 µm, Millipore) and the protein concentration measured using a micro BCA protein kit (Pierce Biotechnology, Illinois, USA).

Fresh plates of RGE-2 cells were prepared and allowed to reach confluency. Media were removed and cell monolayers washed x3 with PBS to remove FCS. Supernatants from amoeba culture were diluted 1:10 with standard culture medium (serum free) and added to the cell monolayers. Observations of changes to the cell monolayer were made at regular intervals and the cell density in duplicate wells was determined by MTT cell proliferation and viability assay at 24 h intervals for 8 days.

Incubation of amoeba culture supernatants caused cytolysis of RGE-2 cells. Various treatments were applied to manipulate this outcome which would provide evidence for the biochemical nature of the cytolysin(s) within the supernatants. In each case the outcome was recorded as 'cytolysis', 'reduced cytolysis' and 'no cytolysis' as used previously. The following treatments were applied:

1. Heat treating supernatants for 30 min at 56°C and 70°C.
2. Incubation of supernatants with proteinase K.
3. Dilution of supernatants to 1:100 and 1:1000 in standard culture medium (serum free).
4. Incubation of supernatants with monoclonal antibodies raised against *N. pemaquidensis.*

A further modification was testing of cytolysis of supernatants collected from gill amoeba incubated with culture medium in the absence of salmon cells and amoeba incubated in culture medium supplemented with 10% v/v Atlantic salmon mucus and 10% v/v Atlantic salmon serum.

Confirmation of CPE by AAHL

Samples of gill-isolated amoebae were sent to AAHL on two different occasions. Two types of cell lines used were CHSE-214 and EPC. The amoeba samples were homogenised by both physical and freeze-thaw method. Solution was filtered on 0.25 µM filter and 140 µL pippetted into respective wells and incubated for 1 hour. Media were added and plates incubated at 15ºC with CO_2 for CHSE-214 or 15ºC for EPC. The plates were checked for CPE every 3-4 days. At day 11 mixture of cells and culture supernatant was passed on a fresh cell line. These plates were checked until the final read on day 11 post-transfer.

Objective 5

To expand *N. pemaquidensis* library of strains

Amoebae isolation from gill material

Gill tissue was collected from both the field (ie. farm sites) and the AGD infection tank at the Aquatic Centre at the School of Aquaculture. Field sites included Bruny Island and the Huon Estuary. At the Huon site gill tissue was dissected and individual filaments immediately placed onto malt yeast seawater (MYS) agar plates (0.1 g malt, 0.1 g yeast, 750 mL filtered seawater, 250 mL reverse osmosis water) then transported back to the laboratory where they were incubated at 20ºC and examined daily for growth of amoebae. At the Bruny Island site whole gills were removed from fish then placed in sterile seawater for transportation back to the laboratory where the culture procedure was carried out as above with the inclusion of the pimaricin (an antifungal compound) and *Stenotrophomonas maltophilia* as a food organism on the MYS plates.

Amoebae were isolated from the infection tank using the method of Morrison *et al.* (2004). Briefly, the entire gill basket was removed from moribund or dead fish then placed in sterile seawater. Individual gill filaments were then dissected and placed into 50 mL tubes of sterile distilled water and gently agitated for 1-2 min before centrifugation at 400 x *g* for 4 min. The supernatant was discarded and the filaments were re-suspended in 0.2 µm filtered seawater and tubes inverted several times to dislodge amoebae from the tissue. The suspension was then poured into petri dishes and left on the bench

for 1 h to allow the amoebae to adhere to the plastic. The liquid was then discarded and the plates washed several times with filtered seawater to clear debris. The adherent cells were removed by addition of trypsin/EDTA (0.05% trypsin, 0.53 mM Na_4EDTA). The cell suspension was then aliquoted into individual cell culture flasks containing the liquid phase culture medium (sterile seawater with antibiotics: 0.001% v/v streptomycin sulphate, 0.001% v/v penicillin, 0.001% v/v carbenicillin, 0.0025% v/v ampicillin, 0.001% v/v erythromycin, 0.63% v/v sulphadiazine and 0.13% trimethoprim and autoclaved *E. coli,* approximately 10^8 cells mL^{-1}) and incubated at 18ºC. Flasks were examined daily and those containing amoebae only were retained.

Amoebae isolation from marine sediments and seacage net material

Marine sediment samples were collected from various sites within specific areas around Tasmania and *N. pemaquidensis* cultured from this material. Areas comprised finfish culture and non-culture sites, these included both estuarine and oceanic locations. The most extensively sampled sites were in the Huon Estuary and on the Tasman Peninsula where samples were collected on 3 and 2 occasions respectively. Other sites, which were sampled once were: Hideaway Bay, Bruny Island, Tinderbox, Tamar estuary, Bicheno and Macquarie Harbour. Sediment sample volumes ranged from approximately 200-800 g. Isolation of amoebae from sediments was achieved by inoculating 3-5 g of sediment material onto MYS agar plates containing pimaricin and which had been seeded with *Stenotrophomonas maltophilia* as a food organism, the plates were then incubated at 20ºC. Amoebae were isolated form seacage net material in the same way with 1-2 cm piece of mesh used instead of sediment.

Sub-culture, identification and cloning

Once amoebae were observed growing on agar, usually within 7-14 days for sediments and net material, they were subcultured by cutting out blocks of agar showing growth and inverting them onto fresh MYS plates. In this way enough cells could be produced to allow identification and maintain the culture. Amoebae were then washed from plates using sterile seawater and suspensions collected, they were identified as *N. pemaquidensis* using 3 detection/ identification methods:

1. Polymerase chain reaction (PCR) – with specific primers developed by Wong, Carson and Elliott (2004) - later shown to cross-react with *N. branchiphila (*Dyková *et al*. 2005).
2. Immunofluorescent antibody test (IFAT).
3. Observation of the parasome, an endosymbiont characteristic of the family, using either a DAPI (4'6-diamino-2-phenylindole) stain or differential interference phase contrast microscopy.

Once cultures were shown to be *N. pemaquidensis* they were cloned by inoculation of a single cell onto a MYS plate and subsequent propagation. To collect a single cell a suspension was diluted serially in 50 µL drops around a sterile petri dish. A drop containing tens of cells was removed and put on a 2% agar plate which was then tilted until the drop ran across and around the plate

leaving a trail, once all the liquid was absorbed by the agar single cells could easily be located and removed individually from the agar.

Objective 6
To implement a long term preservation for *N. pemaquidensis* based on freezing technology

N. pemaquidensis and culture conditions

Isolates of *N. pemaquidensis* used included: PA027 (isolated from Atlantic salmon, *Salmo salar*, in Tasmania by Dr T. Howard 18/4/94), AFSM2V/II and AFSM11/II (isolated from turbot, *Scophthalmus maximus* in Spain by Dr I. Dyková on 9/1/00 and 28/11/00 respectively) and NP 251002 (Isolated by Dr R. Morrison from Atlantic salmon in Tasmania on 25/10/02). All amoebae were maintained on malt yeast seawater (MYS) agar plates and incubated at 20ºC. Sub-culture occurred weekly when dense areas of amoebae growth were cut from the agar and inverted onto fresh MYS plates. To obtain large numbers of cells amoebae were gently flushed from the agar plates with 3 mL of sterile seawater then approximately 200 μL of the suspension was spread onto MYS plates and incubated at 20ºC for 3-4 d. The amoebae were finally harvested as above then cell suspensions were transferred to 50 mL tubes, then washed and concentrated by centrifugation at 400 x *g* for 5 mins.

A virulent strain of *N. pemaquidensis* was collected, partially purified and identified from experimentally infected fish (displaying clinical signs of AGD) using methods described by Morrison *et al.* (2004). The suspension of virulent cells was subjected to the freezing protocol either immediately or after a starvation period of 72-96 h where washed cells were suspended in sterile seawater and incubated at 20ºC or suspended in sterile seawater supplemented with antibiotics (0.001% v/v streptomycin sulphate, 0.001% v/v penicillin, 0.001% v/v carbenicillin, 0.0025% v/v ampicillin, 0.001% v/v erythromycin, 0.63% v/v sulphadiazine and 0.13% trimethoprim).

Freezing protocols

Many attempts were made to preserve a reference isolate of *N. pemaquidensis* (PA027) via cryopreservation based on variation of some parameters including:

1. numbers of cells.
2. age of cells in culture (ie. number of days since sub-culture)
3. volume of suspension.
4. concentration of dimethyl sulfoxide (DMSO) as cryoprotectant.
5. cooling rate.

Methods attempted were:

1. 10^6 cells in 1 mL volume with 1 mL of 10% (DMSO, final concentration 5%), equilibration at room temperature for 25 mins, initial cooling for 1 h at -80ºC, then to liquid nitrogen at -196ºC.
2. 5 x 10^5 cells with 10% DMSO (final concentration 5%) in 1 mL volumes, equilibration at room temperature for 30 mins, exposure for 24 h to liquid nitrogen vapours, then total immersion in liquid nitrogen.

3. Cells harvested from 7, 17 or 21 day-old culture plates, mixed with 10% DMSO as 1 mL volumes (DMSO final concentration 5%) with cell densities 1 x 10^6, 2.5 x 10^5 and 1.3 x 10^5 cells per mL respectively. Equilibration at room temperature for 25 min, then controlled cooling at 1ºC per min using an insulated container (Nalgene ^{TM} cryo 1ºC) placed at -80ºC for 24 h before final placement in liquid nitrogen.
4. 2 x10^6 cells per mL, mixed with 1 mL of DMSO at 15%, therefore the final concentration of cells was 10^6 per mL, and the final concentration DMSO concentration of 7.5% .Suspension was then dispensed into cryovials at 0.5 or 1 mL volumes. Equilibration at room temperature for 20 mins, then controlled cooling at 1ºC per min using an insulated container (Nalgene ^{TM} cryo 1ºC) at -80ºC for 90 mins before total immersion in liquid nitrogen.

For methods 1, 2 and 3 PA027 was the only isolate used. For method 4 all the isolates described above were used.

Method 4 was based on the American Type Culture Collection protocol for freezing 2 reference strains of *N. pemaquidensis* (ATCC 30735 and 50172) and contained the additional instruction to prepare the stock concentration of DMSO on ice to prevent an exothermic reaction occurring that may interfere with the preservation medium.

For all methods amoebae were suspended in sterile seawater and tested for viability prior to freezing but after addition of DMSO, by using trypan blue exclusion or neutral red inclusion as indicator dyes and by re-culturing. After freezing all attempts at recovery were by rapid thawing at 37ºC for 2-3 min then inoculating to fresh MYS plates and by appraisal of cell viability using the indicator dyes. The identities of any surviving cells post thaw and after recovery by culture were confirmed on detection of SSU ribosomal DNA characteristic of *N. pemaquidensis*.

Determination of maintenance of virulence

Atlantic salmon, *Salmo salar*, (127.2 g ± 8.9; mean weight ± SEM) were held in a self-contained non-biofiltered systems consisting of 3 x 80 L tanks (2 fish per tank) supplied from a common 80L reservoir from which seawater was constantly recirculated. Water supplied to the reservoir was 1 µm filtered and temperatures ranged from 16.0 to 16.5°C for the duration of the experiment. The fish were acclimated over a 7 d period from salinity of 0 up to 35‰ and were fed neither during acclimation nor challenge.

To initiate the challenge the first vial of host-derived amoebae, which had been kept at frozen for 72 h, was thawed and viability assessed by trypan exclusion. Thirty millilitres of sterile seawater was added to the cell suspension which was then added proportionately to the challenge tanks and the reservoir at a density of approximately 1180 cells L^{-1}. Time elapsed between thaw and challenge was 1.5 h. Controls included groups of fish exposed to a portion of the same batch of virulent *N. pemaquidensis* prior to freezing (added to tanks

and reservoir at 500 cells L^{-1}) and those exposed to the cryopreservation medium only (seawater with 7.5% DMSO).

The experiment was terminated after 7.5 days for both control groups and 8.5 days for the challenge group when all fish were euthanased with a lethal dose of Aqui-S® ($1:10^4$ v/v). At the termination of the challenge gill samples from all groups of fish (2^{nd} left arch from individual fish) were excised and placed in Davidson's fixative for a minimum of 1 h, examined for gross lesions then transferred to 70% ethanol for routine histological processing. Histology slides were examined for pathological changes and associated amoebae after staining with haematoxylin and eosin. The second vial of host-derived amoebae was thawed after being frozen for 105 days, however cells were found to be not viable based on neutral red inclusion assay, and no challenge was performed.

Objective 7
To develop improved culture systems based on monoxenic and axenic techniques

The reference strain of *N. pemaquidensis* (PA027, isolated by Dr T. Howard, 1994) was used for all experiments. This amoeba was maintained on malt yeast agar (MYS) plates prepared in 75% seawater and seeded with approximately 100 µL of a live *S. maltophilia* cell suspension. The bacterium was grown on nutrient broth no.2 (Oxoid) to dense suspension, washed twice in distilled water by centrifugation then re-suspended in distilled water in 20 mL volumes per 1 g cell pellet weight. The amoeba is routinely sub-cultured every 3-4 weeks by excising sections of agar showing vigorous growth and placing them onto fresh agar plates. Similar procedures were used to transfer the amoeba, firstly onto live *E.coli* except that subculture occurred every 2-3 days and amoeba cells furthest from the inoculation site were selected. For transfer to heat-inactivated *E. coli* antibiotics were incorporated into the MYS plates (see appendix).

To investigate any detrimental effect of the antibiotic cocktail on PA027, the amoeba was inoculated, as inverted agar blocks displaying vigorous growth, onto the following media:

1. Agar only with antibiotics seeded with live *S. maltophilia.*
2. Agar only(no antibiotics) seeded with live *S. maltophilia.*
3. MYS with antibiotics seeded with live *S. maltophilia.*
4. MYS (no antibiotics) seeded with live *S. maltophilia.*

Successful transfer of PA027 from *S. maltophilia* to *E. coli* was confirmed when swabs were taken to inoculate sheep blood agar plates to establish bacterial culture purity and to distinguish between *S. maltophilia* and *E. coli* by oxidase reaction.

To enable production of vast quantities of cells, spread plate techniques, detailed by Howard (2001), were adopted. Briefly, the spread plate technique

entailed harvest of cells from a MYS plate by gentle flushing with approximately 1 mL of seawater, then using this suspension as an inoculum and spreading it across a fresh MYS plate seeded with dead *E. coli*. Confluent growth is normally evident after 3-4 days incubation at 20°C.

Sterilisation of *N. pemaquidensis*

Small-scale assays were conducted in an attempt to remove bacteria present in cultured *N. pemaquidensis.* A liquid phase growth medium (sterile seawater with antibiotics: 0.001% v/v streptomycin sulphate, 0.001% v/v penicillin, 0.001% v/v carbenicillin, 0.0025% v/v ampicillin, 0.001% v/v erythromycin, 0.63% v/v sulphadiazine and 0.13% trimethoprim and autoclaved *E. coli,* approximately 10^8 cells mL^{-1} as a food source) was used as a treatment base then concentrations of antibiotics were increased by 150-300%. *N. pemaquidensis* (strain AFSMII at 10^3 or 10^6 cells in 6 mL media) were placed in 15 mL centrifuge tubes then subjected to increased antibiotic concentrations for 24 and 48 h. Following treatment, amoeba were concentrated by centrifugation (400 x *g* for 5 mins), the supernatant removed and cells re-suspended in sterile seawater. Bacterial presence in cell suspension was detected by inoculation onto Johnson's marine agar (JMA) and Sheih's marine agar (SMA) and subsequent bacterial growth after incubation for 7 days at 18°C. Further variations were the addition of pimaricin (an antifungal agent at 12.5 µg mL^{-1}) and the omission of heat-inactivated *E. coli.*

Objective 8

To develop cell factory capability to produce high density cell suspensions of *N. pemaquidensis*

The isolate in most demand was the reference strain (PA027). Attempts were made to increase the yield of cells firstly by following the method of Howard (2001), where cells were harvested by flooding the plate with sterile seawater and then used as inocula by spreading onto live *S. maltophilia*-seeded MYS plates of 145 mm diameter. Secondly, PA027 were transferred to live *E. coli* and finally the amoeba was grown on autoclaved *E. coli* as spread cultures in 90mm MYS agar plates. Successful transfer of the amoeba from *S. maltophilia* to *E. coli* was confirmed by taking a sample from the MYS plate and inoculating a blood agar plate, then identifying *E. coli* as the only bacterium present.

Objective 9

To develop cell purification techniques to produce pure cell suspension of *N. pemaquidensis* derived from cell culture and gill associated disease.

A detailed description of the method appears under Objective 5 and has been published (Morrison *et al*, 2004).

Objective 10
To implement cell characterisation techniques for strain differentiation
The methods are described in detail in Dyková *et al.* (2005). Summary of methods is presented below. Additionally, a battery of monoclonal antibodies raised against the reference strain PA027 and wild type isolate (gill derived amoeba from UTAS infection tank) were tested by immunofluorescent antibody test (IFAT) against a series of library strains representing non-host environmental and gill-derived isolates. Included was a negative control organism isolated from net material and thought to be *Flabellula* sp. (I. Dyková, pers comm).

Light microscopy and image analysis
Living *Neoparamoeba* trophozoites were observed in hanging drop preparations with an Olympus microscope (BX51) with Nomarski differential interference contrast (DIC) equipment. Images of trophozoites, representatives of individual strains were digitized at 300 dpi by HP Scan Jet 6300C scanner and archived in tiff format for image analysis. Image structure and object analysis software was used for image processing and data acquisition. From each of 20 images selected from the archive based on their quality (given by object and background brightness variance), the following information was extracted: trophozoite area (AR), perimeter (PM), shape (SF), slenderness (EL), dispersion (DP) and extension (EX) factors, and integral brightness (IB). These data were statistically evaluated using principle components analysis (PCA). Morphometry parameters of amoebae shape forms multivariate description of their morphometric space and we have to adopt multivariate statistical methods to simplify and interpret their description or evaluate similarities among amoeba strains. First, principal component analysis (PCA) was adopted for simple two-dimensional visualization of the mutual position of strains in their morphometry multivariate space defined by the variables AR, PM, SF, EL, DP and EX. The morphometry data were log transformed prior to PCA analysis to meet its prerequisites. Second, cluster analysis was computed to evaluate inter-strain similarities according to their morphometry. Representative values of strains were computed for every morphometry variable. Due to asymmetry of data and the presence of outliers within strains, the median was adopted as representative value of variables. The table of median values of morphometry parameters of strains was then normalized prior to cluster analysis to obtain equal weights of all morphometry parameters. Cluster analysis based on the matrix of Euclidean distances of normalized strains data was then computed using complete linkage amalgamation rule and a dendrogram of strain similarities was created. All analyses were performed using Statistica 6 (Statsoft, Inc.).

Transmission electron microscopy (TEM)
Since the generic diagnosis of strains selected using light microscopy as belonging to the family Paramoebidae has to be based on the ultrastructure of the cell surface, homogeneous cultures of all strains were first tested for the absence/presence of surface scales and then cloned for detailed ultrastructural and molecular studies. *Neoparamoeba* cultures were fixed *in situ* by overlaying

with a 3% glutaraldehyde solution in 0.1M Na-cacodylate buffer. Pelleted trophozoites were postfixed with 1% Na-cacodylate buffered osmium tetroxide, dehydrated with a graded acetone series and embedded in Spurr resine. Ultrathin sections were stained with 2% uranyl acetate in 50% methanol, post-stained with Reynold's lead citrate and examined with a JEOL JEM 1010 electron microscope operating at 80 kV.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from clonal cultures of strains using the DNeasy™ Tissue Kit (Quiagen) according to the manufacturer's protocol. Using the same polymerase chain reaction (PCR) protocol as described previously (Fiala and Dyková 2003), SSU rDNA was amplified with universal eukaryotic primers ERIB 1 (5'- ACCTGGTTGATCCTGCCAG - 3') and ERIB 10 (5'- CTTCCGCAGGTTCACCTACGG - 3') (Barta *et al*. 1997). Gel-isolated amplicons were cloned into pCR® 2,1 TOPO cloning vector using the TOPO-TA Cloning Kit (Invitrogen). Sequencing reactions were performed using an automatic sequencer, CEQ™ 2000 (Beckman Coulter), with the CEQ DTCS Dye Kit (Beckman Coulter), according to the manufacturer's protocol.

Alignments and phylogenetic analyses

The set of SSU rRNA gene sequences that resulted from this study were aligned with the whole set of *Neoparamoeba* sequences available in GenBank to date. In total, 33 sequences of 32 *Neoparamoeba* strains of different origin were aligned in the Clustal_X program. Corrections were done by eye using the BioEdit sequence alignment editor. Phylogenetic analyses were performed using the maximum parsimony (MP) and maximum likelihood (ML) methods. The MP analysis was done using heuristic search with random addition of taxa (10 replications) and the ACCTRAN-option. For the ML analysis, the likelihood ratio test (LRT) implemented in the Modeltest v. 3.06 was used to determine the best model of evolution. The best tree was determined using Tree Bisection-Reconnection (TBR) rearrangements. The bootstrap analysis (1000 replicates) was done using the Seqboot in PHYLIP, Version 3.6a3 and the PHYML programs.

Objective 11

To investigate culture strategies to develop infective strains of *in vitro* grown *N. pemaquidensis*

Seawater-adapted Atlantic salmon (approximately 80 g) were placed into autonomous recirculating systems consisting of three 80 L tanks/system (2 fish/tank). Water was maintained at 16.0-16.5ºC and a 25% water change was performed approximately every second day. Three independent inoculations were performed (Table 4).

Fish were euthanased as described and the gills were excised and placed in seawater Davidson's fixative for a minimum of 1 h. Gills were then transferred to 70% ethanol until the second left gill arch was processed for routine histology to investigate presence of AGD.

Table 4. Details of amoebae culture and inoculation duration

Inoculation	Amoebae	Duration of amoebae culture (d)	Number of Passages	Conc. of Amoebae (cells L^{-1})	Duration of Inoculation (d)
1	NP251002	34	4	5020	14
2	NP251002	98	14	59000	19
3	Total amoebae*	3	1	3160	8

* Amoebae harvested directly off the gills of AGD infected Atlantic salmon.

Results/Discussion

Objective 1
To provide knowledge base for development of novel treatments and vaccine

AGD infection (Morrison *et al.* 2004)

The number of gross and histological AGD lesions per gill was proportional to the inoculating concentration of amoebae, indicating that the severity of disease is a function of amoebae density in the water column. After the 14 day inoculation period, all fish in all systems inoculated with amoebae displayed typical AGD-like lesions although the number of lesions was variable within each treatment group. There was a significant linear association between the mean number of gross lesions per gill and the concentration of amoebae inoculated into the systems ($P < 0.05$). Histological evidence of AGD was detected in all treatment groups inoculated with amoebae including an inoculating concentration as low as 10 cells L^{-1}. The relationship between gross gill lesions and amoebae concentration reflected that of the quantitative histological assessment of gills. The proportion of filaments affected by amoebae in histological sections was linearly associated with the inoculating amoebae concentration ($P < 0.05$). However it is possible that at higher concentrations, it would be difficult to retain the linear model empirically due to an amalgamation of lesions. Fish in the negative control group exhibited neither gross nor histological signs of AGD.

Reinfection (Gross *et al.* 2004a)

Fish kept in seawater had lower mortality rates compared to the first time exposed and freshwater maintained group however these data are believed to be biased by on-going mortalities during the seawater maintenance phase. Phagocyte function decreased over exposure time and freshwater maintained fish demonstrated an increased ability to mount a specific immune response. These results suggest that, under the challenge conditions herein described, antigen exposure via infection does not induce protection to subsequent AGD. They also support on-farm observations that fish bathed and allowed to recover from AGD in freshwater are not resistant to re-infection. Under the challenge conditions described there is no resistance provided by prior infection with *Neoparamoeba* sp. regardless of whether the fish are allowed to fully convalesce or if they remain actively infected. AGD appears to alter anterior kidney phagocyte function although it is not known if this is due to migration of phagocytes away from the kidney, inflammatory cytokines, stress or another unidentified effect of infection on phagocyte function.

Immune response (Gross *et al.* 2004b)

In comparison with non-exposed control fish resting respiratory burst responses were suppressed 8 and 11 dpe, phorbol myristate actetate (PMA) stimulated activity was significantly suppressed 11 dpe. Respiratory burst stimulation indices were different only 8 dpe, at which time infected fish had an increased stimulation index, suggesting an initial priming effect of *Neoparamoeba* sp. followed by later desensitization. Variable differences in

phagocytic activity and phagocytic rate following infection were identified. There was an increase in the chemotactic response of anterior kidney macrophages isolated from exposed fish relative to control unexposed fish 8 dpe. Non-specific humoral parameters were not affected by *Neoparamoeba* sp. exposure. Together, these results demonstrate the variable manner in which *Neoparamoeba* sp. infection affects the immune response of Atlantic salmon, and suggest that there appears to be a role for cytokine and/or chemokines in the recruitment of leukocytes to the site of infection and the activation of the systemic phagocytes. However the mechanism by which these systemic effects occur relative to the localised gill infection are yet to be identified.

Simple vaccine (Morrison and Nowak 2005)

During the AGD challenge, mortality began to occur at 7 days-post inoculation and increased steadily thereafter. There was a modest difference in the number of mortalities with consistently lower mortality in the group of fish treated with the placebo control however there was no significant difference between treatment groups using the log-rank statistic for survival data ($P > 0.05$). All mortalities that occurred during the challenge displayed gross signs consistent with AGD such as multifocal white lesions on gill arches (Zilberg and Munday, 2000). Histopathological changes to the gills were also consistent with AGD. No difference between the prevalence of lesions was observed ($P > 0.05$).

Bath administration of antigens from either virulent or avirulent amoebae failed to affect resistance of Atlantic salmon upon subsequent challenge with gill derived amoebae. It is not known if the susceptibility to AGD during challenge was due to a lack of an immune response or that the response was not protective against subsequent challenge. Most likely, either the antigen concentration was limiting, the duration between bathing and challenge was too short for development of a protective immune response or the immune response was not protective.

CpGs (Bridle *et al.* 2003)

Survival of the CpG-ODN 1668 treated fish was significantly ($P < 0.05$) improved compared with the untreated control fish as determined by a log rank test ($P = 0.010$). Survival of both the non-CpG ODN 1720 treated fish and the PBS treated fish was not significantly different from the untreated control fish. At the end of the challenge period (16 days), the presence of pale mucoid patches on the gills, immunocytochemical analysis of gill smears, and gill histology, showed that all the fish were infected with *N. pemaquidensis* and were consequently diagnosed with AGD. Histological examination of the fish treated with CpG-ODN 1668 revealed a more pronounced level of localised inflammation associated with AGD lesions when compared to the other groups of fish. In many instances theses leucocytes could be seen in close association with the amoebae. Additionally, large numbers of interlamellar cysts were observed within AGD lesions of the CpG-ODN 1668 group of fish.

CpG-ODNs are potent activators of the innate immune response in vertebrates. Therefore, the increased resistance to AGD in the present study is possibly the result of a CpG-ODN enhanced innate effector cell response. This study is the first to demonstrate the ability of CpG-ODNs to enhance resistance to disease in fish and highlights its possible use as a disease control treatment or vaccine adjuvant. Additionally, it shows the potential for use of immunostimulants in control and management of AGD.

EcoActiva

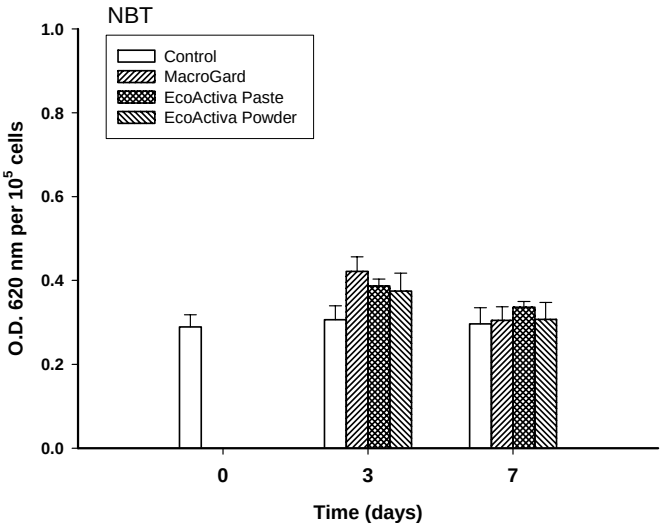
Anterior kidney macrophages isolated from fish sampled at 0, 3, and 7 d post initial feeding of the experimental *β*-glucan feeds were neither directly stimulated (NBT alone) nor primed (NBT + PMA) relative to the control diet.

Moribund fish associated with AGD were observed from 27 d post inoculation and showed obvious clinical signs of AGD infection. Excised gills from the mortalities had gross lesions affecting a large proportion of gill surface area. None of the experimental diets, EcoActiva Paste (1%), EcoActiva Powder (0.3%), or MacroGard (1%), were able to increase the survival of the fish after being infected with 1.13×10^{-3} amoebae L^{-1} (Figure. 4). RPS values of the MacroGard, EcoActiva Paste, and EcoActiva Powder were 14.5, -10, and 6.4, respectively (Table 5).

Table 5. Specific mortality, relative potency (RP), and relative percent survival (RPS) of Atlantic salmon fed experimental diets containing either, EcoActiva Paste (1%), EcoActiva Powder (0.3%), MacroGard (1%), or a control diet (0%), for 1 week before being challenged by the addition of 1.15×10^{3} amoebae L^{-1}. Mortalities in each group of fish were monitored for 72 d post inoculation.

Diet	Total Number of Fish	Number Specific Mortality	% Specific Mortality	RP	RPS
MacroGard	48	28	58.3	1.2	14.5
EcoActiva Paste	48	36	75	0.9	-10
EcoActiva Powder	47	30	63.8	1.1	6.4
Control	44	30	68.2	1.0	

(A)



(B)

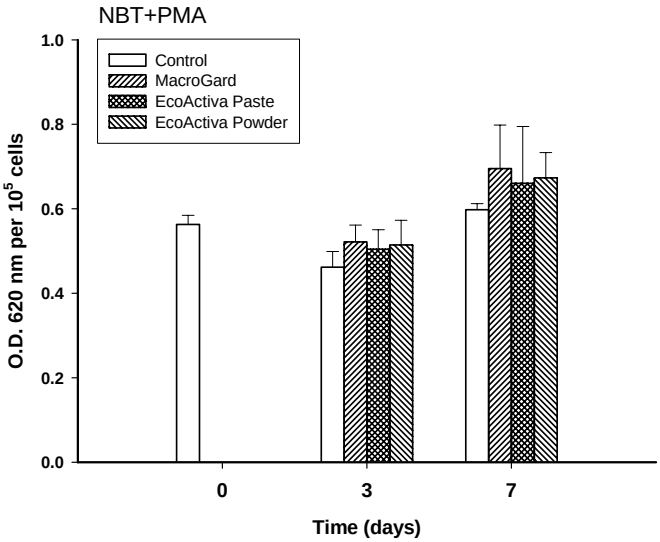


Figure 3. *Ex vivo* respiratory burst activity of Atlantic salmon head kidney macrophages isolated at 0, 3 and 7 days post initial feeding. Atlantic salmon were held in sea water at 16°C and fed one of three commercial *β*-glucan diets or a control diet (no *β*-glucan) over 7 days. At each sampling time fish were euthanased, the head kidney sterilely dissected, and the macrophages isolated over a 34/51% Percoll density gradient. Cell monolayers were established in 96 well tissue culture plates and incubated with NBT (1 mg mL^{-1}) alone (A), or NBT (1 mg mL^{-1}) and PMA (1 µg mL^{-1}) (B) for 60 min. No statistically significant differences were found between the different *β*-glucan diets including the control diet at each sampling time using ANOVA. Data are means (± S.E) of eight wells from four fish.

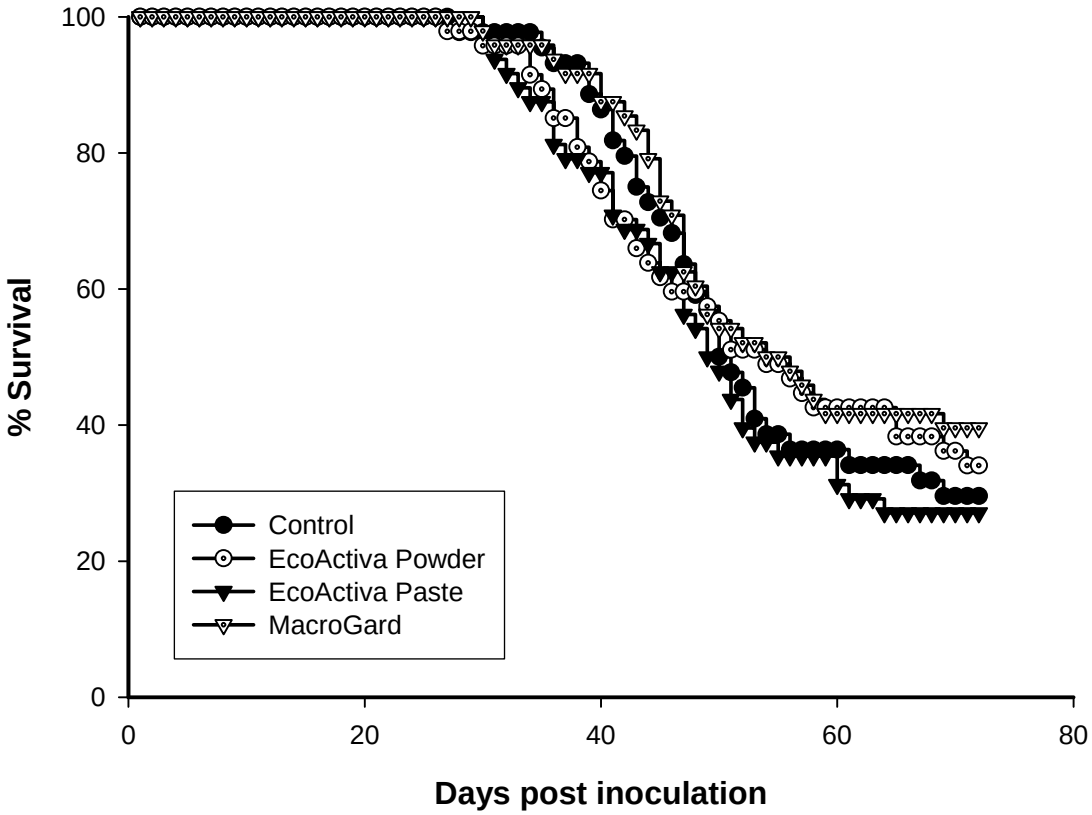


Figure 4. No statistically significant differences were found between the mean survival of triplicate groups of fish fed the four experimental diets and challenged by the addition of 1.15 × 10^3 amoebae L^{-1}. Experimental diets containing either, EcoActiva Paste (1%), EcoActiva Powder (0.3%), MacroGard (1%), or a control diet (0%), were fed to the fish for 1 week before the challenge followed by 3 weeks of the control diet. This 1:3 feeding regime was maintained over the duration of the trial. Survival of each group of fish was monitored over 72 d and Kaplan-Meier survival curves statistically compared using the log rank test (*P*<0.05).

Previous studies have reported enhanced respiratory burst activity of snapper (*Pagrus auratus*) anterior kidney macrophages both *in vitro* (Cook *et al*., 2001) and after dietary administration of 0.1% v v^{-1} EcoActiva Paste (Cook *et al*., 2003). A large amount of variation exists among the concentrations and incubation times various *β*-glucan products require to elicit enhanced macrophage respiratory burst activities *in vitro*. This is further highlighted by species specific responses. For instance, snapper macrophages responded to 0.001-0.1% v v^{-1} EcoActiva Paste after both 1 and 3 h incubation followed by PMA stimulation (Cook *et al*., 2001). Atlantic salmon macrophages incubated with MacroGard for 4 d with 1 µg mL^{-1}, and 7 d with 0.1 and 1 µg mL^{-1}, and then stimulated with PMA had significantly enhanced respiratory burst activities when compared to the control (0 µg mL^{-1}) (*P*<0.05). However, the same study demonstrated that macrophages incubated with 10 µg mL^{-1} MacroGard for 4

and 7 d and then stimulated with PMA had an O_2^- production no different to the control and that 50 µg mL^{-1} was inhibitory (Jørgensen and Robertsen, 1995). Results of a study by Castro *et al*., (1999) demonstrate that at high concentrations (25 – 500 µg mL^{-1}) *β*-glucans can directly stimulate O_2^- production in both turbot (*Psetta maxima*) and gilthead seabream (*Sparus auratus*) anterior kidney macrophages. However, the same study also found that macrophages incubated at similarly high concentrations of *β*-glucans and then stimulated with PMA had greatly reduced respiratory burst activity when compared to the macrophages incubated with *β*-glucans and NBT only. Inhibition of 'priming' following incubation with high concentrations of *β*-glucans led Castro *et al*., (1999) to suggest that high concentration of *β*-glucans can excessively stimulate fish macrophages and after time exhaust the cells.

Evidence that innate immunity might play a part in protective immune responses to AGD has been suggested by Findlay and Munday (1998). Freshwater bath treatments with the addition of levamisole, a known immunostimulant of the innate immune system, have also been shown to reduce mortality due to AGD in laboratory based infections (Zilberg *et al*., 2000). More recently, we have shown that i.p. administration of potent immunostimulatory CpG-ODN is able to increase resistance to AGD in Atlantic salmon (Bridle *et al*., 2003). Therefore, it is not surprising that the lack of *in vivo* macrophage stimulation has translated into a lack of improved resistance to AGD in the present study. As respiratory burst activation was used as a measure of innate immune system activation it is plausible that cells other than anterior kidney macrophages may have responded to the dietary *β*-glucans, or that other innate immune responses have been enhanced. It is also highly likely that the *β*-glucan feed inclusion rates used in the present study were sub optimal and given greater access to resources could have been further optimised in additional dose-response feed trials. In conclusion, although we were unable to demonstrate improved survival to AGD in fish treated with dietary *β*-glucans, the previously mentioned considerations in conjunction with the limited knowledge of the role that the innate immune response plays during AGD infection does not mean that dietary *β*-glucan administration should be dismissed. More importantly, this trial warrants further investigations into both the involvement of the innate immune response during AGD and the determination of the optimal *β*-glucan dietary dose and feeding regime.

<u>EcoBoost</u>

While during the first few days post infection the EcoBoost fed fish showed slightly better survival, there this was not consistent through time and there was no difference between treatments by day 30. Fish from all treatments had signs of AGD.

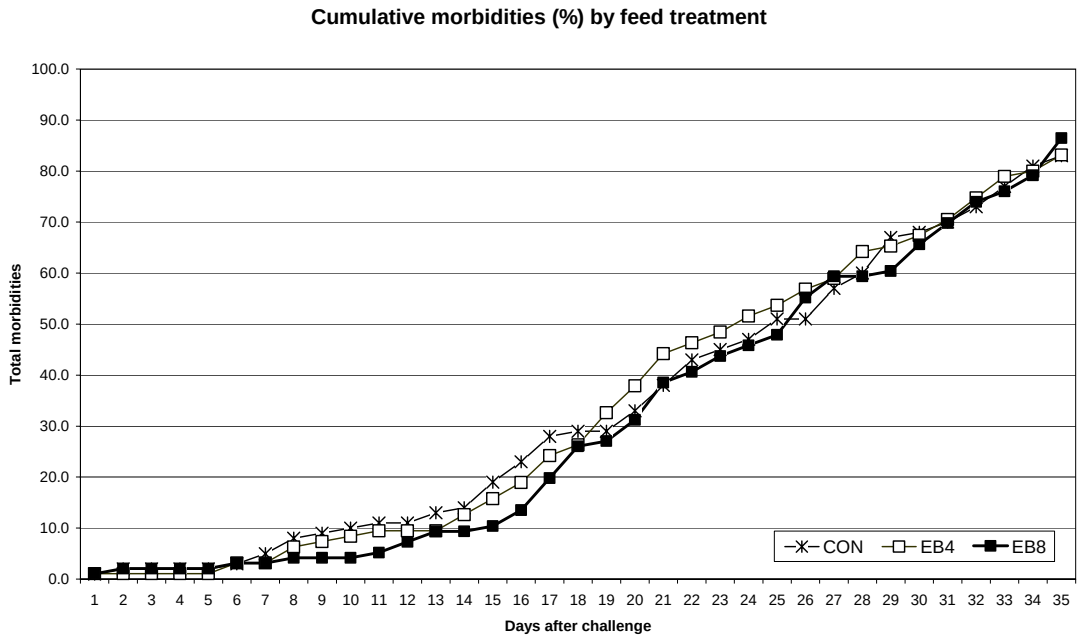


Figure 5. Feeding fish with 4% or 8% Ecoboost incorporated in the diet did not have a significant effect on morbidity of fish infected with AGD.

Inflammation in AGD

AGD-infected Atlantic salmon were found to have significantly increased vascular permeability when compared to uninfected control fish (Figure 6)

Gene expression of interleukin-1β in the liver of AGD infected Atlantic salmon was up to 10 times greater than in the uninfected control fish (Figure 7).

It is proposed that the host response of Atlantic salmon to AGD involves a complex interaction of innate immune responses that are initiated to prevent the fish from further gill injury, to limit the growth of amoebae and activate repair processes to return the gill to normal function. However, by mechanisms not yet fully understood this host response apparently continues eventually resulting in harm to the fish. Based on these preliminary findings further work is necessary to improve our understanding of the interactions of both the local and systemic inflammatory responses, mediators involved and the role they play in the pathogenesis of amoebic gill disease in Atlantic salmon. These studies will hopefully provide insights into preventing this excessive host response and allow for the design of effective AGD treatments.

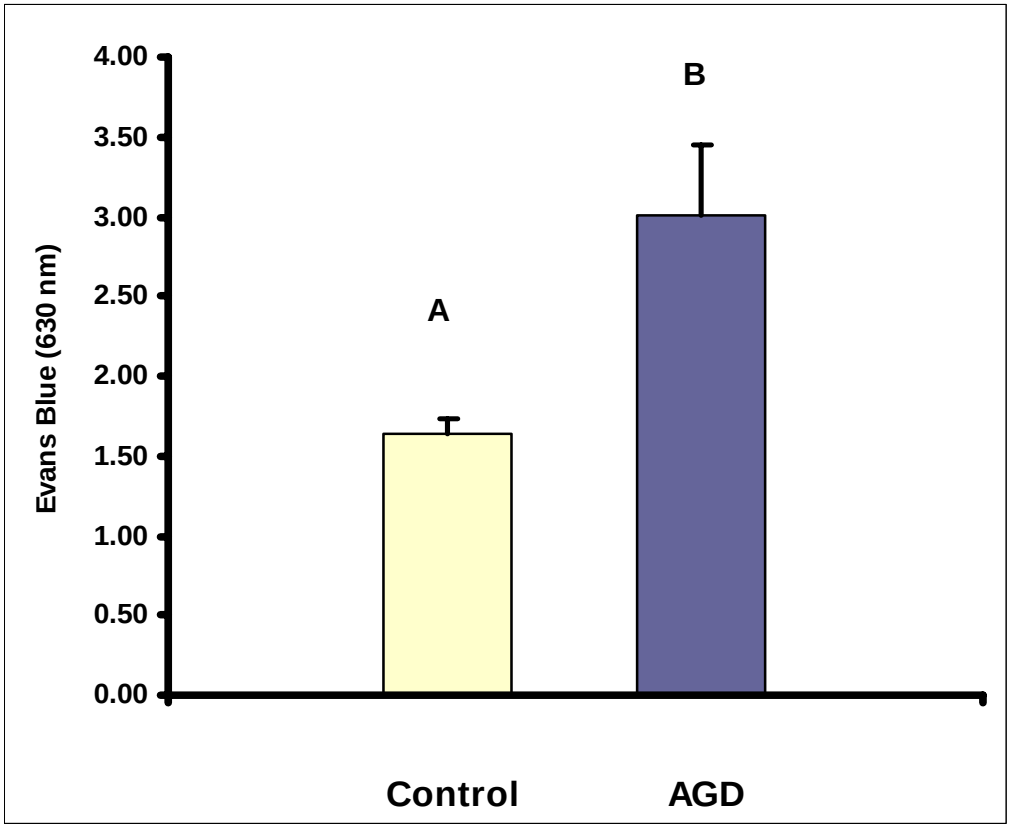


Figure 6. Effects of AGD infection on the vascular permeability in Atlantic salmon measured as Evans blue leakage into the peritoneal cavity. Values are means ± S.E. of n=5 fish. Mean values not sharing letters are statistically different.

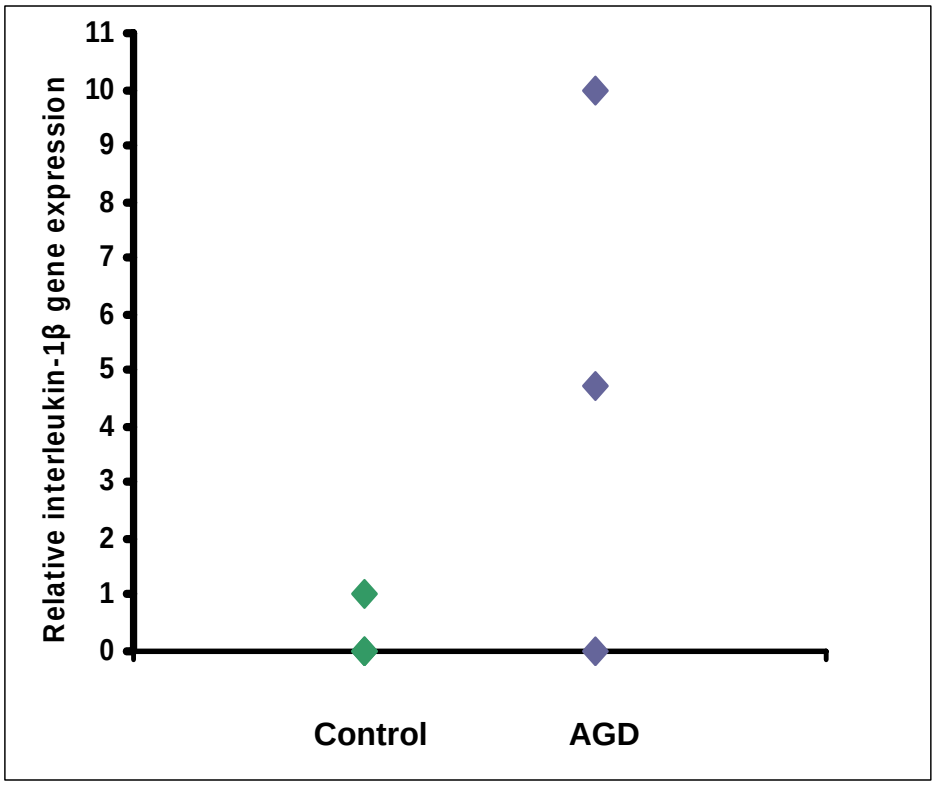


Figure 7. Interleukin-1β gene expression in the liver of AGD infected Atlantic salmon relative to uninfected control fish using real time RT PCR and MGM TaqMan chemistry. Values of 0 represent fish with no detectable interleukin-1β gene expression.

Primary pathogenicity of *Neoparamoeba* (Adams and Nowak 2004a)

AGD only occurred when fish were exposed to viable trophozoites, further confirming the primary role of *Neoparamoeba* and the need for physical contact between the amoeba and host for the disease outbreak.

Sequential pathology (Adams and Nowak 2003, 2004b)

AGD was initially detected histologically at week 13 post-transfer while gross signs were not observed for a further week post-transfer. Significant increases ($P<0.001$) in the proportion of affected gill filaments occurred at weeks 18 & 19 post-transfer coinciding with the cessation of a halocline and increased water temperature at the cage sites. The progression of AGD histopathology, during the sampling period, was characterized by three phases:

1. Primary attachment/interaction associated with extremely localized host cellular alterations, juxtaposed to amoebae, including epithelial desquamation and oedema.
2. Innate immune response activation and initial focal hyperplasia of undifferentiated epithelial cells.
3. Lesion expansion, squamation-stratification of epithelia at lesion surfaces and variable recruitment of mucous cells to these regions. A pattern of preferential colonization of amoebae at lesion margins was apparent during stage 3 of the disease development. Together, these

data suggest that AGD progression was linked to retraction of the estuarine halocline and increases in water temperature. The host response to gill infection with *Neoparamoeba* sp. is characterized by a focal fortification strategy concurrent with a migration of immuno-regulatory cells to lesion-affected regions.

Freshwater bathing cleared lesions of attached trophozoites and associated cellular debris. Subsequent gill re-infection with *Neoparamoeba* sp. was evident at 2 weeks post-bath and had significantly increased ($P<0.001$), in severity, by 4 weeks post bath. No significant difference in gross pathology was observed until 4 weeks post-bath ($P<0.05$). The re-infective progression of AGD was characterized by localized host tissue responses juxtaposed to adhered trophozoites (epithelial oedema, hypertrophy & hyperplasia), non-specific inflammatory cell infiltration (macrophages, neutrophils & eosinophilic granule cells) and finally advanced hyperplasia with epithelial fortification. During the post-bath period, non AGD lesions including haemorrhage, necrosis and regenerative hyperplasia were occasionally observed though no evidence of secondary colonization of these lesions by *Neoparamoeba* sp. was noted. We conclude that pathogenesis, during the inter bath period, was identical to initial infection although the source of re-infection remains to be established.

Objective 2
To identify factors leading to binding of the parasite to fish gills
Antibody approach
In the first two rounds (using PA027) 14 hybridomas produced antibodies that were cross-reactive between surface antigens of PAO27 and wild type parasites. Ten of the 14 Mabs recognize peptide epitopes. Five Mabs were assessed in an attachment assay. No inhibitory antibodies were found.

Hybridoma production round 3 and 4 (using substractive immunisation protocol and whole WT parasites) resulted in fifty-one hybridomas specific to wild type parasites (Table 6). These antibodies have been screened by ELISA (against PAO27 and wild type antigen), immunofluorescence assay (IFAT) and flow cytometry to identify those recognizing wild type-specific antigens expressed on the surface of the parasite. Nine of these hybridomas secreted IgG antibodies, 28 secreted IgM and the rest were undetermined. Only 8 of the Mab exhibit cross-reactivity with PAO27 antigens. Due to a disruption of the CO_2 supply* to the tissue culture incubator, approximately 30 hybridomas were lost or ceased antibody production and had to be discarded. From the remaining 18 (Table 6) the only 2 Mabs that clearly recognise peptide epitopes on the parasites are cross-reactive with PAO27. Of the remaining Mabs, fifteen react with carbohydrate epitopes. The nature of the epitope recognised by Mab 7B5 is still unknown but it is the only IgG Mab that is specific for WT parasites. Mabs 7G5 and 8H4 recognize carbohydrate epitopes but are specific to wild type parasite and exhibit strong reactivity with the surface of the parasite. All 18 hybridoma cell lines have been expanded and frozen for storage in liquid nitrogen, and selected hybridomas have been cloned. From 18 hybridomas

selected as binding to wild type parasites only 3 (16%) exhibit cross-reactivity with PAO27 antigens and 11 reacted with surface antigens. The only 3 Mabs that clearly recognise peptide epitopes on the parasites are cross-reactive with PAO27.

Table 6. Characteristics of secreted Mabs to wild type antigens (pre-cloned hybridomas, fusion #3 and #4).

	Hybridoma	Isotype	ELISA WT	ELISA PAO27	IFAT WT	Epitope	FLOW
1	**5E2** **5E2E4**	**IgG** **IgG**	**0.8** **1.7**	**0.4** **1.6**	**-**	**P**	**+**
2	**6E7** **6E7G10**	**IgG/IgM** **IgG**	**1.2** **2.0**	**0.7** **2.2**	**+++**	**P/P**	**+**
3	**7B5**	**IgG**	**0.7**	**-**	**++/+**	***n/d***	
4	6D6	IgM	1.5	-	++/-	C	+
5	5A6	IgM	1.1	-		C	
6	6C5	IgM	1.3	-	-	C	
7	6G11	IgM	1.2	-	-	C	
8	7B3	IgM	1.3	-		C	
9	7G5	IgM	2.3	-	++	C	++
10	8H11	IgM	1.1	-	+++	C	+/-
11	8H4	IgM	1.8	-	+++	C	+
12	8H5	IgM	2.2	-	+/- or -	C	
13	6E8	IgM	1.4	-	-	C	+
14	4C12	IgM	2.3	-	+++	C	+
15	5E6	IgM	1.4	-	++	C	+
16	7A1	IgM	1.6	-	++	C	-
17	8G6	IgM	1.3	-	+	C	
18	7D6	IgM	2.0	-	+/- or -	C	

P = peptide, C = carbohydrate , *nd* = not determined

In a further attempt to generate high affinity IgG Mabs specific for cell surface antigens on wild type parasites, another fusion was performed on the 4th of May using a modified subtractive immunisation method as outlined in Figure 1. As demonstrated in Figure 2, the objective of suppressing the immune response to PA027 was achieved in that mice administered cyclophosphamide subsequent to immunisation with PA027 failed to produce antibodies to the cultured parasites, while non-cyclophosphamide treated mice mounted a strong antibody response. It was further demonstrated that mice immunosuppressed against PA027 parasites could subsequently respond to immunisation with wild type parasites (Figure 8, mice 1 and 3). These mice were used for fusion number 5, and in order to increase the probability of generating Mabs against peptide epitopes on the surface of wild type parasites the last boosting immunisation was made with sonicated plasma membranes from deglycosylated wild type parasites. The deglycosylated antigen was prepared as follows: wild type parasites were lysed with dH20, treated with N and O –glycosidases (Roche), centrifuged at 100,000g for 1 hour and the pellet disrupted by sonication. The final product represented 6% of the total protein present in the original whole parasite preparation.

The efficiency of this fusion was 100% (ie. all wells of the fusion plates show hybridoma growth) and the results of the first screening by ELISA indicate that at least 18 hybridomas react specifically wild type parasites. These hybridomas are being expanded and will be cloned and characterised.

Hybridoma production round 5 (using substractive immunisation protocol and deglycosylated WT parasites) resulted in 12 hybridomas. Only 2 were specific for WT parasites and both recognize carbohydrate epitopes. Two additional fusions using this protocol have recently been performed and are currently in the screening phase**.**

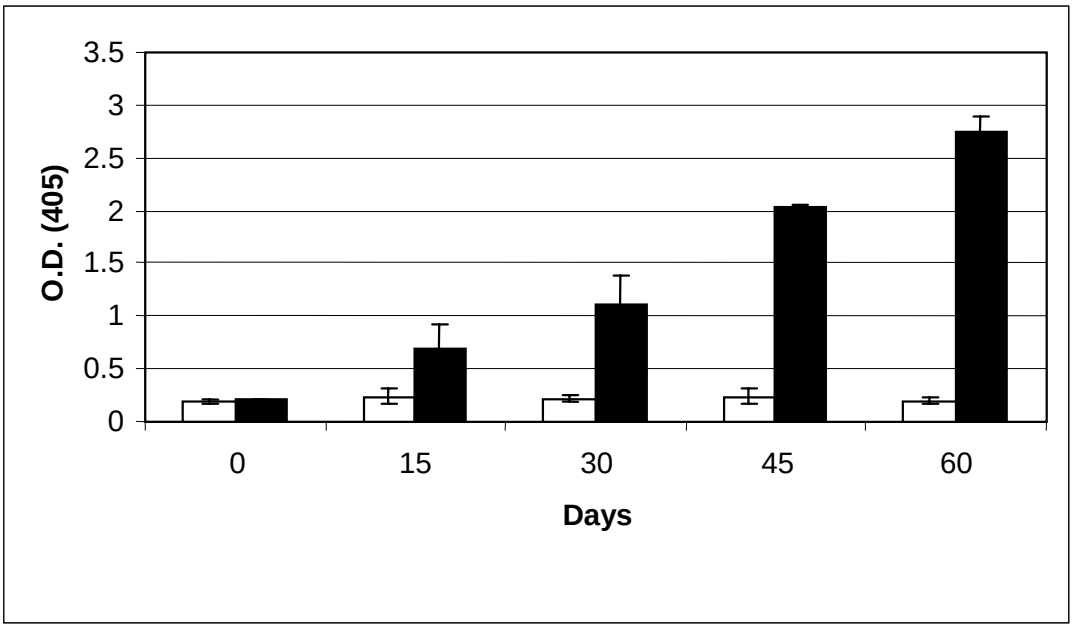


Figure 8. Antibody response to PAO27 parasites (determined by ELISA) of PAO27/cyclophosphamide immunosuppressed mice (white column; n=8) and non-immunosuppressed mice (black column; n=3).

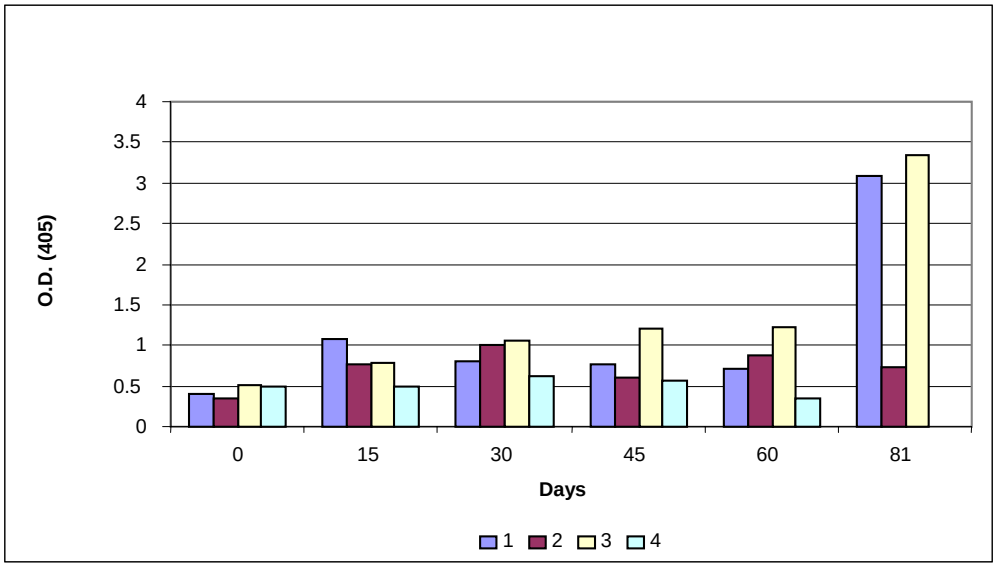


Figure 9. Antibody response to wild type parasites (determined by ELISA) of PAO27/cyclophosphamide immunosuppressed mice. At day 72, two of these mice (numbers 1 and 3) were primed with wild type antigen emulsified in Freund's Incomplete Adjuvant and the antibody response measured at day 81. These mice were subsequently boosted at day 97 with a deglycosylated membrane preparation from wild type parasites and 3 days later the splenocytes were isolated to be used for fusion number 5.

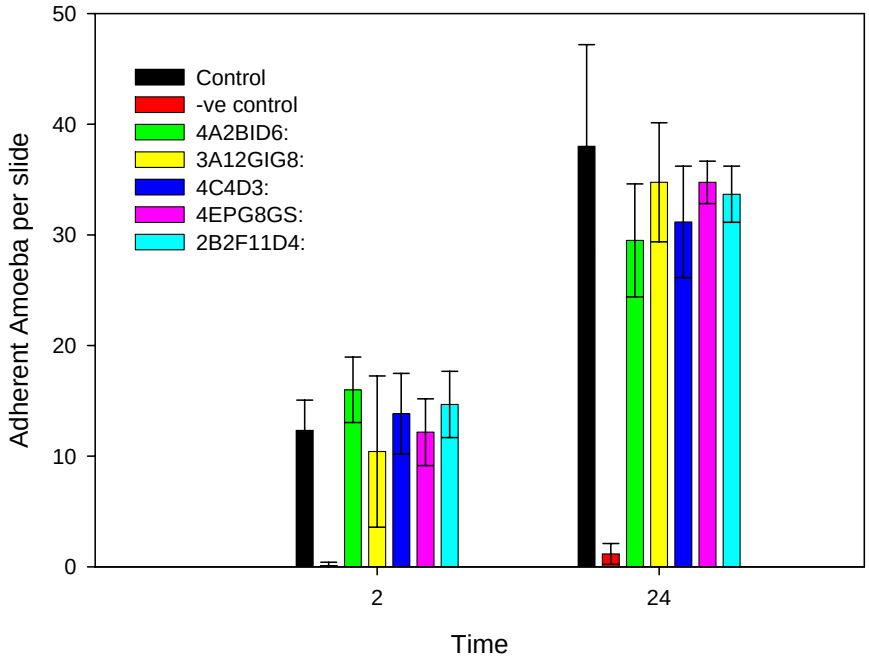


Figure 10. Attachment of isolated gill amoebae to Atlantic salmon gill explants following pre-treatment of amoebae with monoclonal antibodies against *N. pemaquidensis*. Data are mean number of adherent amoebae ± SD in 8 replicate samples. The control consisted of amoebae in seawater with no treatment, and the negative control was amoebae maintained in fresh water.

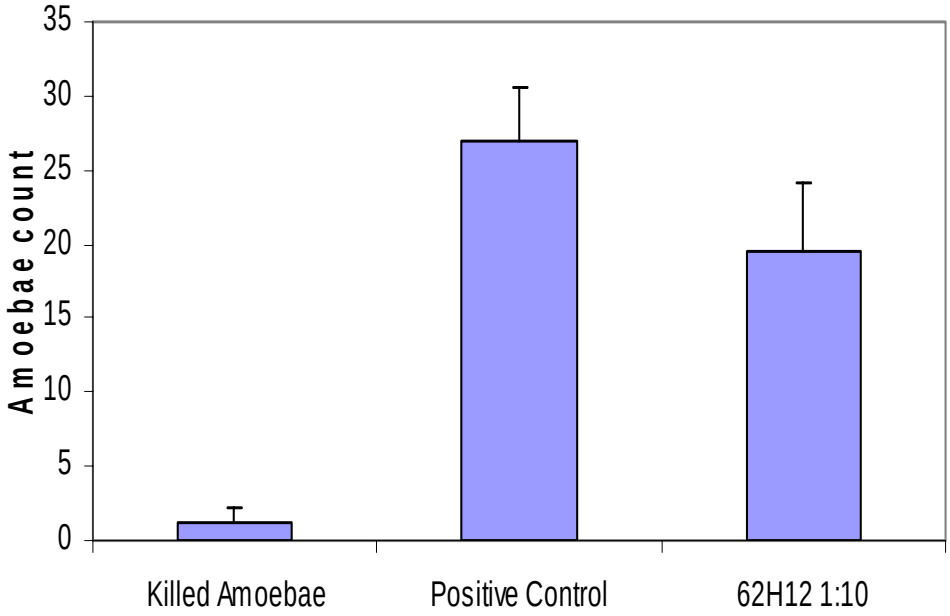


Figure 11. Attachment of isolated gill amoebae to Atlantic salmon gill explants following pre-treatment of amoebae with the pre-cloned monoclonal antibody 2H12. Data are mean ± SD in 8 replicate samples. The control consisted of amoebae in seawater with no treatment, and the negative control was killed amoebae. Statistical analysis was performed using ANOVA test. Preliminary results.

Amoeba attachment to gill explants (Butler and Nowak 2004a)

Infection tank exposure

All gill explants suspended within a tank containing an on-going AGD infection were positive for *Neoparamoeba* spp. at all sample points during the 24 hour experiment. The overall trend was of increasing amoeba number in tissues over time. At 2 hours post incubation those gills pre-treated with the mucolytic agent hyaluronidase showed a significantly greater number (mean 45 ± 13) of amoebae than the other groups ($F=17.98$, df 31, $P<0.001$). However, this level of amoeba colonisation did not change significantly over the course of the experiment. Similarly, those gills that were previously perfused and treated with hyaluronidase had levels of amoeba colonisation that were not significantly different from the hyaluronidase only treated group at the end of the experiment ($F=1.12$, df 31, $P=0.22$). At 12 and 24 hours post incubation both of these groups showed significantly lower amoeba numbers than the other 2 treatment groups. Untreated gills had a mean of 117 amoebae per slide at 24 hours post incubation, and perfused gills a mean of 126 which were not significantly different from each other.

In vitro interaction of gill amoeba and Atlantic salmon gill explants

All gill explants infected *in vitro* with freshly isolated gill amoebae were positive for the parasites at each sample point. Initially, at the first sample point, untreated gills had significantly higher numbers of *Neoparamoeba* sp. than perfused gills but this pattern was not maintained throughout the experiment and at 12 hours post incubation the number colonising perfused gills was significantly greater than that in untreated gills ($F=18.76$, df 31, $P=0.031$). At the end of the experiment there were no significant differences between the numbers of amoeba recovered from these two groups (approximately 38 parasites per slide). Gills treated with the mucolytic hyaluronidase had significantly reduced amoeba attachment.

Objective 3

To identify gill conditions which increase the susceptibility of the fish to AGD

1. Gill damage (Adams and Nowak 2004)

Attachment of *Neoparamoeba* sp. to damaged gill filaments was significantly reduced ($P<0.05$) by 48 h post exposure. These data further confirm and describe the primary pathogenic role of *Neoparamoeba* sp. and the early host response in AGD. They also suggest that lesions resulting from physical gill damage are not preferentially colonized by *Neoparamoeba* sp.

2. Presence of bacteria (Bowman and Nowak 2004, Gopinath-Embar *et al.* 2005)

Gill and mucus samples from fish infected with AGD in the laboratory infection and a sample from field infection were dominated by a geographically widely distributed marine bacterium from genus *Psychroserpens* (Bowman and

Nowak 2004). In the laboratory experiment all amoebae-infected groups (1, 2 and 3) had AGD. However, fish exposed to gram negative bacteria had significantly more filaments with lesions than the other groups ($F = 21.9$, d.f. 3, 73, $P < 0.001$). The majority of histological sections in this group showed the presence of large numbers of gram negative bacteria on the filaments in association with amoeba. There was no difference in percentage of filaments with lesions in fish group 1 and 2; these fish had approximately the same proportion of filaments with AGD lesions (16%). There was no difference in the size of lesions between treatment groups. Typically lesions ranged in size from 3-5 interlamellar units (Fig 12).

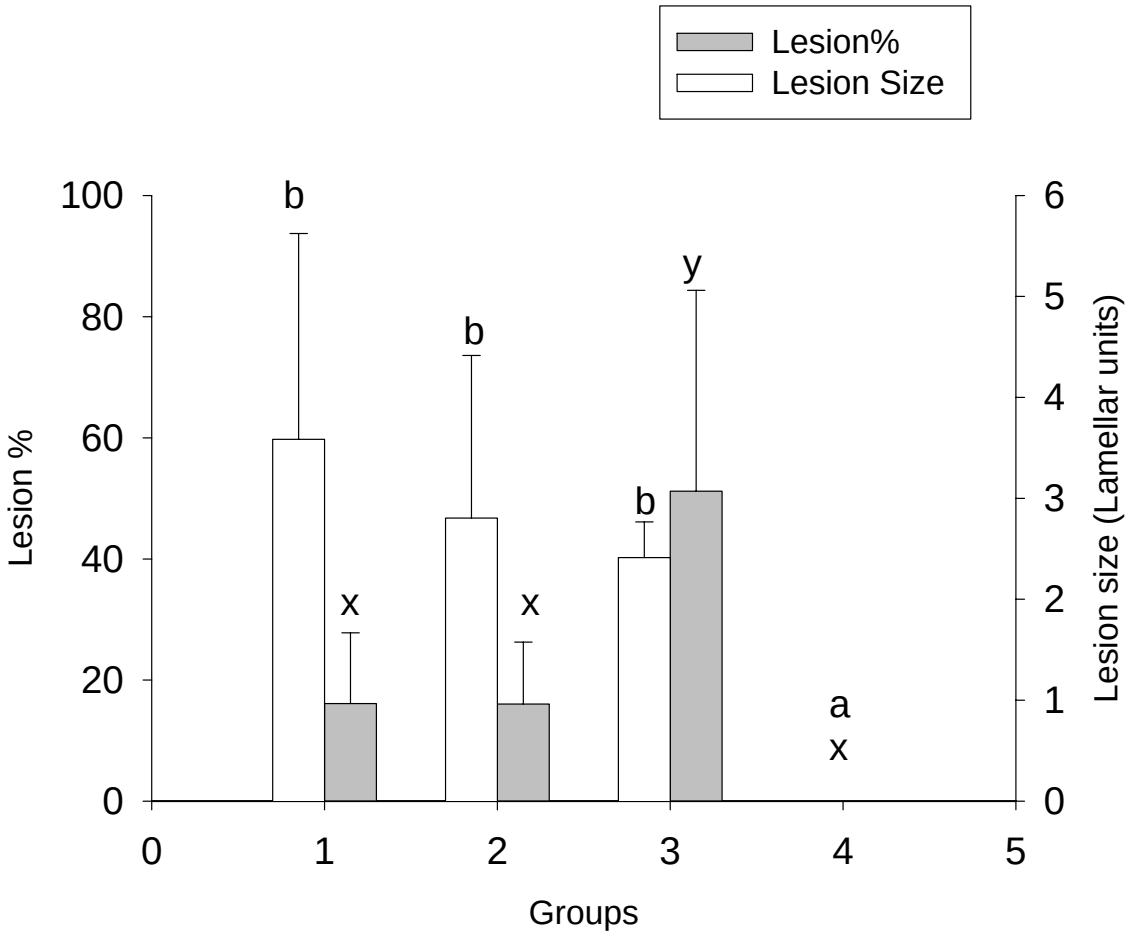


Figure 12. The mean ± standard deviation of percentage of filaments with lesions and number of infected lamellae within in each lesion in treatment groups (Group 1, *Neoparamoeba* sp. ; Group 2, *Staphylococcus* sp. bacteria and *Neoparamoeba* sp; Group 3, *Winogradskyella* sp. and *Neoparamoeba* sp.; Group 4 negative control). Separate analyses were done for lesion percentage and lesion size (lamellar units) and means with different letters above the bars represent groups that are statistically different.

3. Presence of other amoebae

Field study

From the first group of ten AGD positive fish (HAC) we isolated six different strains of flattened amoebae. From the second group (HAC - 20 fish sampled) one strain of flattened amoeba was isolated. The third group included harvest fish (Tassal - 12 fish sampled) and no amoebae were isolated, this may have been due to the harvest procedure, however none of the fish had gross gill lesions. Histology of the fish from the first two groups was also examined, most had AGD lesions but no amoebae present, three fish from group two had paramoebae associated with histological lesions. Flattened amoebae were not seen in histological sections. This is consistent with previous reports in the literature where other amoebae could be isolated from gills of fish with AGD, some of these species (for example flattened amoebae) are easier to culture than *Neoparamoeba* sp.

Experiment 1

Histopathology of pre-control fish (held at full salinity for approx. 2 weeks prior to experimental commencement) revealed a pre-existing amoebic infection, indicated by the presence of hyperplastic lesions with adhered trophozoites, consequently the experiment was terminated (day 8). The pre-existing infection was most probably initiated by contaminated nets or water dispersal from nearby infection trial tanks.

The pre-existing infection confounded the experimental result regarding the potential pathogenicity of the cultured strain of *Platyamoeba* sp. used during the experiment. The lab in which the acclimation and holding phase of experimental fish was performed has since reviewed and addressed the need for suitable cross contamination controls.

Mortalities occurred in two systems toward the end of the infection trial (C1: [day 7 n = 1, day 8 n=2], NP1: [day 7 n = 2]). All fish displayed pronounced scale loss and epidermal erosion/petechial haemorrhage along the ventral flanks, fins, tail and mouth; gross signs of AGD were also evident in the aforementioned fish. Gill histopathology of moribund fish, from all groups, showed moderately advanced hyperplastic lesions with large numbers of attached and adjunct trophozoites. It is likely that a combination of combined dermal and branchial insults were the main factors in the observed morbidity.
All fish, collected after termination of the experiment, displayed gross signs indicative of AGD.

Subsequently, histopathology revealed a similar extent of AGD severity and lesion morphology between groups. No significant difference was found in the percentage of AGD lesion affected filaments (Means: P1 – 63.5% ± 3.5 SE; NP1 - 50.7% ± 7.5 SE; C1 – 63.1% ± 6.2 SE). Trophozoites resembling *Neoparamoeba* sp. (defined by size and detection of endosymbiont) were histopathologically evident upon the majority of gill lesions in all groups. Due to technical difficulties, TEM observations were limited to a single specimen from

group P1 (exposed to infective gill material only). Trophozoites, morphologically identifiable as *Neoparamoeba* sp., were observed in adherence with gill epithelium. Trophozoites consistent with the morphology of *Platyamoeba* sp. were not observed in the sectioned material. Isolation of gill associated amoebae and other organisms are described in table 3. Although detected histologically and by TEM, trophozoites resembling *Neoparamoeba* sp. were not evident in primary cultures from group P1. This was in contrast to histopathological and TEM observations that clearly identified the presence of *Neoparamoeba* sp. in association with the gills.

It would be highly speculative to suggest reasons for this result. It should be recognized that although isolation of gill associated organisms is a valuable diagnostic tool, it should not be construed (on a case by case basis) as fully representative of the sampled gill's micro-fauna. Flattened amoebae and ciliates were present in all groups although neither were observed histologically. Because the experiment was already confounded, primary isolates of the latter two protozoa were not identified beyond the level of their respective families.

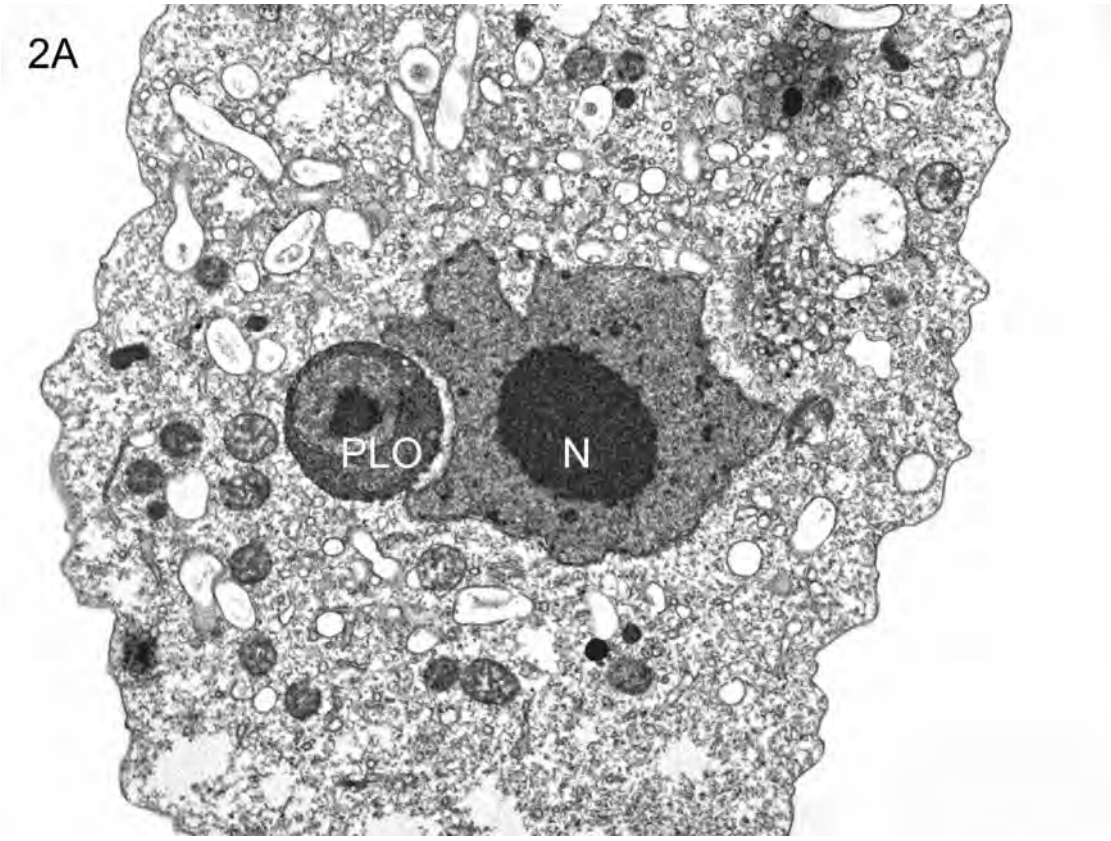


Figure 13A. *Neoparamoeba* sp. from group P1, as indicated by presence of Perkinsiella amoebae-like organism (PLO) partially entwined with the nucleus (N).

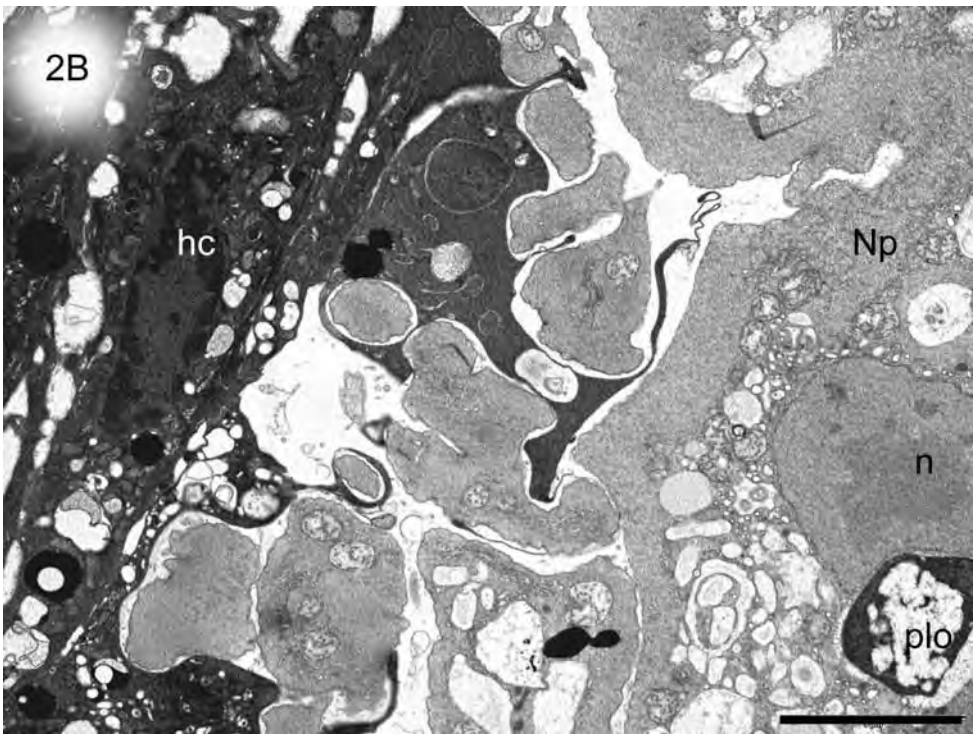


Figure 13B. Trophozoite of *Neoparamoeba* sp. (Np) in contact with necrotic gill epithelium (hc) of salmon from group P1.

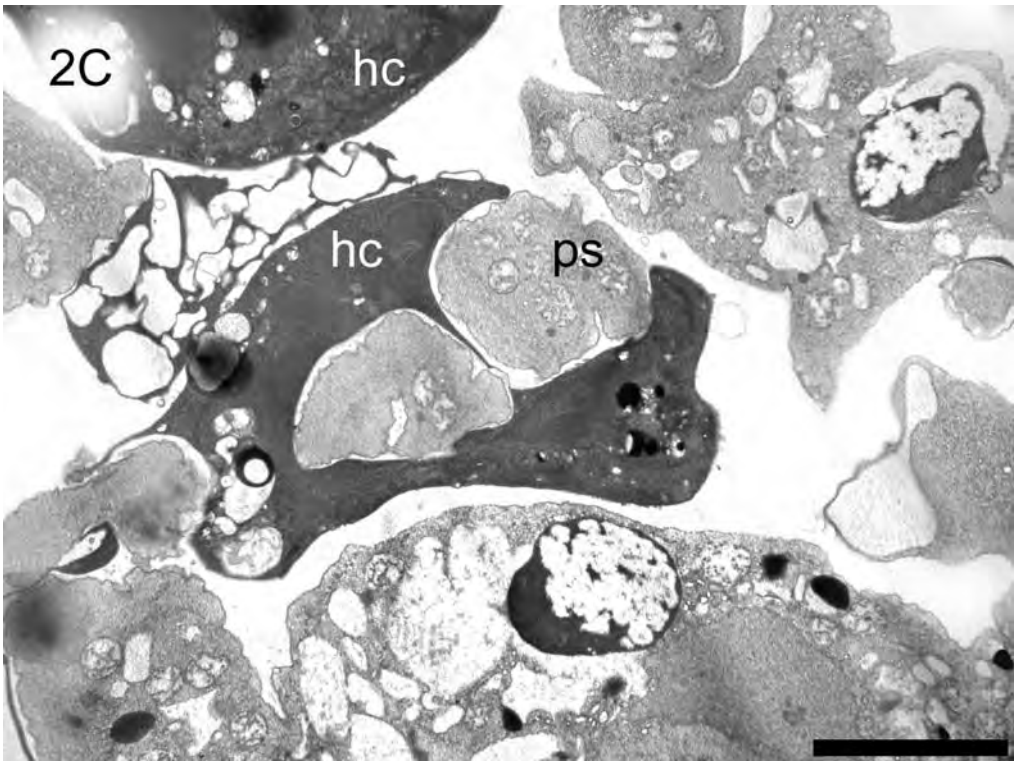


Figure 13C. – Psuedopodia of *Neoparamoeba* sp. (ps) interdigitating with necrotic epithelial cells (hc) of salmon from group P1.

Table 7. – Primary gill isolated micro-organisms post sampling*

Group	Fish No.	Day 5	Day 7	Day 9	Day 11
P1	1		f	f	f
	2		f	f	f
	3		f	f,ob	f
	4			f	f
	5			f	f
	6		f,c	f,c	f
	7		f,c	f,c	f
	8		f	f	f
	9		f	f	f
NP1	1		f	f,np	f,np
	2	c	c	f,c	f,c
	3		f	f	f
	4		f	f	f
	5	f	f	f	f
	6	c	f,c	f,c	f
	7	f,c	f,c	f,c	f
C1	1	c	f,c	f,c	f
	2	c	f,c,np	f,c	f
	3	c	c,np	c	c
	4		np		f
	5		f,np	np	
	6		f,c	f,c	f,c

f = flattened amoebae, c = ciliates, np = *Neoparamoeba* sp.

* organisms detected at 100x magnification on NNA plates

Experiment 2

Groups N2 and P2 were negative, both grossly and histopathologically, for lesions attributable to amoebae. Both groups, however, displayed variable (yet minor) numbers of nodules comprised of mononuclear cells. Such lesions are considered to be a common occurrence following acclimation to full strength seawater (Nowak and Munday 1994; Adams and Nowak 2003). One fish from group P2 displayed a single inflammatory lesion distally spanning two filaments approximately 15-20 interlamellar units (ILU) in length. There was no evidence of a pathogen associated with the lesion and abrasive physical damage cannot be excluded as a possible source of the observed inflammation. Gross and histopathological AGD gill lesions were evident from groups NP2 and C2 although no significant differences were found in the percentage of filaments affected with AGD lesions (Means: NP2 – 40.1% ± 4.8 SE; C2 – 42.2% ± 5.0 SE).

No mortalities or morbidity of experimental fish were noted during the trial. No data were attainable from TEM due to technical difficulties. Small flattened amoebae were observed stereo-microscopically at 14 days post sampling (PCR –ve) in cultures collected from a single fish in two groups (NP2 and C2) and from two fish from group P2. Ciliates and giant trophozoites were not observed in cultures the aforementioned cultures. All other cultures from remaining fish failed to yield any organisms aside from eventual bacterial and

fungal overgrowth. In regard to experiment 1, a reduced microbial presence maybe inferred from the reduced incidence of primarily isolated micro-fauna. However two main factors differed between the experiments. The gill material selected for culture plates was insufficiently moistened after excision and placement upon culture media possibly restricting the ability of gill associated organisms from migrating to the agar. Additionally, in line with animal ethics stipulations, the fish were anesthetized as opposed to cranial puncture, which may have influenced microbial activity upon the gills.

Although histopathologically it would appear that *Platyamoeba* sp. (cultured strain SS8FJ2/l) were not directly pathogenic to Atlantic salmon under experimental conditions, the following points should be considered. It is unclear whether the *Platyamoeba* sp. strain introduced to the water column was firstly able to remain viable and if so whether it was able to successfully colonize the gills. There was no indication histopathologically that the organism was present upon the gills although this may be attributed to fixation which is unable to effectively preserve the mucus upon the gills. Also no gill samples were taken for culture from the source of experimental fish, therefore it could only be assumed that similar type strains are not already present. The only culture evidence of other micro-organisms being present upon the gills was limited to four out of 36 fish. Of these three cultures, in which amoebic morphology was similar, one originated from an experimental fish that was not exposed to the cultured *Platyamoeba* sp. strain. Finally, it has been demonstrated that the virulence of *Neoparamoeba* sp. diminishes following primary isolation and culture (Findlay 2000; Morrison *et al.* 2004) and may potentially be the same for other potentially pathogenic amoebae possibly inhabiting the gills of farmed Tasmanian salmon.

The overall outcomes of the laboratory experiments were largely inconclusive due to a combination of technical difficulties and methodological approaches. If this line of investigation is to be pursued then the study requires refinement, both contextually and technically, by establishing what is achievable and whether a knowledge requirement is essential for understanding AGD in a mitigative context. The role of other amoebae in AGD in Tasmania is multi-faceted and could be broken down into several components.

1. What are the identities, diversities, distributions and relative populations/proportions of different amoebae genera within the gill environment in both healthy and clinically diseased salmon?

2. Can other amoebae induce a detectable pathogenic effect upon salmon gills? (histological evidence of contact with host epithelium and consequent host response)?

3. Can other amoebae induce a pathogenic effect without detection? (ie. with loss of potential aetiological agents due to fixation, only host response is evident).

4. Is there an indirect pathogenic effect? (ie. pre-disposition of the gills to subsequent colonization *Neoparamoeba* sp. and/or mutually beneficial co-existence between genera?).

5. Assuming lack of pathogenic activity, what is the benign role other amoebae upon the gills?

Although tools such as histopathology, culturing and TEM are invaluable for addressing these questions, it was apparent from this study that they may be insufficient in providing a complete answer to the above. Due consideration may need to be given to other forms of investigative techniques and experimental design provided this line of inquiry persists. In particular, procurement of the Olympus Nomarski DIC system would of significant importance for enhanced morphological differentiation of amoebic trophozoites.

It is clear that other amoebae inhabit the gills of AGD affected fish, both in the Tasmanian locale and overseas (Dyková *et al.* 1999, 2000), but evidence is lacking suggesting that mortalities of Tasmanian cultured salmon are attributable to infection caused by amoebae other than *Neoparamoeba* sp. The only field evidence provided in Tasmania, asserting that other amoebae were causative agents of AGD, was presented by Powell *et al.* (2003). However, the inference was made on the basis of differential immunohistochemical staining of crude gill mucus preparations, a method that is subjective and unproven in the field. Additionally, histopathological data were not used for comparison with immunohistochemical findings and no culture isolations were performed to verify the presence of other amoebae. It may be argued that continued investigation of the role of other amoebae in Tasmanian outbreaks of AGD is unwarranted in light of the clear association between *Neoparamoeba* sp. and AGD (Roubal *et al.* 1989; Munday *et al.* 1990, 1993; Clark and Nowak 1999, Adams and Nowak 2003, Adams and Nowak 2004; Morrison *et al.* 2004).

Objective 4
To develop techniques for *in vitro* work on Amoebic Gill Disease
Cell monolayer culture model

Dual-enzyme method for establishment of primary culture from Atlantic salmon gill (Butler and Nowak 2004b)

Primary cultures were established to develop an *in vitro* AGD model. Confluent monolayers developed in 30 of the 32 attempts made using this procedure. Table 8 summarises the lifespan of these cultures. Two cultures continue to persist at passage 92 and 112 and have been classified as RGE-2 (epithelial) and RGF (fibroblastic) respectively.

Table 8. Survival of primary isolations of Atlantic salmon gill cells. A total of 32 isolation attempts were made. The second column indicates the number of cultures that ended during the passage interval, unless otherwise indicated.

Passage	Number of Primary Cultures
Did not survive isolation	2
Did not survive first passage	1
Passage 1–5	1
Passage 6-10	4
Passage 11-20	6
Passage 21-30	7
Passage 31-60	9
Surviving beyond passage 60	2
Total number isolation attempts	32

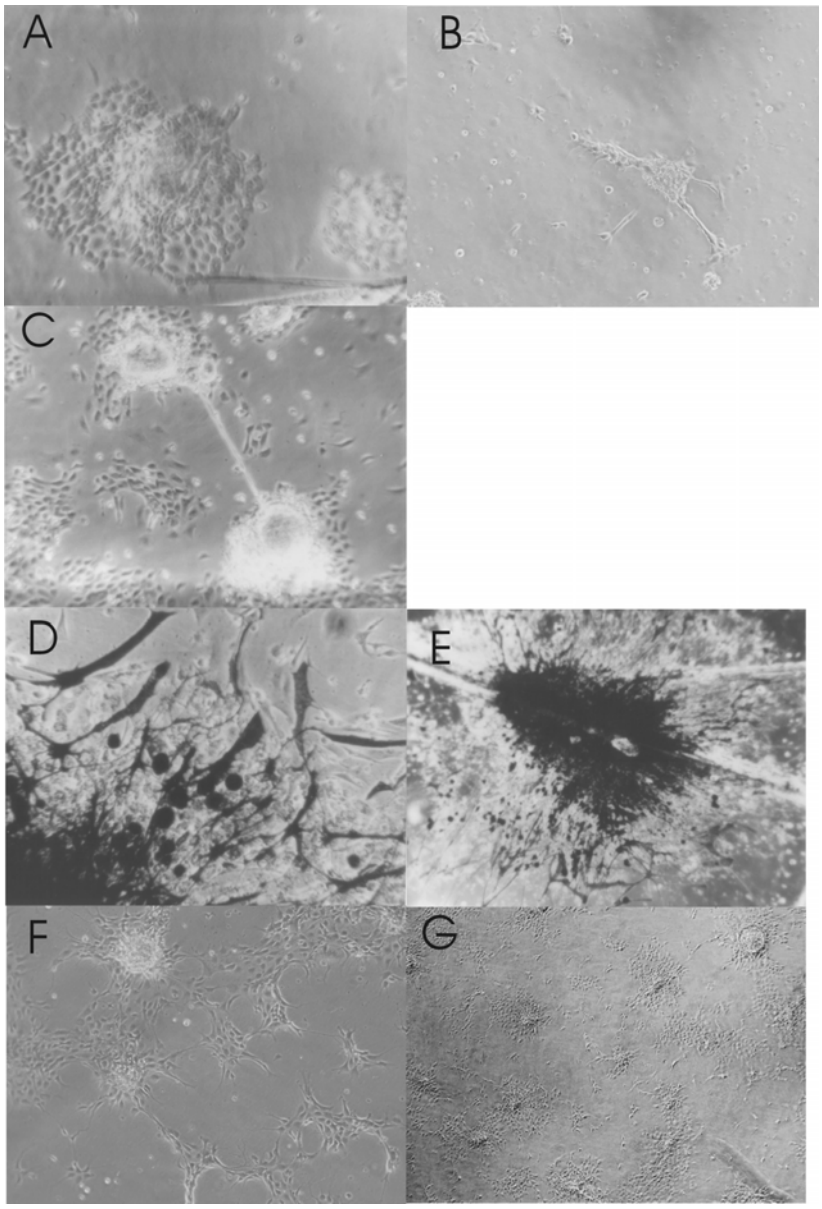


Figure 14. Phase contrast micrographs of primary cultures from Atlantic salmon gill. (A) One day post isolation epithelial-like cells aggregating around tissue debris. (B) One day post isolation, mixed cell types in small and single cell colonies. (C) 5 day-old culture, mixed morphology cells growing around tissue debris and being to form monolayer. (D) Dendritic cells 5 days post isolation. (E) 5 day-old culture of dendritic cells aggregating around tissue debris. (F) - (G) Epithelial cells at passage 1 forming monolayers around larger clumps of non-dissociated cells. Scale bar = 50 µm.

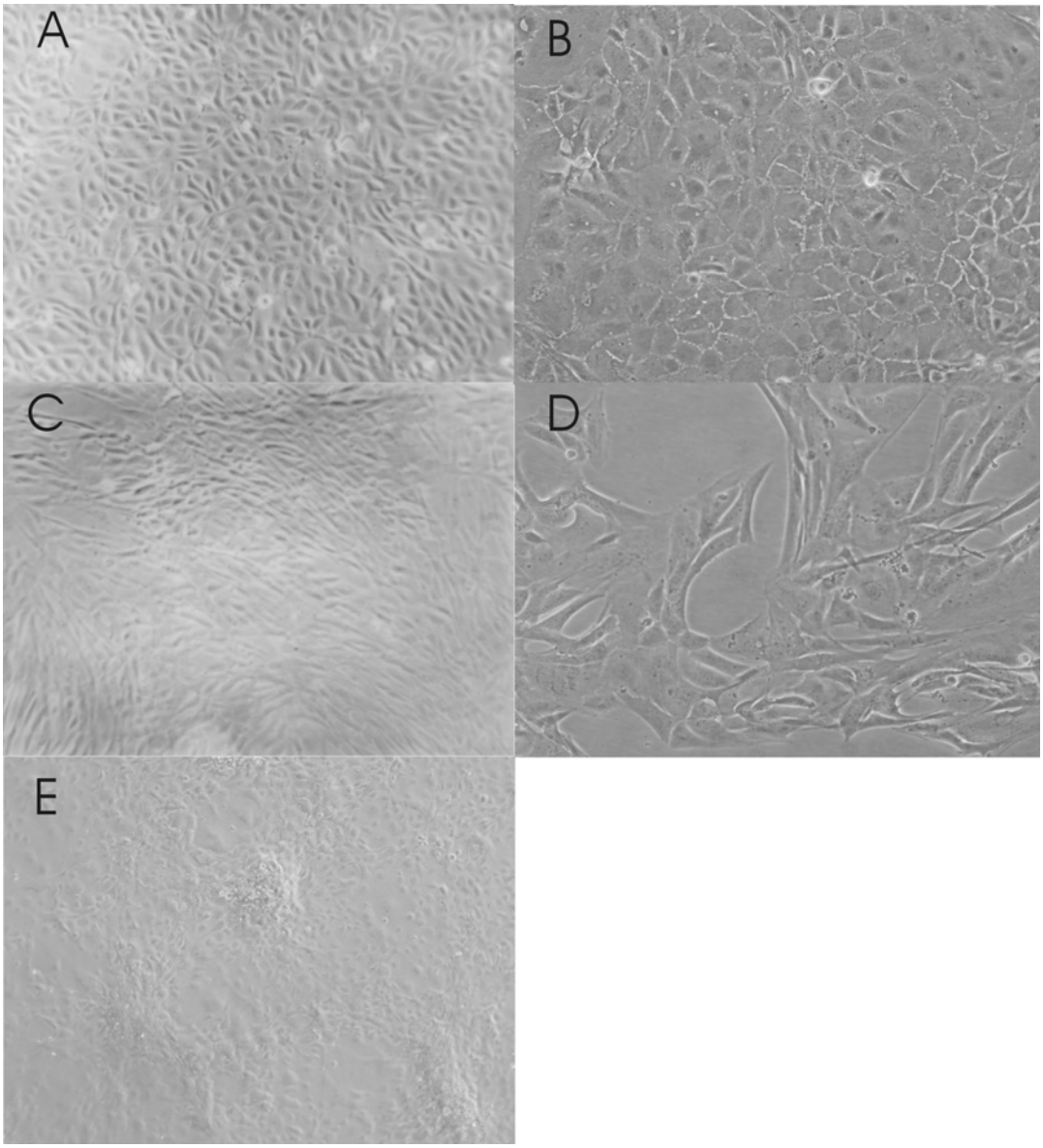


Figure 15. Phase contrast micrographs of primary cultures from Atlantic salmon gill. (A) – (B) Confluent epithelial monolayer passage 8. (C) – (D) Confluent fibroblast monolayer passage 10. (E) Confluent epithelial cells showing cell overgrowth, forming tight associations and dome-like structures. Bar = 50 µm

<u>*In vitro* interaction of gill amoeba and Atlantic salmon gill cells (Butler and Nowak 2004c)</u>

Amoeba growth and survival

At 330 mM kg^{-1} there were no significant changes in the amoeba population when incubated with or without RGE-2 cells ($F=0.712$, df 1,144, $P=0.40$) (Figure 15). In this situation viable amoeba were observed, as determined by trypan blue exclusion assay, but these cells were rounded and displayed no pseudopodia. When incubated in hyperosmotic medium at 780 mM kg^{-1} there was an approximate three-fold increase in amoeba population. Amoeba incubated with epithelial cells showed significantly greater population growth

over time than without cells (F=17.461, df 1,144, P<0.001). Amoeba incubated with seawater culture medium at 1000 mM kg^{-1} showed a six fold increase in population size with RGE-2 cells and a four-fold increase without cells; a rate of growth that was significantly different (F=16.84, df 1,144, P<0.01). Growth over time of these amoebae was significantly greater than amoeba maintained at 780 mM kg^{-1} both with RGE-2 cells (F=16.665, df 1,144, P<0.001) and without cells (F=12.34, df 1,144, P<0.05).

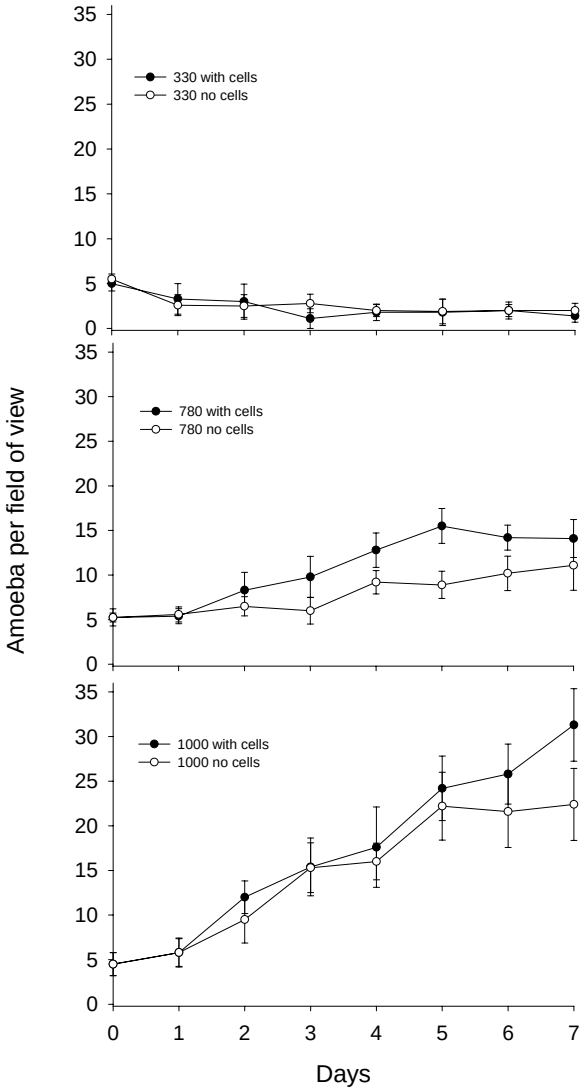


Figure 16. Changes in *Neoparamoeba* sp. population density following incubation at 330m mM kg^{-1}, 780 mM kg^{-1} and 1000 mM kg^{-1} with and without RGE-2 cells. Data are mean number of amoebae per field of view in 10 random fields ± standard deviation.

Effects of amoeba incubation on epithelial cells (Nowak and Butler 2004c)

When amoebae were incubated with RGE-2 cells with standard culture medium there was no significant effect on cell survival or cell morphology, and the monolayer maintained its integrity and confluency (Figure 16). Incubation of RGE-2 cells at osmolality 1000 mM kg^{-1} with or without amoebae resulted in a rapid degradation of the cell monolayer that was complete by five days post-incubation. Subsequently experiments were not performed at this osmolality. Control media at 780 mM kg^{-1} did not significantly affect RGE-2 survival until day 9, following which there was a rapid degradation of the monolayer. The addition of amoebae to RGE-2 cells at this osmolality resulted in rapid breakdown of the cell monolayer. Cell survival was 50% by day 3 and total monolayer degradation occurred by day six. This rate of cell death is significantly greater than that caused by control media at 780 mM kg^{-1} alone ($F=11.52$, df 1,92, $P<0.001$). These apparent cytopathic effects (CPE) developed in multiple foci causing vacuolation and rounding and clumping of cells that became highly light refractive and detached from the culture surface leading to large areas of clearing within the monolayer (Figure 3). Cell lysis in control cultures at 780 mM kg^{-1} was uniform and did not occur in the same focal pattern; similarly the degree of vacuolation was not as evident in these cultures, although cell rounding and clumping did occur. At 330 and 780 mM kg^{-1} amoebae were predominately seen associated with, but not attached to the surface of the cell cultures. At 780 mM kg^{-1} some pseudopodia formation was observed but for the most part amoebae appeared flattened and polygonal and were distinct from the rounded amoebae seen at lower osmolality. At 1000 mM kg^{-1} amoebae attached to the culture surface were similarly flattened and polygonal but with clear extended pseudopodia, those within the culture medium were typically spherical with multiple spindle-like pseudopodia.

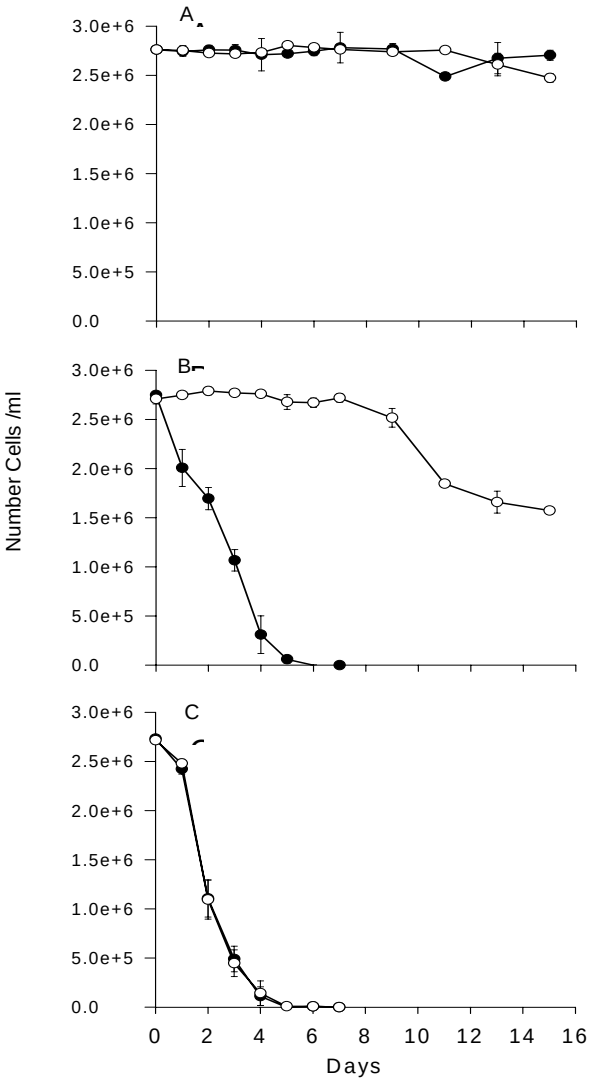


Figure 17. The effects of incubation with *Neoparamoeba* sp. on RGE-2 cell density at 3 osmolalities. (A) 330 mM kg^{-1}, (B) 780 mM kg^{-1}, (C) 1000 mM kg^{-1}. Data represent the mean cell number per mL from 4 replicate cultures ± standard deviation where (○) is the cell number without *Neoparamoeba* sp and (●) is the cell number following incubation with *Neoparamoeba* sp.

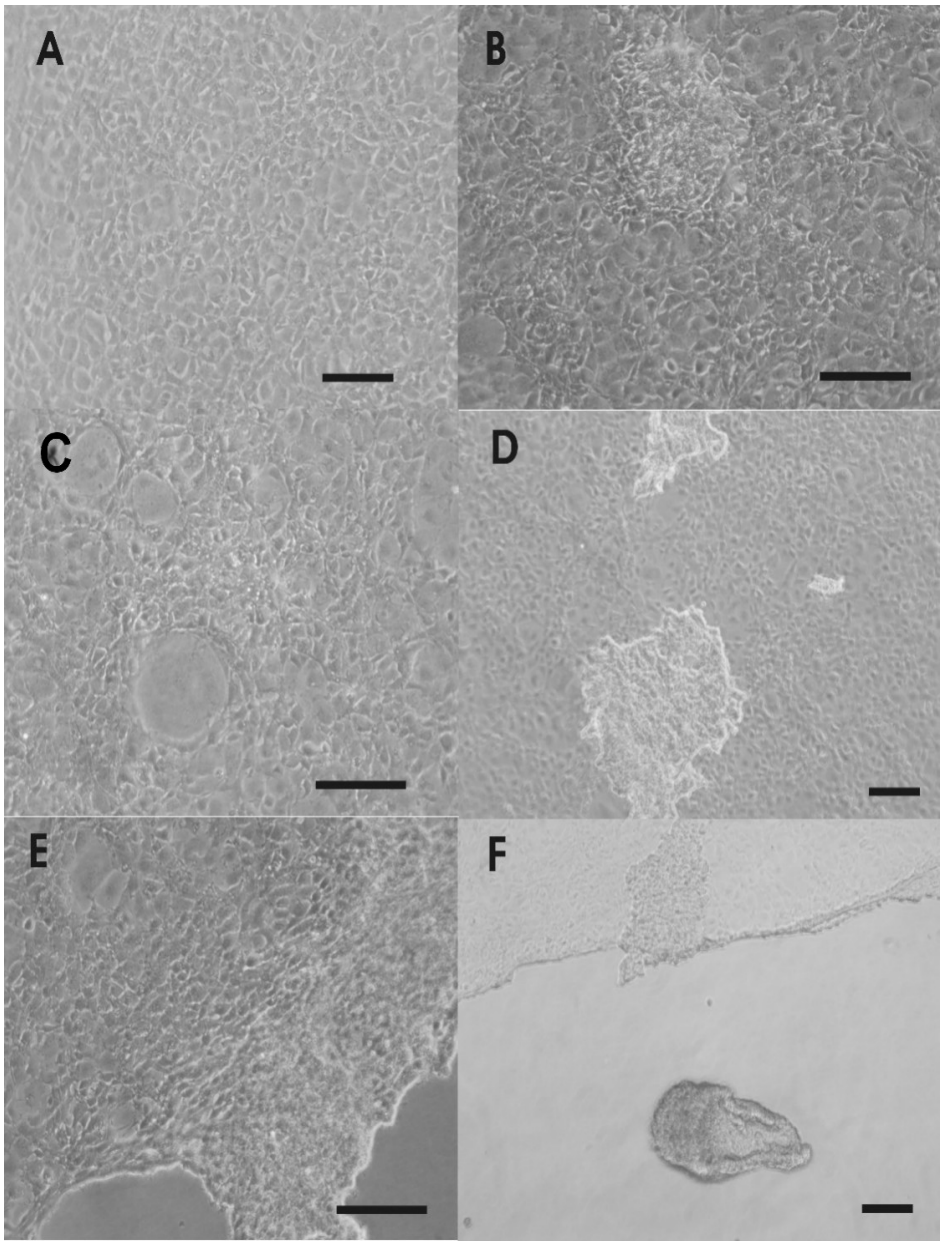


Figure 18. Cytopathic effects (CPE) observed in RGE-2 epithelial cells following the incubation of *N. pemaquidensis* at 780 mM kg^{-1}. (A) Cell monolayer prior to addition of amoebae (B) 2 hours post-incubation with amoebae (C) 8 hours post-incubation (D) 12 hours post-incubation (E) 24 hours post-incubation (F) 48 hours post-incubation. Bar = 50µm

Effects of amoeba incubation on other fish cell lines

Data represent the number of days at which complete cytolysis and 50% cytolysis (data in parentheses) of cell monolayers occurred (Table 9). The assay duration was 8 days.

Table 9. Number of days when complete cytolysis (50% cytolysis) occurred at three different osmolalities.

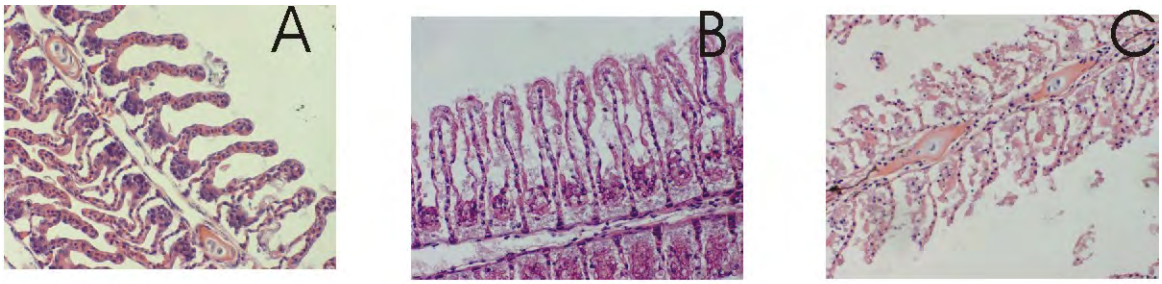
Cell Line	Cytolysis 330 mM kg^{-1}	Cytolysis 780 mM kg^{-1}	Cytolysis 1000 mM kg^{-1}
RGF	No cytolysis	4 (2.8)	4 (1.6)
ASE-w	No cytolysis	4 (2.4)	4 (1.3)
AS-6	No cytolysis	4 (2.4)	4 (1.9)
CHSE-214	No cytolysis	4 (2.5)	4 (0.9)
RTG-2	No cytolysis	>8 (3.0)	4 (1.1)
BF-2	No cytolysis	6 (1.8)	4 (1.3)
EPC	No cytolysis	5 (3.1)	4 (0.9)

Confirmation of CPE by AAHL

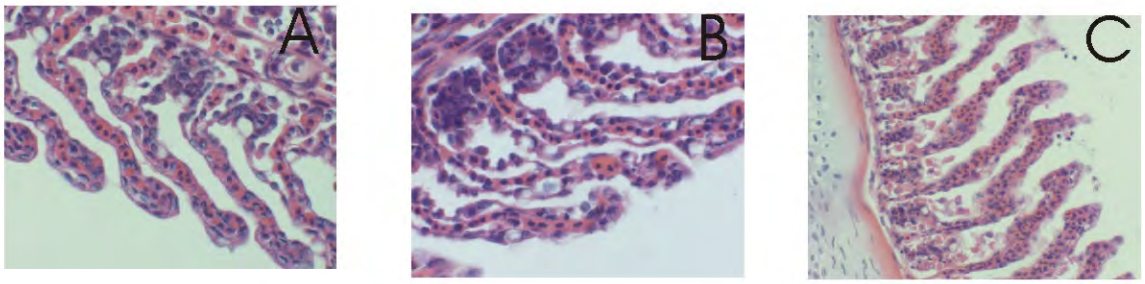
There was no effect (no CPE detected) of gill-isolated amoebae on CHSE-214 and EPC cell lines. This further suggest that high osmolality used in previous experiments (780 or 1000 mM kg^{-1}) significantly contributed to any cell damage observed. Furthermore, it suggests that there is no consistent physical association between *Neoparamoeba* sp. and viruses causing CPE in CHSE-214 and EPC cell lines. This suggests that *Neoparamoeba* sp. does not carry viruses pathogenic to fish.

Gill explant culture model

Explants of gill tissue retained structure and cellular organisation for 3-4 days in seawater and up to 12 days in culture media. Tissue degradation is evident but only becomes widespread at the end of its viable life. Whilst structurally intact, the biochemical products of tissue breakdown, especially when the extent of the breakdown is significant, may have consequences for the assay of amoeba interaction. For this reason all assays of interaction and attachment were restricted to 24h post explantation at which point there were no visible indications of tissue breakdown in either explants maintained in seawater or culture medium. This model was used to test monoclonal antibodies developed in Objective 2 and role of mucus investigated under Objective 3.



Gill explants in seawater. A, 1 day; B, 2 days; C, 3 days



Gill explants in cell culture medium. A, 1 day; B, 3 days; C, 8 days,

Figure 19. Gill explants.

Application of models to *in vitro* study of gill amoeba

Incubation of amoebae and amoebae culture products cause cytolysis of RGE-2 cell monolayers within 5 days. Table 10 summarises the outcomes from pre-treating gill amoeba and amoeba culture supernatants prior to assays of this cytolytic capacity. Outcomes are scored as either causing complete cytolysis within 5 days of incubation ('cytolysis'), causing incomplete cytolysis within 5 days ('↓ cytolysis') or causing no cytolysis ('no cytolysis').

Table 10. Results of *in vitro* study of interaction between amoebae and RGE-2 cell monolayers.	
Test	**Outcome**
Amoeba Amoeba products Amoeba products (x2 passage)	Cytolysis (5 days) Cytolysis ↓ cytolysis
Treatment of culture products	
Dilution 1:10 1:100 1:1000	Cytolysis ↓ cytolysis No cytolysis
Heat 56°C (30min) Heat 70°C (30min)	↓ cytolysis No cytolysis
Proteinase k	No cytolysis
Amoeba in seawater (no cells) Amoeba in seawater plus salmon serum	No cytolysis No cytolysis
Treatment of amoeba	
Heat 56°C (30 min)	No cytolysis
Proteinase k Proteinase k plus 24 hr recovery in seawater	No cytolysis ↓ cytolysis
Mabs against *Neoparamoeba* sp. (4)	Cytolysis

Objective 5
To expand *N. pemaquidensis* library of strains

The library has now been expanded to include 2 strains isolated from the gills of turbot from the Mediterranean (isolated and supplied by Dr Iva Dyková), 6 strains from gill material from Atlantic salmon in Tasmania, 4 strains from marine sediments from various sites around Tasmania and 3 isolated from net material taken from seacages housing Atlantic salmon (full list follows this section).

The identity of these isolates has since been confirmed using sequence data from the SSU rRNA gene and the information has been included in phylogenetic studies currently being performed by collaborators (Dr Iva Dyková and associates) at the Academy of Sciences of the Czech Republic. All strains were examined by light and electron microscopy for ultra structural and morphological differences but no differences were noted between strains for theses parameters (see Objective 10). Results and images were presented as a poster at the 11th Annual Meeting of the European Association of Fish Pathologists in Malta in 2003 (Crosbie *et al*, 2003). All the strains have been

cloned and are currently maintained in culture and have been cryopreserved (see Objective 6).

Culture collection of marine amoebae held at School of Aquaculture
The culture collection currently holds 14 strains of neoparamoebae isolated from Tasmania (all cloned, 12 of *Neoparamoeba pemaquidensis* and 2 of *Neoparamoeba branchiphila*), 2 strains of *Neoparamoeba pemaquidensis* from UK (water isolates), 2 strains of *Neoparamoeba pemaquidensis* from the US (one fish isolate, one water isolate) and 2 strains of *Neoparamoeba pemaquidensis* from Spain (both fish isolates). Additionally, we have six clones of non-paramoeba species (flat amoebae). *Neoparamoeba pemaquidensis* isolated from Tasmania includes fish isolates (5), sediment isolates (4) and net isolates (3). The total number of amoeba isolates is 26.

Neoparamoeba pemaquidensis
Reference cultures supplied by Culture Collection of Algae and Protozoa (CCAP):

1560/4- isolated from seawater (F.C. Page, Wales, 1976)
1560/5-isolated from seawater (F.C. Page, Wales, 1976)
sub-cultured every 4-5 weeks

Reference cultures supplied by American Type Culture Collection (ATCC):

50172- isolated from coho salmon, *Oncorhynchus kitsutch*,
(M.L. Kent, USA, 1988)
30735- isolated from seawater (T.K. Sawyer, USA, 1971)
sub-cultured every 4-5 weeks

Reference Isolates supplied by Dr I. Dyková:

AFSM2V/II- isolated from turbot, *Scophthalmus maximus* (I. Dyková , Spain, 9/1/2000
AFSM11/II- isolated from turbot (I. Dyková, Spain, 28/11/2000)
Tasmanian Isolates:

PA 027- isolated from Atlantic salmon, *Salmo salar,* (T. Howard, Dover, 18/4/1994
NP251002- isolated from Atlantic salmon (R. Morrison, UTAS, 25/10/02
GILLNOR 1 - isolated from Atlantic salmon (P. Crosbie, Bruny Island, 9/8/02)
GILLNOR 2 - isolated from Atlantic salmon (P. Crosbie, Bruny Island, 9/8/02)
SEDCB 1- isolated from sediment (P. Crosbie, Bicheno, 7/4/02)
SEDTC 1- isolated from sediment (P. Crosbie, Tamar Estuary, 29/4/02)
SEDST 1 – isolated from sediment under seacage, Tassal, Stringers Cove, (P. Crosbie, Tasman Peninsula, 14/11/02)
NETC1- isolated from net at HAC (C. Bagley, Huon Estuary, 23/1/03)
NETC 2- isolated from net at HAC (C. Bagley, Huon Estuary, 23/1/03)
NETH2T3- isolated from net at HAC (C. Bagley, Huon Estuary, 15/7/02)
SED 5A- isolated from sediment (P. Crosbie, Tasman Peninsula, 19/3/02)
WTUTS –isolated from Atlantic salmon (R. Morrison and K. Mc Carthy, UTAS, July 03)

Neoparamoeba branchiphila
NRSS- isolated from Atlantic salmon (I. Dyková and R. Morrison, UTAS, 5/11/02)
ST4N- isolated from Atlantic salmon (I. Dyková), Huon Aquaculture, Dover, 5/11/02

Platyamoebae/Vanella
S58FS2
SMA17
SB6LI
MSPE
RSSF
RSL

Objective 6
To implement a long term preservation for *N. pemaquidensis* based on freezing technology
Freezing methods 1, 2 and 3 were not successful, there was no cell survival or recovery by culture post thaw. However, all isolates except the host-derived virulent strain survived the ATCC freezing protocol (method 4) and subsequent thaw with viability ranging between 44.5 and 71.5% (Table 11).

Table 11. Viability, based on trypan blue exclusion, and culturability of 4 isolates of *Neoparamoeba pemaquidensis* after a periods of at least 24h and 100d in liquid nitrogen. Freezing volumes were 0.5 mL and cells densities were 2 x 10^6 cells per mL with 7.5% DMSO as a cryoprotectant.

Isolate	Viability (%)		Recovery by culture (after each period)
	+24h	+100d	
NP 251002	n.a.	54	Yes
PA027	60.5	71.5	Yes
AFSM2V/II	49.3	44.5	Yes
AFSM11/II	79	66	yes

n.a. Not available, entire suspension was plated to MYS post thaw

The first attempt to freeze and thaw host-derived neoparamoebae and then induce AGD in salmon failed. Although trypan blue exclusion indicated that approximately 80% of the cells were viable no AGD was induced when these cells were inoculated to tanks at densities of 1180 cell L^{-1}. The control group infected with a portion of the cell suspension prior to freezing (at 500 cell L^{-1}) all presented with AGD.

A second cryovial from the same batch was thawed and again viability was estimated at 72% (trypan blue exclusion). However, there was no obvious adherence of cells to a glass slide. Rapid adherence to glass is characteristic behaviour of both cultured and freshly host-derived amoebae. The cells were then added to 30 mL liquid phase culture medium in flasks without inactivated *E. coli* and incubated for 48 hours. Flasks were monitored closely for signs of adherence to the surface but none was observed after 48 h. Cells were judged to be non-viable and no experimental infection was attempted. Trypan blue exclusion was abandoned as a viability assay for post freeze/thaw cells as it only indicates that the cell membrane retains integrity, not necessarily viability. The neutral red inclusion assay was then adopted as the dye is taken up and is actively transported into vacuoles and vesicles indicating at least some function of cellular mechanisms.

Objective 7
To develop improved culture systems based on monoxenic and axenic techniques

Transfer of isolate PA027 from *S. maltophilia* to *E. coli* was successful. After 5 passages purity plates indicated a monoculture of a bacterium that was identical in colonial morphology to a known *E. coli* monoculture, oxidase negative and presumably *E. coli* (*S. maltophilia* is oxidase positive). This was confirmed when the bacterium was identified as *E. coli* using a biochemical test strip (Microbact 12E and12A).

However, passage onto MYS plates containing antibiotics and seeded with heat-inactivated *E. coli* was not successful. Growth of amoeba only seemed to occur when the effect of the antibiotics was ameliorated and live *E. coli* carried over in the inoculum propagated, usually after 4-5 days of incubation. It was previously determined that the antibiotics themselves were not detrimental to growth of the amoeba as results from trials where PA027 was incubated with live *S. maltophilia* on MYS plates with and without antibiotics showed little difference in amoebic growth after one day. This observation is supported by Dr Richard Morrison who routinely grows the reference isolate NP251002 in liquid culture using the same antibiotic cocktail. Similar observations were made by Kent *et al*. (1988) and Howard (2000). It seems that PA027 can grow in the presence of antibiotics when MYS plates are seeded with live *S. maltophilia* but not when seeded with heat-inactivated *E. coli.* However, in the absence of antibiotics PA027 grows vigorously on MYS plates seeded with dead *E. coli,* this system is in fact used to rapidly culture large numbers of the amoeba. The strategy in attempting to axenise PA027 is to culture the amoeba on an inactivated bacterium then to transfer to an acellular nutrient medium.

This method has been shown to produce nominally surface sterile *N. pemaquidensis.* Antibiotic concentrations at 250% of the base in a 48 hour treatment time level successfully removed culturable bacteria from the cell suspension with a non-lethal effect on the amoeba (Table 12). On transfer of the suspension to fresh malt yeast agar plates, supplemented with heat-killed

E. coli added as a food source, the amoeba recovered. There was a clear association of amoeba cell concentration, and antibiotic concentration, with the level of bacterial elimination.

Table 12. Bacterial growth (+ positive, - negative) in *N. pemaquidensis* suspension (strain AFSMII; 10^3 cells) on Sheih's marine agar (SMA) and Johnson's marine agar (JMA) media following 48 hours incubation.

	Base media	Seawater control	Base + 150%	Base + 200%	Base + 250%	Base + 300%
SMA	+	+	+	+	-	-
JMA	+	+	+	-	-	-

Treatments using the anti-fungal agent pimaricin and 10^6 amoeba cells suggested that there maybe some increase in bacterial removal (Table 13.). The data in Table 12 show the base + 300% treatment nominally sterilised the cells, however this was not the case for the same treatment when 10^6 amoebae cells were used unless pimaricin was added (see Table 13). Additionally, the base + 200% treatment using 10^6 amoebae cells was also effective with the pimaricin supplement. Pimaricin is not known to be an antibacterial but may act synergistically in the media resulting in bacterial death.

Table 13. Bacterial growth (+ positive, - negative) in *N. pemaquidensis* suspension (strain AFSMII; 10^6 cells) on Sheih's marine agar (SMA) and Johnson's marine agar (JMA) following 48 hours incubation. (P = Pimaricin).

	Base media	Seawater control	Base + 200%	Base + 200% P	Base + 300%	Base + 300% P
SMA	+	+	+	-	+	-
JMA	+	+	+	-	+	-

Sterilisation treatments where autoclaved *E. coli* were omitted are presented in Table 14. Interestingly, in all treatments where *E. coli* was removed there was bacterial growth even at the base + 300% level which was effective for 10^6 amoeba cells previously.

Table 14. Bacterial growth (+ positive, - negative) in *N. pemaquidensis* suspension (strain AFSMII; 10^6 cells) on Sheih's marine agar (SMA) and Johnson's marine agar (JMA) following 48 hours incubation.

	Base media	Seawater control	Base + 200% - E.coli	Base + 200%	Base + 300% - E. coli	Base + 300%
SMA	+	+	+	-	+	-
JMA	+	+	+	-	+	-

A possible explanation is that inactivated *E. coli* cells replace live bacteria in vacuoles as they are digested by the amoeba, but in the absence of *E. coli* there may be a moderation in feeding behaviour and bacteria internalised prior to treatment may be retained, following transfer to agar media, cells resume division and remnant bacteria are released. Absence of bacteria with addition of *E. coli* in treatment may be a result of amoebae feeding during treatment, bacteria internalised prior to treatment being digested or released to the treatment media during cell division, or feeding, and eliminated. An alternative explanation is that there is an element of luck involved in whether all the bacteria are either digested or neutralised by the antibiotics. It would only take very small numbers of viable bacteria to survive and re-contaminate the suspension following treatment.

Objective 8

To develop cell factory capability to produce high density cell suspensions of *N. pemaquidensis*

N. pemaquidensis (PA027) was grown on MYS plates with a live bacterium, *S. maltophilia*, as a food source. Sub-culture occurred every 3-5 weeks by cutting a block of agar showing a dense area of growth and inverting onto freshly bacteria-seeded MYS plates of 90mm diameter then, as they multiplied, amoebae would slowly radiate out from the inverted agar block. Attempts were made to increase the yield of cells firstly by following the method of Howard (2001) where cells were harvested by flooding the plate with sterile seawater then used as inocula by spreading onto live *S. maltophilia*-seeded MYS plates of 145 mm diameter. This method did increase the yield but was found also to increase chances of contamination with other bacteria and fungi. After transfer of PA027 to live *E. coli* and following the spread plate technique overgrowth by *E. coli* became an issue, thereafter heat-inactivated *E. coli* were used. The spread plate system currently fulfils requirements for amoebae of other research groups with yields in the order of 10^6 cells per 90 mm plate after 3-4 days incubation.

Objective 9

To develop cell purification techniques to produce pure cell suspension of *N. pemaquidensis* derived from cell culture and gill associated disease.

<u>Culture systems</u>

As noted above current requirements for the cultured cells are being met. There has been a slight change in focus for collaborators and the demand for the cultured *N. pemaquidensis* has decreased. When cultured cells are required they are supplied in a relatively clean state with *E. coli* contamination only. Collaborators are aware of this and can account for it in interpretation of results.

Improved isolation methods of host-derived *Neoparamoeba* sp.

Techniques have been developed which allow isolation of a relatively clean preparation of *Neoparamoeba* sp. from the gills of salmon. Previously amoebae were collected by being scraped from the gills in a mixture containing mucus and host cells. Such preparations consisted of trophozoites of varying viability and when used to induce laboratory infections resulted in variation in the kinetics and severity of amoebic gill disease. The refined isolation technique, developed by Dr R. Morrison, takes advantage of the adherence ability of the cells and yields a cleaner and more homogeneous suspension of cells. A more detailed description of the method appears under Objective 5 and is published (Morrison *et al.* 2004)

Access to the partially purified preparations continues to allow further *in vitro* work on the organism such as:

- accurate counts of cells to determine an infective dose under defined conditions (ie. tank size, density of fish) and then induce laboratory-based amoebic gill disease.
- investigation into maintenance of virulence over time after removal from the host.
- investigation into cryopreservation techniques to maintain viability and infectivity.
- development of cell sterilisation techniques.
- allow study of cell attachment to gill explants or fish cell lines.
- production and subsequent screening of monoclonal antibodies directed against *Neoparamoeba* sp. antigens.

Objective 10

To implement cell characterisation techniques for strain differentiation

None of the monoclonal antibodies tested was strain-specific. Although there was a range of reactivities noted across strains, there appears to be no distinct epitope unique to any isolate regardless of origin (see Table 15). Discounting isolate numbers 5, 7, 13 and 14 (2 gill and 2 sediment isolates), the reaction patterns of Mabs against strains look very similar. There were no Mabs that were reactive solely with net or sediment–derived isolates, however there were some Mabs which were solely reactive with at least 1 gill isolate. As 2 Mabs were weakly reactive with the negative control amoeba more screening is required against a range of other amoebae.

A total of 18 *Neoparamoeba* strains were characterised both morphologically and using the SSU rRNA gene sequences as molecular markers (Dyková *et al.* 2004). The results are presented in detail in the manuscript and are summarised below. Nine strains were isolated from gills of farmed Atlantic salmon, *Salmo salar* L., six from sediments sampled in areas of sea-cage farms and three from net material of sea-cages. The newly obtained sequences extended substantially the dataset of *Neoparamoeba* strains available for phylogenetic analyses, which were used to infer taxonomic relatedness among 32 strains morphologically assigned to this genus. In

addition to the *N*. *pemaquidensis* and *N*. *aestuarina* clades, phylogenetic analyses clearly distinguished a third clade with sequences from six strains. Members of this clade are characterised as representatives of a new species *N*. *branchiphila* n. sp. Size differences and great diversity of the shape were recognised within clonal populations of trophozoites derived from each strain. However, morphological discrimination of strains is almost impossible.

Anecdotal observations of sensitivities in water-isolated and host-isolated *Neoparamoeba* sp. to two different antifungal chemicals (amphotericin and pimaricin) suggested that this factor may be a useful marker. It had been noted that water-derived isolates had much slower growth when pimaricin was added to the MYS agar plates, but this was not the case for the host-derived isolates. To test this clonal isolates from the CCAP and the ATCC were cultured on MYS plates containing either antifungal chemical. In all cases the amoebae grew regardless of origin or type of antifungal chemical.

Objective 11
To investigate culture strategies to develop infective strains of *in vitro* grown *Neoparamoeba* sp.

NP251002 fails to induce gross or histopathological symptoms of AGD in Atlantic salmon

NP251002 were inoculated into fish holding tanks at concentrations well in excess of that routinely used to generate experimental AGD infections {Zilberg and Munday, 2001, Morrison *et al.* 2004). In addition, the duration of inoculation was well in excess of the time normally taken to elicit gross signs of AGD in fish kept in the systems described here (unpublished observations). Despite the concentration and duration of inoculation, neither gross nor histological signs of AGD were detected in fish inoculated with NP251002 after 34 days (4 passages) or 98 days (14 passages) in culture. While a single amoeba was detected in the gills of fish inoculated with NP251002 after 34 days in culture, no host-inflammatory reaction consistent with AGD was observed.

Short term cultured gill associated amoebae trophozoites elicit AGD in Atlantic salmon

The failure of NP251002 to elicit AGD could be interpreted as:

- the selection of an avirulent strain of *Neoparamoeba pemaquidensis.*
- an inoculation period that was too short.
- the down-regulation of putative virulence factors.
- the inhibition of virulence by the culture conditions.

To address these issues at least in part, amoebae were isolated as described (Morrison *et al*, 2004) and placed in culture with or without *E. coli* for 72 h. These cells were then used to inoculate recirculation systems housing AGD naïve fish. At 8 d post-inoculation, fish inoculated with amoebae cultured with (6/6 fish) or without (6/6 fish) *E. coli* displayed gross signs of AGD which was later confirmed by histology. Again, negative control fish showed neither gross nor histological signs of AGD.

Table 15.
Reactivity of monoclonal antibodies (Mab) with fourteen isolates of amoebae.

Mab	1	2	3	4	5	6	7	8	9 *	10	11	12	13	14
2B2F1D4	-	-	-	-	+++	-	+++	-	-	-	+	-	-	+++
4E8G8G5	-	-	+	-	+++	-	+++	-	-	-	-	-	++	-
3A12G1C8	+++	+++	+++	+++	+++	+++	+++	+++	-	+++	+++	+++	+++	+++
4C4D3	-	-	-	-	+++	-	+++	-	-	++	-	-	-	-
3D3F11	-	-	-	-	+	-	-	-	-	-	-	-	-	-
6G11H10	-	-	-	-	+	-	-	-	-	-	-	-	-	-
4C12	-	-	-	-	-	+	-	-	-	-	-	-	-	-
4A2B1	-	-	-	-	+++	-	+++	-	-	+	-	-	-	++
7G5E1211	+++	+++	+++	+++	+++	+++	+++	++	-	++	++	++	-	+++
7A1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3H2G12	-	-	-	-	-	-	-	-	-	-	-	-	-	+
7B5F11	++	+	++	++	++	++	++	++	-	+	++	+	-	++
6E7	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	++	+++	++	+++
4G9B1	+	+	+	+	+	++	+	+	+	+	-	+	-	+
8H11	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- no fluorescence, +++ strong, ++ medium, + weak

Isolate identification and origin:
1. AFSMII/II (turbot NW Spain- I Dyková)
2. AFSM2V/II (turbot NW Spain- I Dyková)
3. Gilnor 1 (salmon, Nortas)
4. Gilnor 2 (salmon, Nortas)
5. PA 027 (salmon, Saltas, Dover)
6. ST 4N # (*N. branchiphila* salmon, HAC- I Dyková)
7. WT UTS (salmon- isolated from infection tank Utas, cultured by UTS)
8. NP 251002 (salmon Utas-R. Morrison)
9. * Net C3 (non-Neoparamoeba sp.)
10. Net C2 (net material HAC- C. Bagley)
11. Net C1 (net material HAC- C. Bagley)
12. Net H2T3 (net material HAC- C. Bagley)
13. Sed TC1 (sediment Tamar estuary)
14. Sed ST 1 (sediment Stringers Cove)

Benefits

This project directly benefits the Atlantic salmon industry in Tasmania by contributing to vaccine development research, novel treatments research and epidemiology investigation undertaken under the AGD subprogram within Aquafin CRC. These benefits include improved knowledge of AGD and new methods which are now applied in further AGD research. This project provided background information and techniques for the current AGD projects under Aquafin CRC. Aquatic Animal Health research in Australia benefited from this project through training of five PhD students and three postdoctoral fellows.

This project has indirect benefits for other aquaculture sectors, particularly those based on marine pen culture. It provides methods and knowledge which can be applied to other gill diseases or parasitic diseases. Some of these methods have been already applied in the research on histopathology caused by Southern Bluefin Tuna gill parasites and kingfish gill parasites.

Further Development

Our research on host-pathogen interaction in AGD has developed a number of methods for AGD research that can be easily applied to research on other gill diseases or parasitic diseases. These methods are now available to collaborators and other researchers. Fish Histopathology workshops could be used to further distribute these methods. The manual for gill histopathology and immunohistochemistry could be published as a CDROM for more general distribution.

While our project resulted in significant findings, it also raised some questions. These will be addressed in the new research projects within AGD research under Aquafin CRC.

Two major areas require investigation from a host–pathogen perspective for the impact of AGD to be ameliorated. Firstly, it is essential that more economic and effective treatment options are identified and secondly, experimental vaccines need to be developed and tested. This is critical to the evaluation of alternative treatment options in the interim and to the eventual development of an efficacious vaccine in the longer term. The project should follow two distinct paths: performance of research and provision of services to other AGD projects, and will comprise three key elements:

1. Development of a robust, well-defined challenge model, which is the research component.
2. Use of the model to perform challenge tests, which will be a major service component for the vaccine development project.
3. Provision of the organism to collaborators, which is an important service component to all the AGD projects, including this one. This element also has a research component in that cryopreservation techniques will be refined.

Supply of infective material and a means of controlled testing of candidate vaccines are integral to success of AGD research. Vaccine development requires identification of specific antigens from the pathogen that will elicit a protective immune response in the host, hence the need for significant quantities of infective material. Similarly, success of the treatment of AGD investigation is dependent on supply of cells for initial screening of a battery of potential therapeutants *in vitro* before attempting field trials. The research component of the proposal, which is the development of a standard AGD challenge method that can be used in experimental tanks, is essential for the success of these projects. We need to be able to consistently induce AGD in fish to economically appraise alternative treatments and candidate vaccines before moving onto costly field trials. Inducing controlled experimental infections is widely recognised as one of the cornerstones of vaccine development.

We have identified three key research areas for use of immunomodulation to ameliorate amoebic gill disease (AGD) of Atlantic salmon. Immunomodulation may enhance productivity by significantly reducing the number of freshwater baths required to treat AGD. Three lines of inquiry will be followed:
1. Preliminary evidence suggests that immunostimulation with the novel CpG oligonucleotides can enhance resistance to AGD (Bridle *et al*. 2003). The existing data set will be expanded by following up the CpGs results, and investigating other novel naturally occurring immunostimulatory compounds and optimising the use of these immunostimulants.

2. While immunostimulation has been identified as a means of reducing the effect of AGD on Atlantic salmon, results of this project have led to the hypothesis that in fact it may be the inability of the host to control pro-inflammatory signals upon attachment of amoebae that leads to AGD-associated death. A human analogy of this hypothesis would be the induction of a hypersensitive/allergic type reaction by a skin irritant. In the case of humans, topically or orally delivered immunomodulators are often used to control the host reaction. Hence this sub-project will also continue investigating the role of inflammation in AGD. In particular the sub-project will assess the ability of immunomodulating compounds to prevent the inflammatory response and perhaps death. This may provide a possible solution to AGD. Therefore this aspect of the project will have both fundamental and applied outcomes in that we will have a greater understanding of the inflammatory response in fish, opening lines toward treatment.

3. Crude vaccine trials will be included to provide clear evidence whether vaccination is possible. While so far all crude vaccine trials have been unsuccessful, antigen dose was low, adjuvants used were not the most effective, time from vaccination to challenge was usually short, an antibody immune response to vaccine was not investigated, transfer to sea water occurred soon after vaccination. All these factors may have adversely affected the result. There is a need for further crude and partially purified vaccine trials under optimised conditions, including testing of serum antibody to confirm that the vaccine has induced an antibody response.

The approach to each of these elements will be very much applied but based upon fundamental knowledge gained from this report. This new project will significantly contribute to achieving sustainable aquaculture in Australia through reduction of economic impact of diseases in farmed fish, development of environmentally friendly approaches to disease management and training aquaculture industry and researchers in the fields of fish immunology. It will also provide innovative, collaborative and commercially-focused research to meet needs of Australian finfish industry. Furthermore, it will significantly contribute to capacity building in the area of fish health and fish immunology. Thus, these projects fit well into the Aquafin CRC mission and strategy.

Planned Outcomes

The outcomes of this project are beneficial both directly to the industry as well as indirectly by providing information and material to the other subprojects. The methods developed are directly adopted for investigation of vaccine and/or improved treatment against AGD. The technology has been transferred to research groups involved in vaccine development and research on novel treatments.

Specifically this project developed:

- methods for *in vitro* research.
- methods for investigation of host-pathogen interaction.
- knowledge about host-pathogen interaction in Amoebic Gill Disease.
- information for development of risk forecasting model, for example the relationship between the number of amoebae in the water and severity of AGD lesions.

These outcomes were achieved through specific project's outputs including:

- the development and validation of standard protocols for *in vitro* techniques.
- the development of a standard protocol for improved culture techniques for *Neoparamoeba* sp*.,*
- the development of strain characterisation,
- the adaptation of methods for histopathology and immunohistochemistry for AGD research,
- the identification of factors responsible for parasite attachment to the gills.

This project has achieved its outcomes and contributed to improved treatments and disease management as a result of better understanding of host-pathogen interaction.

Conclusion

Objective 1

To provide knowledge base for development of novel treatments and vaccine

We have significantly increased the knowledge base for development of novel treatments and vaccines. In particular:

- We showed a relationship between the number of amoebae in the water and severity of AGD.
- We have investigated AGD pathology, both in the laboratory and in the field.
- AGD case definition was developed.
- We have increased our understanding of the immune response in AGD.
- Injection with CpGs delayed outbreak of AGD, suggesting that immunostimulants have a potential in reducing impact of AGD.
- However, in laboratory experiments, three commercially available immunostimulants when used according to manufacturers instructions, failed to reduce outbreaks of AGD.
- Atlantic salmon was not protected against AGD by previous exposure to the disease.
- Similarly, exposure of salmon to amoeba antigen by immersion did not reduce impact of AGD during an experimental challenge.

Objective 2

To identify factors leading to binding of the parasite to fish gills

Monoclonal antibodies against the parasite were developed and characterised. These antibodies will be used to identify and characterise molecular mediators of the amoeba attachment to the gills. If these antibodies can block the binding of the parasite, it could form basis for a vaccine development.

Objective 3

To identify gill conditions which increase the susceptibility of the fish to AGD

We have identified presence of mucus as necessary for amoebae attachment. Mechanical abrasion and subsequent damage reduced number of amoebae attaching to the gills over 48 hours. There is no evidence for role of amoebae other than *Neoparamoeba* sp. in AGD outbreaks. Preliminary results suggest that AGD lesions can develop on gills sterile at the time of infection, however, by day 12 the extent of lesions was greater on nonsterile gills or gills of fish pre-exposed to bacteria. Furthermore, gill and mucus samples from fish infected with AGD in the laboratory infection and a sample from field infection were dominated by a geographically widely-distributed marine bacterium from genus *Psychroserpens*. The possibility of involvement of this bacterium and others in AGD requires further investigation.

Objective 4
To develop techniques for *in vitro* work on Amoebic Gill Disease
Gill explant method and cell monolayer method developed in this project are currently used for evaluation of monoclonal antibodies produced by UTS.

Objective 5
To expand *N. pemaquidensis* library of strains
Current library contains twenty cloned strains of *Neoparamoeba* sp., fourteen isolated from Tasmania, all of them except for one during this project. These strains were characterised using electron microscopy and molecular techniques. The library has strains isolated from gills, sediments and net material.

Objective 6
To implement long term preservation for *N. pemaquidensis* based on freezing technology
Cryopreservation method (using liquid nitrogen) was successfully developed for cultured amoebae with survival post-thaw ranging from 40 to 60%. Amoebae freshly isolated from the gills showed much lower survival than cultured amoebae using the same methods. Further research is required to develop cryopreservation methods for virulent amoebae.

Objective 7
To develop improved culture systems based on monoxenic and axenic techniques
Primary reference strain PA027 is now grown in monoxenic conditions. Surface sterilisation of *Neoparamoeba* sp. has been successful after 48 hours exposure to a liquid medium containing a cocktail of antibiotics and heat-inactivated *E. coli*. Increasing this concentration by 200% removed all culturable bacteria as judged by lack of growth on Sheih's marine agar and Johnson's marine agar.

Objective 8
To develop cell factory capability to produce high density cell suspensions of *N. pemaquidensis*
We developed methods of isolation and purification of amoebae from the gills and improved culture techniques. We have continued to supply our collaborators with high density suspensions of gill isolated and cultured amoebae.

Objective 9

To develop cell purification techniques to produce pure cell suspension of *N. pemaquidensis* derived from cell culture and gill associated disease.

Host-derived amoebae are isolated by the use of distilled water, adherence and washing. Neoparamoebae from culture are supplied in relatively pure state with *E. coli* contamination only.

Objective 10

To implement cell characterisation techniques for strain differentiation

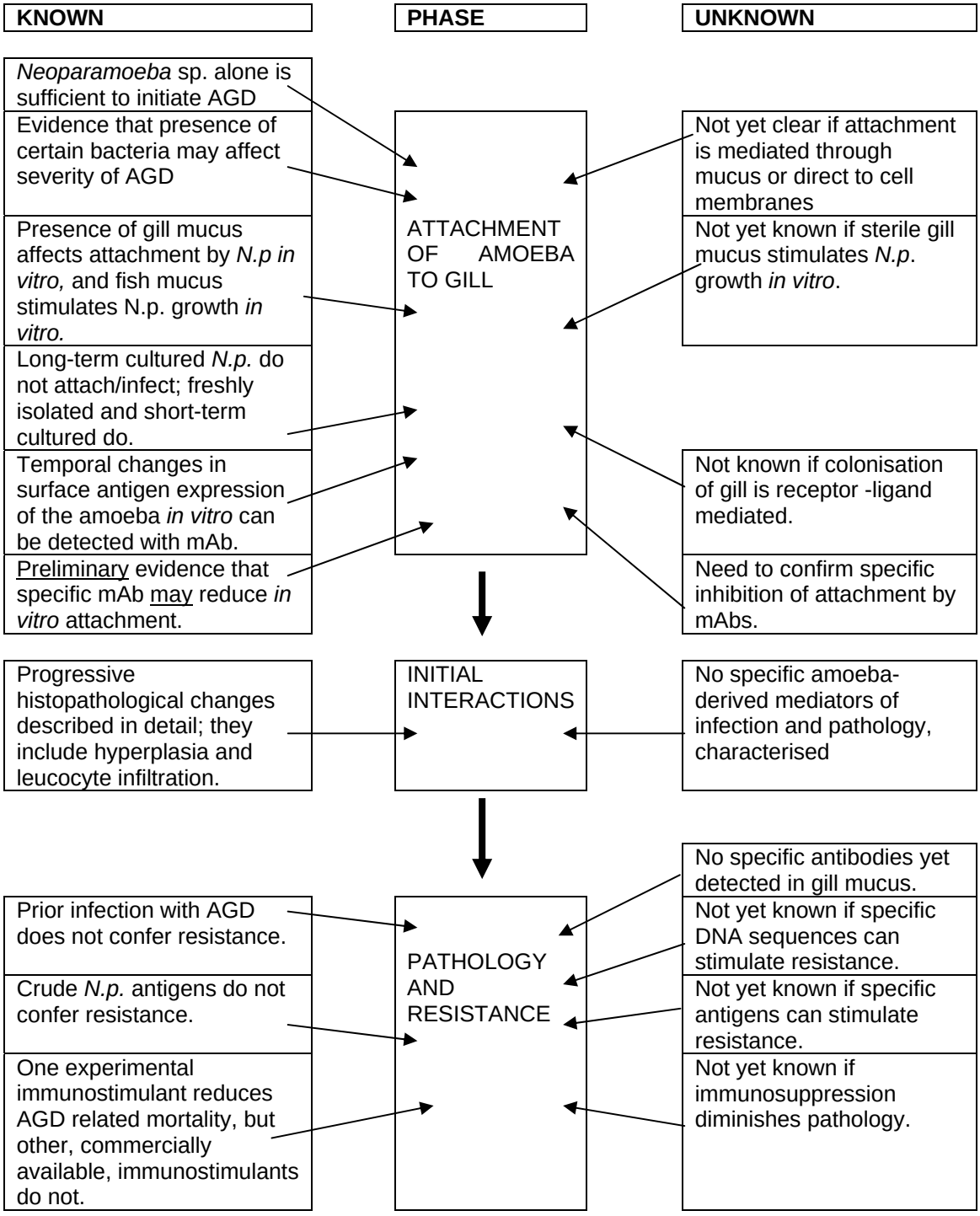
Morphological and molecular techniques were used to characterise isolates of amoebae. New species (*Neoparamoeba branchiphila*), isolated from salmon gills in Tasmania, turbot gills in Mediterranean, sediments and nets in Tasmania, was described as a result of the characterisation of cloned isolates.

Objective 11

To investigate culture strategies to develop infective strains of *in vitro* grown *N. pemaquidensis*

Gill isolated amoebae that were cultured for a short time (up to 24 days) caused gross lesions in Atlantic salmon during laboratory infection. Atlantic salmon skin mucus has been evaluated as a substrate for amoebae growth. Amoebae isolated from the gills grew at a much faster rate with skin mucus than without. Ciliates, flagellates and bacteria contaminate cultures therefore there is a need to “sterilise” amoebae (as described in objective 7) before placing them in culture with sterilized supplements (eg. Gamma irradiated skin mucus). Also amoebae supplemented with mucus grew much faster than cells supplemented with *E. coli*.

SUMMARY OF HOST-PATHOGEN INTERACTIONS IN AMOEBIC GILL DISEASE OF ATLANTIC SALMON



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Appendix 1

INTELLECTUAL PROPERTY

The intellectual property and valuable information arising from this report are:

1. Copyright of this report

Appendix 2

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Appendix 3

AGD - case definition

Prepared by Mark Adams, Barbara Nowak, Jeremy Carson and Stephen Pyecroft

Case definition is a set of standard criteria for deciding whether an individual study unit of interest has a particular disease or other outcome of interest. The study unit can be an individual fish, a cage, a site or a whole farm. Case definition is an essential part of any disease study.

Individual fish has been the study unit in AGD research so far. In experiments and diagnostic research, fish have been considered positive for AGD if characteristic AGD lesions with associated paramoebae were present in histological sections. Sensitivity of the histological test can be greatly enhanced if an area of grossly affected tissue is fixed for histology and not a randomly selected sample or a standard gill arch.

Case Definition:	Amoebic gill disease is defined as the presence of amoebae with parasomes that are in association with characteristic histological changes in gill tissue.

If the fish is in a very early stage of infection, no lesion may be present, only paramoebae attached to the gill tissue. If paramoebae are present but there are no lesions then there is no amoebic gill disease, only amoebic infection. Therefore, if fish are positive only for pathogen (histology or IFAT) or antigen (immunodotblot) these fish are not AGD positive, but only amoeba positive or antigen positive. If the test is for pathogen or antigen presence only, at least gross gill changes should be present for the fish to be declared AGD positive.

Interpretation of diagnostic tests is very important, including knowledge of fish history. For example, if the fish was sampled very soon after freshwater bathing, only AGD lesions may be present without any paramoebae. Some tests have defined applications, IFAT was developed for cage diagnosis without fish sacrifice and, because of sampling error, may be less suitable as a diagnostic test for individual fish.

Use of a whole suite of tests (immunodotblot, IFAT, histology, gross lesions) may be confusing as these tests have different specificities and sensitivities. However, if one test is used for pathology (gross lesions or histopathology) and the second one for pathogen presence (IFAT) and they are both positive it establishment of a diagnosis of amoebic gill disease is more certain.

White patches are a sign of a syndrome indicating some form of gill insult or trauma. In Tasmania this syndrome is mostly due to AGD. Development of white patches is not evidence alone of AGD and confirmatory testing is essential to reach a diagnosis of AGD. White patches are very useful for farm management and at an operational level, but for scientific research however, reliance should not be made solely on white patches as these hyperplastic lesions may arise from a variety of gill insults unrelated to AGD.

There is a need for validation of existing tests and further development of new quantitative diagnostic tests. A manual of sampling techniques could be developed for the salmon industry to train new employees and as a reference for experienced farm staff.

Table 1. Reliability and validation of diagnostic tests for AGD. All tests results are affected by the observer's experience, time post-bathing, prevalence of AGD, stage of infection.

Test	Reliability/field validation	Factors affecting test results
Histopathology	high/gold standard	stage, gill selected, section selected, fixative, staining
IFAT	high/validated	antibody, sampling site, operator's skills
Immunodotblot	low/partially validated through IFAT	antibody, bleach type, standard (positive and negative controls), sample volume
Culture/PCR	low/not validated	sample site, microbial community, primer design
Gross signs	medium-high/validated	season, annual variation, cause, differentiation, environment, external biotic factors
Wet preparation	low/no correlation	differentiation of cells, volume of mucus, sampling technique

Table 2. Test combinations to meet the essential requirements of the case definition

No.	Physical signs test	Proxy test for *N. pemaquidensis*
1	Histopathology	None
2	Patches	IFAT
3	Patches	Immunodot blot
4	Patches	Culture-PCR

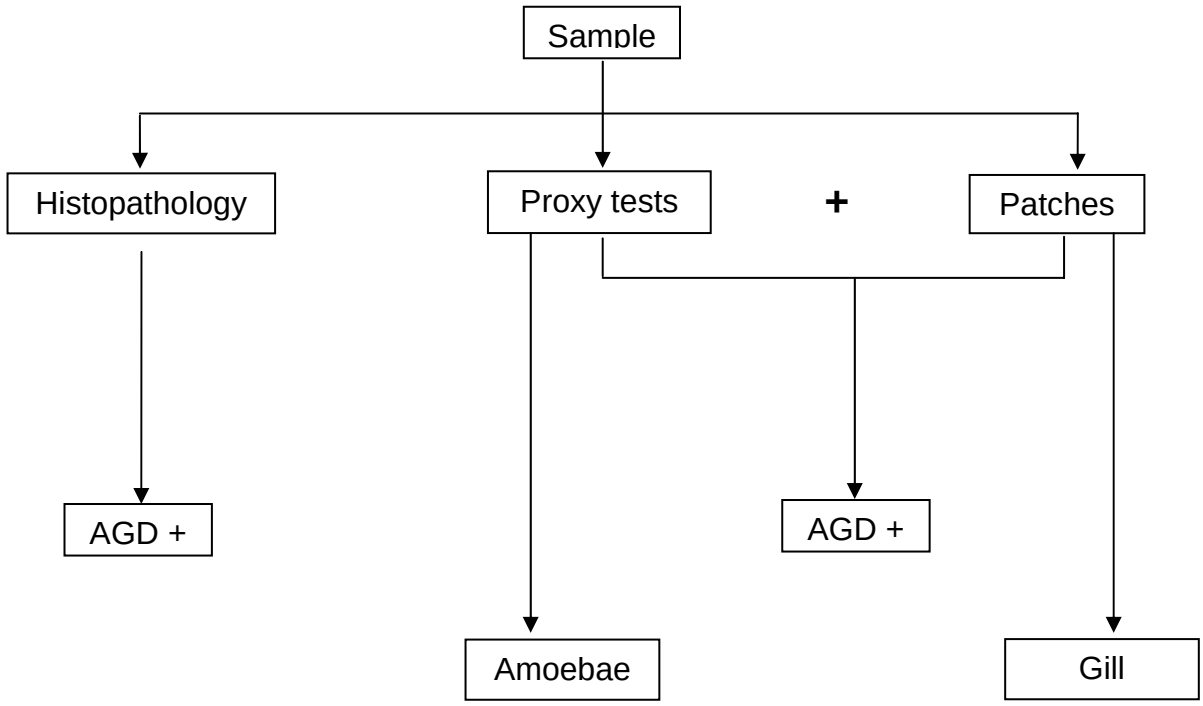


Figure 1. Test combinations and outcomes to establish a diagnosis of AGD

AGD Case Definition – Histopathological features of amoebic gill disease (AGD).

Prepared by Mark Adams

Histopathological verification of AGD is the only diagnostic tool available, to researchers and industry alike, that successfully demonstrates both:

- Presence of the primary pathogen (*Neoparamoeba* sp.)
- Inflammatory host response to infection with the above organism.

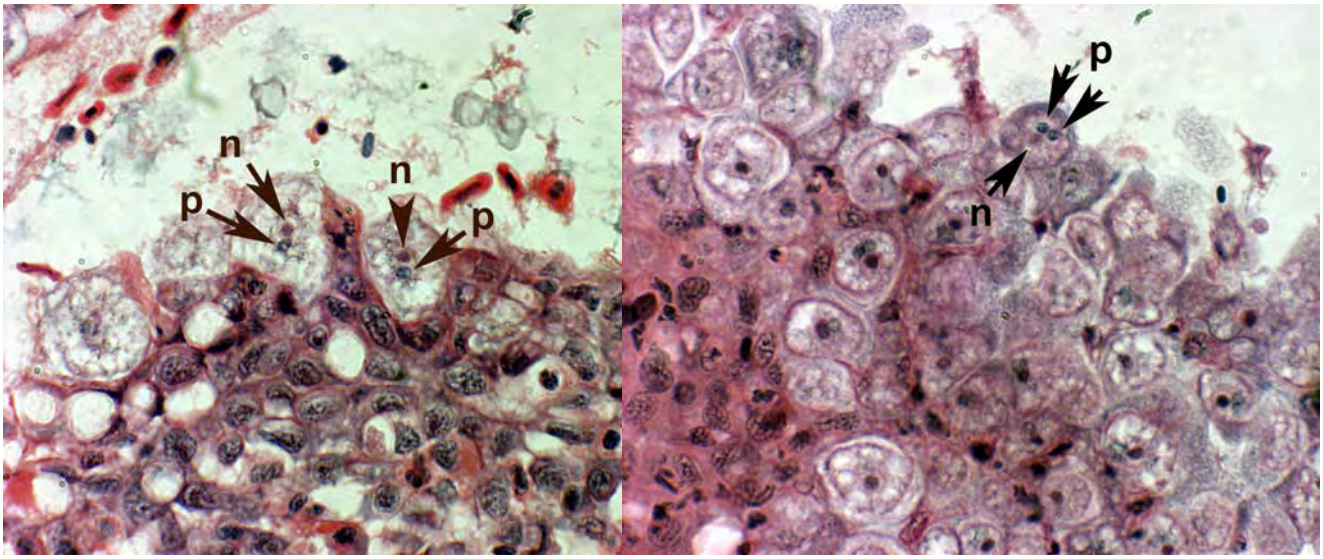
Diagnosis of AGD affected salmon is made on the basis of the presence of the following histopathological features:

- Presence of *Neoparamoaeba* sp. (recognition of nucleus and "parasome")
- Presence of hyperplastic gill lesions* in association with the above organism.

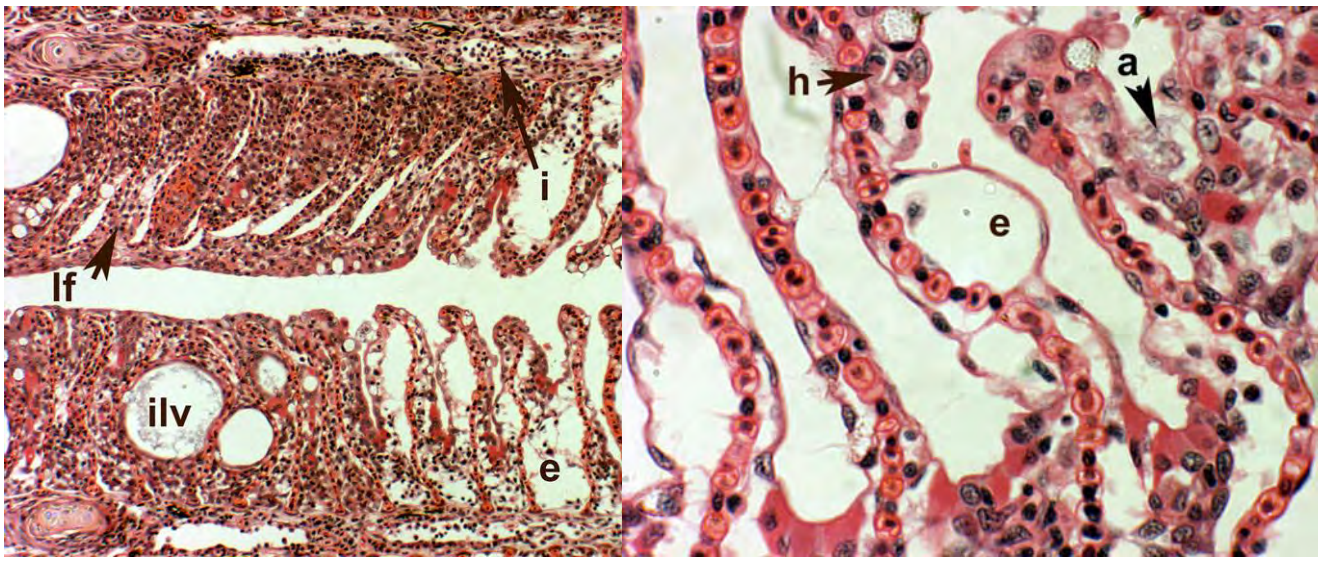
*Hyperplastic gill lesions will typically involve a combination of the following pathological features depending upon the ontogeny (early v advanced), nature (acute v chronic) and sectional plane of the lesion if present:

- Epithelial hyperplasia and resultant lamellar fusion
- Leucocyte infiltration of CVS and hyperplastic regions
- Oedema, epithelial desquamation/hypertrophy
- Interlamellar vesicle formation (sometimes referred to as cysts or crypts)
- Spongiosis, mucous cell hyperplasia and hypertrophy.

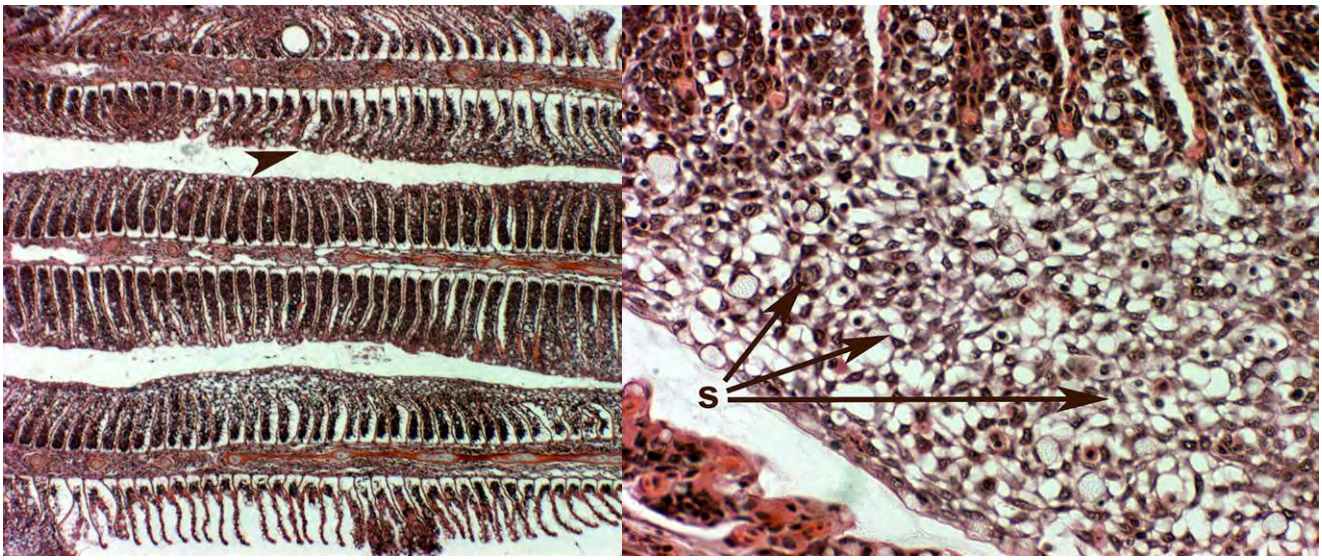
Figures 1 and 2. *Neoparamoeba* sp. upon lesion surfaces, note presence of nucleus (n) and “parasome” (p), amoeba (a) and epithelial desquamation/hypertrophy (h).



Figures 2 and 3. Histopathological features including filamental and lamellar oedema (e), hyperplasia resulting in lamellar fusion with undifferentiated epithelial cells (lf), infiltration of the central venous sinus with leucocytes (i), formation of interlamellar vesicles (ilv), attached.



Figures 4 and 5. Advanced AGD lesions showing severe hyperplasia and fusion of lamellae across many filaments. Mucous cells feature prominently (arrow heads). Spongiosis evident within an advanced lesion (s).



Appendix 4

Amoebic Gill Disease - Microscopy, Histopathology and Immunohistochemistry Protocols
Adams, M.B. and Nowak, B.F.

Amoebic Gill Disease: Microscopy, Histopathology and Immunohistochemical Protocols

Mark B. Adams and Barbara F. Nowak

Tasmanian Aquaculture
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FISHERIES
RESEARCH &
DEVELOPMENT
CORPORATION

Aquafin CRC

General Procedures for Histology and Electron Microscopy

HISTOLOGY

Gill removal, embedding and sectioning

1. Anaesthetise fish in AQUI-S™ (0.25%) until gill ventilation has ceased.

2. Remove the entire gill case carefully avoiding any mechanical abrasion of the filament regions. Remove blood and other debris by gently wash the gills in 0.2 μm filtered seawater (Figure 1.1.1) and place into seawater Davidson's fixative for no longer than 72 hours. (Transfer to 70% ethanol for longer term

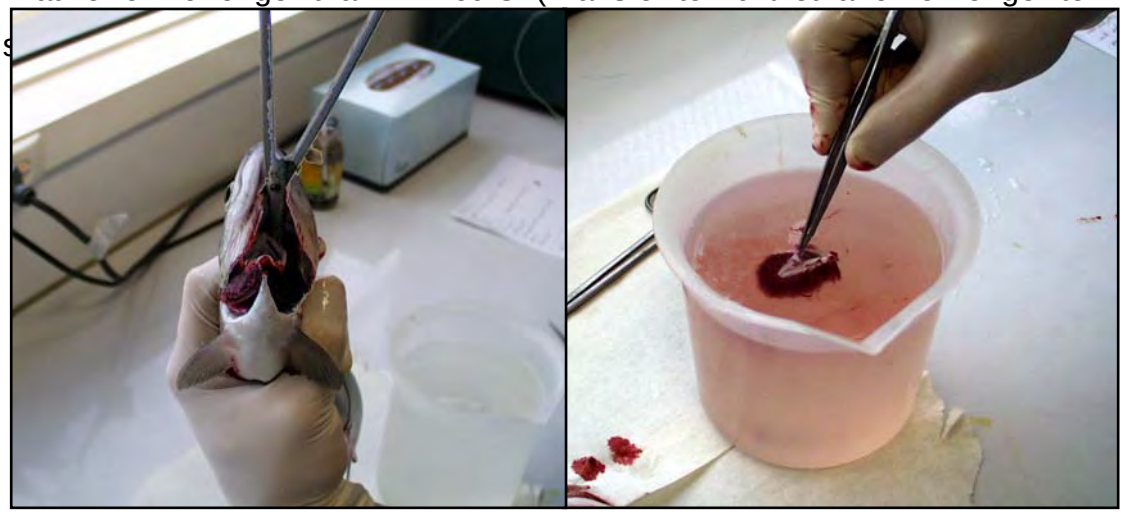

Figure 1.1.1 - Removal and washing of gill case from salmon smolt.

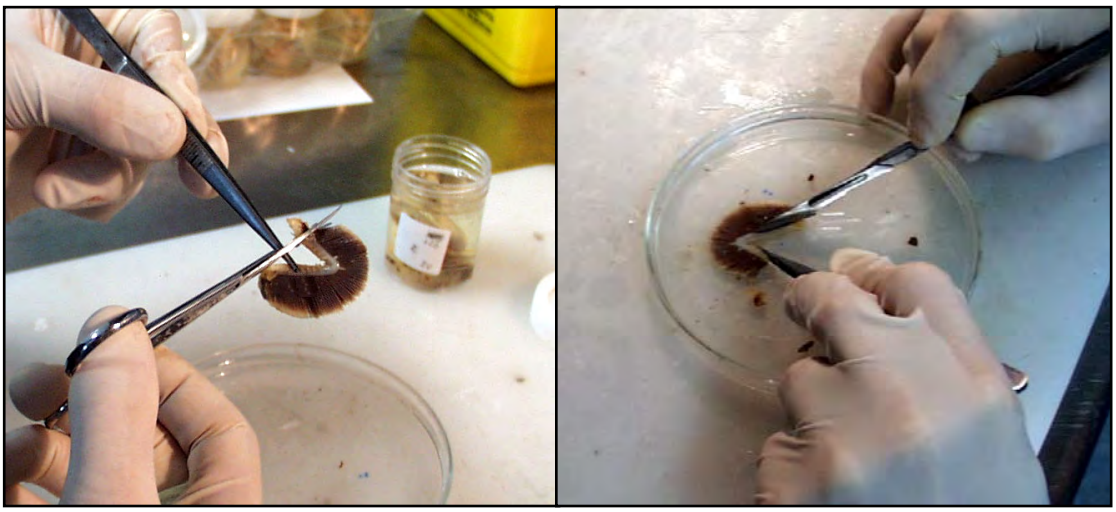

Figure 1.1.2 - Post-fixation trimming of excess tissue.

3. Place a single arch (generally the 2nd left) in a glass Petri dish containing 70% alcohol. Trim any excess tissue and carefully remove gill arch with a scalpel ensuring that the filaments are not injured by mechanical abrasion (Figure 1.1.2).

4. Transfer gill to a tissue cassette fitted with a foam biopsy pad (Figure 1.1.3). Place another pad on top and seal the cassette. The inclusion of biopsy pads prevents any lateral distortion to the gill during the infiltration process. Larger gills may only require one pad.

5. Dehydrate in a graded series of alcohol, clear with xylene or xylene substitute and infiltrate with paraffin at 60°C.

6. The following steps require a paraffin embedding machine. Remove each arch from their respective cassettes and place into heated cassette moulds partially filled with paraffin (Figure 1.1.3).

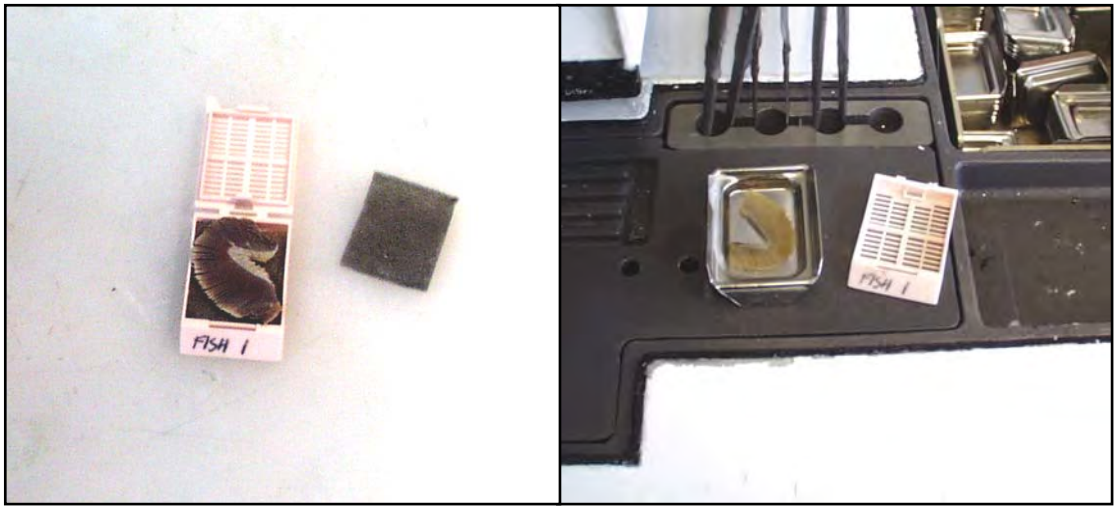

Figure 1.1.3 - Tissue cassette with gill prior to dehydration and wax infiltration (on left). Cassette mould partially filled with paraffin and second left gill arch orientated so that the anterior hemibranch is subsequently sectioned.

7. Orientate so that either the anterior or posterior hemibranch is consistently placed downward (Figure 1.1.3). Move the mould onto the cooled surface and gently press the arch so that it is flush with the bottom of the well (Figure 1.1.4.). Place the cassette body onto the mould (labelled side should point to the left) and fill with paraffin.

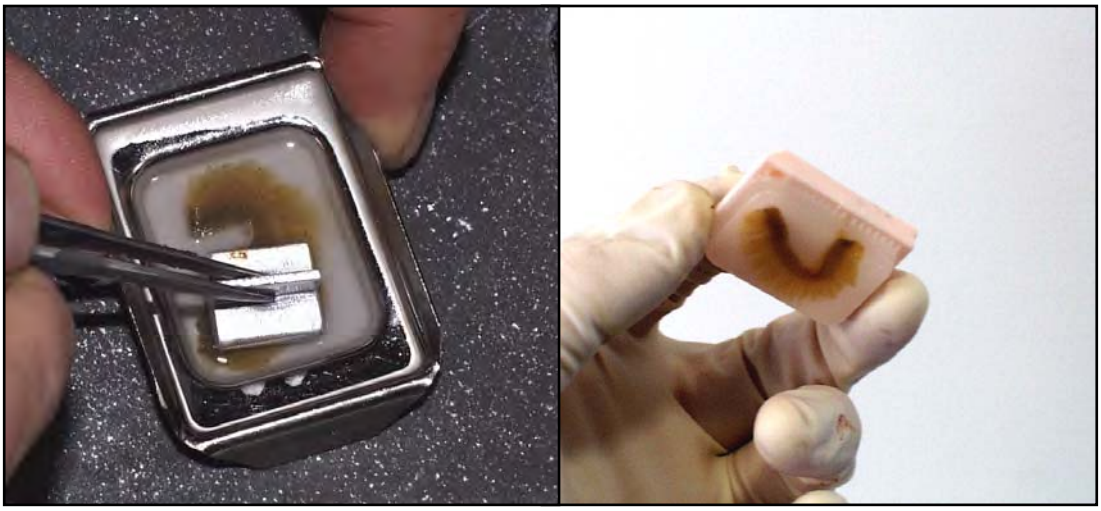

Figure 1.1.4 - Gentle pressing of the gill into a flush position with the bottom of the cassette mould (on left). A completed block after cooling and removal from the cassette mould.

8. Remove the cooled blocks from the moulds and remove all excess wax. Section at 5 μm using a microtome (Figure 1.1.4). Transfer and float sections in a heated (38-40^{o}C) water bath until any creasing has disappeared.
9. Retrieve sections using frosted slides (for labelling) coated in Poly-L-lysine for optimal adherence of tissue sections (Figure 1.1.5).

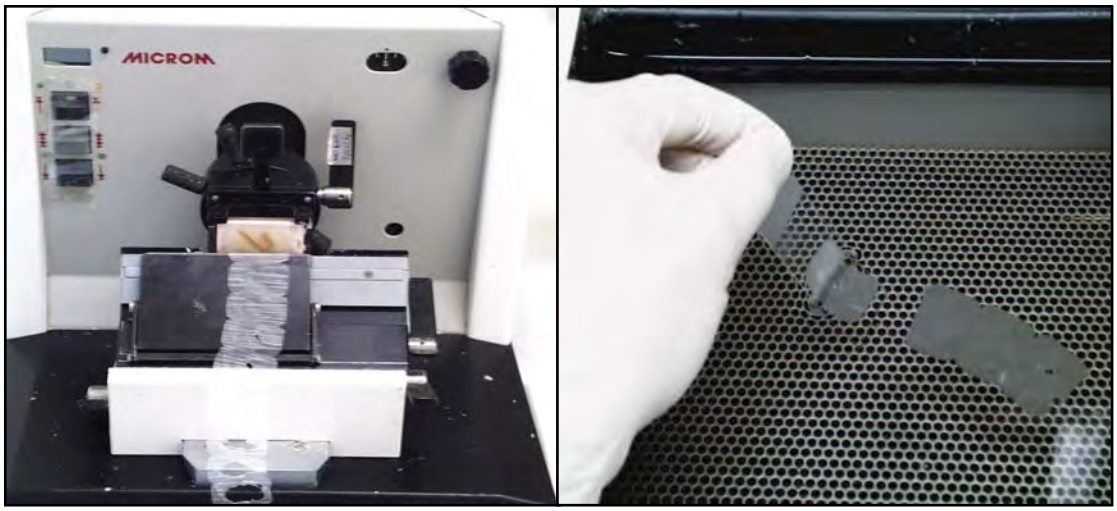

Figure 1.1.5 - Sectioning of block (5 μm) creating a ribbon of sections prior to transfer to a water bath. Retrieval of a single section from a heated water bath (on right).

The ideal plane of section will be within 200-400 μm of the block surface depending on the size of the fish from which the gills were taken. Dry slides overnight at room temperature for standard histological staining.

Dewaxing, staining and mounting.

1. Rinse in xylene or xylene substitute (2 x 5 min)
2. Rinse in 100% alcohol (2 x 5 mins)
3. Rehydrate in graded alcohol series (95% - 70% - 50% - dH_2O, 3 mins each) or slowly replace 100% alcohol with dH_2O over 15 minutes.

Figure 1.1.6 - Manual method for hydrating tissue sections (on left). An automated stainer facilitates automatic rehydration, staining, dehydration and clearing of tissue sections (on right).

4. Proceed immediately with chosen staining protocol.
5. Dehydrate in 95% & 100% alcohol (5 mins) respectively
6. Clear in xylene or xylene substitute (1 min)
7. Mount sections using DPX mountant. Do not let sections dry prior to mounting (Figure 1.1.7). Dry completed slides at room temperature overnight.

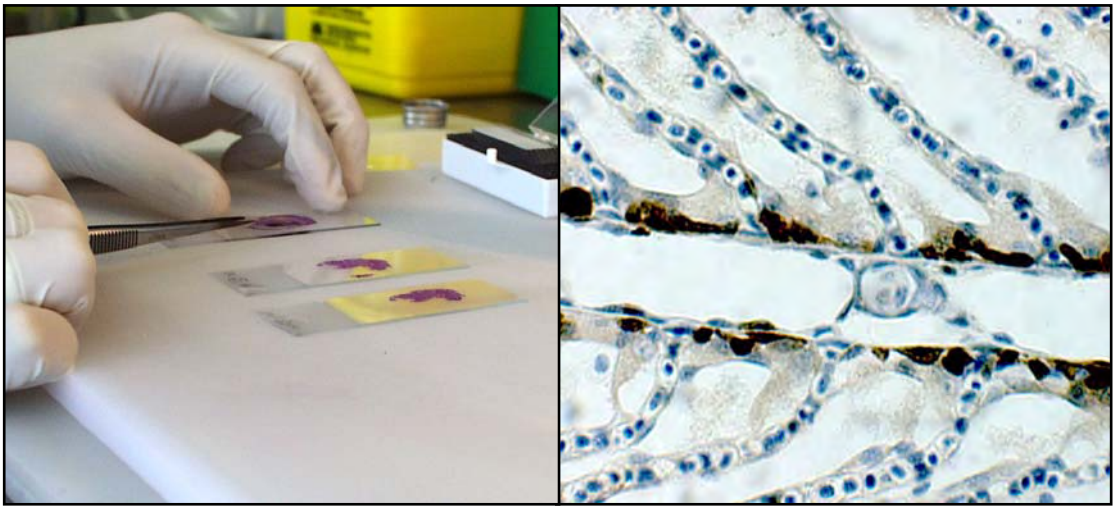

Figure 1.1.7 - Mounting a cover-slip onto a slide using DPX mountant (on left). A histological section showing correct orientation. The central venous sinus (containing a small cartilage protrusion) is clearly visible.

8. View under light microscope. For quantification of cell populations, only count cells from filament regions where the central venous sinus is visible (Figure 1.1.7). This ensures that counts and interpretations are consistently made from a virtually identical plane of section.

NB. - See appendix for further information.

Gill removal, fixation, drying & coating

1. Anaesthetise fish in AQUI-S™ (0.25%) until gill ventilation has ceased.
2. Remove the entire gill case carefully avoiding any mechanical abrasion of the filament regions. Remove blood and other debris by gently wash the gills in 0.2 μm filtered seawater and place gills into a glass Petri dish containing filtered seawater.
3. For SEM, remove a 0.5 – 0.75 cm portion of the arch, containing gross pathology, with surgical scissors and tweezers ensuring that the filaments are not injured by mechanical abrasion (Figure 1.2.1).

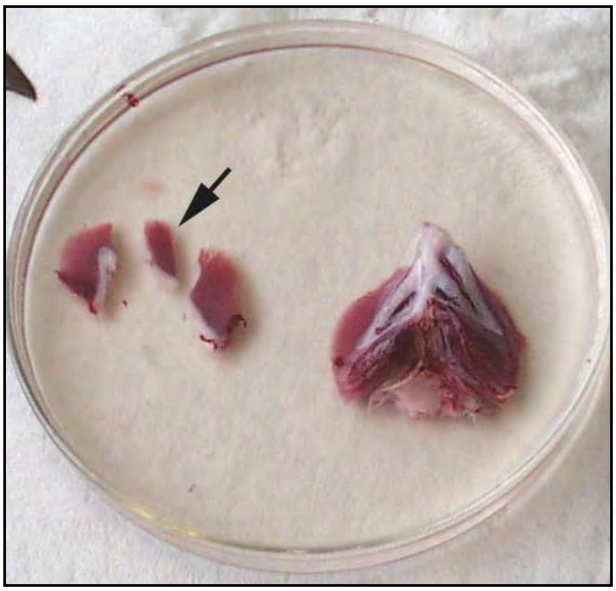

Figure 1.2.1 – A gill arch showing a small removed portion (indicated by arrow) suitable for Scanning Electron Microscopy fixation.

4. Transfer samples to Karnowsky's fixative (sucrose added) (1-2 hours at 4^{o}C).
5. After fixation gently wash samples in 0.1M phosphate buffer (2 x 5 mins)
6. Working within a fume hood, transfer samples to 1% osmium tetroxide in 0.1M phosphate buffer (2 hours). NB – Osmium tetroxide is extremely toxic.
7. Gently wash samples with distilled water (2x 5mins).

8. Transfer to 70% alcohol (samples may be held for storage at this time if necessary).

9. Progressively dehydrate specimens to 100% ethanol. Final ethanol step should be repeated (3 x 5 mins).

10. Specimens are then critical point dried. See machine user manual for specific instructions. Attach dried specimens carefully to a SEM mounting using carbon tape.

11. Sputter coat specimens in with gold. See machine user manual for specific instructions.

12. Store specimens in a dry, preferably vacuum sealed container prior to viewing under a SEM.

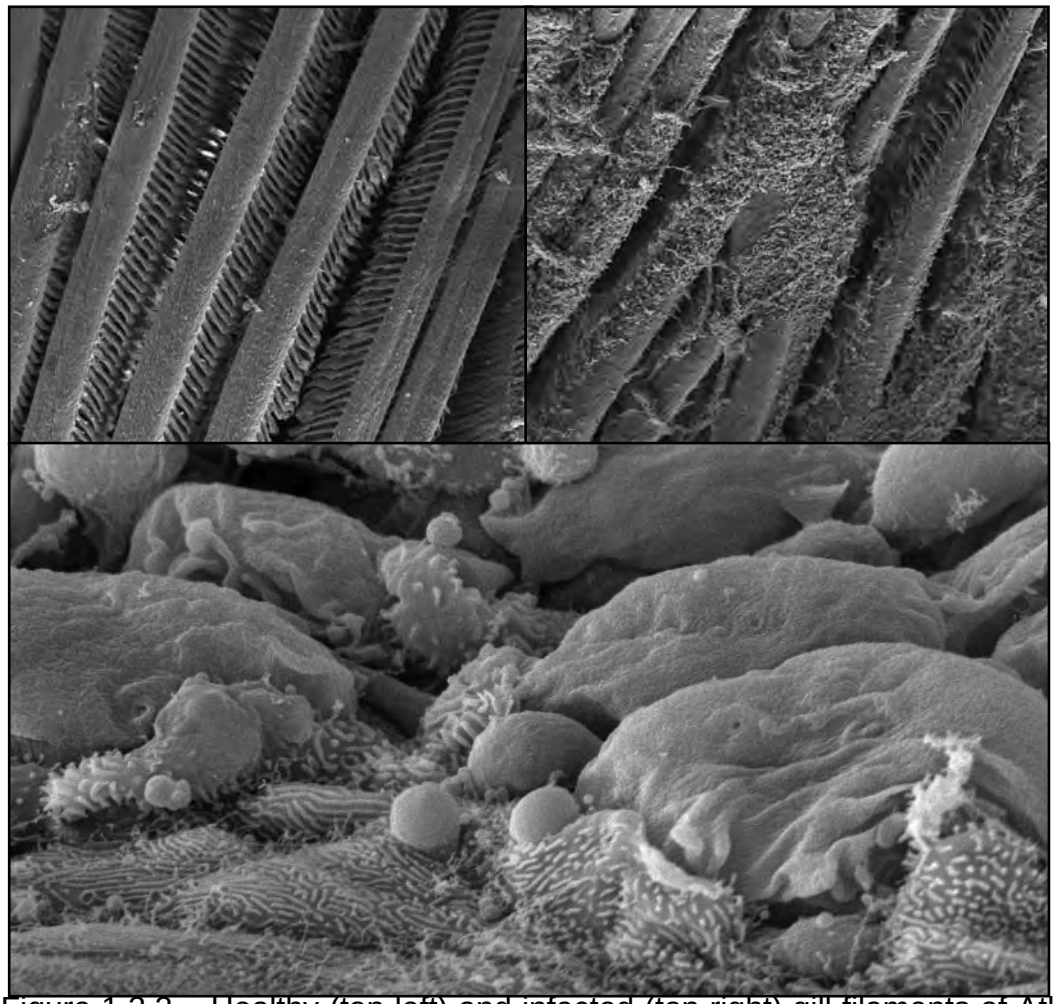

Figure 1.2.2 – Healthy (top left) and infected (top right) gill filaments of Atlantic salmon viewed under an SEM. Note attachment of amoebae on hyperplastic epithelium (bottom).

Hematoxylin and Eosin Stain

Description

A general purpose stain used for diagnostic histopathology. Enables the detection of most tissue abnormalities and limited differentiation of cellular structure.

Fixation and Processing

Gills are removed immediately following terminal anaesthesia and placed in seawater Davidson's Fixative for 24 hours. Prepare gills for embedding as previously described.

Protocol

1. De-wax and re-hydrate sections.
2. Mayer's hematoxylin (3 min).
3. Tap water (30 secs).
4. Scott's tap water (30 secs).
5. Tap water (30 secs).
6. Eosin Solution (3 x 30 secs).
7. Differentiate in 95% alcohol (3 x 30 secs).
8. Dehydrate in 100% alcohol (4 x 30 secs) and clear with xylene (1 min).
9. Mount slides using DPX or similar.

Results

Nuclei and basophilic structures (eg. chondrocytes) stain blue to dark purple. Cytoplasm and remaining tissue will satin varying shades of pink to red. Amoebae usually stain purple and can generally be found on hyperplastic tissue (Figures 2.1.1 & 2.1.2).

Additional Comments

There are many variations to the H & E protocol although the net results are generally very similar. Most histological manuals will describe the various situations where a particular variation maybe more suitable than others.

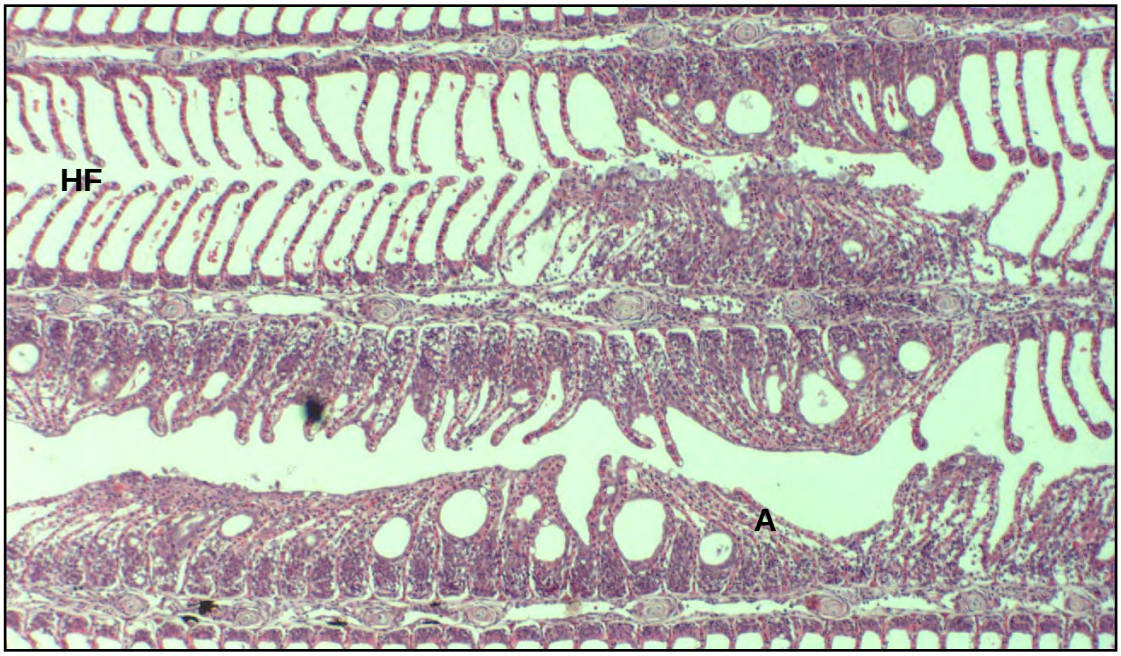

Figure 2.1.1 - Hematoxylin and eosin staining of a salmon gill showing healthy filaments (HF) and multiple AGD lesions (A).

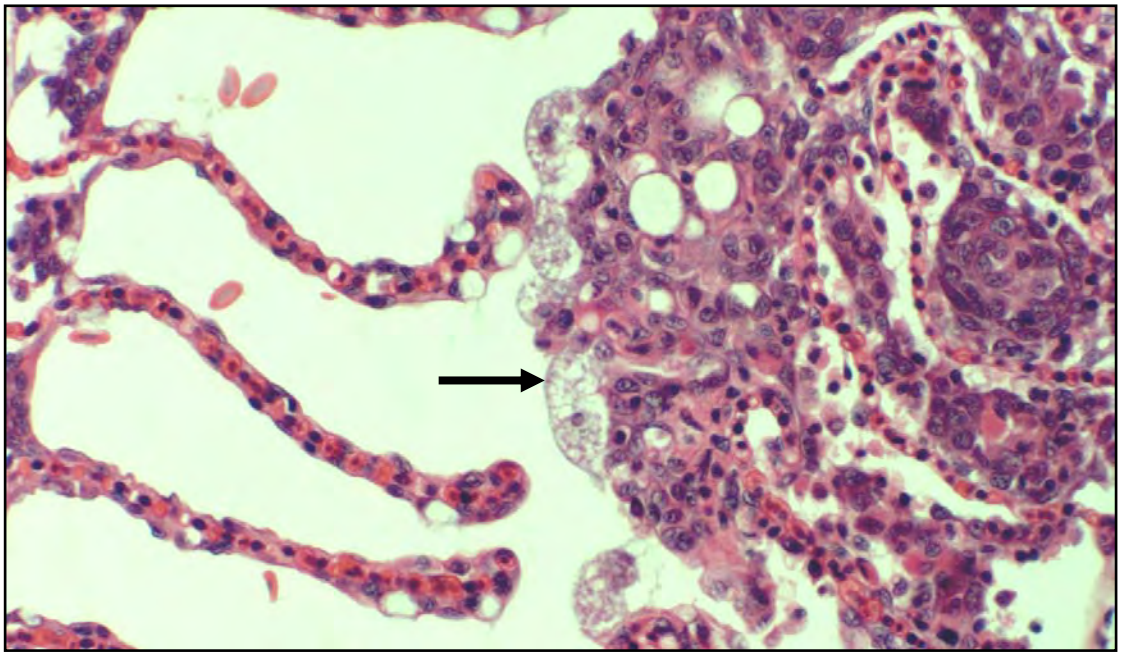

Figure 2.1.2 – Closer view of an AGD lesion showing attachment of amoebae to hyperplastic tissue (arrow).

Combined Periodic Acid-Schiff (PAS) / Alcian Blue (AB) Technique

Description

This protocol is used for identification of mucous cells and is able to distinguish between neutral and acidic mucins. Differentiation of acidic mucins into sulphated and carboxylated structures can be achieved by varying the pH.

Fixation and Processing

Gills are removed immediately following terminal anaesthesia and placed in seawater Davidson's Fixative for 24 hours. Prepare gills for embedding as previously described.

Protocol

1. De-wax and re-hydrate sections.
2. Alcian blue solution at selected pH (5 mins).
3. Wash in distilled water (1 min).
4. Periodic acid solution (5 mins).
5. Wash in distilled water (1 min).
6. Schiff's reagent (15 mins).
7. Wash in running water (10 mins)
8. Counterstain briefly with Mayer's hematoxylin (5 – 10 secs) and rinse.
9. Dehydrate in 100% alcohol (4 x 30 secs) and clear with xylene (1 min).
10. Mount slides using DPX or similar.

Results

At pH 2.5, cells containing acidic mucins will be blue, neutral mucins are pink and a mixture of both types will be purple. At pH 1.0, sulphated mucins stain purple while other mucins (neutral or acidic carboxylated) stain magenta. (Figures 2.2.1 & 2.2.2).

Additional Comments

To count the total number of mucous cells, sections stained at pH 2.5 should be used. Carboxylated mucosubstances can be identified at pH 4.0.

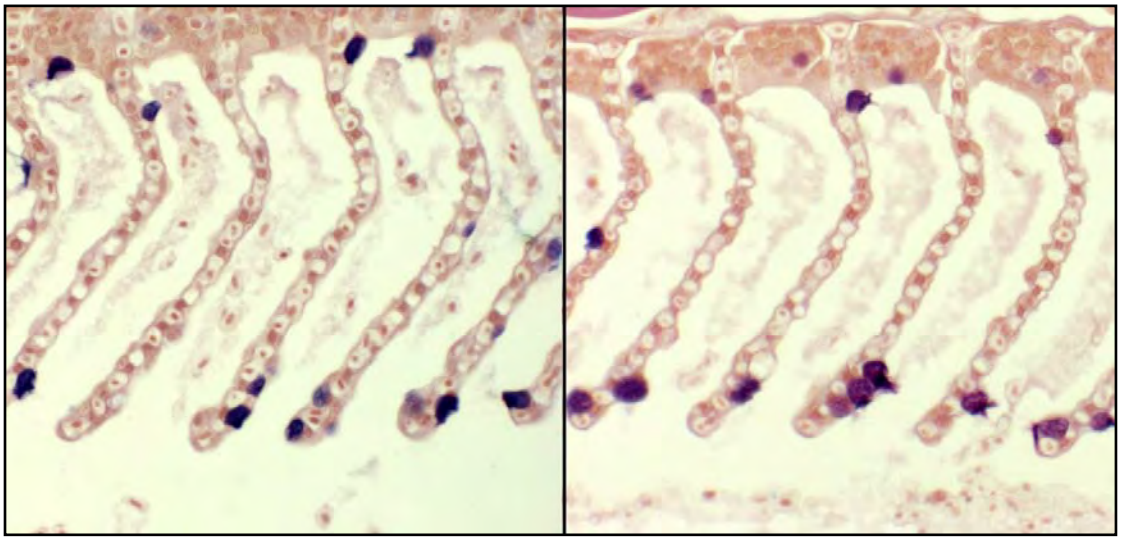

Figure 2.2.1 – Healthy filament stained at pH 2.5 (left) showing acidic (blue) mucous cells. On right are mucous cells stained at pH 1.0 showing mucous cells with sulphated mucins (purple).

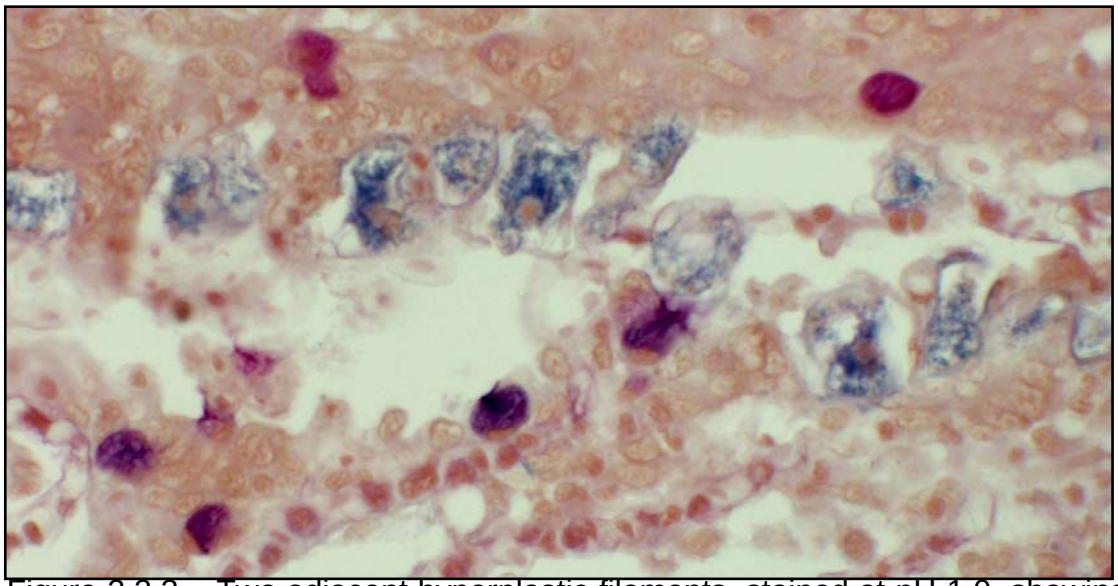

Figure 2.2.2 – Two adjacent hyperplastic filaments, stained at pH 1.0, showing attached amoeba (large blue cells). Purple cells in bottom half of plate are mucous cells with sulphated mucins. Two magenta mucous cells containing either carboxylated or neutral mucins are seen toward the top of plate.

Proliferating Cell Nuclear Antigen (PCNA)

Description

An immunohistochemical assay that enables the identification of proliferating cells in gill tissue sections. An antibody (clone PC10) against PCNA (a protein directly involved in DNA synthesis) is used to localize cells in all active phases of the cell cycle.

Fixation and Processing

Gills are removed immediately following terminal anaesthesia and placed in 10% neutral buffered formalin. Samples maybe stored in this fixative for up to two years. Prepare gills for embedding as previously described. Use silane coated slides and cut sections at 5 μm.

Protocol (optimized using Zymed™ PCNA Staining Kit – Cat. No. 93-1143)

1. De-wax and re-hydrate sections.
2. Perform Heat Induced Epitope Retrieval (HIER) by pouring 500 ml of citrate buffer solution pH 6.0 into a one litre microwave proof vessel with a perforated snap lock lid. Load slides into a plastic, 20 slot slide holder and immerse in buffer. Replace lid and microwave on high (700 watts) for 12 minutes. Allow container to stand for twenty minutes at room temperature.
3. Block with 3% H_2O_2 in 100% methanol (10 mins)
4. Rinse in distilled water (1 x 5 mins).
5. Rinse in PBS (1 x 5 mins).
6. Apply blocking solution (10 mins). Do not rinse.
7. Apply primary antibody at room temperature (1 hr)
8. Rinse in PBS (3 x 2 mins)
9. Apply streptavidin/peroxidase solution (10 mins)
10. Rinse in PBS (3 x 2 mins)
11. Apply DAB solution (5 mins)
12. Rinse in distilled H_2O (30 seconds)

13. Apply Mayers hematoxylin (1-2 seconds) and rinse immediately in tap water.
14. Blue sections in PBS (30 seconds)
15. Rinse in distilled water (1 x 2 mins)
16. Dehydrate, clear and mount in DPX mountant.

Results

PCNA positive cells will exhibit a dark brown stained nucleus. Nuclei of negative cells will counterstain blue.

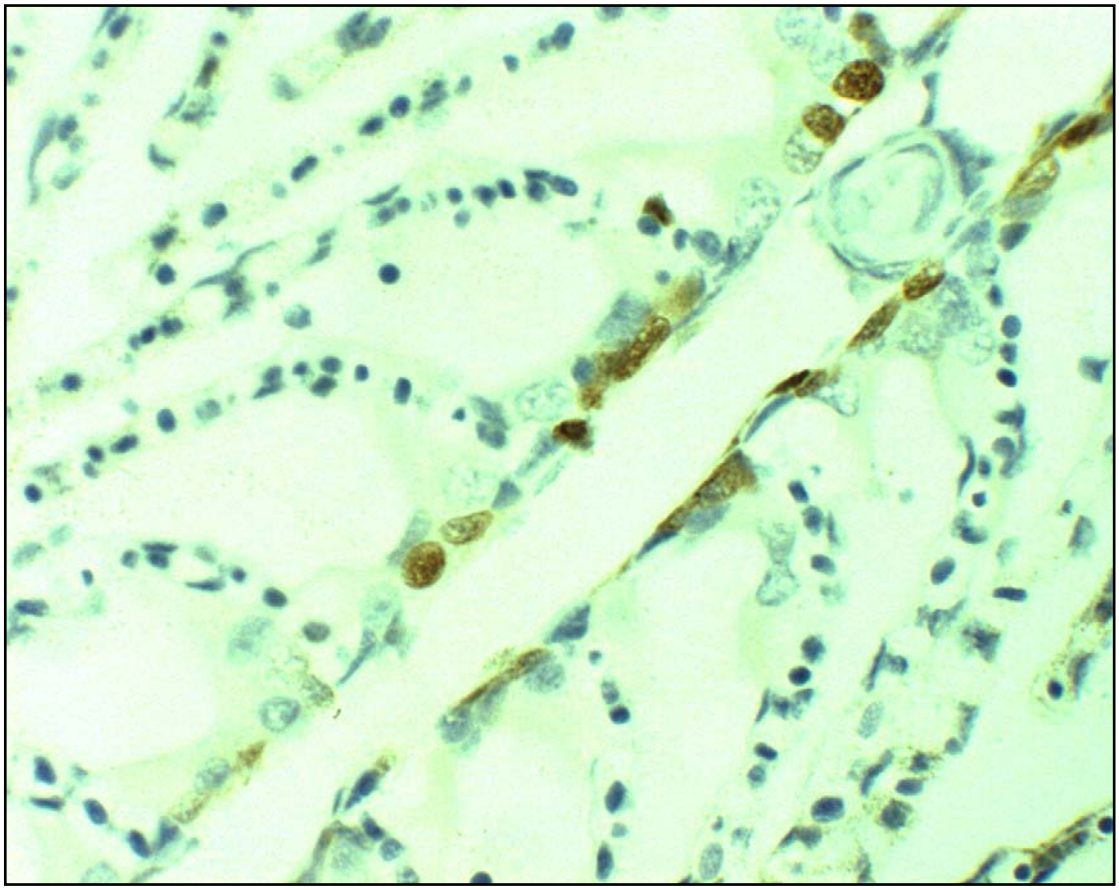

Figure 2.3.1 – PCNA stained filament from a salmon one day after transfer to brackish water. The nucleus PCNA positive cells stains brown as opposed to hematoxylin counterstained tissue (blue).

Additional Comments

DAB mixture must be prepared fresh and protected from light at all times. It may be preferable to use either a pap pen or smaller arch sections to conserve

the amount of antibody used. Crosscheck block with a routine stain (H&E) to ascertain the region with best plane of section.

Chloride Cell Identification

Description

An immunohistochemical assay that enables the identification of active chloride cells in gill tissue sections. This assay uses an antibody (clone μ5) against Na^+K^+ATP-ase, an enzyme that plays a crucial role in branchial epithelial ion transport.

Fixation and Processing

Gills are removed immediately following terminal anaesthesia and placed in seawater Davidson's Fixative for 24 hours. Prepare gills for embedding as previously described. Use silane coated slides and sections cut at 6 μm.

Protocol (optimized using Vectastain ABC Kit – Cat. No. PK-4002 and Roche DAB substrate – Cat. No. 1 718 096)

1. De-wax and re-hydrate sections.
2. Perform HIER as previously described.
3. Block with 3% H_2O_2 in 100% methanol (20 mins).
4. Rinse in distilled water (1 x 5 mins).
5. Rinse in PBS (1 x 5 mins).
6. Apply blocking solution (20 mins). Do not rinse.
7. Apply primary antibody at room temperature (1 hr).
8. Rinse in PBS (3 x 2 mins).
9. Apply streptavidin/peroxidase solution (30 mins).
10. Rinse in PBS (3 x 2 mins).
11. Apply DAB solution (2 mins).
12. Rinse in distilled H_2O (30 seconds).

13. Apply Mayers hematoxylin (1-2 seconds) and rinse immediately in tap water.
14. Blue sections in PBS (30 seconds).
15. Rinse in distilled water (1 x 2 mins).
16. Dehydrate, clear and mount in DPX mountant.

Results

Nucleus and cytoplasm of chloride cells will stain dark brown. Other tissues counterstain blue.

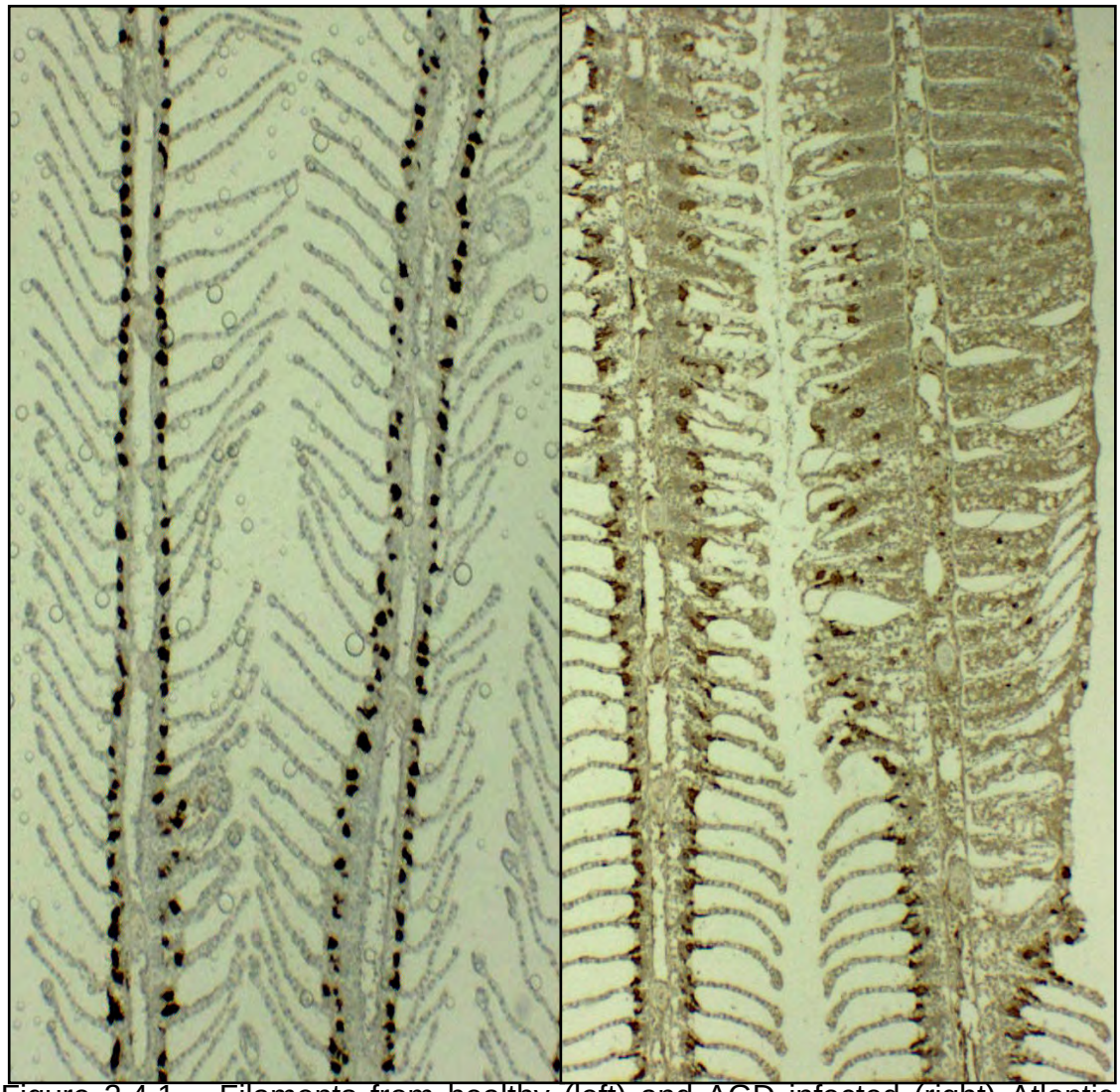

Figure 2.4.1 – Filaments from healthy (left) and AGD infected (right) Atlantic salmon. Na^+K^+ATP-ase positive cells (chloride cells) are stained dark brown whilst other tissue counterstains blue.

Additional Comments

It may be preferable to use either a pap pen or smaller arch sections to conserve the amount of antibody used. Crosscheck block with a routine stain (H&E) to ascertain the region with best plane of section. Unless using an auto-immunohistostainer, it is recommended that only 20 slides are handled per incubation to ensure consistent results.

APPENDIX:

Fixative, dye and buffer recipes:

Mayer's Haematoxylin

Haematoxylin	1 g
Distilled water	1000 ml
Aluminium ammonium sulphate	50 g
Sodium iodate	0.2 g
Citric acid	1 g
Chloral hydrate	50 g

Dissolve haematoxylin, aluminium ammonium sulphate and sodium iodate in distilled water and leave this mixture to stand overnight at room temperature. Add chloral hydrate and citric acid, mix and boil for 5 mins. Cool and filter.

Eosin

1 % Aqueous eosin Y	100 ml
1 % Aqueous phloxine	10 ml
95% Ethanol	750 ml
Glacial acetic acid	4 ml

Phosphate buffer pH 7.4

0.1M Sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$)	34 ml
0.1 M Disodium hydrogen phosphate (Na_2HPO_4)	166 ml

Citrate Buffer

0.1M Citric acid	9.5 ml
0.1M Citrate	41.5 ml

Phosphate Buffered Saline

0.1M Phosphate buffer pH 7.4	1000 ml
Sodium chloride	9 g

Modified Karnowsky's Fixative for Marine Organisms

Paraformaldehyde	2 g
25% Glutaraldehyde	10 ml
0.1 M Calcium chloride ($CaCl_2$)	2.5 ml
Sucrose	20 g
Distilled water	to 100 ml
0.2M Phosphate buffer (pH 7.4)	

Heat paraformaldehyde in 25 ml distilled water until solution reaches 60^{o}C - 70^{o}C then add 2-3 drops of 1M NaOH to clear the solution. Add glutaraldehyde, buffer and $CaCl_2$ solutions. Add sucrose and bring final volume to 100ml with distilled water then stir to dissolve.

Seawater Davidson's Fixative

95% Ethanol
37 – 40% Formaldehyde
Glacial acetic acid
0.2 µm filtered seawater
Mix at 3:2:1:3

10% Neutral Buffered Formalin

37 - 40% Formaldehyde	100 ml
Sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$)	4 g
Disodium hydrogen phosphate (Na_2HPO_4)	6.5 g
Distilled water	900 ml

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Appendix 5

In vitro research

Prepared by Rick Butler, Richard Morrison and Barbara Nowak
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In vitro research has been used for long time due to experimental and ethical reasons. It allows for large replication and reduction of variability, reduces need for killing animals and allows working on valuable species. The two-stage *in vitro* to *in vivo* research program is widely regarded as the best approach for systematic biological research and in medical science research. There are numerous examples of this strategy in the literature including that used for investigations into immunostimulant efficacy in fish (eg. Burrell & Williams, 1998) or effects of treatments (Tafalla *et al.* 1999). Knowledge gained from *in vitro* studies can be applied directly to the *in vivo* component however without that initial knowledge the research is largely dependent on serendipity. Our fish health research group has certainly used the two step program with success. For example the *in vitro* benefit of β-glucans immunostimulation on snapper macrophages was the impetus for *in vivo* trials (Cook *et al.* 2001). Snapper fed β-glucans coated pellets at low (winter) temperature were significantly larger than fish fed a control diet (Cook *et al.* 2003). Similarly immunostimulatory CpG oligonucleotides (bacterial DNA motifs) immunostimulated Atlantic salmon leucocytes *in vitro* (Jorgensen *et al.* 2001a; Jorgensen *et al.* 2001b) and the same CpGs have recently been shown to enhance resistance of Atlantic salmon (*Salmo salar*) to challenge against amoebic gill disease (Bridle *et al.* 2003). Another example of successful application of *in vitro* research is work done on the effects of cortisol on fish immune response. *In vivo* experiments indicated immunosuppression, reduction in leucocyte proliferation, reduced numbers of antibody producing cells and decreased level of antibodies (reviewed by Weyts *et al.* 1999). This was explained when subsequent research *in vitro* showed inhibition of lymphocytes proliferation, reduced antibody production and increased apoptosis of B lymphocytes (reviewed by Weyts *et al.* 1999, Verburg *et al*. 1999). Thus, *in vitro* research can be used to understand effects at cellular, tissue and organ level.

In vitro models of fish tissues have been used extensively in the study of physiology and toxicology, and to a lesser extent health and immunology. Whilst most of these models have been based on commercially important species due to the increase in aquaculture of high value fish, many, particularly those investigating aquatic toxicology have used tissues from benthic and demersal species such as flounder, sole, eel, cod, plaice and dab. Such models allow the interpretation of the effects of specific perturbations on the target animal, be that the effect of a pollutant or a disease causing organism, since they lack the 'whole tissue' complexities that might otherwise mask the effect *in vivo*. For many the lack of the full suite of systemic and tissue responses to a

perturbation allows the study of particular metabolites or metabolic pathways and such research often does not need to interpret findings in the context of the whole animal. However, for those researchers whose study is at the animal level there is a requirement for the prior validation of the *in vitro* methodology as an appropriate model of the in vivo situation. Such validation can be an exhaustive series of manipulation and testing of each possible component that might influence the outcome and because of this for many this process is simplified to answering the question "is the outcome *in vitro* comparable to that which is expected *in vivo*". It may be surprising to some that the answer is often yes even when using the simplest of models which can, at least at the cellular level, serve as approximate indicators of the likely outcome within the animal. More complex *in vitro* models, it is argued, increase the predictability of the outcome from "approximate" to "accurate", but this is often dictated by the type of study, and the complexity of the biological process being modelled. In most cases *in vitro* models fall into 2 main groups, those that use suspensions or monolayers of isolated host cells, and those that use freshly excised host tissues. More recently complex reconstructed tissue models have also been developed that are finding applications in fish physiology and disease research.

Monolayers of cultured fish cells are most extensively used for the isolation of viral pathogens of commercially raised species. However, they have been used by several authors to study bacterial diseases, and in particular to model early pathogenesis. Del Corral (1975) and Zaldiver (1985) used cultured fish cells to study the invasion mechanisms of *Flavobacterium columnaris* and were able to correlate the virulence of the organism with its ability to adhere to epithelial cells *in vitro*; adherent strains being the most virulent pathogens. Similarly, Lopez-Doriga *et al.* (2002) used carp epithelial cells to characterise the invasion mechanisms and cell surface receptors required for successful invasion of host cells by *Photobacterium damselae* (formerly *Pasteurella piscicida*). These authors are now in the process of investigating potential vaccine antigens that they hope will block these proteins and prevent bacterial invasion. The most ubiquitous and consequently often the most problematic fish bacterial pathogens are of the genus Vibrio and these have also been studied extensively *in vitro* to describe their pathogenesis and strain variation and to develop and test methods of immunotherapy (Wang and Leung 2000). Whilst *in vitro* research of this nature is not extensive that which has been performed has been judged comparable to the *in vivo* situation and has in recent years formed the basis of immunological study for many bacterial pathogens.

There are obvious limitations to the use of substrates of a single cell type, especially the loss of, or change in type, distribution and accessibility of specific cell surface receptors through the selective processes and subsequent de-differentiation that occur in cell culture. More importantly when considering the modelling of interactions within

complex tissues such as the gill is the loss of cellular organisation, architecture and cell-cell communication. For this reason several authors have investigated the use of explanted host tissue excised from the animal and used as part of *in vitro* studies. Most of the work has made use of fish gills and skin as part of disease and physiological study because of their exposure to the external environment. Their structure and functions within a living animal also make them suitable tissues for explantation since there are tolerant to environmental changes and are less affected than some other tissues by the trauma of the explant procedures. Fish gills have been used primarily in the study of gill associated bacterial disease (Decostere *et al*., 1999a; Decostere *et al*., 1999b; Decostere *et al*., 2000), but more recently have been employed in the study of the infective mechanisms of *Neoparamoeba pemaquidensis* (Butler & Nowak, unpublished data). In the latter case data is still being collected and correlation to observations *in vivo* is not appropriate at this time, however, perfused explanted gill models from both rainbow trout and common carp have shown high correlation to the progression of flexibacteriosis *in vivo*. These tissues have been used to investigate the biochemical interactions between pathogen and host as well as those environmental factors such as salinity, temperature and water chemistry that are known to affect the progression of the disease *in vivo*. The models have accurately mimicked the expected outcomes and have in addition been able to map the changes in bacterially expressed proteins during *in vitro* infections within sub-optimal environments that are currently being tested as possible candidate antigens for the development of vaccines against *F. columnare*. This technology has been adopted by several other groups working on bacterial gill diseases as a simple and feasible model of studying early pathogenesis of bacteria and evaluating bacterial virulence.

Explanted skin models have been used in the study of several metazoan parasites, but with limited and variable success. Butler *et al.* (1997 & 1999) found that sea lice, *Lepeophtheirus salmonis* that *in vivo* attach to host skin within the first 24 hours of contact did not attach to explanted Atlantic salmon skin until 72 hours post incubation. Whilst attachment did eventually occur, these animals had insufficient energy reserves to go through metamorphosis and died within 5 days of attachment. However, the same authors had success with the same parasite that was maintained on an artificial skin substrate. Using a reconstructed salmon skin model composed of primary cultures of skin epithelial cells and dermal fibroblasts parasite attachment was observed within 24 hours of incubation. Furthermore, the parasites performed the normal set of settlement behaviours observed *in vivo* and began to feed on the artificial substrate. Metamorphosis did not occur, but the model allowed the extended maintenance of the parasite for up to 12 days. Similar complex reconstructed tissues, this time using gill derived material have been employed by fish physiologists and aquatic toxicologists (Gilmour *et al*., 1998; Wood *et al*., 1998; Wood & Part,

1997). These models have added additional layers of complexity by the introduction of specialised tissue architecture such as chloride cells and have produced comparable results of physiological responses and ion transport at the gills to those obtained using more traditional whole animal models.

While often there is a need for validation of cellular, tissue or organ models, *in vitro* research can be successfully used in immunology, physiology and toxicology. It is particularly useful when animals are too valuable or a large range of treatments and replicates is necessary or biological variability has to be reduced. There are numerous examples in scientific literature on application of *in vitro* research to aquaculture issues. A number of Aquafin CRC scientists have a significant expertise in the application of *in vitro* research to fish health and physiology.

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Aquafin CRC participants shown in bold

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Appendix 6

Results from Honours project undertaken by Kristy McCarthy "Changes in the antigenic profile of cultured Neoparamoeba sp., causative agent of amoebic gill disease in farmed salmon." (UTS 2003)

The objectives of the Honours project undertaken this year by Kristy Mc Carthy were:

1. Comparison of the antigen profiles of various *Neoparamoeba pemaquidensis* isolates.
2. Characterisation of antigens recognised by Mab generated against *Neoparamoeba pemaquidensis*.
3. Identification of antigens differentially expressed between infective and non-infective parasites during *in vitro* culture.

The results of this project have provided evidence of antigenic differences between cultured and wild type (freshly isolated) parasites. In addition, this project has revealed that the antigen profile of cultured parasites depends on the method of culture. Thus, significant differences in antigen profile were observed when the same isolate was cultured in liquid medium versus on a solid (agar) substrate (Fig 1).

Monoclonal and polyclonal antibodies directed against surface antigens of cultured *Neoparamoeba*. were used to identify differences in the antigen profile of wild type and cultured parasites. Three different wild type isolates were established in liquid cultures and sampled at 24 hrs, 5, 10, 15, 20, 25 and 30 days in culture. Immunoblot techniques were used to identify differential antigen expression using two different polyclonal sera, one against fixed PAO27 parasites and the other against a sonicated preparation from wild type organisms.

Monoclonal antibodies specific for surface proteins of cultured *Neoparamoeba* were used in IFAT to assess the development of specific antigens of the parasite in the three different cultures (Table 1). Immunoblots, using the polyclonal sera against PAO27 or wild type parasites, identified significant changes in antigen expression after fifteen days of *in vitro* culture of wild type parasites (Figure 2). The use of monoclonal antibodies specific for the cultured parasite demonstrated progressive exposure/expression of antigens specific to the cultured parasite (Table 1).

These results indicate that the antigen profile of wild type parasites changes with the period in culture. These changes may result from differential antigen expression or exposure of cryptic or masked antigens during *in vitro* culture.

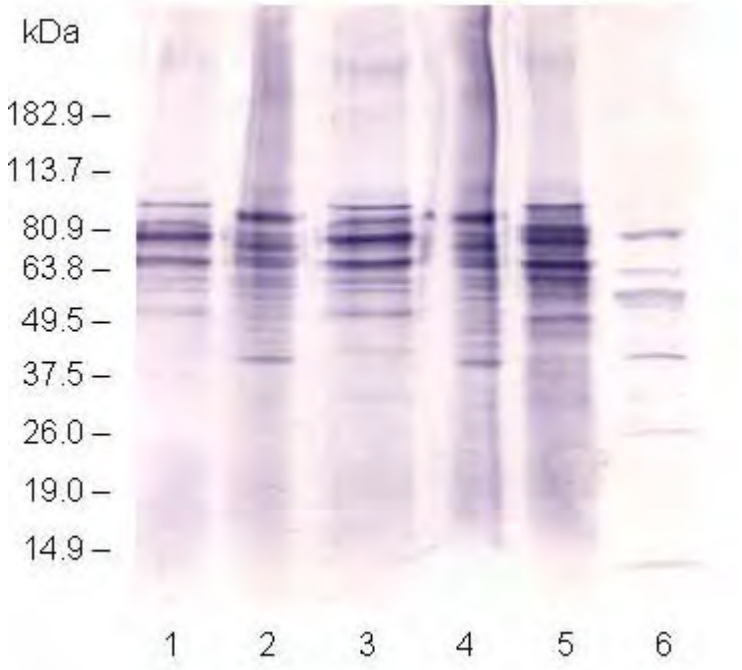

Figure 1 – Immunoblot profile of *Neoparamoeba* antigens probed with mouse anti - PA027. Antigen extracts from $NP2510_{liquid}$ (Lane 1), $NP2510_{agar}$ (Lane 2), $PA027_{liquid}$ (Lane 3), $_{PAO27agar}$ (Lane 4), freshly isolated *Neoparamoeba* (Lane 5) and *E. coli* antigens were resolved on 4-20 % gradient SDS-PAGE gel under reducing conditions and transferred onto nitrocellulose.

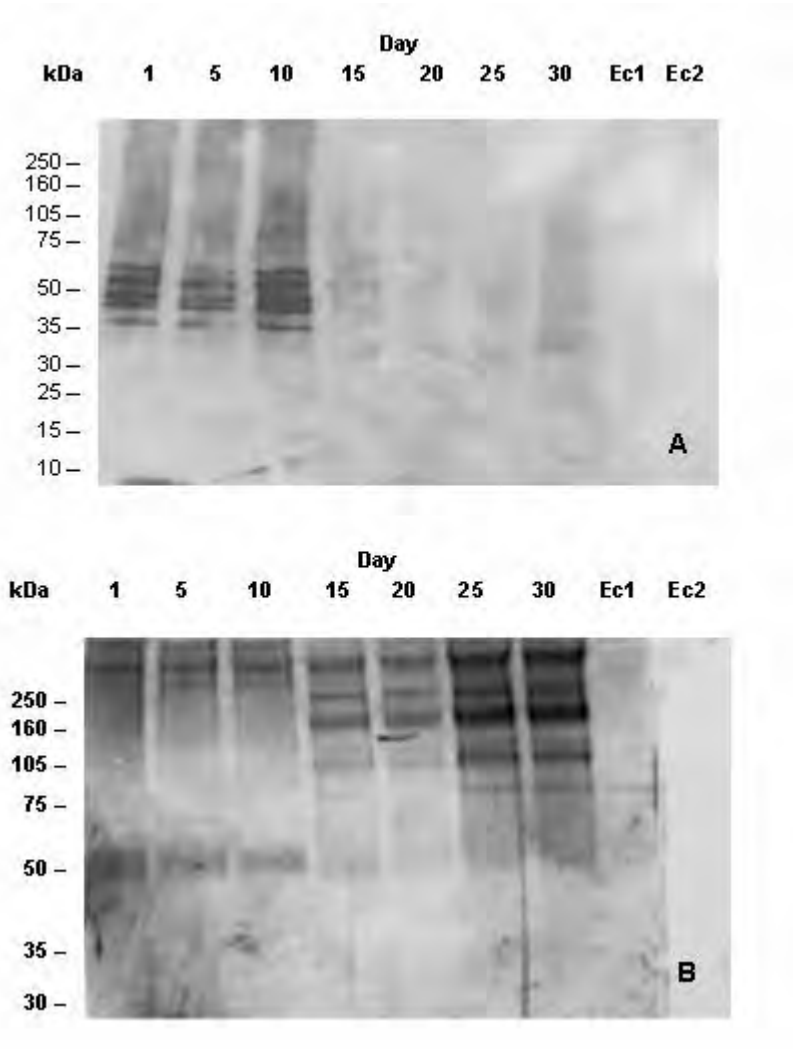

Figure 2 - Enhanced chemiluminescence detection of E1 antigens at days 1, 5, 10, 15, 20, 25, 30 and *E. coli* antigen controls (E.c1 and Ec2) probed with mouse anti-freshly isolated *Neoparamoeba* (Panel A) and with mouse anti - PA027 (Panel B). Samples from replicate cultures (E2 and E3) yielded similar results. Antigens were subjected to SDS-PAGE under reducing conditions prior to probing with the specified antisera

Table 1 – Immunofluorescence reactivity profiles of anti- PAO27 mAbs with freshly isolated parasites cultured over a period of 30 days.

	Day 0			Day 1			Day 5			Day 10			Day 15			Day 20			Day 25			Day 30			PA027		
	E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3			
(-) control IgM	-	*nd*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(-) control IgG	-	*nd*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(+) control	+	*nd*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4G9	+	*nd*	+	+	++	++	+	++	++	+	++	++	++	++	++	++	++	++	++	+++	+++	++	+++	+++	++	+++	+++
3H2	-	*nd*	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++	+	+	++	+	+	++	+	+
4B1	-	*nd*	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+++	+	+	+++	+	++	++	+	+
2B2	-	*nd*	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	++	+	+	++	+	++	++	++
2H9	-	*nd*	-	-	-	-	-	-	+	-	-	+	+	-	+	+	+	+	+	+++	+	++	+++	++	++	+++	+++
4A2	-	*nd*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++	+	+	+	++	++	++	++	++
4D12	-	*nd*	-	-	-	+	-	-	+	+	-	+	+	-	+	+	-	+	++	+	+	++	+	+	++	+	+
3D3	+	*nd*	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	++	++	+	++	+	+	++	+	+
3F7	-	*nd*	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	++	++	+	++	+	+	++	+	+
4E8	+	*nd*	+	+	++	+	++	++	+	++	++	+	++	+	+	++	++	+	++	++	+	++	+	+	++	+	+
3A12	-	*nd*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++	++	++
5C1	-	*nd*	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++	++	++
1A2	-	*nd*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++	++	++

Percentage of IFAT positive parasites

0%	<50 %	50 % to 60 %	70 % to 90 %	100 %

[1] E1, E2 and E3 represent three independent cultures of freshly isolated parasite

Appendix 7

Ectoparasites - immune response and vaccine development

Satellite Workshop

3rd International Symposium on Fish Vaccinology

3rd International Symposium on Fish Vaccinology

Satellite Workshops

1. Ectoparasites - immune response and vaccine development

Friday 11, 14:00-17:00, Grieghallen

Half day workshop sponsored by Cooperative Research Centre for Sustainable Aquaculture of Finfish (Aquafin CRC). This workshop will cover recent advances in studies of fish immune response to ectoparasites and development of vaccines against external parasites. A presentation on development of tick vaccine for livestock and potential lessons for aquaculture vaccines against ectoparasites will be given by Dr Chris Prideaux, CSIRO Australia. Current research on monogenean trematodes infestation, white spot, sea louse and Amoebic Gill Disease will be covered by invited speakers. We will discuss main challenges in these research areas and ways to overcome them. We are hoping that the workshop will lead to development of future collaborations.

Organisers:
Dr Barbara Nowak (University of Tasmania, Australia)
Dr Frank Nilsen (Institute of Marine Research, Norway)

Please send a mail to fishvaccinology@veso.no to register!

This workshop is sponsored by:

Workshop program:

'What can the aquaculture industry gain from the development of a tick vaccine for cattle?' - Chris Prideaux

'Responses in fish hosts against monogenean infections' - Kurt Buchmann

A genomic approach in the development of vaccine against the salmon louse – *Lepeophtheirus salmonis*. - Frank Nilsen

'Potential for immunomodulation to control Amoebic Gill Disease in Atlantic salmon' - Barbara Nowak

'Host responses against the fish parasitizing skin ciliate *Ichthyophthirius multifiliis*' - Kurt Buchmann

Discussion

What can the aquaculture industry gain from the development of a tick vaccine for cattle?

Chris Prideaux
Aquafin CRC; CSIRO Livestock Industries, Private Bag 24, Geelong, Vic., 3220. Australia. (chris.prideaux@csiro.au)

The tick *Boophilus microplus* is a major ectoparasite of cattle in many parts of the world including Australia. Death can result from heavy infestations *per se*, or by tick transmission of anaplasmosis and babesiosis.

Chemicals have been used extensively to control ticks and have been partially successful, but this approach suffers from a number of negatives including environmental and residue problems, the emergence of high levels of chemical resistance in the field, the need for frequent administration, and high costs. The use of biological control measures such as pasture spelling and the selection of tick resistant breeds of cattle have also been used to reduce the impact of this disease on industry. The development of a vaccine to enhance immunological protection to ticks would be a major advancement in the control of this disease.

Prolonged exposure of cattle to *B. microlus* allows the development of some level of immunity, but this is not sufficient to prevent production losses. As a result the development of natural immunity through vaccination would be of little advantage, and what is required is the development of an artificial immunity following vaccination.

The development of effective treatments for cattle ticks has many parallels with the search for reliable treatments for ectoparasites of fish; a number of these will be discussed with particular reference to amoebic gill disease of Atlantic salmon.

Responses in fish hosts against monogenean infections

Kurt Buchmann
Department of Veterinary Microbiology, Section of Fish Diseases, Royal Veterinary and Agricultural University, Frederiksberg, Denmark

It has been estimated that the taxon Monogenea comprises more than 24,000 species. The majority are parasitic on teleosts. This makes this class one of the most successful fish parasite groups suggesting that evasion mechanisms are well developed among monogeneans. Nonetheless, during the latest 70 years a number of studies have elucidated the capability of fish hosts to mount a response against these parasitic infections. A range of papers have been presented demonstrating acquired or innate resistance of fishes belonging to many teleost orders to these platyhelminths.

Best characterized is the response in cyprinids, anguillids, percids and salmonids. Attempts to vaccinate fish against monogenean infections have been conducted at several occasions with some success. Despite this the mechanisms involved in the responses are not clearly defined. Recent work has elucidated various factors of the host immune system which are likely to be at least partly responsible for the anti-monogenan activity in teleosts. Thus, humoral factors such as antibodies, complement components and lectins are known to be active in host-monogenan associations. Cellular factors comprise leukocyte action and mucous cell activity. It is probably an intricate interplay of various factors which is responsible for the host response and a model of the reactions in the telost epidermis is presented and discussed.

A genomic approach in the development of vaccine against the salmon louse – *Lepeophtheirus salmonis*.

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The salmon louse (*Lepeophtheirus salmonis*) is a marine ectoparasite infecting salmonids. After the onset of salmon aquaculture, infections with the salmon louse soon emerged as a significant problem and is currently the main disease in Norwegian Aquaculture and several other countries. In order to control this parasite various insecticides are used in both bath and oral treatment. However, experience with similar drugs against insects has shown that resistance development may be a significant problem. In addition, the use of large amount of these drugs may also raise environmental issues. Vaccines against bacterial and viral diseases have contributed significantly to the success for the salmon aquaculture industry. Similarly, an efficient vaccine against the salmon louse would be a large advantage towards the control of this highly important parasite in marine salmon farming.
Vaccine development against ectoparasites is challenging. Currently one commercial available vaccine exists which is against the cattle tick (*Boophilus microplus*). Lessons from vaccine development against ticks and other arthropods show that using one or a few purified antigens gives the best results in trial vaccines. In order to find one of the relatively few suitable vaccine antigens in an organism like the salmon louse, a broad approach is needed. With the recent development in genomic technology an approach utilising these tools may be appropriate in order to identify protective antigens in the salmon louse. An overview of our strategy will be outlined together with some results obtained so far. These issues will be discussed in view of some requirements needed to succeed in making a vaccine against the salmon louse.

Potential for immunomodulation to control Amoebic Gill Disease in Atlantic salmon

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Amoebic Gill Disease (AGD) is the most serious health problem in sea-cage culture of salmonids in Tasmania, Australia. Currently, freshwater bathing is the preferred treatment for AGD, however it is expensive and relies on availability of good quality fresh water. So far trials of simple vaccine preparations against AGD have been unsuccessful. Inconsistent results have been reported with use of immunostimulants such as levamisole and glucans. Recent research has shed some light on the immunomodulation potential in AGD. Here, we report an increased resistance against AGD following administration of CpG oligodeoxynucleotides and as a result of pre-exposure to AGD. Fish treated with CpG ODN 1668 (i.p. injection of 50 µg $fish^{-1}$) and challenged with AGD six days later, showed significantly greater survival ($P<0.05$) and a more pronounced level of localised inflammation associated with AGD lesions than untreated control fish and non-CpG ODN 1720 treated fish. In another experiment, fish re-infected with AGD four weeks post-treatment showed improved survival in comparison to naive fish challenged with AGD. While there were changes in immune response during the infection, there was no statistically significant difference between the immune response of naive and re-infected fish (which were held in fresh water post infection) at the beginning of the challenge ($P>0.05$). Culture supernantant stimulated lymphocytes proliferation in naive fish. The implications of these findings for use of immunostimulants and vaccine development against AGD will be discussed.

Host responses against the fish parasitizing skin ciliate *Ichthyophthirius multifiliis*

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It has been known for more than a century that teleosts respond effectively against the ciliate *Ichthyophthirius multifiliis.* Fish surviving a primary invasion do normally exhibit a high degree of protection to challenge exposure by infective theronts. Several studies have been conducted in order to elucidate the mechanisms involved in this antiparasitic immunity. Thus, investigations have been performed with various host groups ranging from anguillids via cyprinids to salmonids and although they all respond it is not clear if the response sequences are identical in these taxa. Both humoral and cellular factors are likely to play a role in the intricate interactions between host and parasite. The action of specific antibodies to i-antigens have clearly been demonstrated. In addition, complement factors and lectins are known to affect parasitic stages as suggested by *in vitro* studies. Further, additional innate factors in fish may take part in the humoral response pattern. Cellular elements have also been suggested as active components in the over all response. Leukocytes ranging from granulocytes to non-specific cytotoxic cells have been demonstrated to possess effector mechanisms against various stages of the skin parasite. Based on the well-known development of immunity in fish against Ich various experimental vaccines (comprising crude extracts of parasitic stages and recombinant vaccines including DNA-vaccines) have been developed. The applicability of these vaccines in practical fish farming will be discussed.

Appendix 8

PRESENTATIONS MADE IN RELATION TO THIS REPORT

Nowak, B., Powell, M. and Carson, J. (2001) AGD in mariculture (a round-table discussion session) 10th International Conference of the European Association of Fish Pathologists at Trinity College in Dublin, September 2001, Ireland.

Adams, M., Ellard, K. and Nowak, B. (2002) Sequential pathology and gross lesion histopathology of amoebic gill disease (AGD) in farmed Atlantic salmon *Salmo salar* L. The Second Scientific Conference of the Atlantic Salmon Aquaculture Subprogram 8th July 2002 – Hobart, Tasmania

Nowak, B., Douglas-Helders, M., Gross, K., Bridle, A., Morrison, R., Crosbie, P., Bagley, C., Adams, M., Butler, R. and Carson, J. (2002) Amoebic gill disease – research highlights. The Second Scientific Conference of the Atlantic Salmon Aquaculture Subprogram 8th July 2002 – Hobart, Tasmania

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Cytokines as immunostimulants in farmed fish and their role in the pathogenesis of Amoebic gill disease in salmonids. (Bridle, Butler & Nowak)
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Bridle, A., Butler, R and Nowak, B. (2003) Pathophysiological relevance of inflammation and innate immunity in amoebic gill disease in Atlantic salmon (*Salmo salar*). The Third Scientific Conference of the Atlantic Salmon Aquaculture Subprogram. 21 May 2003. Hobart, Tasmania.

Butler, R. and Nowak, B. (2003) Models for *in vitro* study of *Neoparamoeba pemaquidensis*; an update. The Third Scientific Conference of the Atlantic Salmon Aquaculture Subprogram. 21 May 2003. Hobart, Tasmania.

Crosbie, P., Carson, J. and Nowak, B. (2003) Cryopreservation of *Neoparamoeba pemaquidensis*. The Third Scientific Conference of the Atlantic Salmon Aquaculture Subprogram. 21 May 2003. Hobart, Tasmania.

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Villavedra, M., To, J., Hutson, K., McCarthy, K., Broady, K., Smith, N. and Raison, R. (2003) Identification of attachment factors of *N. pemaquidensis*: Monoclonal antibody approach. The Third Scientific Conference of the Atlantic Salmon Aquaculture Subprogram. 21 May 2003. Hobart, Tasmania.

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Bridle, A. and Nowak, B. (2003) Pathophysiology relevance of inflamation and innate immunity in amoebic gill disease in Atlantic salmon. Aquafin CRC Conference, October 2003, Adelaide.

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McCarthy, K., Villavedra, M., To, J., Morrison, R., Crosbie, P., Broady, K. and Raison, R.L. (2003) Changes in the antigenic profile of *Neoparamoeba* species. Aquafin CRC Conference, October 2003, Adelaide.

McCarthy, K., Villavedra, M., To, J., Morrison, R., Crosbie, P., Broady, K. and Raison, R.L. (2003) Changes in the antigenic profile of *Neoparamoeba* species. RNSH/UTS Research Meeting. November 2003, Sydney.

Appendix 9

PUBLICATIONS ARISING FROM THIS REPORT

Nowak BF, Powell MD, Carson J, Dyková I (2002) Amoebic Gill Disease in the marine environment. Bulletin of European Association of Fish Pathologists, 22, 144-147.

Adams, M.B., Nowak, B.F. (2003) Amoebic gill disease (AGD): sequential pathology in cultured Atlantic salmon (*Salmo salar* L.). Journal of Fish Diseases, 26, 601-614.

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Gross, K., Morrison, R.N., Butler, R., Nowak, B.F. (2004) Atlantic salmon (*Salmo salar* L.) previously infected with *Neoparamoeba* sp. are not resistant to re-infection and have suppressed macrophage function. Journal of Fish Diseases, 27, 47-56.

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Morrison R.N., Nowak, B. (2005) Bath treatment of Atlantic salmon (Salmo salar) with amoebae antigens fails to affect survival to subsequent amoebic gill disease (AGD) challenge, Bulletin of European Fish Pathologists, submitted.

Gross, K.A., Alcorn, S., Murray, A., Morrison, R., Nowak, B. (2005) The effect of sonicated *Neoparamoeba* sp. on the chemiluminescent and chemotactic responses of leucocytes isolated from Atlantic salmon, Chinook salmon and rainbow trout, Journal of Fish Diseases, submitted.

Embar -Gopinath, S., Butler, R., Nowak, B. (2005) Influence of gill colonising bacteria on development and severity of amoebic gill disease in Atlantic salmon. Diseases of Aquatic Organisms, submitted.