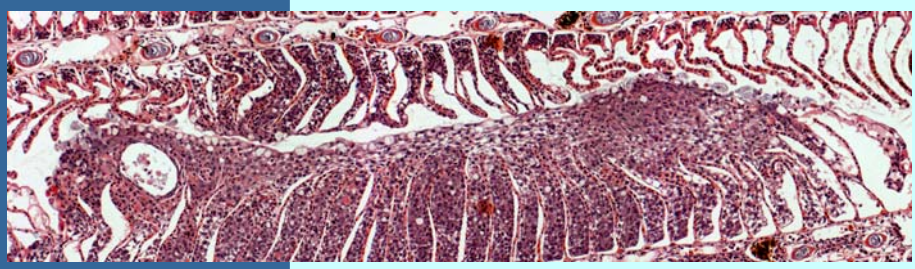
Final Report

MODEL DEVELOPMENT FOR EPIDEMIOLOGY OF AMOEBIC GILL DISEASE

Barbara Nowak, Marianne Douglas-Helders, Carley Bagley, Philip Crosbie, Mark Adams, Richard Morrison, Rick Butler and Jeremy Carson

August 2004

Aquafin CRC Project 3.4.4
(FRDC Project No. 2001/245)



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Atlantic Salmon AQUACULTURE SUBPROGRAM

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PRINCIPAL INVESTIGATOR: Dr B. Nowak
ADDRESS: School of Aquaculture
Tasmanian Aquaculture and Fisheries Institute
University of Tasmania
Locked Bag 1370
Launceston Tasmania 7250
Telephone: 03 63243814 Fax: 03 63243804

OBJECTIVES:

1. Identification of *Neoparamoeba pemaquidensis* reservoirs.
2. Identification of risk factors of AGD including the spatial relationship between infected and uninfected cages.
3. Development of a pilot surveillance system.

NON-TECHNICAL SUMMARY:

OUTCOMES ACHIEVED

This project has increased our understanding of epidemiology of Amoebic Gill Disease, characterised risk factors for this disease and identified gaps in our knowledge and methods. It formed a discussion platform, which considered AGD data collection and sharing within the industry, including the development of a database. It provided training in the area of epidemiology to the researchers (including one PhD student and one postdoctoral fellow) and the industry and will improve interpretation of disease patterns and farm data. This project builds a basis for future studies in aquaculture epidemiology.

Amoebic Gill Disease (AGD) is the main health problem affecting salmon industry in Southern Tasmania. To improve management of fish with AGD on the farms, the industry needs better understanding of AGD epidemiology. This will provide a basis on which to develop strategies for new treatment or vaccine application in the future.

We have identified reservoirs of the amoeba causing AGD in Atlantic salmon in Tasmania. The amoebae were widespread and easily isolated from marine and estuarine sediments and cage netting. There was no apparent relationship between the presence of the amoebae in the environment and AGD prevalence in the cages. Development of methods to isolate amoebae from water samples and to accurately quantify the amoebae is needed if the relationship between the amoebae in the environment and AGD outbreaks is to be fully understood. However, computer simulations suggested that once fish in a cage are infected

with AGD, they become the main source of amoebae and other reservoirs become insignificant.

Risk factors for AGD outbreaks were reviewed. New risk factors were identified on the basis of laboratory experiments, field trials, limited farm data and the industry's perception. Main environmental factors included high salinity and increased temperature. *In vitro* growth of the amoeba was affected by increased copper sulphate concentrations, low salinity and low cell densities.

Effects of farm activities, such as movement of cages to a fallowed site or use of antifouling paint on cage netting, on AGD were evaluated. Movement of cages to a new site after bathing reduced the impact of AGD on these cages. While amoebae were isolated more often from the cages on which antifouling paints were used, it did not seem to affect AGD prevalence in these cages. A draft protocol of best husbandry techniques that reflected both industry practices and our current understanding of the disease was developed.

Gross gill checks used by the industry to evaluate the need for freshwater bathing and the general gill status of the fish were compared to histological evaluations. This comparison confirmed that experienced farm personnel can identify gross lesions caused by AGD. This allows farm data, including gross gill scores to be used for AGD research. Sometimes, however the agreement between gross gill scores and histology worsens, most likely due to environmental factors affecting gross gill appearance. In general, AGD diagnosis based on gross gill score gives more positive results than histological diagnosis.

This project provided specialised training in introductory and advanced epidemiology, sampling design, design of field trials and data analysis to all stakeholders, including researchers, government veterinarians and industry. Potential for AGD data sharing and analysis for the whole industry was discussed. A trial AGD database was set up, however industry showed no interest in using it. A computer model was developed based on data from one farm collected during 2003. While the simulations provided interesting insights and identified knowledge gaps, the model could not be validated using data from the same farm from 2002, proving that it has serious limitations. Increased understanding of the disease outbreaks dynamics is needed before a predictive model of AGD can be developed.

KEYWORDS: **Amoebic Gill Disease, Atlantic salmon, aquaculture, epidemiology.**

Acknowledgments

This study formed part of a project of Aquafin CRC, and was supported from the Australian Government's CRCs Program, the Fisheries R&D Corporation and by other CRC participants. We would like to thank the 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) and Dan Fisk (Tassal). This project would not be possible without their help. Dr Chris Baldock, Dr Angus Cameron, Dr Evan Sergeant from AusVet Animal Health Services provided training, advice and expertise. We would like to thank Mick Attard and Dr Mark Powell for providing infected fish from AGD infection tank during this project. Dr Kathy Ophel-Keller (SARDI) has provided her expertise and resources to develop PCR method for quantification of the amoebae in the sediment and net samples. We thank Catriona Macleod and Susan Forbes (Aquafin CRC Environment Program, TAFI) for providing sediment samples, environmental results and valuable discussions. 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 Sonja Saksida (Sea to Sky Veterinary Services) provided a valuable advice and shared her knowledge and observations on epidemiology of salmon diseases. 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) 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 formed part of the Research Program of the CRC for Sustainable Aquaculture of Finfish (Aquafin CRC) and employed funds from the CRC's Commonwealth Grant and by FRDC and other CRC Participants. This project was part of the Amoebic Gill Disease Subprogram.

Health is one of the major issues in aquaculture. Sick fish not only increase production costs, sometimes to the point where an industry becomes non -viable, but also adversely affect the image of the aquaculture industry. For example, infectious salmon anaemia (ISA) resulted in some salmon companies in Scotland going into receivership. Additionally, medications used to treat diseases of cultured fish often result in increased production costs and can negatively affect the markets.

In Australia, salmon aquaculture is relatively disease free, giving us a marketing advantage. However Amoebic Gill Disease (AGD) is a major threat to the economic viability of the salmon industry in Tasmania. Its financial impact can reach $11 million per annum, or 9.4% of the industry input value. Competition from imported salmon increases the urgency to improve cost-efficiency of production. The current practice of freshwater bathing is effective, but in warmer years with low rainfall (when freshwater supplies are low, salinity in estuaries increases, and AGD pressure is high) bathing becomes less practicable and even more costly. Its frequent use also adversely affects growth and production.

While AGD is a major problem for the industry, risk factors for outbreaks of this disease are largely unknown. Considerable progress has been made since the start of the CRC for Aquaculture funded PhD project on Aetiology of AGD (July 1999 - June 2002), however we were still unable to determine the best husbandry practices or establish a forecasting model for the industry.

Some of the major findings included: identification of dead fish as a disease reservoir, wild fish of other species being low risk, and fouling as an additional potential reservoir of the pathogen. While certain antifouling paints may increase risk of AGD, there is little information about other paints or types of netting. The significance of bottom sediments and biota as reservoirs of the pathogen is not understood. The effect of basic husbandry procedures such as cage towing on AGD prevalence also remains unknown.

The main beneficiary of this project is the Tasmanian salmon industry. The current epidemiological project will provide information for development of a surveillance system and decision aids for management of AGD. Additionally, it will provide a template for future epidemiological studies of diseases affecting aquaculture species. As a consequence it provides spillover benefits to other aquaculture industries in Australia, including FRDC Project 2003/225 on Effects of husbandry on SBT health, which uses components developed during this project (such as epidemiology training and database strategy) and an epidemiological approach to study blood flukes and their effects on SBT.

Need

The Australian salmon industry is free from the major infectious diseases that affect salmon industries in other countries. Amoebic Gill Disease is the most significant infectious disease affecting the Tasmanian salmon industry. While this disease occurs in other countries, it does not appear to be as severe as in Australia.

The disease-related fish mortalities are greatly reduced by freshwater bathing, however the disease treatment results in greater production costs and reduces the Australian salmon industry's ability to compete in international markets. While development of a vaccine to protect against the causative agent is a desirable solution for the industry, it must be appreciated that this is a challenging task and requires a long-term view. In the meantime, the industry needs more knowledge of AGD epidemiology and more tools to improve management of fish with AGD on the farms, where improved AGD treatment and control will decrease the risk of large outbreaks of AGD and reduce production costs. Improved understanding of the epidemiology of the disease will also provide a valuable basis on which to develop strategies for vaccine application in the future.

The ultimate goal of our work is to develop an AGD surveillance system that can provide information about the host, agent and environment which can be used to manage or prevent the disease. These results will also improve our understanding of factors contributing to AGD outbreaks and will assist the development of a best industry practice protocol aiming at the reduction of AGD effects on the Tasmanian salmon industry. Information from this surveillance system will facilitate better decisions on the timing of treatments as well as provide long-term data for analysis to identify additional management strategies and minimise disease risks and economic impacts. Specifically, farm-level surveillance data will eventually provide a basis for measuring spatial and temporal trends in AGD occurrence in both salmonids and potential reservoirs as well as environmental and production factors associated with changes in AGD occurrence. Analysis of retrospective data will permit the identification of improved management strategies as well as providing farm managers with a more reliable basis on which to make decisions on prevention and control. This project will provide ways of value adding to the data collected by the salmon industry in general while protecting the interests of individual companies with respect to privacy of commercial information.

Objectives

Objective 1.
Identification of *Neoparamoeba pemaquidensis* reservoirs.

Objective 2.
Identification of risk factors of AGD including the spatial relationship between infected and uninfected cages.

Objective 3.
Development of a pilot surveillance system.

Methods

Objective 1. Identification of *N. pemaquidensis* reservoirs.

Method development for detection of *Neoparamoeba* sp. in environmental samples

The main challenge was a lack of any method for quantification of the viable pathogen in the environment. All the environmental samples were cultured and then any presumed *Neoparamoeba* sp. isolated and confirmed using a combination of three methods: DAPI stain (morphology of nucleus and presence of parasome), IFAT (morphology and reaction with antiPAO27) and PCR (species specific, developed in FRDC Project 98/209). This system precludes false positives, identifies specifically only viable amoebae and generally works well with sediment samples and netting, although *Neoparamoeba sp.* is difficult to culture and under unfavourable conditions can easily be overgrown by flattened amoebae, resulting in false negatives. Unfortunately, this method does not seem to work well for water samples and so far all water samples have been negative. Furthermore, the current detection methods are not quantitative, making any comparisons difficult. Dotblot samples quantified by Most Probable Number (MPN) were used for water samples in the past (Douglas-Helders *et al* 2003b), however this method was not validated for water and could produce false positives as the antibody used in the test cross-reacts with other species of amoebae which could be present in the environmental samples. We attempted to develop new methods for quantification of *Neoparamoeba* sp. in environmental samples, including quantitative PCR and flow cytometry. Full reports are presented as Appendix 5 and Appendix 6.

Identification of reservoirs - sediments

Sediment samples were collected around Tasmania, within salmon farms and from areas where there was no salmon farming. Samples were collected from 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 (Figure 1). Sediment sample volumes ranged from approximately 200 to 800 g. Isolation of amoebae from sediments was attempted using techniques described by Page (1983). Briefly, 3-5 g of sediment material was smeared onto Malt Yeast Seawater Agar (MYS) plates (0.1 g malt, 0.1 g yeast, 750 mL filtered seawater, 250 mL reverse osmosis water, 250 µL pimaricin) which had been seeded with *Stenotrophomonas maltophilia* as a food organism, then incubated at 20ºC and examined every 5-6 days.

When sufficient numbers of amoebae were cultured, three identification methods were employed to confirm the presence of *Neoparamoeba* sp. Firstly, the amoebae were harvested from the MYS plates using sterile seawater, a small volume of the suspension was then placed onto a slide and allowed to dry before an immunofluorescent antibody test (IFAT) (Howard and Carson, 1993) was

performed to detect *Neoparamoeba* sp. Secondly, another portion of the suspension was used as a wet slide preparation for observation of the characteristic parasome using either differential interference contrast (DIC) microscopy or after staining with 4'6-diamino-2-phenylindole (DAPI) to highlight the nuclei and parasomes. The staining protocol with DAPI was adapted from that of Howard (2001), briefly 200-500 µL of the cell suspension was incubated with formalin (37% formaldehyde) to a final concentration of 3% v/v and 10-25 µL of DAPI solution (0.05 mg per mL reverse osmosis water) for at least 30 min in the dark. After incubation a wet mount of the suspension was prepared and examined with a fluorescent microscope with a filter block in the UV excitation range. Thirdly, the final proportion of the harvested cell suspension was subjected to a DNA extraction procedure (Wilson and Carson, 2001) then a polymerase chain reaction (PCR) using *Neoparamoeba* sp. (cross-reacting with both *Neoparamoeba pemaquidensis and Neoparamoeba branchiphila,* Dyková *et al*, 2005) -specific primers of the 18S rDNA gene sequence (Elliott *et al*., 2001, Wong *et al*. 2004). Presence of *Neoparamoeba* sp. was considered to be confirmed when a positive result was recorded for all three detection methods.

Relationship between the presence of *Neoparamoeba* sp. and AGD prevalence

This study was based on three field surveys. One started in November 2002 and finished in May 2003 and examined fish, sediments and net samples collected on monthly basis from two sites (Garden Island and Flathead Bay, both Huon Estuary). As the fish sampling was not terminal, a fish positive for AGD was defined as a fish having gross gill lesions and having a mucus sample test positive in a dotblot test for *Neoparamoeba* sp.

Four pieces of net per cage and two sediment samples per cage were collected on each occasion. The net and sediment samples were cultured on MYS agar. Any amoebae were subcultured and identified using PCR, IFAT and DAPI. If all three tests gave positive results, the sample was considered positive for *Neoparamoeba* sp.

The second survey was done in 2004. Two sites (Garden Island and Flathead Bay, both Huon Estuary) were sampled in January and March. Baseline sediment samples were collected for both sites in November 2003 when the sites were empty. Two more sites (both at Tasman Peninsula, 4 cages from each site, 10 fish from each cage) were sampled in March 2004 only. The fish were sampled terminally and the standard scientific case definition for AGD was used: a fish was considered positive if it had AGD lesions with paramoeba present on them on the basis of histological section stained with haematoxylin and eosin. Two net and three sediment samples were collected for each cage. Net and sediment samples were treated the same way and results interpreted the same way as in the previous survey.

The third field trial was performed at a salmon farm in the Tamar estuary, where AGD has so far not been detected. Four of the farm's nine cages were selected as

trial cages. On these trial cages two net panels of 50 X 50 cm were attached in mid-March at five meters depth; one made of steel and the other nylon. A base-line sampling of 20 fish for each cage (20 for dot blot analysis and 10 for histological examination) was performed and completed prior to attachment of the net panels. Length and weight was recorded for each fish. The sediment samples were taken at two of the outer corners of two of the trial cages and inoculated onto four replicate MYS plates. After one month the trial cages were sampled again for the presence of *Neoparamoeba* sp. on fish, sediments and netting. Twenty fish were sampled for each trial cage; 20 for dot blot analysis and 10 for histological examination. Four replicate sediment samples were taken for each of the two outer corners of each trial cage, and inoculated onto MYS plates and incubated at 18°C for up to two weeks. For each net panel, four squares of each corner of the panel were cut and inoculated onto individual MYS plates and incubated at 18°C for up to two weeks.

Objective 2. Identification of risk factors of AGD including the spatial relationship between infected and uninfected cages.

Rotation trial - field (Douglas-Helders et al 2004 a)

The rotation trial was a repeat of a similar trial in the previous year (2000/2001) and studied the effects of the placement of cages with fish to sites that were devoid of fish (fallowed) for a short period of time, ranging from four days to 97 days. Methods for this trial are described in Douglas-Helders et al (2004a). Briefly, data for these cages were compared with control cages that remained on sites, which had not been devoid of fish, for any of the duration of the trial. Samples were collected from December 2001 to April 2002. To determine if any treatment effect was due to the movement of cages to the fallowed sites, the direct effect of towing on AGD prevalence was tested. In the short towing trial, twenty fish from five towed cages were sampled directly before and after a short tow. The towing speed was on average 2.8 km/h for all towed cages, and the towing time never exceeded five hours. To assess the effect of time between the two samples for each towed cage, five stationary control cages were sampled at the same time as the towed cages, with the same interval time between the two samples. Out-of-season Atlantic salmon smolts with mean weight of 94.0 g (SE 4.6) were introduced to a salmon farm in the Huon Estuary, Tasmania, Australia. All trial fish were fed with commercial salmon pellets (Skretting, Australia) of various sizes according to fish size, on the Aquasmart TM demand feeding system.

Signs of clinical disease were assessed monthly, using the routine Tasmanian salmon farmers gill assessment method, by examining at least 20 fish for the presence of AGD related mucous patches. A score of severity of infection was estimated for each sea cage based on the number of fish examined that were infected and the degree of AGD infection for each fish (A. Steenholdt, pers. com). This scoring system was consistently used during the trial, and determined the need of freshwater bath treatment for all cages. At an overall moderate to heavy

infection level in a cage, fresh water bath treatments were administered and all cages within one treatment group bathed in succession. Fish were transferred into cages with clean nets after freshwater bathing at all times. The number and timing of freshwater baths was recorded within internal farm data management systems for each trial treatment group. AGD prevalence for each cage was determined using pathogen specific dot blot analysis of gill mucus samples (Douglas-Helders et al 2001).

The effect of each treatment on general fish performance was determined by comparing weight gain and mortality data from farm records. Weight gain data were obtained either by manual weight checks or using the Vicass system (SIGMA Technologies, Canada). For manual weight checks 40 to 60 fish were used; and the mean biomass for the sea cage estimated by dividing the total biomass by the number of fish sampled and multiplying the figure by the approximate total number of fish in the cage.

Data were checked for homogeneity of variance and normality before performing a Student's t-test to determine differences. Any significant difference due to treatment was determined by comparing data from the two treatments within each trial at the final sampling, and between the two years within each treatment for the rotation trial. Mortality data were expressed in percentages through dividing the cumulative number of mortalities at the completion of the trials by the initial numbers of fish in the cages at commencement of the trials. Weight gain data were analysed as the cumulative biomass of each cage from which the biomass of the cage at the start of the trial was subtracted. Results of all statistical analysis were considered significant when $P \leq 0.05$.

Antifouling paint trial- field

This trial was run from April 2002 until September 2002, with trial cages placed at the Garden Island site of the Huon Aquaculture Company. Six cages of 120 m circumference were used in this trial. Nets from two of these cages were treated with Hempel® (copper based paint) antifouling paint, nets from two other cages were treated with Netclear® (lanolin based paint) and the remaining two cage nets left untreated. The treatment followed manufacturer instructions. All the nets including the untreated control were hung in water for a minimum of 2 weeks before being stocked with Atlantic salmon.

Every month, for a total of six months, 20 fish per trial cage were crowded, dip-netted and anaesthetised using clove oil, so gills could be examined to determine a gross gill score and sampled for dot blot analysis. The dot blot samples were stored on ice while in the field and then stored at -20°C until analysis. In addition, a 4 X 4 cm net filament was cut from each of the netting at five meters depth and placed into a sterile container by divers. These samples were inoculated onto four replicated MYS plates immediately upon arrival on shore, placed for up to two weeks at 18°C in an incubator once in the laboratory, and tested for the presence of *Neoparamoeba* sp. by PCR technique. For analysis a positive fish was defined

as a fish with a positive gross gill score and a positive result for dot blot analysis. AGD prevalence for a cage was calculated as a percentage from the 20 sampled fish.

Segments of 0.5 mm net filaments (approximately 4 x 4 cm) were cut from the nets by divers and placed in containers underwater. Initially net samples were taken from 0, 5 and 10 m depths. As there was no difference in the presence of amoeba at different depths, subsequent samples were taken at 5 m depth. Upon reaching the shore individual segments were placed on a MYS plates, labelled and sealed.

Initially a chi-square test compared the total AGD prevalence within treatments to identify whether the cage data could be pooled. A comparison of total AGD was made over time regardless of the treatment type (6x2 table) and between the treatments regardless of time (3x2 table) using a chi-square test. Within each sample time total AGD for each treatment was compared against the other treatments (2x2 table) using a chi-square test. The presence of *Neoparamoeba* sp on net segments was analysed over time regardless of treatment and between the treatment types regardless of time by a means test. A two-tailed student t-test was used to analyse the presence of *Neoparamoeba sp* on net segments in relation to AGD prevalence.

Effects of environmental factors on the survival of *N. pemaquidensis in vitro*

Aquaculture in Tasmania is mostly performed in relatively sheltered estuaries. These estuarine environments contain a variety of habitats in which *Neoparamoeba pemaquidensis*, the AGD-causing protozoan, may or may not survive.

Tasmania is divided into two regional zones, one where AGD is present and one where this disease is absent. Ecological data to rationalise this distribution are lacking.

In *in vitro* trials, one to three *N. pemaquidensis* strains (PA027, AFSM11/II and a fresh isolate) were exposed to three different concentrations of ammonium sulphate (0.1, 10, and 100 mg/L) in filtered seawater, five concentrations of copper sulphate (10, 100, 1,000, 10,000, and 100,000 µM) in seawater, a solution of copper sulphate (0.56 µM) and tannin (30 mg/L) in seawater, and lastly five different cell densities 625, 1250, 2500, 5000, and 10000 cells/mL), three salinities (38‰, 27‰, and 15‰) and three temperatures (4°C, 13°C, and 18°C). All trials used sterile filtered seawater seeded with *N. pemaquidensis* as a positive control. A field trial investigated the survival of *N. pemaquidensis* in waters sourced from AGD-free (Tamar Estuary and Macquarie Harbour) and AGD-positive zones (Huon Estuary and North West Bay), and water analysis (total copper, dissolved calcium and magnesium, dissolved organic carbon) performed in order to explain any differences. Upon arrival in the laboratory each water sample was divided into three sub-samples prior to the *in vitro* testing. Two of the sub-samples were treated to destroy any possible viable *Neoparamoeba* cells present in the water samples by freezing to -80°C and thawing at room temperature prior to use, while

the other sub-sample was left untreated and stored at 4°C until use. One of the treated sub-samples was adjusted to the highest salinity of all samples taken, at a salinity of 36‰, and the other treated sub-sample was used unadjusted.

Objective 3. Development of a pilot surveillance system.

Validation of gross gill checks (Adams et al 2004)

Gross pathological assessment of amoebic gill disease (AGD) is the only non-destructive, financially viable method for rapid and broad scale disease management of farmed Atlantic salmon (*Salmo salar* L.) in Tasmania. However, given the presumptive nature of this diagnosis, the technique has been considered questionable. We investigated the degree of conformity between clinical signs and histological lesions observed in a commercial setting. Three groups of Atlantic salmon (n = 42, n = 100, n = 100 respectively) were collected from various farm sites in southern Tasmania between December 2001 and April 2003. Gross lesions and histological lesions were assessed and AGD diagnosis compared using kappa statistics.

Development of a pilot computer model

Research on amoebic gill disease (AGD) in Tasmania comprises field and laboratory studies aimed at identifying cost-effective options for prevention and control. While substantial progress has been made and many aspects of AGD are now better understood, integrating current and future knowledge about the disease to develop improved prevention and control remains a complex challenge. A prerequisite to identifying the relative importance of different risk factors is an understanding of the temporal patterns of AGD at the cage level. This is best achieved by developing a computer model of AGD which can be used to explore the potential impacts of putative risk factors. This should also lead to a better understanding of how historical company data can be best used.

The objective was to develop a computer model to reflect changes in fish prevalence of AGD over time in a single cage to better understand the epidemic behaviour of the disease and to provide a basis for evaluating the likely effect of putative risk factors and interventions such as fresh water bathing.

Conceptual model

Parasite distributions within a natural population of hosts are frequently "overdispersed" – most of the hosts have a low burden while a few have very high burdens. Because of this it is common to model parasitism as the number of parasites per host in the population with the distribution of parasites per host modelled as a negative binomial distribution. However, in the case of salmon it may be more useful to model the relative frequency of fish with different gill scores.

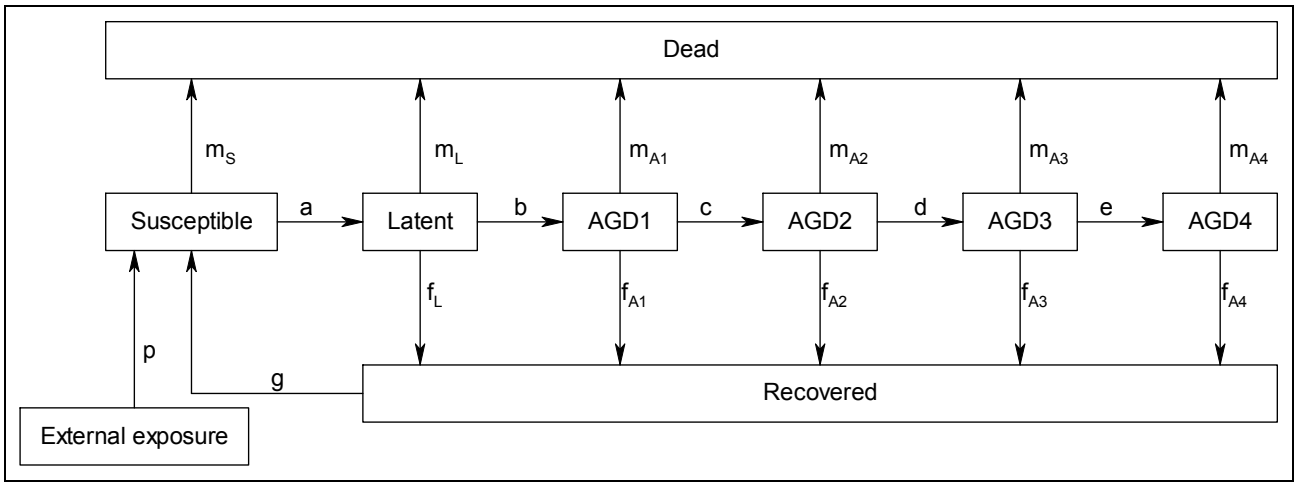
To do this, a state-transition approach was developed using an MS Excel spreadsheet. This was based on the conceptual model with specification as set

out below. A deterministic model was developed, with no allowance for random variation, rather than a stochastic one, which would incorporate random elements. The model was refined following discussions with AGD researchers and salmon industry representatives. It is a modified SIR (Susceptible-Infected-Recovered) model, in which Susceptible fish become infected and move through a number of States (stages) of infection, depending on various transition probabilities. A time step of one week was used and the model simulates a 52 week (1 year) period.

Under this concept, Susceptible (uninfected) fish are exposed to the parasites and become latently infected and then progress through several stages of severity indicated by gill scores of 1 to 4 (AGD1 to AGD4). Susceptible and latently infected fish have a gill score of 0.

Figure 1 outlines the conceptual model proposed to simulate epidemics of AGD within a single salmon cage.

Figure 1: Conceptual model for an SIR model of AGD infection in farmed salmon



Transitions are indicated by arrows linking the various states. Lower case letters associated with each transition indicate the relevant transition parameters for each step. Transition parameters (b – m) can be estimated as the probability of the transition occurring during a one-week period. Alternatively, parameters b – e and g can be estimated as 1/(mean stage duration).

Under this model, it is assumed that fish can recover from any stage of the disease but do not remain permanently immune and subsequently revert to a Susceptible State. It is also assumed that the rate of recovery varies depending on the severity of infection. The rate of new infections is proportional to the cumulative burden of AGD in the population measured in terms of AGD4 equivalents and to the number of effective contacts that occur between fish, where an effective contact is contact sufficient to transmit infection if one of the fish is infected. The model also allows for infection from an exogenous source depending

on the probability of external exposure (p). To start the model an initial number of fish in each infected State must be specified, unless p > 0, in which case infection can enter the population from external sources.

Model specification

In the model, fish can exist in any of the States shown in Figure 1 and listed in Table 1:

Table 1: Summary of infection states modeled in AGD model

State	Description
S	Susceptible (uninfected and not resistant to infection)
L	Latent (infected but no detectable lesions)
A1	AGD score 1
A2	AGD score 2
A3	AGD score 3
A4	AGD score 4
R	Recovered (and resistant to reinfection)
D	Dead

Transitions were modelled to occur between states as shown in Figure 1, and according to the transmission probabilities shown in Table 2.

Table 2: Transition parameters for AGD model

Parameter	Description	Calculation
a	Contact rate	Average number of effective contacts per fish each week
b	Transition from L > A1	Inverse of duration of L in weeks
c	Transition from A1>A2	Inverse of duration of A1 in weeks
d	Transition from A2>A3	Inverse of duration of A2 in weeks
e	Transition from A3>A4	Inverse of duration of A3 in weeks
f_S	Recovery rate by State	Probability of recovery for fish in State S
g	Transition from R>S	Inverse of duration of R in weeks
p	External exposure rate	Probability of exposure to external infection
m_S	Mortality rate by state	Probability of death for fish in State S
I	I = Total A4 equivalents	$A1/I_{A1} + A2/I_{A2} + A3/I_{A3} + A4$

The model was fully specified by the following series of equations:

$N = S+L+A1+A2+A3+A4+R$
$I_{A1,A2,A3}$ = A4 equivalent cases for A1 to A3
I = Total A4 equivalents
$I = A1/I_{A1} + A2/I_{A2} + A3/I_{A3} + A4$

$New_t = aS_{t-1}I_{t-1}/N_{t-1}$
$L_t = L_{t-1} + New_t - L_{t-1}(m_L + b + f_L) + pS_{t-1}$
$A1_t = A1_{t-1} + bL_{t-1} - A1_{t-1}(m_{A1} + c + f_{A1})$
$A2_t = A2_{t-1} + cA1_{t-1} - A2_{t-1}(m_{A2} + d + f_{A2})$
$A3_t = A3_{t-1} + dA2_{t-1} - A3_{t-1}(m_{A3} + e + f_{A3})$
$A4_t = A4_{t-1} + eA3_{t-1} - A4_{t-1}(m_{A4} + f_{A4})$
$R_t = R_{t-1} + f_LL_{t-1} + f_{A1}A1_{t-1} + f_{A2}A2_{t-1} + f_{A3}A3_{t-1} + f_{A4}A4_{t-1} - R_{t-1}(g + m_R)$
$D_t = D_{t-1} + m_LL_{t-1} + m_{A1}A1_{t-1} + m_{A2}A2_{t-1} + m_{A3}A3_{t-1} + m_{A4}A4_{t-1} + m_SS_{t-1} + m_RR_{t-1}$
$S_t = S_{t-1} - New_t - m_SS_{t-1} + gR_{t-1} - pS_{t-1}$

Parameter estimates

Data for pens of salmon from 2003 were analysed to determine patterns of AGD infection, bathing and mortality over time. Initial parameter values for the model were estimated from available data, or where adequate data estimates were not available, were based on discussions with AGD researchers and industry representatives, or on trial and error to produce realistic model output.

Model starting conditions

The model was started with a pen of 50,000 fish, of which 5 were assumed to be infected (State = A4) at the start of the modelled period (t = 0).

Contact rate (a)

The contact rate (a) is the average number of fish-contacts that a fish makes each week. Contact rates for SIR models can be estimated from transmission studies, and for diseases of terrestrial animals are usually relatively low (<5 contacts per time period). Empirical estimates of contact rates for AGD were not available for inclusion in the model. However, because of the stocking density and the opportunity for indirect transmission of AGD through the aquatic environment in which the fish are grown, contact rates for AGD were assumed to be high, and an initial value of a=200 was used.

Transitions from L>A1, A1>A2, A2>A3, A3>A4 (b, c, d & e)

Again, no empirical data for transition rates for AGD were available, so rates were estimated to provide a fairly rapid progression of infection, as appears to be the case in the field. For the initial model, values of b = 0.2, c = 0.33, d = e = 0.5 were used. These values reflect a slower progression early in the infection process (5 weeks for State = L, compared to 3 weeks for State = A1 and 2 weeks for State = A2 and State = A3). These values also result in an average period of 12 weeks from infection to a fish becoming maximally infected (State=A4)

Recovery rate by State (f_S)

Recovery from infection and reversion to susceptibility were included in the model for completeness. However, there is no evidence for spontaneous recovery from AGD, so the recovery rate was assumed to be zero for all States ($f_S = 0$).

Transition from R>S (g)

For completeness, any recovered individuals were assumed to revert to susceptibility in the time period following recovery (g = 1).

External exposure rate (p)

Although the model allows for a proportion of fish to be exposed to external challenge with AGD during each time period, the importance of external challenge is still unclear. Therefore, external challenge was assumed to be zero (p = 0) for the initial model.

Mortality rate by State (m)

Background mortality rates in farmed salmon appear to be approximately 0.1 to 0.2% per week. It is also assumed that AGD only causes significant mortality in fish with severe lesions (State = A4). For all States except A4, weekly mortality rate was set to m_S = 0.001 (0.1%), while for State = A4, m_{A4} = 0.1 (10%). This value implies that fish with A4 lesions will (on average) survive for about 10 weeks.

Infection equivalents for States A1 – A3

To allow lower level infections (A1 – A3) to contribute to the level of exposure for Susceptible fish, the contribution of these fish to the total infectious load must be calculated. For the initial model, the contribution of each level was assumed to be half that of the level above. Therefore, 2 A3 fish were assumed to be equal to 1 A4, 4 A2 fish were equal to or 1 A4 (or 2 A3) and 8 A1 fish contributed the same amount to exposure as 1 A4 (or 2 A3 or 4 A2).

Effect of treatment

Treatment of AGD is by fresh-water bathing to flush the amoebae off the gills of affected fish. In general, most affected fish are assumed to recover and lesions resolve after bathing, at least in the short term. It is unknown whether fish recover completely after bathing, or whether amoebae remain in the gills of affected fish in low numbers, virtually returning them to the Latent State (or to other infection States). The model was constructed to allow consideration of a proportion of affected fish in any State reverting to any of the previous States following bathing. However, considering that AGD lesions appear to resolve temporarily before returning, and that AGD-levels in affected pens can build up very rapidly again after treatment, it was assumed for the initial model run that all affected fish revert to Latent State following treatment, and that none revert to Susceptible (or other States).

For the initial simulation, no treatment was applied to the pen. A second simulation was also done for comparison, where treatment was assumed to be applied when the mortality rate in the pen reached about 0.5% per week.

Comparison of simulation results with real data for 2002

Data for 29 pens of salmon for the 2002 year were summarized to allow comparison of patterns in the real data with model output for bathing frequency, as discussed below. Nine of the 29 pens were selected at random for detailed analysis of prevalence, AGD score and mortality rates. Analyses were undertaken for the period up to 52 weeks post-stocking for each pen. Mortality rates were calculated across all subdivisions of the original pens, while prevalence and AGD score were summarised separately for the subdivided (A and B) pens.

Results/Discussion

Objective 1. Identification of *N. pemaquidensis* reservoirs.

Method development for detection of *Neoparamoeba* sp. in environmental samples

While some progress has been achieved with the quantitative PCR method, it was only for samples spiked with very high number of cultured amoebae (clone P027) and not for gill-isolated amoebae. Dr Kathy Ophel-Keller's report is attached as Appendix 5.

The practical level of sensitivity of flow cytometry to detect *Neoparamoeba* sp. was shown to be approximately 2 organisms per mL. This was based on concentrating 5 L water samples. However, in the field, no viable *Neoparamoeba* sp. could be detected in such samples, collected from salmon cages. Five litre samples of water were collected from within and around sea cages containing salmon in an area with a know history of AGD. These samples were concentrated by centrifugation, stained with rabbit anti-PA027, but no organisms could be detected in flow cytometric analyses. This would suggest that the concentration of *Neoparamoeba* sp. in these water samples was less that 2/ml or that the amoeba was lost during the processing of the samples. If flow cytometry is to be used to detect *Neoparamoeba* sp. in the water column, then other technologies must be developed to process large volumes of water. Presence of salt in the water would also interfere with the flow cytometry and a sample processing protocol would have to be developed. Further refinements would be required before it can be used on environmental samples. Main advantages of this method include objective appraisal of viability/specificity. Lack of easy access to a flow cytometer (Pathology, University of Tasmania, Hobart) may limit applications of this technique. Full report is included as Appendix 6.

Identification of reservoirs - sediments (Crosbie et al 2003)

In virtually all cases the sediments yielded amoebae within 7-14 days and *Neoparamoeba* sp. was shown to be present in sediments from all areas (Table 3), including those where there is no history of AGD in farmed salmonids and where salinities fluctuate (i.e. Tamar estuary and Macquarie Harbour). The only site where *Neoparamoeba* sp. was not detected was the in-shore reference site at Macquarie Harbour. It is therefore reasonable to conclude that this amoeba is ubiquitous in the marine environment in Tasmania. It is also of interest to note that sediment types ranged from sand to much finer, denser, organically-rich and anoxic material. The isolation by culture indicates not only the presence of *Neoparamoeba* sp. in marine sediments but also its viability.

The fact that many sediment samples did not yield *Neoparamoeba* sp. is thought to be a sensitivity issue and there is also a reasonable possibility of false negative results as *Neoparamoeba* sp. can be difficult to culture (I. Dyková pers com). It is likely that the amoeba is more common in Tasmania than these results would indicate and a lack of detection does not necessarily imply absence. Even though

there was no attempt made to concentrate any amoebae which may have been in the sediments, and only a small amount of sediment was used to inoculate the MYS plates, a significant proportion of sediments nevertheless yielded *Neoparamoeba* sp.

This study indicates that, as in other parts of the world, *Neoparamoeba* sp. is a common marine amoeba in the Tasmanian coastal environment. The relationship between presence of *Neoparamoeba* sp. and disease outbreaks is not clear and the virulence of the sediment strains is not known. Quantitative detection methods would need to be used to investigate the relationship between *Neoparamoeba* sp. density and AGD occurrence.

Table 3. *Neoparamoeba* sp. detection in sediments sampled from various sites around Tasmania and their amoebic gill disease (AGD) status (Crosbie et al 2003).

AREA	Date	n	Positive (%)	AGD status
Stringer's Cove, farm	19/02/02	12*	75	Positive
	02/07/02	12*	58	
	04/08/02	12*	83	
Nubeena, farm	19/03/02	4*	50	Positive
	10/09/02	6*	0	
Hideaway Bay, farm	19/04/02	5	20	Positive
Tamar Estuary	23/04/02	4	25	Negative
Tinderbox, farm	09/05/02	4	25	Positive
Bruny Island, farm	27/05/02	4	75	Positive
Macquarie Harbour, farm	17/10/02	9	11	Negative
Bicheno, non- finfish farm site	07/04/02	4	50	Negative
Macquarie Harbour, reference site	06/05/02	5	0	Negative

*At these farms samples were supplied in duplicate, therefore n = 12 refers to duplicate samples from 6 sites within the farm, similarly n=4 or 6 means 2 or 3 sites within the farm

Relationship between the presence of *Neoparamoeba* sp. and AGD prevalence

In the first trial, no *Neoparamoeba* sp. was detected in the net samples in December 2002. The greatest number of positive net samples was reported in February 2003, corresponding with the increase in salinity. This was consistent for both sites. Only one net sample from Garden Island was positive for *Neoparamoeba* sp. in May. The effect of sampling time was statistically significant

(P=0.0468). The pathogen was rarely detected in the sediment. One sediment sample was positive at each site in January. The prevalence of AGD was greatest in January at Flathead Bay and February at Garden Island. There was no significant correlation between AGD prevalence and prevalence of positive net samples (r=-0.1397, n=40) of sediment samples (r=0.0547, n=40). AGD prevalence in cages with positive nets was 53.33% and in cages with nets negative for *Neoparamoeba* sp. AGD prevalence was 61.6%, this difference was not statistically significant (t-test, df=39, P=0.4448)

In the second trial, most sediment samples were positive in November 2003, before fish were introduced to the site. In contrast to sampling during previous summer *Neoparamoeba* sp. was very commonly isolated from sediments, however AGD prevalence was much lower than the year before. Mean AGD prevalence for cages with positive nets was 13.75% and for cages with negative nets 16.67%, this difference was not statistically significant (t-test, df=13, P=0.8057). There was no significant correlation between AGD prevalence and presence of *Neoparamoeba* sp. in nets (r=-0.1505, n=14) or sediment samples (r=-0.2533, n=14). The increased incidence of isolation of *Neoparamoeba* sp. could have been due to improved isolation methods and an increase in the experience of the staff.

Results of the histological examination from the third trial showed that all fish from the Tamar site were negative for the presence of *Neoparamoeba* sp.. This result was confirmed with a negative result for all dot blot samples. IFAT and DAPI stains of the culture of sediments and netting showed that 62 out of a total of 68 samples were positive for IFAT and 60 out of 68 positive for DAPI. Interestingly, no growth has been observed on the steel netting panels. The absence of AGD in this area while *Neoparamoeba* sp. was found both in sediments and nets emphasises the crucial role of the environment in the expression of AGD in cultured fish.

We could not find any relationship between AGD prevalence and presence of *Neoparamoeba* sp. in net or sediment samples in either of these two surveys. This may be due to the high probability of false negatives (as *Neoparamoeba* sp. is hard to culture) or very imprecise quantification (only as percentage of positive samples with low number of samples 2-4). There is no method available for quantitative detection of *Neoparamoeba* sp. in water. Finally, the AGD model suggested that once the fish are infected, the external reservoirs are not significant, so it may be that our results are true despite many limitations.

Potential reservoirs for *Neoparamoeba* sp. are summarised in Table 4. It is obvious that this amoeba is a widespread organism, present on a variety of substrates and in the water. However, few experiments have been done to investigate if amoebae from these reservoirs can infect fish. Virulence of strains isolated from different reservoirs is also not known. This would be difficult to investigate as only a few cells are isolated and the amoebae lose virulence in culture.

Table 4. Potential reservoirs for *Neoparamoeba pemaquidensis.* "No" for confirmed infection from the reservoir means current lack of experimental data, and not that we have scientific evidence that *Neoparamoeba* sp. from this reservoir cannot infect fish.

Potential reservoir	*Neoparamoeba* sp. presence	Confirmed infection from the reservoir
AGD infected fish	Yes	Yes
Water	Yes	Yes
Dead fish	Yes	Yes
Sea cage netting	Yes	No (one experiment negative)
Biofouling	Yes	No (one experiment negative)
Sediment	Yes	No (no experiments done)
Wild fish species	No	No (no experiments done)

Objective 2. Identification of risk factors of AGD including the spatial relationship between infected and uninfected cages.

Cage rotation trial - field (Douglas-Helders et al 2004a)

The AGD prevalence in the rotated cages (final prevalence in 2002 28.4%) was below that of the stationary cages (final prevalence in 2002 60.1%) at all times in both years (Douglas-Helders et al 2004a). However, no statistical difference in AGD prevalence between the two treatment groups was detected (P=0.072). Maximum AGD prevalence occurred in January for both treatment groups. The mean number of days between freshwater baths was longer for rotated cages (35 days) than for stationary cages (29.2 days). The period between freshwater baths in the rotated cages was statistically significantly longer than in stationary cages when data of the two years were pooled (P=0.037). Also, the weight gained in the rotated cages was statistically significantly greater at the completion of the trial than in the stationary cages (P=0.041). The cumulative mortality rate of the rotated cages was not affected by treatment (P=0.436). The cumulative mortality at the end of the trials was 2.06% (SE 0.68) for the rotated cages and 2.88% (SE 0.76) for the stationary cages. Towing of the cages from the short towing trial did not directly affect the AGD prevalence (P=0.111). The mean AGD prevalence at commencement and completion of the short towing trial was 61.7% (SE 14.2) and 71.2% (SE 16.0) for the towed cages, and 44.6% (SE 11.3) and 42.3% (SE 15.8) for the non-towed control cages.

The results of the cage movement study confirmed previous results, that cage rotation during summer and autumn leads to a reduced freshwater bathing frequency and higher biomass gain in the moved cages. Although due to the bad weather in 2002 the rotation schedule did not exactly follow the plan, an obvious effect of the rotation was apparent, with the mean AGD prevalence of the rotated cages lower than in the stationary cages at all times. Interestingly, a similar pattern was found as in the previous year, with a maximum AGD prevalence peak in January for both groups. This seems to imply that when paramoebae numbers in the environment or AGD prevalence are extreme, site rotation is not effective. However, in any other case site rotation is a good strategy in reducing AGD prevalence in pen-reared salmon. Both trials were done at the time of greatest risk of AGD (starting in early summer and finishing in autumn).

Antifouling paint trial- field

Results showed that both treatment ($P = 0.002$) and time ($P < 0.001$) had significant effects on the AGD prevalence. Overall, fish in control nets had the lowest AGD prevalence (42.50%), while there was no significant difference between fish from Hempel (58.33%) or lanolin nets (64.17%). Significantly higher AGD prevalence was found in fish grown in lanolin nets in April, May, July and August compared to the control nets, while AGD prevalence was greater in Hempel nets than control nets in May, July, August and September. Month had significant effect on AGD prevalence with July, August and September showing greater AGD prevalence than April, May and June. *Neoparamoeba* positive net samples were found in June (1 Hempel net, 1 control net), July (2 Hempel nets and 1 control net), August (1 Hempel net), and September (1 Hempel net). Maximum AGD prevalence was seen in July (40.8 %).

Despite the Hempel nets being most often positive for *Neoparamoeba* sp., no relationship between AGD prevalence and presence of *Neoparamoeba* sp. could be found ($P = 0.310$). This could have been due to the potentially false negative results of cultures, through competition between microorganisms in the samples and possibly non-optimal growth conditions.

The AGD prevalence results presented are in agreement with the results obtained in previous studies where AGD prevalence was significantly greater in Hempel-treated nets (Douglas-Helders et al 2003a). The study presented here was run over a period of six months, while the previous study was performed over a period of ten weeks. Interestingly, the higher frequency of *Neoparamoebae* presence on nets in the present study is in agreement with results found in the previous study (Douglas-Helders et al 2003a).

Effects of environmental factors on the survival of *N. pemaquidensis*

It was apparent that the three *N. pemaquidensis* strains were affected at different levels when exposed to certain environmental factors, which raises the question of variability in pathogenicity and virulence between strains in different environmental conditions. Exposure to increasing concentrations of ammonium sulphate did not affect the growth compared to sterile and filtered seawater ($P = 0.550$). Exposure

to increasing copper sulphate concentrations significantly affected survival of the amoeba ($P < 0.001$), as did low salinity ($P < 0.001$), low cell densities ($P = 0.0005$), and water sourced from Macquarie Harbour ($P < 0.001$). Interestingly, there was no effect of temperature on the protozoan survival ($P= 0.655$). The results of the field study showed that survival of *N. pemaquidensis* exposed to Macquarie Harbour waters was significantly reduced. As expected, Macquarie Harbour water showed the significantly highest total copper concentrations, highest dissolved organic carbon concentrations, lowest salinity, and the lowest dissolved calcium and magnesium concentrations. Both the *in vitro* study and the field study suggest that Macquarie Harbour is free from AGD because of its hydrographical (resulting in a lower average salinity in the Harbour) and chemical characteristics (such as total copper, dissolved calcium and magnesium concentrations). However, AGD has not been observed in the Tamar Estuary even though *N. pemaquidensis* has been detected, which in this study could not be explained by the factors tested. This suggests that factors other than the ones tested may be necessary for the onset of AGD. The Tamar Estuary is characterised by the steepest tidal gradient and the highest catchment freshwater runoff compared to the other sites studied, resulting in high water turn-over. It may be that this factor reduces the residence time or contact opportunity of *Neoparamoeba* sp. with farmed fish and prevents AGD from occurring in the estuary. In addition, only one farm site is present in the Tamar Estuary at this point in time and therefore this area has a comparatively low total biomass of cultured fish. A better understanding of the ecology of *N. pemaquidensis* gained from these trials will enable better control and prevention strategies for Tasmanian salmon growers.

Disease is usually a complex interaction between host, pathogen and environment. Risk factors usually contribute significantly to disease outbreaks so controlling risk factors can often control the disease itself. Identification of key risk factors can lead to realistic control options. However, the most recent results of our experiments, showing a linear relationship between numbers of *Neoparamoeba* sp. in the water (Morrison et al, 2004) suggest that except for salinity and temperature, other risk factors may not be as significant as we previously believed. Some of the risk factors reviewed here include the reservoirs for *Neoparamoeba* sp., host susceptibility and effects of the environment.

Table 5. Summary of identified and perceived risk factors for amoebic gill disease outbreaks, attributable to the pathogen, host and environment (↑ = increased risk; ↓ = decreased risk, no = no effect on risk). From Douglas-Helders et al (2004). Identified factors were identified in laboratory experiments and field trials. Perceived factors are based on a questionnaire and discussions with the industry.

Variable		Effect on risk	Reference
Pathogen factors: identified			
	Reservoirs		
	Water column	Yes, ↑	Zilberg *et al*., 2001, Douglas-Helders *et al*., 2003a
	Other AGD infected fish	Yes, ↑	Munday *et al*., 2001
	Mortalities left in sea cage	Yes, ↑	Douglas-Helders *et al*., 2000
	Netting/ biofouling	Yes, ↑	Tan *et al*., 2002
	Sediment	Unknown	Crosbie *et al*., 2003
	Wild fish	No	Douglas-Helders *et al*., 2002
Infective up to 14 days		Yes, ↑	Douglas-Helders *et al*., 2003a
Surfaces with min. negative charge		Yes, ↑	Martin, 1987
Salinity > 15 ppt		Yes, ↑	Douglas-Helders *et al*., 2004c
High dissolved calcium and magnesium concentrations in water		Yes, ↑	Green, 2003, Douglas-Helders *et al*., 2004c
Artificial light/continuous dark		No	R. Morrison, unpublished
Temperature		No	Douglas-Helders *et al*., 2004c
Copper concentrations in water		No	Douglas-Helders *et al*., 2004c
Ammonia concentration in water		No	Douglas-Helders *et al*., 2004c
Pathogen factors: perceived			
Bacterial loads in water		Yes, ↑	Douglas-Helders *et al*., 2004c
Suspended matter in water		Yes, ↑	Douglas-Helders *et al*., 2004c
Other gill-colonizing protozoa		Yes, ↑?	Dyková *et al*., 2003
Adaptation to fresh water		Yes, ↑?	Parsons *et al*., 2001
Gill-associated bacteria		Yes, ↑?	Bowman & Nowak, 2004
Increase in virulence		Yes, ↑	Findlay *et al*., 2000
Host: identified			
Species		Yes	Munday *et al*., 2001
Lack of immunity		Yes, ↑	Aklaghi *et al*., 1996, Zilberg *et al*., 2001, Gross *et al*., 2004
Loss of genetic diversity		No	Elliott & Reilly, 2003
Mechanical damage to gills		No	Adams & Nowak, 2004
Host: perceived			
Ploidy status (triploid vs diploid)		Yes, ↑?	Nowak, 2001
Poor general gill health		Yes, ↑?	Munday *et al*., 2001
Age/ size fish		Yes, ↑?	Nowak, 2001
Sexual maturation		Yes, ↑?	

Variable	Effect on risk	Reference
Swimming behaviour	Yes, ↑?	Douglas-Helders *et al*., 2003b
Environment: identified		
High average temperature	Yes, ↑	Clark & Nowak, 1999
High average salinity	Yes, ↑	Clark & Nowak, 1999, Douglas-Helders *et al*., 2004c
Season	Yes	Clark & Nowak, 1999, Douglas-Helders *et al*., 2003b
Low rainfall	Yes, ↑	Clark & Nowak, 1999
Environment: perceived		
Little exposure to freshwater runoff	Yes?, ↑	Douglas-Helders *et al*., 2004c
Lack of tidal flows	Yes?, ↑	Foster & Percival, 1988, Douglas-Helders *et al*., 2004c
Site depth/ bottom type	?	Douglas-Helders *et al*., 2004b
Environment: Husbandry: identified		
Copper paint treated netting	Yes, ↑	Douglas-Helders *et al*., 2003c
Geographical positioning of farms	Yes	Clark & Nowak, 1999, Parsons *et al*., 2001, Douglas-Helders *et al*., 2004 b,c
Intensive culture conditions	Yes, ↑	Dyková *et al*., 1995
Prophylactic bathing	Yes, ↑	Douglas-Helders *et al*., 2004a
Release of untreated fresh water from bath	Yes, ↑	Parsons *et al*., 2001, Clark *et al*., 2003
Hard fresh water for bathing	Yes, ↑	Powell & Clark, 2003, Green, 2003, Douglas-Helders *et al*., 2004c
Use of levamisole in freshwater bath	No	Clark & Nowak, 1999
Presence of dead fish in sea cage	Yes, ↑	Douglas-Helders *et al*., 2000
High cage/ farm density	Yes, ↑	Nowak, 2001, Douglas-Helders *et al*., 2004b,c
Immuno-stimulation	Yes, ↓	Bridle *et al*., 2003
Regular net changes	Yes, ↓	Clark & Nowak, 1999
Fallowing	Yes, ↓	Douglas-Helders *et al*., 2004a
Environment: Husbandry: perceived		
Multiple year class site	Yes, ↑?	Munday *et al*., 2001
Surplus feeding rates	Yes, ↑?	
Food characteristics	Yes?	
Fish density	Yes, ↑?	Zilberg *et al*., 2000
Freshwater origin	Yes?	Parsons *et al*., 2001, Douglas-Helders *et al*., 2004b
Artificial lighting	No?	C. Bagley, unpublished

Objective 3. Development of a pilot surveillance system.

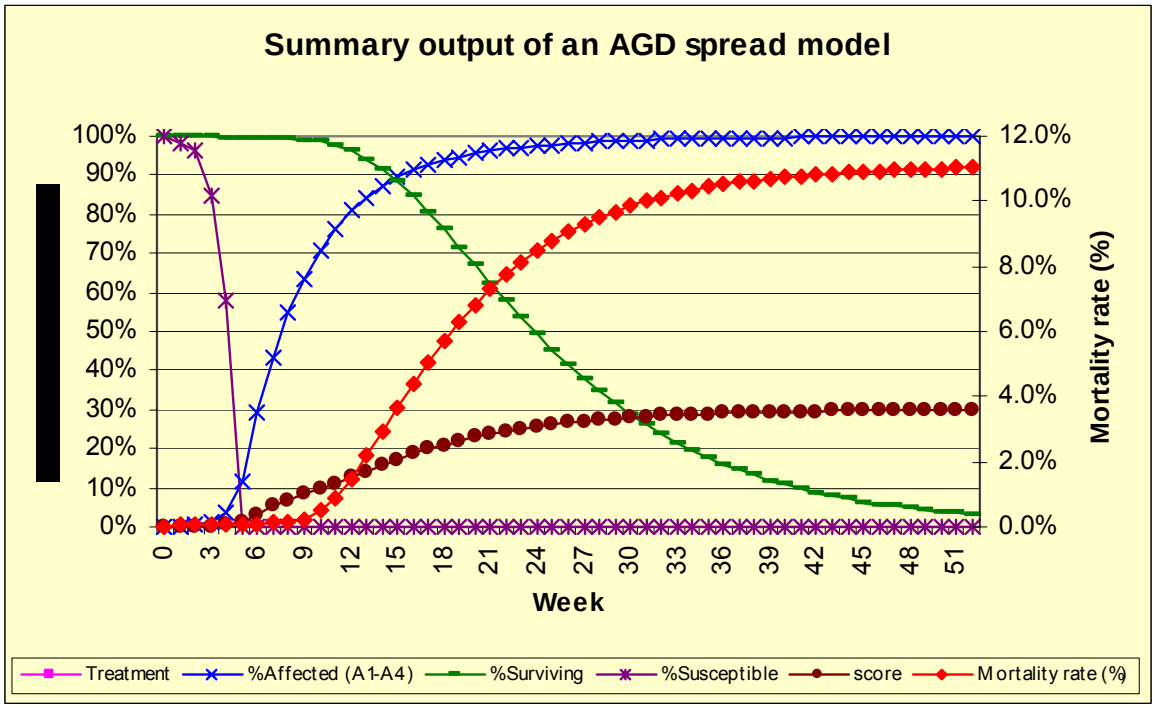
Validation of gross gill checks (Adams et al 2004)

Micro-stereoscopic analysis showed that grossly affected tissue regions correspond to areas of hyperplastic lamellae fusion generally in association with attached *Neoparamoeba* sp. We also compared agreement between gross signs of AGD and histopathological diagnosis. Kappa analysis indicated moderate to good agreement between methods (k = 0.52 – 0.74). Individual cases of disagreement were further scrutinised and several factors were found to influence the level of agreement between the two methods. Stage of disease development, lesions derived from other pathogens, assessor interpretation and experience, sampling methods, histological technique and/or experience were potential confounding factors. Thus, the clinical diagnosis based on gross gill lesions is acceptable as a farm monitoring tool. Removal of grossly affected tissue and subsequent histological examination is recommended to augment diagnostic accuracy.

Development of computer model

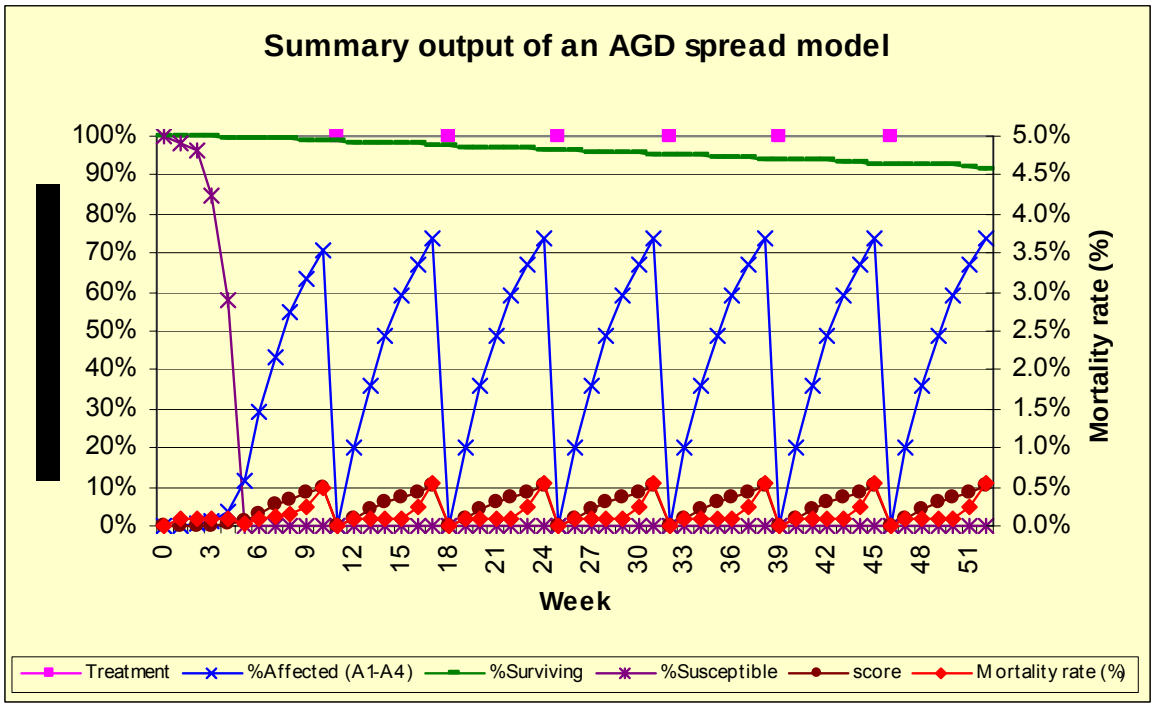
The results of the initial simulations are summarized in Figures 2 and 3. Briefly, for the parameter estimates used for these simulations, the number of Susceptible fish declined rapidly to zero within 5 weeks for both scenarios. The percentage of fish with lesions reached 70% after 11 weeks and 90% after 16 weeks if left untreated, at which time a total of 10% of fish had died. The weekly mortality rate remained below 0.5% for the first 10 weeks, after which it rose rapidly, reaching 5%/week after 17 weeks if untreated. Without treatment, 50% of fish had died by Week 24 of the simulation.

Figure 2: Summary output of an AGD model, assuming no treatment was applied



For the treatment scenario, the first treatment was administered in Week 11, and treatment had to be repeated every 7 weeks if weekly mortality rate was to be kept at or below about 0.5% per week. This resulted in 6 treatments during the year and a cumulative mortality for the year of <10%.

Figure 3: Summary output of an AGD model, where treatment was applied when the mortality rate in the pen was ≥0.5%



COMPARISON OF SIMULATION RESULTS WITH REAL DATA

Summary of 2003 data

Data for 28 pens of salmon for the 2003 year were summarised to allow comparison of patterns in the real data with model output, as discussed below. Where appropriate, six representative pens were used for comparison, rather than all pens.

Bathing frequency

Under the simulation described above, the first bathing was required after about 11 weeks, and subsequent bathings at about 7-week intervals. Bathing data for the first 6 baths for 28 pens for which data was available for 2003 are summarised in Table 6. The time to the first bath averaged 12 weeks, but varied from 6 to 25 weeks. Intervals between subsequent bathings averaged 5 – 10 weeks, but ranged from 3 to 22 weeks. Although the modelled intervals are similar to the mean values from 2003 data, the model does not exhibit the same degree of variability as was observed in the real data.

Table 6: Interval to first bathing and between subsequent bathings for 28 pens of salmon during 2003

Interval	Mean	Range
Weeks to Bath 1	12	6 - 25
Weeks to Bath 2	6	3 - 19
Weeks to Bath 3	10	4 - 22
Weeks to Bath 4	7	4 - 11
Weeks to Bath 5	5	4 - 6
Weeks to Bath 6	6	5 - 7

Prevalence

Prevalence of infection in affected pens is measured by inspection of a sample of 20-30 fish and calculating the percentage of fish inspected that have visible lesions due to AGD. In the model, prevalence was calculated as the percentage of simulated fish in States A1, A2, A3 or A4, because these States would be expected to have lesions visible. For the modelled scenario, prevalence rose slowly to about 70%, and then dropped to 0% after treatment, before rising again to about 70%, which corresponded to a mortality rate of about 0.5% per week. In a sample of 6 representative pens from 2003, prevalence generally peaked at between 70 and 100% prior to bathing and then dropped an unknown amount before rising again for the next bathing. Inspections are not routinely undertaken soon after bathing, and many affected fish still have resolving lesions during this

period, making estimation of prevalence difficult. Simulated prevalence in the model generally peaked at a lower level than was observed in the data, and then rose rapidly again following treatment.

AGD Score

A summary AGD Score can also be calculated from the results of periodic inspections, using a simple formula:

AGD score = 0.1 X proportion with mild lesions + 0.2 X proportion with moderate lesions + 0.3 X proportion with severe lesions

This results in a scale of severity from 0 to 0.3, depending on both the proportions of affected fish and the severity of lesions in those fish. For the model, State A1 was classified as mild lesions, A2 as moderate lesions and A3 and A4 were classified as severe lesions.

The simulated score from the model in an untreated pen reached 0.2 when the weekly mortality rate reached about 4% (see Figure 3). In the treated simulation, score remained consistently low, peaking at about 0.1 when mortality rate reached 0.5%, prior to treatment. In contrast, in data for selected pens from 2003, scores fluctuated from close to zero soon after bathing up to 0.20 – 0.25 prior to the next bath, when mortality rates were often in the range 0.1 – 0.3%. AGD score calculated from the model was generally lower than AGD scores from 2003 data at comparable times.

SENSITIVITY ANALYSIS

A sensitivity analysis was run for the model, to determine which parameters were likely to be most influential in the final result. For these analyses, individual parameters were changed, while the remaining parameters were kept constant at their initial values.

Contact rate (a)

Contact rate was varied to 20 and 1,000 contacts per week, while holding other parameters constant. At a contact rate of 1,000, infection spread more rapidly, so that there were no Susceptible fish left after 3 weeks (instead of 5), and the first bathing needed to be one week earlier. For a Contact rate of 20, it took 14 weeks for the number of Susceptible fish to decline to zero, and the first bathing could be put back to about 17-18 weeks. Mortality rates and subsequent bathing intervals remained virtually unchanged for this wide range of assumed contact rates.

Transition rate L>A1 (b)

Doubling the transition rate for Latent to A1 from 0.2 to 0.4 resulted in an increased prevalence (peaking at almost 100%) and AGD score (peaking at about 0.15) and a reduction in the interval between baths to about 6 weeks. Conversely, halving the transition rate resulted in lower peak prevalence (50%).and AGD score (0.9) and slightly longer inter-bathing intervals (8 weeks).

Transition rate A1>A2 (c), A2>A3 (d) and A3>A4 (e)

Doubling these transition rates individually resulted in a reduction in the interval between baths to about 6 weeks and a slight decrease in peak prevalence (about 60%), while halving them resulted in slightly longer inter-bathing intervals (8 weeks) and a slight increase in peak prevalence (80%).

Halving of all the rates together resulted in an increase in inter-bathing interval to about 11 weeks, in the interval to first bathing to 16 weeks and in a slight reduction in peak prevalence (about 60-65%). Doubling all the rates together resulted in a slight decrease in inter-bathing interval to about 6 weeks, in the interval to first bathing to 9 weeks and in an increase in peak prevalence to about 90%. For this scenario, peak AGD scores also increased to about 0.2 prior to bathing.

Mortality rate

Doubling (to 0.2) or even quadrupling (to 0.4) the mortality rate for fish in the A4 State had little effect on prevalence or inter-bathing intervals. However, higher mortality rates resulted in very sudden increases in mortality in the simulated pen, so that timing of bathing became much more critical if it was to prevent excessive losses occurring. Conversely, halving the mortality rate resulted in a slower build-up of losses, providing increased flexibility in timing of bathing.

Infection equivalents of A1-A3 States

The numbers of fish in each of the A1, A2 and A3 States required to equal one fish in the A4 State were increased to a four-fold relationship (A1=64, A2=16 and A3=4), so that fish in the earlier stages of infection made a relatively smaller contribution to the overall level of exposure for Susceptible fish. This increase resulted in a slowing of the initial spread of infection, so that it took 8 weeks for the number of Susceptibles to decline to zero, and 13 weeks to the first bath. It did not affect the subsequent inter-bathing intervals or peak prevalence reached.

Treatment effect

If treatment resulted in 90% of infected fish reverting to Susceptible and 10% to Latent (instead of 100% Latent), the inter-bathing interval was extended to 9 weeks instead of 7, but peak prevalence and AGD score remained unchanged. Alternatively, if treatment was assumed to result in 50% of fish reverting to A1 and 50% to Latent, the inter-bathing interval was reduced to 5-6 weeks, and timing of bathing became more critical to prevent rapidly increasing losses.

Effect of external exposure

The model was varied to assume that there were no infected fish initially, but at either 1% or 0.1% of fish were exposed to an external source of infection each week. These changes resulted in the first bath being delayed by 1 week (for p = 1%) or 3 weeks (for p = 0.1%), with no change to subsequent inter-bathing intervals, peak prevalence or AGD score estimates.

Based on the above analysis, this model appears to provide a reasonable approximation of the behaviour of AGD in a salmon population, although

prevalence and AGD score were generally underestimated. However, the usefulness of the model is constrained by lack of knowledge of key parameters such as contact and transition rates. Modelling of AGD is further complicated by the apparent variation in behaviour of the disease depending on season, whether pens are lit or not and other factors (temperature, salinity, etc). This degree of variability is very difficult to model in a simple deterministic model such as that presented here.

Although a more complex model could be developed using a stochastic approach (incorporating random variation) and allowing temporal variation in parameters, this would add significant complexity to the model, which would not be supported by our current knowledge of the values and behaviour of important parameters driving AGD infection.

Despite the limitations of this model, some useful conclusions could be drawn from the model.

1. Assuming a relatively high contact rate (>100 contacts/fish/week) virtually all fish in a pen have been exposed to infection within the first 5-7 weeks after introduction of infection. Even at quite low contact rates (20 contacts/week) all fish have been exposed by about 14 weeks.

2. Ongoing external exposure to infection was relatively unimportant once a pen became infected, at an assumed high contact rate among fish within the pen.

3. The assumed effectiveness of treatment by bathing has a substantial impact on the inter-bathing interval, depending on the proportions of affected fish that revert to Susceptible (uninfected), Latent or A1 States. The higher the proportion of fish that remain infected and in A1 or Latent States the shorter the interval until another bath is required.

4. At higher assumed mortality rates for fish in A4 State (0.2/week), timing of bathing was critical to avoid a rapid increase in the number of deaths occurring.

5. Simulated prevalence of clinically affected fish (States A1-A4) of 70-90% was reached prior to bathing, where bathing was imposed when weekly mortality rates approach 0.5%. These levels were slightly lower than peak prevalence levels observed in affected pens in 2003.

Bathing frequency for 2002

Under the simulation described above, the first bathing was required after about 11 weeks, and subsequent bathings at about 7-week intervals. Bathing data for the first 8 baths for 29 pens for which data were available for 2002 is summarised in Table 7. The time to the first bath averaged 15 weeks, but varied from 5 to 32 weeks. Intervals between subsequent bathings averaged 7 – 13 weeks and ranged from 3 to 22 weeks.

The modelled intervals are consistently shorter than the mean values from the 2002 data, and there was considerable variability between pens and between bathings in the real data that did not occur in the model. Model input parameters could be modified to produce longer inter-bathing intervals, similar to those observed in the data, however, current knowledge of the disease and its epidemiology is insufficient to reliably reproduce the observed variability.

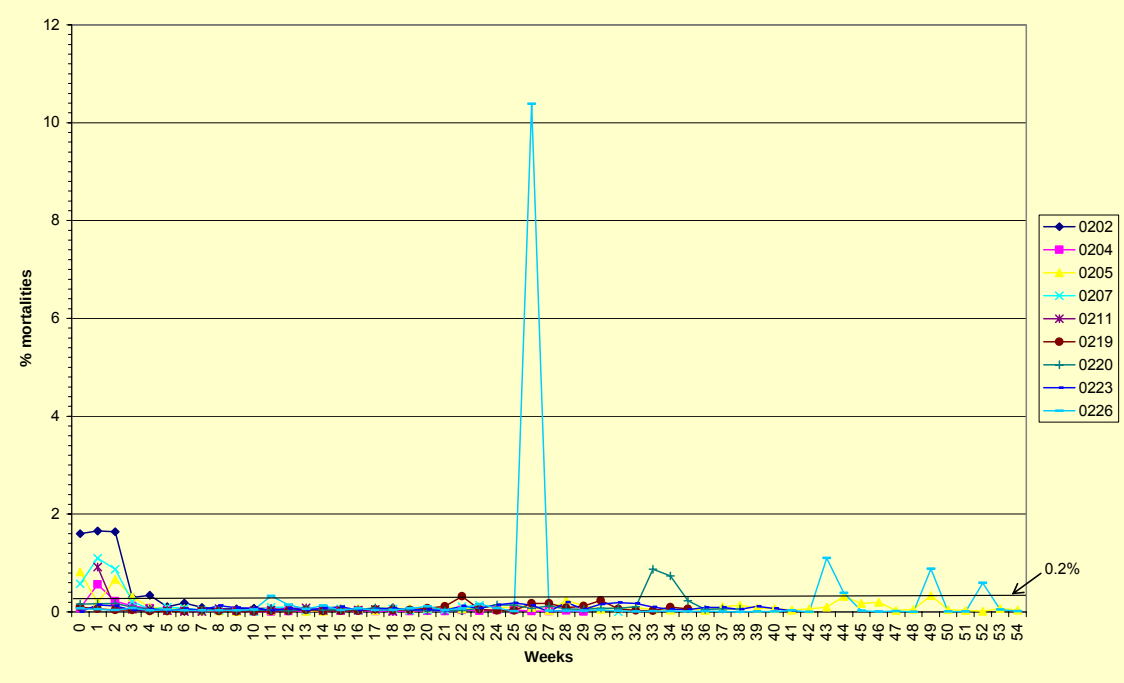
Table 7: Interval to first bathing and between subsequent bathings for 29 pens of salmon during 2002

Interval	Mean	Range
Weeks to Bath 1	15.2	5 - 32
Weeks to Bath 2	8.5	3 - 20
Weeks to Bath 3	8.2	3 - 24
Weeks to Bath 4	6.6	4 - 14
Weeks to Bath 5	7.3	4 - 17
Weeks to Bath 6	11.1	5 - 24
Weeks to Bath 7	13.4	5 - 22
Weeks to Bath 8	8.5	7 - 10

Mortality rate for 2002

In the modelled scenario, the mortality rate in infected pens remained at background levels (0.1%) for some weeks after bathing, before rising as the prevalence and AGD score in the pen increased prior to the next bathing. In contrast, in the 9 pens examined from the 2002 data, mortality rates remained consistently low (<=0.2%) except for occasional mortality 'spikes' in a couple of pens (see Figure 4).

Figure 4: Weekly total mortality rates for 9 selected pens from 2002.



Prevalence for 2002

Prevalence of infection in affected pens is estimated by inspection of a sample of 20-30 fish and calculating the percentage of fish inspected that have visible lesions due to AGD. In the model, prevalence was calculated as the percentage of simulated fish in States A1, A2, A3 or A4, because these States would be expected to have lesions visible. For the modelled scenario, prevalence rose slowly to about 70%, and then dropped to 0% after treatment, before rising again to about 70%, which corresponded to a mortality rate of about 0.5% per week.

In a sample of 9 representative pens from 2002, prevalence generally peaked at between 80 and 100% prior to bathing and then dropped an unknown amount before rising again for the next bathing. Inspections are not routinely undertaken soon after bathing, and many affected fish still have resolving lesions during this period, making estimation of prevalence difficult. Simulated prevalence in the model generally peaked at a lower level than was observed in the data, and then rose rapidly again following treatment.

In addition, in several pens, extended periods of variable prevalence were observed, when prevalence varied in a range from about 30% to 70%, sometimes declining to 20%, without treatment. These periods extended in some instances for >6 weeks, and up to 10-12 weeks. In contrast, modelled prevalence dropped following bathing and then increased steadily until the next bathing.

AGD Score for 2002

A summary AGD Score can also be calculated from the results of periodic inspections, using a simple formula:

AGD score = 0.1 X proportion with mild lesions + 0.2 X proportion with moderate lesions + 0.3 X proportion with severe lesions

This results in a scale of severity from 0 to 0.3, depending on both the proportions of affected fish and the severity of lesions in those fish. For the model, State A1 was classified as mild lesions, A2 as moderate lesions and A3 and A4 were classified as severe lesions.

In the modelled scenario, AGD score remained consistently low, peaking at about 0.1 when mortality rate reached 0.5%, prior to treatment. In contrast, in data for selected pens from 2002, scores fluctuated from close to zero soon after bathing up to >0.20 prior to the next bath. As was observed for prevalence, extended periods of fluctuating AGD scores without bathing were also observed.

CONCLUSION

Although the current model provides some useful insight into the behaviour of AGD in farmed salmon, it is unable to adequately simulate the complex epidemiology of this disease. There are several reasons for this limitation:

1. Firstly, the model itself is a very simplistic representation of a complex biological system, and therefore results in an over-simplified version of reality.

2. The model is deterministic in nature, so that it produces only a single 'average' outcome for any set of input values. In contrast, a stochastic model would allow random variability in model outcome (as observed in the data) but at the expense of a substantially more complex model.

3. Parameter values for model inputs are largely unknown and were therefore derived as best-guesses or by trial-and-error from attempts to fit the model to 2003 data. Better knowledge of true parameter values would allow improved design and fit of the model.

4. There are epidemiological aspects of the disease (for example effects of temperature, lighting and salinity) which have a major impact on disease occurrence, but are still not well understood. Such effects could account for extended periods when AGD was present but not progressing in some pens. These factors are not represented in the model at all and would be very difficult to incorporate without making it substantially more complicated.

It might be possible to construct a more complex model that provided a better representation of AGD dynamics in farmed salmon, but this would depend on an improved understanding of the epidemiology of the disease, quantitative estimates of the impact of potential risk factors on AGD progression and improved parameter estimates for the model. Currently, there is no understanding of contact rates and transition parameters under varying conditions of salmon culture. Overall, the model has highlighted some specific deficiencies in knowledge that could be targets for future research.

Benefits

Epidemiology research is crucial for our understanding of disease patterns, causation and risk factors. The Tasmanian salmon industry will benefit directly from this research project. Additionally, methods and approach developed will be applicable to other aquaculture industries, in particular those based on finfish cage-culture. Currently, FRDC project 2003/225 is using a similar approach for studying Southern Bluefin Tuna (SBT) health, in particular epidemiology of blood fluke infections of SBT. Additionally, this project provided long-term training to a postdoctoral fellow and a PhD student and short term training to other researchers, fish health practitioners in public service and industry representatives. Thus, this project significantly improved our capacity in the area of aquaculture epidemiology. We facilitated meetings and discussions with the salmon industry, resulting in a better understanding of farm trials, farm data collection and utilisation. While there was no consensus on common access to data, and a farm database was not built, individual companies were able to apply information from this project at the company level. A draft best husbandry protocol for AGD was developed in collaboration with salmon industry and will be continuously updated by other AGD projects in the Aquafin CRC program. It has educational value and sets a benchmark reflecting our current understanding of AGD and best management strategies. This project significantly increased our knowledge of AGD epidemiology and identified the knowledge gaps in this area.

Further Development

While this project has increased our understanding of AGD epidemiology, it has also identified knowledge gaps. The main challenge for future research on the relationship between the presence of the amoebae in the environment and AGD outbreaks is the accurate detection and quantification of *Neoparamoeba* sp. in environmental samples. Furthermore we are not able to estimate contact rates and transition parameters, necessary for the development of a model for the epidemiology of this disease.

Results of this project have been widely disseminated throughout the Tasmanian salmon industry through industry meetings, workshops and through the Aquafin CRC and Atlantic Salmon Aquaculture Subprogram conferences. The results can be exploited commercially by individual companies if they choose to adopt any of the suggested strategies. A number of attempts were made to develop an industry-wide approach to AGD. While a discussion paper on value adding to farm data and a trial database were provided to the industry, there was no support for the development of an industry database for AGD.

A draft best husbandry protocol was developed and it will be continuously updated by other AGD projects in the Aquafin CRC program. It has educational value and reflects our current understanding of best management strategies. Finally, gaps in our understanding of AGD identified in this project, including effects of husbandry procedures not fully investigated here (for example the use of artificial lights and effects of stock type) are investigated by FRDC project 2004/214, which commenced in July 2004.

Planned Outcomes

Improved prevention and management of AGD based on sound epidemiological knowledge

Research results showed that some husbandry methods (for example fallowing) may reduce the impact of AGD on salmon industry. For other methods we confirmed the lack of impact on AGD (for example preliminary trials suggest lack of impact of antifouling paints on AGD and lack of impact of artificial light on AGD). We have also reviewed and identified risk factors affecting AGD outbreaks. This information could result in improved prevention and management of AGD, provided that the benefits from the change in husbandry practices outweigh the costs of this change.

Epidemiology courses and industry workshops resulting in sound interpretation of disease patterns and farm records

The following courses were provided:

Introduction to Epidemiology for Aquaculture Workshop - 5-7 November 2001, Hobart. Workshop conducted by Dr Chris Baldock, AusVet, attended by 12 industry representatives, 1 CSIRO representative, 2 TAFI representatives, 2 DPIWE representatives, no cover fee.

Advanced Epidemiology Workshop - 30.09 - 4.10.2002, Launceston. Workshop conducted by Dr Angus Cameron, AusVet, attended by 3 salmon industry representatives (Dr Jo Sadler, Dr Dom O'Brien, Innes Weir, 3 CRC researchers, 4 CRC PhD students, 1 CRC technician, 2 DPIWE fish health experts, no cover fee.

Design and analysis of field trials - 3-4 February 2003, Launceston. Workshop conducted by Dr Chris Baldock, AusVet, attended by Dr Marianne Douglas-Helders, Dr Rick Butler, Ms Carley Bagley, Dr Stephen Pyecroft (DPIWE), Dr Cameron Bell (DPIWE), Dr Barbara Nowak, no cover fee.

Identification of risk factors for disease outbreaks - 11-13.03, Launceston. Workshop conducted by Dr Chris Baldock, AusVet, attended by Dr Marianne Douglas-Helders, Dr Rick Butler, Ms Carley Bagley, Dr Stephen Pyecroft (DPIWE), Dr Cameron Bell (DPIWE), Dr Barbara Nowak, no cover fee.

Introduction to Epidemiology, 1-3 December 2003, course leaders: Dr Marianne Douglas-Helders and Dr Rick Butler, attended by 12 participants, including three from salmon industry.

Disease Investigation, 18-19 August 2003, Launceston, course leader/presenter: Dr Chris Baldock AusVet, attended by 6 participants, including 2 DPIWE staff.

Industry workshops and pilot surveillance system resulting in maximisation of benefits from farm records

Following a number of industry workshops, individual farm visits, a discussion paper on maximising benefits from farm records and a trial database, the salmon industry decided that there was no interest in this area of the project. Individual advice was provided to interested companies on the improved use of their data. The discussion paper is provided as Appendix 2.

AGD outbreaks forecasting potential as a consequence of improved epidemiological knowledge

While a model was developed based on limited farm data provided, it has some serious limitations, including inability to simulate outbreaks in other years. Currently available data do not allow estimation of contact rates and transition parameters under varying conditions in salmon cages.

A sound basis for future epidemiological studies in aquaculture industry

This project provided a basis for future epidemiological research in the aquaculture industry. A similar approach and methods are used in Aquafin CRC - FRDC project 2003/225 *"Effects of husbandry methods on SBT health"*. In particular, the database approach, epidemiology training and research methods to study epidemiology of blood flukes have been used in the new project.

This project also provided long-term epidemiology training for one postdoctoral fellow and one PhD student who can continue working in the area of aquaculture epidemiology.

Scientifically based best husbandry protocol for control of AGD

A scientifically-based best husbandry protocol was prepared and distributed to the salmon industry for comments. Any comments received before the completion of this report have been included in the current draft. The draft is provided as Appendix 4 in this report. This draft will be continuously updated by other AGD projects within Aquafin CRC to reflect our understanding of the best management of AGD on the salmon farms.

Conclusion

Objective 1. Identification of *Neoparamoeba pemaquidensis* reservoirs.

During the project a second species of *Neoparamoeba* sp. was described. It was determined that none of the methods used could differentiate between these two species. As both species *Neoparamoeba pemaquidensis* and *Neoparamoeba branchiphila* are associated with AGD and have been isolated from the gills of salmon with AGD in the field and in experimental infection tank, we consider that they are both of interest. Methods to differentiate between these two species are currently being developed, however all previous research (unless done *in vitro* on known clones) results have to be considered applicable to both species and not just *Neoparamoeba pemaquidensis*.

Two seasons of field sampling identifying reservoirs of amoeba focused on sediment and netting. Sediment was also investigated in more detail on two salmon leases. It is obvious that *Neoparamoeba* sp. is a common organism which can be consistently isolated from marine sediments.

The main challenge remains quantification of *Neoparamoeba* sp. in the environmental samples. Some progress has been achieved, but only with samples spiked with very high numbers of cultured amoebae (clone PA027).

Even with limited methods we could show that *Neoparamoeba* sp. is present in environmental samples even in areas which are negative for AGD. However, lack of quantitative methods could be the reason why no relationship could be detected between the presence of the amoeba in the environment and AGD outbreaks.

Objective 2. Identification of risk factors of AGD including the spatial relationship between infected and uninfected cages.

Cage rotation after freshwater bathing significantly improved fish performance, including increased time to freshwater bath and increased weight gain. Amoebae were more consistently isolated from nets painted with copper-based antifouling paints, however no relationship between presence of amoebae on the nets and severity of AGD could be found. Field trials investigating the effects of artificial lights and the relationship between maturation and AGD outbreaks are continuing. *In vitro* studies on the sensitivity of *Neoparamoeba* to water quality factors (including salinity, temperature, copper and tannins) have been completed, and confirmed that exposure to copper and low salinity are the main factors affecting growth of the amoeba.

Objective 3. Development of a pilot surveillance system.

After discussions with industry it was decided that instead of development of a pilot surveillance system, which required industry participation in an AGD database and provision of farm results, a predictive model would be developed. The model reflects changes in prevalence of AGD over time in a single cage and improves understanding of the epidemic behaviour of the disease. This model is based on expert knowledge (from both industry and researchers) and was developed in collaboration with researchers and salmon industry. One company provided limited production data from one year for one stock type to assist in model development. This model is presented as a part of this report. It has a limited value due to our inability to validate it on another data set from a farm from a different year. This suggests that our current knowledge and available data do not allow estimation of contact rates and transition parameters under varying conditions in a salmon cage. At the same time, general dynamics of the disease description resulting from this model can be accepted. The process highlighted some specific deficiencies in our knowledge of AGD epidemiology which could be targets for future research.

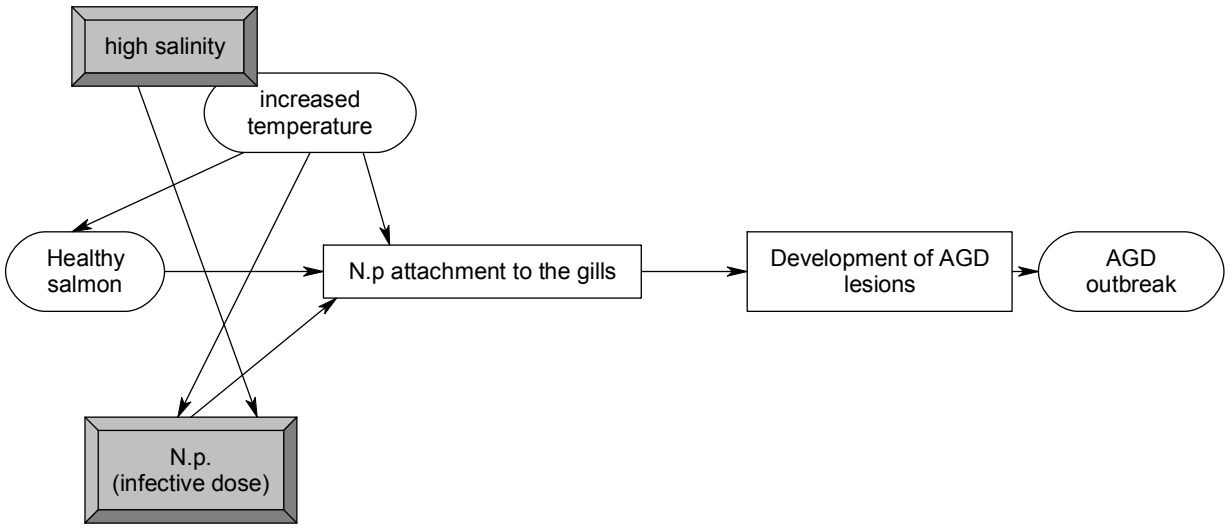


Figure 5. Flow chart showing our current understanding of AGD outbreaks. Shaded 3D rectangles are most significant in an AGD outbreak. While AGD outbreaks can occur in low temperatures, they do not happen unless salinity is high. N.p. - *Neoparamoeba* sp.

References

Adams, ,M.B., Ellard, K., Nowak, B. (2004) Gross pathology and its relationship with histopathology of amoebic gill disease (AGD) in farmed Atlantic salmon *Salmo salar* L. Journal of Fish Diseases, 27, 151-161.

Aklaghi M, Munday BL, Rough K, Whittington RJ (1996) Immunological aspects of amoebic gill disease in salmonids. Dis. Aquat. Org 25, 23-31

Bridle, A.R., Butler, R., Nowak, B.F. (2003) Immunostimulatory CpG oligodeoxynucleotides increase resistance against amoebic gill disease in Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases, 26, 367-371.

Clark, A. and Nowak, B.F. (1999) Field investigations of Amoebic Gill Disease in Atlantic salmon, *Salmo salar* L., in Tasmania. Journal of Fish Diseases, 22, 433-443.

Crosbie, P.B.B., Nowak, B.F., Carson, J. (2003) Isolation of *Neoparamoeba pemaquidensis* Page, 1987 from marine and estuarine sediments in Tasmania. Bulletin of European Association of Fish Pathologists, 23, 241-244.

Douglas-Helders, M., Nowak, B., Zilberg, D. and Carson, J. (2000) Survival of *Paramoeba pemaquidensis* on dead salmon: implications for management of cage hygiene. Bulletin of European Association of Fish Pathologists 20, 167-169.

Douglas-Helders, M. Carson, J., Howard, T., Nowak, B., (2001) Development and validation of a new dot blot test for the detection of *Paramoeba pemaquidensis* (Page) in fish. Journal of Fish Diseases 24, 273-280.

Douglas-Helders, M., Dawson, D., Carson, J. and Nowak, B.F. (2002) Wild fish are not a significant reservoir of Amoebic Gill Disease, Journal of Fish Diseases, 25, 569-574.

Douglas-Helders, M., Tan, C.F.K., Carson, J., Nowak, B.F. (2003a) Effects of copper-based antifouling treatment on the presence of *Neoparamoeba pemaquidensis* Page 1987 on nets and gills of reared Atlantic salmon (*Salmo salar*), Aquaculture, 221, 13-22.

Douglas-Helders, M., O'Brien, DP, McCorkell BE, Zilberg D, Gross A, Carson J, Nowak B (2003b) Temporal and spatial distribution of paramoebae in the water column - a pilot study. Journal of Fish Diseases, 26, 231-240.

Douglas-Helders G.M., Weir I.J., O'Brien D.P., Carson J., Nowak B.F. (2004a) Effects of husbandry on prevalence of amoebic gill disease and performance of reared Atlantic salmon (*Salmo salar* L.). Aquaculture, 241, 21-30.

Douglas-Helders, M, Saksida, S, Nowak, B.F (2004b) Questionnaire-based risk assessment for amoebic gill disease (AGD) and evaluation of freshwater bathing efficacy of reared Atlantic salmon (*Salmo salar* L.) Diseases of Aquatic Organisms, in press.

Douglas-Helders, M., Nowak, B.F., Butler, R. (2004c) The effect of environmental factors on the distribution of *Neoparamoeba pemaquidensis* in Tasmania. Journal of Fish Diseases, submitted.

Dyková I, Figueras A, Novoa B (1995) Amoebic gill infection of turbot, *Scophthalmus maximus*. Folia Parasitol. 42, 91-96

Dyková I, Fiala I, Lom J, Lukeš J (2003) *Perkinsiella amoebae*-like endosymbionts of *Neoparamoeba* spp., relatives of the kinetoplastid *Ichthyobodo*. European Journal of Protistology 39, 37-52.

Dyková, I., Nowak, B.F., Crosbie, P.B.B., Fiala, I., Pecková, H., Adams, M., Macháčkova, B., Dvořáková (2005) *Neoparamoeba branchiphila* n. sp. and related species of genus *Neoparamoeba* Page, 1987: morphological and molecular characterisation of selected strains. J. Fish Diseases, in press.

Elliott, N.G., Wong, F., Carson, J. (2001) Detection and abundance of Paramoeba species in the environment. FRDC Project 98/209 report. 68 pp.

Foster C, Percival S (1988) Paramoebic gill disease, Occurrence of *Paramoeba* in Tasmania. Saltas Aquanotes 15, Dover, Tasmania

Green, T.J. (2003) Exploitation of water chemistry in the treatment of amoebic gill disease (AGD). *Honours thesis*, 112 pp., University of Tasmania, Launceston, 2003

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.

Howard, T., Carson, J. (1993) Progress report: are there alternatives to freshwater treatment of AGD?. Saltas R & D Review Seminar, Hobart, Tasmania.

Howard, T. (2001) Paramoebiasis of sea-farmed salmonids in Tasmania - a study of its aetiology, pathogenicity and control. PhD thesis, University of Tasmania, 367 pp.

Martin RE (1987) Adhesion, morphology, and locomotion of *Paramoeba pemaquidensis* Page (Amoebida, Paramoebaidae): Effects of substrate charge density and external cations. J. Protozool. 34 (4), 345-349

Morrison, R.N., Crosbie, P.B.B., Nowak, B.F. (2004) The induction of laboratory-based amoebic gill disease revisited. Journal of Fish Diseases, 27, 445-449.

Munday BL, Zilberg D, Findlay V (2001) Gill disease of marine fish caused by infection with *Neoparamoeba pemaquidensis*. J. Fish Dis. 24, 497-507

Nowak, B. (2001). Qualitative evaluation of risk factors for Amoebic Gill Disease in Atlantic salmon. In: Proceedings of the OIE International Conference on Risk Analysis in Aquatic Animal Health, Paris, France, 8-10 February, 2000. (Ed: Rodgers, C.J.). Office International des Epizooties (O.I.E.), 148-155.

Page FC (1983) Marine Gymnamoebae. Institute of Terestial Ecology, Culture Centre of algae and protozoa, pp. 1-54, Cambridge, UK

Parsons, H., Powell, M., Fisk, D. and Nowak, B. (2001). Effectiveness of commercial freshwater bathing as a treatment against Amoebic Gill Disease in Atlantic salmon. Aquaculture, 195, 205-210.

Powell MD, Clark GA (2003) *In vitro* survival and the effect of water chemistry and oxidative chemical treatments on isolated gill amoebae from AGD affected Atlantic salmon. Aquaculture 220, 135-144

Tan, C., Nowak, B.F. and Hodson, S.L. (2002) Biofouling as a reservoir of *Neoparamoeba pemaquidensis* (Page 1970), the causative agent of amoebic gill disease in Atlantic salmon. Aquaculture, 210, 49-58.

Wilson, T., Carson, J. (2001) Rapid, high-throughput extraction of bacterial genomic DNA from selective-enrichment culture media. Letters in Applied Microbiology 32, 326-330.

Wong, F.Y.K, Carson, J., Elliott, N.G. (2004) 18S ribosomal DNA-based PCR identification of *Neoparamoeba pemaquidensis,* the agent of amoebic gill disease in sea-farmed salmonids, Diseases of Aquatic Organisms 60, 65-76.

Zilberg D., Findlay V.L., Girling P., Munday B.L. (2000) Effects of treatment with levamisole and glucans on mortality rates in Atlantic salmon (*Salmo salar* L.) suffering from amoebic gill disease. *Bulletin of the European Association of Fish Pathologists* 20, 23-26.

Zilberg, D., Munday, B.L. (2001) Responses of Atlantic salmon, *Salmo salar* L., to *Paramoeba* antigens administered by a variety of routes. Journal of Fish Diseases 24, 181-183.

Appendix 1
INTELLECTUAL PROPERTY

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

1. Copyright of this report

Appendix 2
STAFF

Principal Investigator:
Barbara Nowak
School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania

Co-investigators:
Marianne Douglas-Helders
School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania

Phil Crosbie
School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania

Mark Adams
School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania

Richard Morrison
School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania

Rick Butler
School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania

Carley Bagley
School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania

Jeremy Carson
DPIWE, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania

Consultants:
AusVet Animal Health Services
Sea to Sky Veterinary Service

Appendix 3

Industry Options Paper on the Improved Use of Farm Records within the Tasmanian Salmon Industry

Prepared for Aquafin CRC by
Drs Angus Cameron and Chris Baldock
AusVet Animal Health Services
June 2002

BACKGROUND

The aggregation and collective use of information to benefit an industry without detriment to individual participants has been a feature of livestock production for some time and there are now examples in aquaculture. For example, systems have been developed in British Columbia (the Cooperative Assessment of Salmonid Health or CASH Program) and the University of Prince Edward Island's Animal Productivity & Health Information Network (http://www.aphin.upei.ca). Modern information management and internet technologies have greatly enhanced the value of such systems in recent times by providing web access to summary information while preserving the confidentiality of individual producer information through advanced electronic security mechanisms. By using secure web-based information systems, producers remain totally in control of confidential information in much the same way as occurs with internet banking.

The benefits extend well beyond the use of the actual information. Industry-based information systems also provide a platform to improve communication and build cohesiveness within an industry. In addition, system management leads to consensual processes for developing industry-wide standards for a whole range of measurement types. In particular, an industry-wide system provides the opportunity to improve:

- Record keeping to a minimum accepted level;
- Information flows throughout the industry;
- Production, health and financial management;
- Investigation of industry-wide problems such as AGD;
- Standardisation of common measurements.

In the case of the salmon industry in Australia, the relatively small number of producers provides a real opportunity to put in place a system that will contribute to industry sustainability and competitiveness well into the future.

An information system is a system for the collection, processing, storage, analysis, reporting and practical use of information. This paper deals with the options for the modification or extension of existing information systems, and for the development of new systems within the Tasmanian salmon industry. Focusing on i*nformation systems* rather than *farm records* allows a more complete understanding of all aspects of information processing.

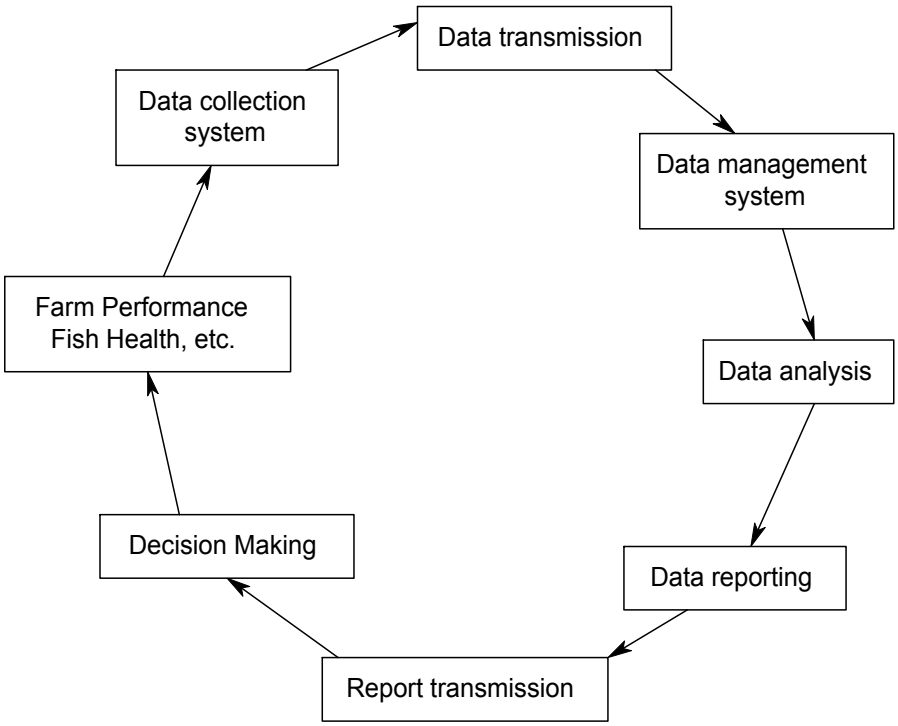
Industry prerequisites

Production and health issues are complex, multi-factorial and can require a long-term approach to finding solutions to particular problems such as AGD. Industry needs to be mature, internally cooperative and forward thinking to develop and implement an industry-based information system as it will take a long-term commitment. In addition, industry must be willing to provide direction and management for the system.

Individual companies must believe that improved information management has benefits that exceed costs and be willing to contribute both time and money to the project. They must have the capability to reliably record specifically agreed data and relevant staff must have appropriate computer expertise.

INFORMATION SYSTEMS

All salmon farms within Tasmania have existing information systems. All are different, and all have various advantages and disadvantages. Some are more sophisticated than others but all share the same basic components, as shown diagrammatically below.



All information generated on a farm is based on some aspect of the farm performance, and often related to the fish performance. This data are collected and transmitted to a data management system. Once analysed, reports are produced. The most important part of the system which is often implicitly assumed, but needs to be explicitly stated, is the use of the results of the analysis to make decisions, which in turn have an impact (preferably positive) on the farm performance that was being measured in the first place.

The development of an information system normally follows a logical step-wise progression:

- Identify the problem (current or potential)
- Identify indicators that enable one to detect the presence and magnitude of the problem
- Identify data sources required to calculate those indicators

- Identify mechanisms for the collection and management of those data sources.

This is an example of how, ideally, the development of an information system should be problem driven. The opposite approach is a data driven information system – a certain amount of data is available, and one asks what problems can it be used to address. The data driven approach to information system development (as is implied by the title of this options paper), runs the risk of ending up with an inefficient or unnecessary result – a solution in search of a problem.

To avoid this trap, this discussion will instead try to adopt the problem-driven approach to information system development. However, instead of focusing on the problems that have already been identified by producers (and in response to which the current information systems have been developed) it will explore other potential or unrecognised problems, and present options for information system development that may help address those problems.

In summary, the ideal approach is:

Here is a problem, what information do I need to solve it?

An undesirable alternative is:

Here is some information, what sort of problem will it help me solve?

The approach taken in this paper is:

What are some potential or unrecognised problems that we may need address, and what information may be need to address them?

It is worth clarifying some of the terminology used in relation to information systems, particularly the distinction between data and information. Data are the raw facts or measurements that are collected, for instance the weight of a fish*. Information is the result of analysis of data, and is the basis upon which decisions can be made. In this case, the* growth rate *represents information, derived from the analysis of weight data from numerous fish, at different times. It can be compared with growth standards to determine if a problem exists, and to plan appropriate responses to the problem.*

OBJECTIVES

As discussed, the structure of an information system is dependent on its overall objective, or the problem(s) which it is trying to address. This section will discuss a number of alternative objectives for salmon information systems. It is possible for a single system to meet a number of different objectives, but in designing a system, each objective needs to be clearly identified. One of the listed objectives, supporting on-farm management, represents the main purpose of existing farm-level information systems. All the others, however, have one important feature distinct from the existing systems - they require farms to share information at the industry level. Sharing of data is required for a range of reasons, but is more important in the Tasmanian salmon industry than many other comparable industries. This is because variation in production is high, and the amount of data that can be gathered in each farm is relatively low. In order for each farmer to get

some understanding of what is 'normal', examination of their own farm records is not sufficient. Instead it is necessary to use data from a larger population to overcome the large inherent variability and start to identify trends operating at the industry level. Issues arising from, and options to achieve, data sharing are discussed later in this paper.

The range of possible objectives for information systems considered are:

- On-farm management
- Benchmarking of production and financial performance
- Disease monitoring and control (endemic diseases)
- Disease surveillance (new and emerging diseases)
- Market support - product quality
- Industry support - welfare and environmental management
- Research

On-farm management

It is likely that the prime objective of most existing systems is to support on-farm management. This is because, on the establishment of a farm, day to day management decisions are the most obvious and pressing reason for collecting information.

As all farms are familiar with information systems for on-farm management, there is no need to describe such systems in detail. However, there are two issues that should be highlighted. The first is the approach used to analysing data for decision making. While some data analysis that is currently used is likely to be very sophisticated (such as the interpolation algorithms used for estimating biomass), other aspects of the data analysis may be overly simplistic, and interpretation of the data may be less rigorous that desired. An epidemiological approach to data analysis will provide greater confidence in the interpretation of the data. In essence, this involves adjusting the raw data to take into account other factors that may be influencing the observations, such as the population size, the season or other confounding factors.

For example, farms collect mortality records, classified by the apparent cause of mortality. In some cases, this data are only analysed by calculating the percent mortality due to the different causes over different time intervals. While this indicates the relative importance of the different causes, it does not, of itself provide any direct management advice. Time series analysis and other modelling techniques offer the potential to identify normal seasonal or production cycle patterns of mortality that may not be immediately apparent on the examination of raw data. Combination of the data with other environmental or management records may provide clues to the causal factors, and modelling may provide indications of the relative importance of a range of factors working together. Using these approaches, it may be possible to predict times of high risk for particular mortalities, and introduce management changes to minimise these risks.

The second issue relates to data management. Most farms are currently using computerised systems, but some still depend on manual paperwork. Despite the up-front cost of investing in hardware, establishing systems and training staff, the time and efficiency savings of using a computerised data management system will outweigh these costs in any commercial enterprise. This is largely due to the ability of computerised systems to use the same data for multiple different types of analysis instantly, using pre-programmed procedures. The time involved in performing repeated calculations manually means that either staff costs increase unreasonably, or, more likely, the data are not thoroughly analysed, and management decisions are based on a poor understanding of the true situation on the farm.

Benchmarking

In order to achieve optimal production, and maximum sustainable economic return from an enterprise, it is necessary to identify areas of production and economic performance that fail to achieve optimal levels. These weaknesses are identified by regularly measuring key production and performance indicators, and comparing them with standard targets. Where achieved production or performance falls below the target, the reasons need to be investigated and steps taken to correct any problems.

Implicit in this concept is the existence of standard production or performance targets, or benchmarks. However, in a relatively small and relatively new industry, such benchmarks are not yet defined. The use of standards from other parts of the world is inappropriate because the physical and economic environment in the Tasmanian industry is unique. Similarly, variations between seasons (both climatic and economic) mean that the use of fixed benchmarks is often inappropriate. The only way to assess a farm's performance accurately, is to compare it to the performance of other similar farms in the same area, at the same time. This requires continual re-evaluation of key indicators or benchmarks, and ongoing comparison of farm performance with these industry indicators.

The development of an information system with the objective of allowing the development and use of industry level benchmarks requires that appropriate indicators be first identified. A wide range of indicators are available, and any system should use a variety of different indicators to reflect the different factors that impact on farm profitability. A benchmarking system can also have a narrow, defined target - for instance examining only one key indicator, such as mortalities. However, in order to gain the maximum benefit from industry benchmarks, they should reflect the overall objectives of the industry. Where the industry objective is to achieve maximum sustainable profit for farmers, economic indicators should be included in the benchmarking. Examples include indicators such as the gross cost of production per kg harvested, profit per kg harvested etc. These should be supported benchmarks for the key components that contribute to profit, including health, growth, input costs etc.

Generation of benchmarks relating to farm performance requires that detailed information on farm performance be shared. However, as discussed below, this does not mean that farms need give other (competitor) farms access to their confidential information. Detailed farm-level data are used to generate benchmarks. These benchmarks are shared between farms, not the data used to generate them. This allows individual farms to confidentially evaluate their performance against that of their peers.

Disease monitoring and control

In this context, disease monitoring is used to refer to the ongoing collection and analysis of data related to diseases known to be present in the area, for the purposes of detecting changes in the distribution, level or impact of the disease. Monitoring may be designed to determine when a disease becomes a significant enough threat to warrant an intervention, or may be used to evaluate the effectiveness of an intervention. It may also be used to develop predictive indicators of the risk of disease, which allow action to be taken before the disease actually causes a significant problem.

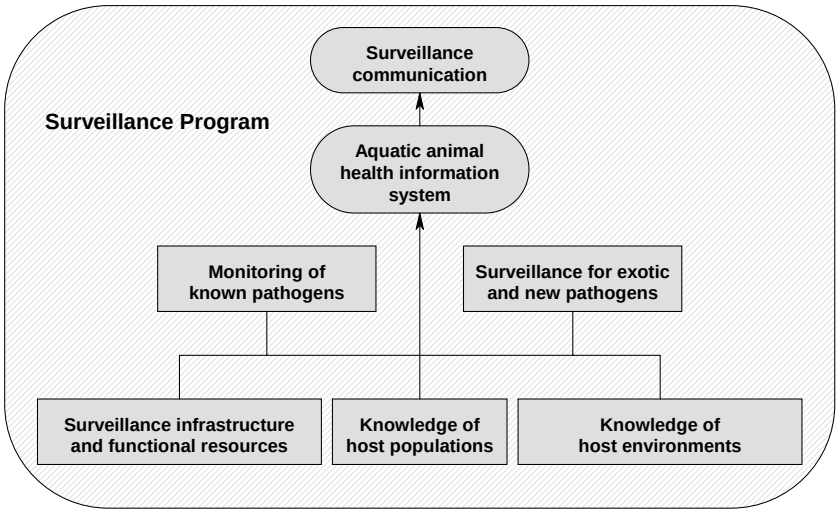
While monitoring performance (including disease as one indicator of performance) is a major component of a system for on-farm management, disease, and many factors which contribute to disease, are rarely confined to a single farm. In order to understand the distribution of disease and associated factors, it is necessary to look beyond the individual farm. Effective disease monitoring systems therefore rely on the sharing of data between farms.

Disease surveillance

In the narrow sense, disease surveillance relates to activities which aim to detect the incursion or emergence of a disease which was not previously present in an area. If effective, surveillance provides early warning of new diseases, allowing effective responses to be mounted before the disease becomes widespread or causes major problems.

In the broader sense, the term surveillance is used to encompass both surveillance and monitoring.
Disease surveillance is an integral and key component of all government aquatic animal health services who rely heavily on industry for information. Surveillance information is important for early warning of diseases, planning and monitoring of disease control programs; provision of sound aquatic animal health advice to farmers; certification of exports; international reporting and verification of freedom from diseases. It is particularly vital for animal disease emergency preparedness.

The structure of a surveillance program is stylised in the diagram below. As can be seen, a comprehensive approach includes having available a lot more information than just disease occurrence data.



By its nature, disease surveillance depends on the integration of data from across the area of interest. A national surveillance program requires the sharing of information from all farms with the relevant species, as well as data from wild stocks.

Market support

An important objective for an information system is to provide the data required to support continued or expanded market access. The objectives discussed previously have been related to ensuring the ability of farmers to successfully produce their product. This one relates to ensuring farmers can market the product.

There are two major areas of information required for effective market support. The first is information to demonstrate freedom from specified diseases of interest to the importing country. While closely related to disease surveillance, there is a subtle difference between early detection of disease incursions and providing evidence that a disease does not exist.

The second information area relates more specifically to product quality. Examples include provision of data demonstrating that the product is free from potentially harmful substances, such as chemicals, drugs or toxins such as heavy metals. This may be achieved either at the input or output stage, by recording detailed information on the composition and quality of all farm inputs, or by testing the product prior to sale.

Industry support

Whereas market support ensures the continued demand for product, industry support aims to ensure the continued viability of the industry within the domestic context. A range of pressures and threats may face farmers at an industry level, based on concerns ranging from environmental and welfare to social and political. One objective of a forward-looking information system may be to collect data which may be used to support arguments defending the industry against potential

future attacks. Monitoring these type of factors, and taking action when, for instance, potentially harmful environmental or welfare situations arise, will provide a good basis for responsible self-management of the industry, and strong defence against unreasonable moves for externally imposed controls.

Research

The last objective of an information system to be discussed is the collection of information for the purposes of research. It is rarely justifiable to establish a farm or industry-level information system solely for the purpose of possible future research. Instead, it is more common to take advantage of information collected for one or more of the other reasons listed above, and use it for research purposes. In most cases, detailed research will require the collection of different and more detailed information than is required for the other objectives. As a result, additional components often need to be added to existing information systems for the duration of a research project. These should be carefully designed with specific reference to the research objectives.

DATA COLLECTION AND MANAGEMENT

In establishing or expanding an information system, the data that are collected should be determined primarily by the objectives of the system. However, it is clear from the previous discussion that a system may have multiple objectives, and that some of the same data may contribute to a number of different objectives. Data collection is always associated with some cost. Decisions on what data collect depend on an assessment of the value of that data in achieving one or more objectives, the relative importance of the different objectives, and the costs of collecting the data. For instance, laboratory examination of individual sick fish is a relatively expensive data source. If the only objective of an information system is routine on-farm management, there may be little justification for the routine submission of samples to a laboratory. However, if disease monitoring, disease surveillance, market support or research form part of the objectives of the information system, the value of diagnostic information is far greater, and the expense more easily justified. In fact, seeking a diagnosis on cases of disease, usually through laboratory examination, is an essential component of a system with the objective of monitoring disease or market support through demonstration of freedom from disease.

CURRENT DATA MANAGEMENT

Most production and health recordings are made on a cage basis using day sheets. It would therefore seem that the cage is the most useful unit for the purposes of data management and analysis for epidemiological purposes. Data management systems range from highly sophisticated databases to paper-based systems, although all enterprises which were visited maintained some records in an electronic format. The more simple electronic systems were based on MS Excel spreadsheets. All systems use some form of mathematical model to forecast fish weights from feed inputs and the number of fish in the cage. Forecast weights are verified by periodic weightings. Although all sites have been accurately surveyed, none of the sites visited maintains exact location and

movement details of cages suitable for input into a Geographical Information System. All farm sites are required to have an environmental survey once every six months and these results in a report and an underwater video.

Tassal has a complex, relational database used to handle all production, health and environmental records. The system is called Infosal and was developed in-house using the Oracle database management software package. It has been in operation since 1996. Tassal decided to develop their own system after investigating a package in common use in the northern hemisphere called Superior. The system is capable of handling a vast array of data and operates over a wide-area network with farm data entered at the individual farms. The database has numerous routine reports. The software packages, Impromptu and PowerPlay are used to query the database for special analyses.

Aquatas have developed a production data management system in MS Excel. A new file is used for each cage for each month with files linked to provide long-term summaries. AGD data are recorded in Lotus Approach (database management software for PCs like MS Access) and are therefore not directly linked to production and mortality data for easy analysis. AGD is recorded by cage with score distributions and bathing dates.

Nortas use a commercial database package known as Fish Management System. It is a modification of a system developed in Australia for pearl oysters and has a crude site mapping facility to track the position history of cages. Data are recorded on a fish group/cage basis as well as the area of the farm where they are located. All data from each farm are now entered in the one location from hand-written data collection forms filled out at the different farms. Each farm is then provided with a weekly list of the last bathing date for each cage and the date of last sampling for AGD screening. The software package, Seagate Crystal is used for report generation. Nortas uses square cages in groups (system cages) at fixed sites whereas other companies have round cages which are more easily quarantined from one another.

Seafarms is a smaller operation. Again, data are recorded for each cage. Feeding and mortality details are hand recorded and entered into an MS Excel spreadsheet. Feed conversion ratios and stocking densities are determined monthly. Weather, dissolved oxygen (5 m) and temperature (5 m) are recorded at one site each day on paper but not computerised. Gill checks are undertaken once a month and recorded on the same sheet as weights but not computerised. Bathing dates and differential mortalities are also recorded and stored as paper files. This company is reported to have bathe less and generally to be less affected by AGD.

OPTIONS FOR CONSIDERATION

This section briefly outlines some of the issues related to the various options for improving information systems for the Tasmanian salmon industry. The options

and issues listed are not designed to be completely exhaustive but simply to highlight some of the major possibilities, opportunities and issues.

1. No data sharing

This option describes the status quo, where there is no structured collation or sharing of data between farms. This option implies that the only objective of the information system is to achieve effective on-farm management, and that the other objectives listed above are not currently industry priorities. In the absence of data sharing there are several options for improved use of data within individual farms. These options are also available if other objectives and data sharing were adopted at the industry level.

1.1 Improved on-farm analysis

This would involve a critical analysis of the specific information needs of individual farms, and an assessment of the data and data analysis techniques available to meet those information needs. Examples of improved techniques include the integration of multiple data sources, time-series or quality control analytical techniques, and modelling approaches for risk factor assessment and risk prediction.

1.2 Data collection and management techniques

There are several options for on-farm data collection and management. The first is to use a completely paper based system. The disadvantages of this include the time required to manage and analyse the data, problems retrieving historic data, and difficulty integrating and analysing multiple datasets. Computerised systems overcome these problems. When using computerised systems, on-site data recording can be achieved in a number of ways. The first is the manual recording of data on standard paper forms, and later data entry from the forms into the computer. This is considered the default approach. In some cases, automated data capture may be possible (eg in environmental monitoring), whereby a monitoring device either logs data for periodic electronic download, or has a direct connection to the computer database. This is only an option for those data sources which lend themselves to automated capture. A third option involves the use of handheld portable data terminals for on-site data capture. These units are specialised battery operated data capture devices designed for direct data entry away from a computer. One common application is in large retail stock-take operations (eg in supermarkets). These devices are programmable and can be customised to allow rapid, simple data entry of on-site observations. Current unit costs range between $500 for the simplest up to about $3000 for a water-resistant, shock-proof model with a large memory. These units can be combined with bar-code readers, GPS units or other automated data capture devices, and data can be downloaded to a computer via an infra-red link, modem, or direct cable connection. Key advantages of these systems include removing the time, labour and potential errors introduced by manually re-entering data from paper records, and rapid access to data, as well allowing the primary data collector to verify the accuracy of the data instantly.

2. Data sharing

The main option to maintaining the status quo, and pre-requisite to adopting any of the objectives listed above other than on-farm management, is to develop an information system that involves sharing of data between farms. There is a wide range of options and issues raised by this, some of which are discussed below.

2.1 Standards

In order to gain the potential benefits from shared data, for benchmarking, monitoring, surveillance, market support and so on, it is essential that the data from different farms can be compared and evaluated in a consistent manner. For this to be possible, it is necessary to first develop a set of agreed data standards. The normal process is to identify those data items that are desired to be shared or centrally collated, and then formulate a formal standard definition for each data item. Current examples of non-standardised data items include environmental measurements (many taken at different water depths) and gill scores. Standardisation does not necessarily mean that every farm has to do everything in exactly the same way, but simply that data from different farms are compatible and suitable for cross-industry analysis. While the simplest long-term solution to the use of different gill scoring systems is for all farms to adopt the same standard system, a short-term solution is to develop a translation table that can convert the scores used in each farm into a standard, comparable score. The process of standardisation is best achieved through the cooperative development of documented set of standards which are incorporated into each farms operational manuals. The standards need to cover not only data definitions but all aspects of the information system including recording and reporting frequencies and formats.

2.2 Range of data

The range of data that is handled by a shared-data information system depends on the objectives of the system, the relative importance of the different objectives, and practical and cost constraints associated with the collection of different data. As a rule of thumb, it is usually wisest to collect the minimum number of data items required to meet the identified objectives. Each data item under consideration should be carefully evaluated to determine its relative value, and how it will be used to achieve the desired system outcomes. There is often a temptation to collect large amounts of data, simply because it is available. If these data cannot be shown to contribute tangibly to the objectives, it only increases the cost of the system and dilutes the effort of those involved in collecting, managing and analysing the data. Brief examples of the main data categories that may be included for the different listed objectives are shown below:

2.2.1 On-farm management

Health, production, and management.

2.2.2 Benchmarking

Production, health, economic performance.

2.2.3 Disease monitoring

Disease and associated disease factors including management, production as an indicator of disease.

2.2.4 Disease surveillance

As for disease monitoring, focus on differential diagnosis and exclusion of disease.

2.2.5 Market support

Freedom from disease (as for disease surveillance), and product quality, including details of all production inputs and product safety and quality testing.

2.2.6 Industry support

Identification of the key industry threats and collection of monitoring data for key indicators of performance in each area. Eg. Environmental degradation, fish escapes, incidents causing potential suffering for animal welfare, etc.

2.2.7 Research

The data required for research needs to be determined in light of the specific research project.

2.3 Level of data to be shared

One important option that is raised when data are shared is the level of data that are shared. Data may be exchanged either in a raw, un-interpreted, un-summarised form, or selected key indicators derived from analysis of the raw data can be exchanged.

2.3.1 Raw (un-interpreted data)

There are two main advantages of the use of raw data. The first is that it removes the requirement for complex data analysis from the data provider, and similarly the requirement for standardisation in data analysis. The second, and more important advantage, is that raw, disagreggated data are available for multiple different types of analysis, and re-calculation of different indices. Analysed data can only indicate one result. For instance, monthly mortalities may be reported, but a sudden environmental change may indicate that weekly analysis of the data are required to clearly understand the impact of the change. Reanalysis of raw data poses no problem, but this is not possible if only monthly summaries are presented. Other advantages include increased transparency and the ability to assess data quality. It is strongly recommended that any system developed be based on the exchange of raw data.

2.3.2 Selected key indicators (analysed data)

The advantages of exchanging only analysed data include: the need to transfer much small volumes of data; lower data analysis requirements for the centralised data management system; and increased privacy and control over the data from individual farms. The first of these two advantages are not relevant when using automated digital data transfer and analysis systems. The issue of privacy will be discussed below.

2.4 Mechanism of sharing

There are a number of options for the physical flow of information from farms.

2.4.1 Exchange between farms (bilateral)

One option is the bilateral exchange of data between farms. This approach requires the establishment of bilateral relationships between all farms intending to share data, and fails to meet any privacy concerns. It will not be further considered.

2.4.2 One farm performs centralised analysis

An alternative is to nominate one farm as the data manager for the industry, and all data are submitted to that one farm. This approach has the advantage that larger farms are likely to have the existing staff, data management hardware and software required to manage the data, as well as the background experience in the industry to provide valid interpretation. It may also address some of the privacy concerns, as most farms will not have direct access to information supplied by other farms. However, the one coordinating farm will have access to all farms' data, and this may well raise concerns.

2.4.3 Third party (independent) performs analysis

The third option is for all farms to submit their data to an independent third party, with expertise in data management and analysis. The advantage of this option is that the organisation with access to individual data has no vested interest in using that data inappropriately. In other industries the use of an independent data manager has shown to be able to overcome many problems, including privacy concerns, organisational jealousy, and inadequate resourcing.

2.5 Data transmission

The options for data transmission include non-digital formats and digital formats. Non-digital formats include verbal reporting of data to a central site by telephone, and mailing or faxing hard-copy (either hand written or computer generated reports. In all cases this involves the re-keying of data, leading to increased time and cost requirements, as well as increasing the risk of data entry errors.

Digital data transmission systems involve the sending of disks through the mail, direct connection between two computers using a modem and telephone line, and using the Internet to transmit data, either as an attachment to an email, or through direct connection and incorporation into a database. As a generalisation, increased levels of automation require increased up-front development costs, but result in more significant cost savings during system operation, as they remove the need for ongoing routine manual data processing. While privacy is an obvious concern, there are mechanisms that ensure that the transmission of data across the Internet is highly secure, either through high-level encryption of attachments, or secure connections such as those used in Internet banking.

2.6 Data Repository

One requirement of effective use of shared data is the existence of a centralised data repository. There are a number of technical considerations as to the software used to manage the data which will not be dealt with here. However, there are two main options as to the siting of the data repository. One is to place it on a stand-alone computer, the other on a computer connected to and accessible from the Internet. While the later raises security concerns, once again, technologies exist to provide a very high level of security for data on the Internet. The main advantage of a web-accessible database is that it is able to provide interactive real time reporting. The underlying purpose of any information system is to provide information, so the nature of the reporting system is a prime concern. This is considered below.

2.7 Local data storage and submission

Options for data management on each of the participating farms have already been identified as either paper or computerised records. Computerised data can be managed by a range of different software, including word processors, spreadsheets, and generic or customised databases. Databases are the most appropriate tools for managing the types of data under discussion, as they are able to ensure greater consistency that spreadsheets. However, both spreadsheets and databases are able to integrate with centralised data management systems.

2.8 Privacy

It is important that adequate safeguards are built into any system to ensure that the privacy of data from individual farms is not compromised. If users have confidence in the privacy controls, even the most potentially sensitive information can be included in an information system, if it is able to contribute usefully to the objectives of that system. For example, financial performance benchmarking would provide very valuable information to farms, allowing them to evaluate the relative efficiency and profitability of their enterprise. This would require farms to submit financial records, naturally a highly confidential and sensitive form of data. However, if users develop confidence that no data within a system can ever be individually accessed or identified by other interested parties, then they may chose to include this as one aspect of a system.

Data collated into a centralised system, as is required to achieve the benefits of the objectives listed above, must be managed and analysed by a data administrator. This data administrator must be required to meet minimum privacy standards, and these standards will be easier to meet if the administrator is independent of the industry, as opposed to being a member of the industry. However, other than the administrator, there is no need for anybody other than the farm submitting the data to have access to the individual data.

This can be achieved through a clear separation of data submission and data reporting functions of a database. A system can be established so that once data go in, access can be fully controlled. This may range from the data being

accessible only to the administrator and the submitter, to encryption of the data meaning that nobody, including the administrator, is able to access that data. This data submission and data storage function is independent of the data analysis and reporting function of the database, in which data from many farms may be summarised to produce the required outputs.

One option presented in such a system is the point at which data are de-identified. The first option is to store information on the source of the data, but ensure that the data is fully de-identified during reporting. The second option is to de-identify the data during data submission, so that no identifying data is stored in the system at all.

2.9 Reporting

The final issue to be considered in this discussion is the mechanism used for reporting. The nature of the reports needs to be carefully designed to best meet the objectives of the system. The delivery of the reports should meet the specific requirements of the users of the system. For instance, regular hard copy report may be mailed to each participating farm, or transmitted over the Internet by email. Alternatively, reports may be made available to participating farms through an Internet web site. This last option means that farmers are able to get access to the most up-to-date information whenever they require it.

Reports may also follow a standard, pre-defined format, or could be user-customisable. This would mean that the user was able to identify the specific indicators, reporting period and so on that was of interest. This enables the information system to be used as a practical tool for investigating and solving problems. It does, however, required web-based reporting supported by a real-time on-line database.

RECOMMENDATIONS FOR IMPROVED COLLECTION AND USE OF DATA

Decisions on the most appropriate way to support and develop the industry are rightly the responsibility of the members of the industry. This options paper has been prepared to present the industry with some of the issues surrounding data management. The main decision required is the extent to which the industry needs to work together to ensure profitability and long-term sustainability. A range of challenges currently exist, for example in the form of disease problems. However it is likely that even more challenges will arise over the next few years. Decisions are required to effectively meet these challenges, and good decisions require good information.

This paper has avoided discussing whether pH or water temperature should be recorded once or twice a day or at 1 or 5 metres. Identification of the long-term objectives of the industry and the information required to support those objectives is much more important. While these decisions can only be made by the industry the authors would like to make the following recommendations:

- That due consideration be given to including aspects of all the objectives listed in this document as part of the objectives for an industry information system.

- That an information system to meet the identified objectives be developed by and for the industry. It is likely that this system will need to be progressively developed, and made more comprehensive as participants gradually gain greater confidence in both the security and benefits of the system. However, coordinated development will be made easier if a blueprint for the future is established at the outset.

- That industry information standards to be developed and adopted.
- That the technical details of any information system be developed based on a clear statement of current and future objectives and in close collaboration with all industry participants

Appendix 4

Best husbandry protocol - draft framework

- **Introduction**
- **Monitoring and Control of AGD**

  Monitoring

  - How is AGD diagnosed?

  - How frequently should a population be monitored for AGD?

  Control

  - What is the current recommended treatment for AGD?

  - What factors affect the success of freshwater bathing?

  - How effective is freshwater bathing in treating AGD?

  - How long can one expect the reduced paramoeba load to last?
- **Reducing incidence of AGD on the Atlantic salmon farm**

  -What are the risk factors associated with AGD?

  - Primary environmental risk factors

  - Secondary environmental risk factors

  - Management associated risk factors

  - How can these factors be reduced?
- **Reducing the spread of AGD within an area of Atlantic salmon farming**

  - What are the risk factors that appear to spread AGD between Atlantic salmon farms?

  - How can these factors be minimised?

  - What factors should be incorporated into area management for AGD?
- **In summary**

INTRODUCTION

Amoebic gill disease (AGD) is the most serious health problem in marine cultured Atlantic salmon in Tasmania. AGD is caused by the protozoan pathogen *Neoparamoeba pemanquidensis* (will be referred to as paramoeba in the following document). In addition to Tasmania, AGD has been reported in several other countries including Ireland, Chile, France, New Zealand and the United States.

In Tasmania, the paramoeba appears to be ubiquitous in the marine environment and can be found on the gills of Atlantic salmon year-round. However, clinical signs and elevated mortality rates are primarily seen in the summer and autumn. Mortality as a result of AGD in smolts can reach up to 10% per week, while in larger salmon the rate can be as high as 4% per week. The costs to the Tasmanian salmon aquaculture industry is high, not only as a result of the mortality attributed to AGD, but also due to losses in production due to poor growth associated with the disease, as well as the high costs of treatment.

Because of the ubiquitous nature of the paramoeba and since AGD appears to be endemic to parts of Tasmania, eradication of the disease by stock destruction is not an option. Therefore, to ensure sustainability of the aquaculture industry and the environment in which it operates, a management strategy must be established to control AGD. Best practices outlined in the following report will detail methods for disease management, pathogen avoidance and finally, pathogen load mitigation on the Atlantic salmon farm. Also, the role of area management strategy will be discussed as a method to further control AGD.

The most currently published scientific data were used to compile this report. This report should be reviewed and updated as new information becomes available.

MONITORING AND CONTROL OF AGD

Monitoring

How is AGD diagnosed?

- **Clinical signs**

  Clinical signs of AGD include lethargy, with the salmon swimming closer to the water surface, and respiratory distress, often noted by flaring of the operculum. Divers often note subpopulations of fish facing into the net on the side closest to the tide ("tv watching"). A decrease in appetite and increased mortality rates are also seen with AGD.

- **Gross signs**

  Increased mucous and white patches are seen on the gills of fish with AGD. Grading systems to determine the level of infection has been developed by each company, for example Tassal's scale has 5 levels of infection (Table 1).

Table 1 – Gross grading method for AGD (Tassal Pty Ltd)

Infection Level	Description
Clear (0)	Gills appear clear, healthy red
Very Light (1)	1 white spot/light mucous
Light (2)	2-3 white spots/ small mucous patch
Medium (3)	>3 white spots/ established thickened mucous patch
Heavy (4)	Established lesions / 2+ thickened mucous patch

- **Diagnostic tests**

  Various methods are available for the detection of the paramoeba: these include examination of wet mounts taken from gills, histology, IFAT and PCR. However since the paramoeba can be found on the gills year-round, the decision to treat is usually based on other factors, rather than the presence of the paramoeba. Those factors are: an increased number of fish exhibiting clinical signs, the observation of gross signs and logistics of the scale of operation. The scale of treatment of the whole site may significantly influence the timing of treatment on some farms.

How frequently should a population be monitored for AGD?

- **Monitoring of mortalities should occur on a year-round basis**

  However, surveillance should increase to include moribund and apparently healthy salmon during the summer and autumn months when incidences of AGD are likely to increase. However, increased mortalities usually means that the infection has been allowed to develop too far. Gill checks are undertaken by some farms regularly, for example Tassal checks every pen once a month. At some times the frequency of gill checks may increase to as often as once a week if there is a suspicion that AGD progression is fast. Ceasing of feeding can also be a useful tool leading up to bath treatment.

Control

What is the current recommended treatment for AGD?

- **Freshwater baths are recommended for controlling AGD**

  Other products such as levamisole, chloramine-t (including chloramine-t in sea water), and hydrogen peroxide have been tried, with mixed results, but are not currently recommended for commercial use.

What factors affect the success of freshwater bathing?

- **Bath water quality**

  Studies found that the paramoeba grows poorly at salinities below 10 ppt and that fresh water with high levels of calcium and magnesium ($\geq$200mg/L) enhanced survival of the paramoeba. Therefore, for best results, fresh water, with low hardness and low calcium and magnesium levels should be used for bathing.

  The length of the treatment should be at least 2 hours with oxygen being supplemented to ensure a minimum of 100% saturation is present throughout the cage during treatment.

- **Number of treatments**

  A single treatment does not appear to be adequate for controlling and managing AGD. Freshwater treatments are currently ongoing management strategy while the fish are in the sea. The number is related to the disease progression and gill check results. There is perception that a second freshwater bath provides a longer period of relief to the salmon.

- **Return to clean nets**

  Paramoeba has been found even on a lightly fouled nets. Therefore, in an attempt to reduce exposure to the paramoeba, all salmon should be placed in clean nets post-treatment.

- **Separation from infected fish**

  Following bathing the cage with bathed fish should be moved reasonable distance (but yet unknown) from heavily infected fish in other cages.

How effective is freshwater bathing in treating AGD?

- **Freshwater bathing does not cure AGD**

  Freshwater baths do not cure the salmon of AGD but only control it by decreasing the paramoeba prevalence, decreasing the number of live paramoeba and decreasing the number of mucous patches.

  Re-infestation from the paramoeba that survive the treatment is not only possible but probable.

How long can one expect the reduced paramoeba load to last?

- **10 days to 4 weeks**

  This is affected by multiple factors, including time of the year and pre-bath paramoeba load. Originally, published literature found it took 4 weeks for pre-treatment infection levels to return after a freshwater bath. More recent literature cites that pre-treatment infection levels can return after only 10 days post-treatment. This may be an indication that freshwater bath treatments are resulting in the selection of paramoeba that are more tolerant of freshwater. It may also indicate that a significant amount of the problems with AGD result from the spread of these tolerant strains within the Atlantic salmon populations, rather than exposure to 'new' strains of the paramoeba. This cycling could result in a further reduction in the effectiveness of freshwater baths and needs to be broken.

REDUCING INCIDENCE OF AGD ON THE ATLANTIC SALMON FARM

What are the risk factors associated with AGD?

Primary environmental risk factors

- Full-strength sea water
  Clinical AGD outbreaks are normally associated with full salinity sites (35ppt), while brackish sites appear to be free of the paramoeba.

- Warm water temperatures
  AGD appears to be more prevalent in the summer/autumn months when water temperatures climb above 12ºC. Clinical AGD appears in Atlantic salmon in water temperatures ranging from 12ºC to 20ºC with AGD mortality rates increasing when water temperatures climb above 16ºC.

- Farming regions
  Atlantic salmon farming occurs in several pockets around Tasmania, however AGD appears isolated to a few areas. Sites located in the southeastern part of Tasmania appear to have more issues with AGD.

Secondary environmental risk factors

- Low rainfall
  High rainfall that lowers the salinity of surface water may reduce the risk of AGD.

- No nearby freshwater source
  Local freshwater sources may help reduce the salinity of a site and decrease the risk of AGD. Presently, freshwater baths are used to control AGD; therefore local fresh water sources are also essential for treatment.

- Low level of dissolved oxygen
  Studies have found that AGD-affected salmon show reduced survival in poorly oxygenated water.

- Low current areas

  Strong currents have been shown to dislodge the mucus in AGD-affected patches, suggesting that they may reduce the severity of AGD.

Management associated risk factors

- **Mixed year class sites**

  It is known that the paramoeba can be found on the gills of salmon year-round, with disease usually occurring in summer and autumn, and that Atlantic salmon do not appear to develop resistance to the infection. It is also recognized that freshwater bath treatments are not 100% effective in killing and removing the gill paramoeba. It has been determined that infected Atlantic salmon are a reservoir for AGD. Therefore entering naive salmon into a site while still containing these reservoirs increases the risk of spreading the infection to these naive salmon.

  It has also been shown that the paramoeba is capable of surviving, in a dormant phase, in seawater for up to 14 days. As a result, it is essential to recognize that exposure to the paramoeba can occur even after the removal of infected salmon, therefore emphasizing the importance of appropriate cleaning and fallowing between year class entries.

- **Close proximity to other Atlantic salmon sites**

  Since Atlantic salmon are considered a significant reservoir for AGD, and the paramoeba is capable of surviving in seawater for 14 days, it is quite possible that infection can travel via water currents to sites located downstream from infected salmon populations. A conducted survey showed that sites in areas where AGD prevalence was higher were in closer proximity to each other than in areas of low or no AGD.

  When there are multiple year classes within an area, sites with older AGD-infected salmon can potentially infect younger, unexposed salmon in nearby sites due to their proximity. It is a cycle that could continue indefinitely.

- **Poor smolt quality and early maturing strains**

Atlantic salmon with deformities that affect respiratory efficiency and strains of salmon with early maturing rates develop AGD signs earlier and are at higher risk of succumbing to AGD.

- **Nets treated with copper antifoulant**
  The presence of paramoeba on copper-treated nets is typically much higher than on non-treated nets. Nets treated with antifoulant may act as a reservoir for the paramoeba. However, there is no scientific evidence that AGD severity is related to the presence of amoebae on the nets. Results of two field studies are contradictory and therefore inconclusive.

- **Fouled nets**
  It has been found that the paramoeba requires a surface area to replicate. Appropriate surfaces include cage nets where paramoeba have been found to inhabit even lightly fouled, untreated nets; therefore, fouled nets must be considered a reservoir for AGD.

  Fouled nets also reduce water exchange within a pen, thereby reducing water quality. This has the potential of negatively impacting the survival of salmon with AGD.

- **Salmon escapes**
  To date, there have been no wild fish reservoirs found for AGD. However, infected Atlantic salmon are known to act as reservoirs and therefore escaped salmon, which are infected, may pose a risk to surrounding populations. Salmon escapes can occur during fish handling events such as during net changes, freshwater treatment, and harvesting. Escapes can also happen as a result of equipment failure or unforeseen events such as storms and predation.

- **Salmon mortalities**
  Research has found that the paramoeba remains on the gills of dead Atlantic salmon for up to 30 hours post mortem and therefore, is a potential source of infection.

- **Poor rearing practices**

  Factors that increase stress on fish may affect the salmon's ability to survive AGD. These include high pen and site densities, as well as poor fish health.

Potential reservoirs of paramoeba

This table summarises our current knowledge of environmental reservoirs of the paramoeba.

Table 2 - Potential reservoirs for *Neoparamoeba pemaquidensis.* No for confirmed infection from the reservoir means current lack of experimental data, not that we have scientific evidence that paramoeba from this reservoir cannot infect fish.

Potential reservoir	*Neoparamoeba pemaquidensis* presence	Confirmed infection from the reservoir
AGD infected salmon	Yes	Yes
Water	Yes	Yes
Dead fish	Yes	Yes
Sea cage netting	Yes	No (one experiment negative)
Biofouling	Yes	No (one experiment negative)
Sediment	Yes	No (no experiments done)
Wild fish species	No	No (no experiments done)

How can these risk factors be reduced?

- **Reduce exposure to high salinity and warm water temperatures**
  When selecting new farm sites, it is better to avoid locations with full-strength seawater and high water temperatures or carry out further investigations to ensure the location is clear of the paramoeba. Special consideration should also be given to the secondary environmental risk factors, since their presence may increase the severity of AGD.

- **Avoid areas known to have AGD**
  Do not locate new farms in areas known to have AGD. Farm sites already established in high-risk environments must implement farm practices that help reduce and control AGD.

- **Monitor the environment**
  A good understanding of the local environment and its patterns enables a site to set up management practices to handle expected, less than optimal, environmental conditions such as periods of traditionally low dissolved oxygen levels. In order to develop this knowledge base, sites must routinely monitor and record environmental parameters including salinity, water temperature and dissolved oxygen. These measurements should be monitored at various depths, for example at the surface, at 3 to 5 m and at the cage bottom, throughout the year.

- **Maintain single year class sites**
  This will prevent the spread of AGD from infected salmon to unexposed salmon. Since the paramoeba is able to survive for up to 14 days in seawater, to control exposure between year classes, sites should be cleaned and then remain fallow for a minimum of two weeks before restocking.

- **Good smolt quality**
  To improve survival from AGD, ensure that all smolts entered onto sites have good mouth, gill and operculum conformation.

- **Genetics**

  Avoid stocking triploids in areas where AGD is prevalent, as they appear to be more sensitive to AGD; this may be related to increased prevalence of gill deformities. Early maturing strains should also not be used in high-risk AGD areas because they appear to be more sensitive to infection. However, this can be reversed in late summer by more frequent bathings earlier in the season.

- **Net management**

  Nets treated with antifoulant should only be used in areas of low to no risk of AGD. However, a balance is needed between the ensuring a good flow and use of non-antifoulant nets. There is no confirmed link between the presence of paramoebae on the antifouled nets and increased prevalence of AGD.

  Areas of moderate to high risk of AGD should use only untreated nets. The frequency of untreated net changes depends on several factors, including the level of fouling, and the physical characteristics of the site: current, water flow and dissolved oxygen. In areas with a moderate to high risk of AGD, more frequent net changes are recommended in an effort to ensure that nets are clean during periods of higher AGD prevalence (November to April).

  All fouled nets, especially those from moderate and high-risk AGD areas, should be cleaned at land-based facilities using fresh water.

- **Prevent Atlantic salmon escapes**

  Salmon escapes as a result of high-risk activities, such as net changes, can be reduced with proper planning and care during implementation. To reduce escapes resulting from unforeseen situations, use only equipment well suited for the site, make sure all nets are of an appropriate mesh size for the Atlantic salmon they contain, and perform regular net inspections for holes.

- **Regular retrieval and removal of mortalities**

  Routine mortality retrieval should be part of regular good management practices.

  A minimum retrieval of once per week should be adequate when mortality rates are low. However, because the paramoeba can survive on the gills of dead fish, it

is recommended that mortalities be collected on a daily basis during an AGD outbreak.

Mortalities should be examined for cause of death, then placed into a leak-free container and disposed of on land.

- **Good husbandry practices**
  Sites and cages should be stocked and grown at densities that promote optimal growth and good welfare in the population. The appropriate rearing density for a site must take into consideration the environmental characteristics of that site. For instance, a well-oxygenated site with moderate current may allow for higher cage/site density than a site with low flow and seasonal problems with dissolved oxygen levels. Similarly, areas with higher AGD risks may need to be stocked at lower densities than other areas. It has been suggested that high stocking density can contribute to AGD outbreaks.
  Fish health should be monitored regularly by fish health specialists.

REDUCING THE SPREAD OF AGD WITHIN AN AREA OF ATLANTIC SALMON FARMING

What are the risk factors that appear to spread AGD between Atlantic salmon farms?

- High-risk salmon farming regions
- Close proximity to other Atlantic salmon sites
- Multiple year classes within an area

How can these factors be minimised?

- **Area management**

The purpose of area management is to avoid, control and contain a disease within its boundaries. The boundaries of a management area are usually based on the oceanographic conditions, as well as the presence or absence of disease within the area. Area management systems are used in Norway and Scotland to manage sea lice and Infectious Salmon Anemia (ISA) and have been proposed in Canada as a way to control the spread of Infectious Haematopoietic Necrosis (IHN).

Management area agreements are made among the stakeholders sharing an area. Two important goals of an area management plan would be improved control in areas of moderate and high AGD prevalence and avoiding the spread of AGD into areas where it is currently not a problem. The level of integration in management practices amongst the stakeholders could be based on the expected risk of contracting AGD, for example high and moderate risk areas would have closer integration.

What factors should be incorporated into area management for AGD?

- **Single year class**

  Since there is no treatment that effectively cures Atlantic salmon of the infection, and the paramoeba is able to survive for up to 2 weeks in seawater, infected Atlantic salmon populations remain the primary risk factor for AGD within an area. It is therefore essential that areas coordinate synchronized year class production,

so that all sites are stocked/restocked at approximately the same time, in an effort to reduce transmission of the paramoebae from one population to another.

- **Fallowing of an area**
  In order to reduce exposure, management areas must synchronize the cleaning and fallowing of all sites in the area. Fallowing dates should be agreed upon between all parties and should be obligatory.

- **Synchronized or strategic freshwater treatments**
  Since freshwater baths are very labour intensive and time consuming, synchronizing the timing would be impossible. The effect of release of the bathing water on spread of paramoebae is not understood. If the bathing was killing all amoebae, it might be possible to treat sites located upstream prior to treating the downstream sites in order to reduce the potential of a downstream spread of the paramoebae.

- **Movement of fish**
  Salmon should not be moved from moderate or high AGD areas to areas of no or low AGD, especially if they contain the environmental risk factors.

- **Exchange of information**
  Information exchange may include fish health reports, mortality rates, and treatment information.

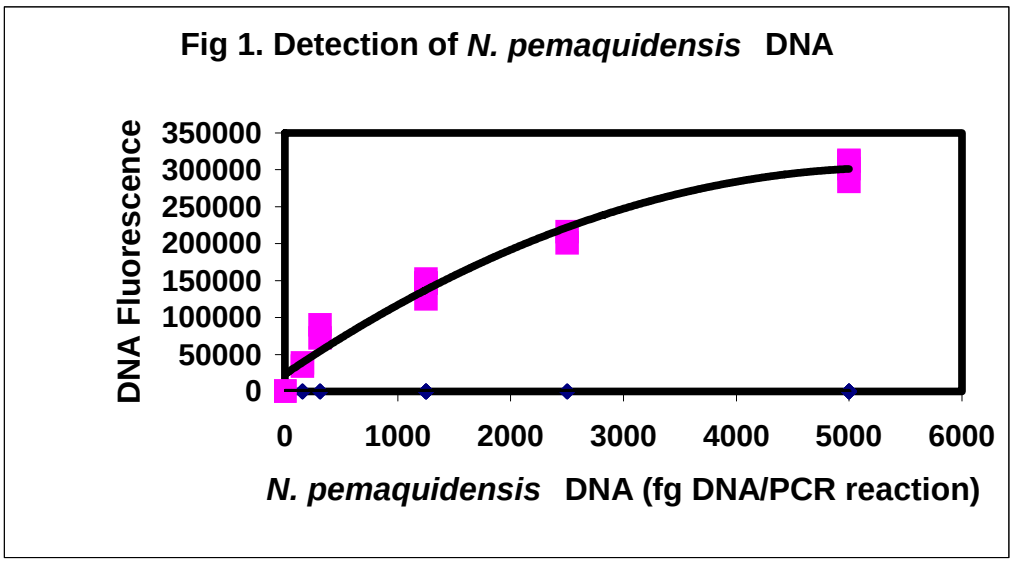
IN SUMMARY

At present, improved control against AGD will only occur as long as each individual farm makes an effort to reduce the risk factors associated with the disease. An area management scheme may also improve the managing of AGD within an area. As well, more research is required to develop improved methods of prevention or a more effective treatment against AGD.

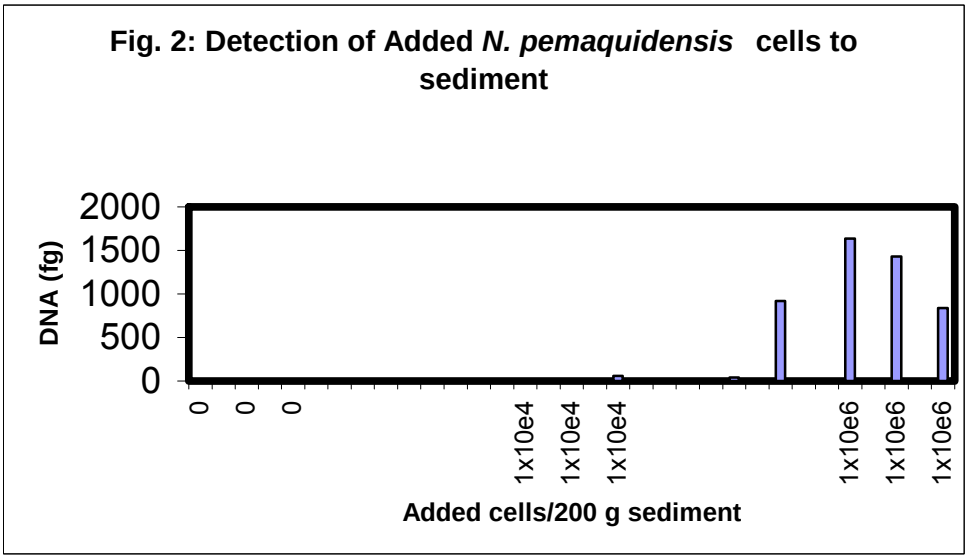
Appendix 5

Report on *Neoparamoeba pemaquidensis* DNA Detection in sediments
Based on report written by Dr Kathy Ophel-Keller

1. Quantitative detection of *Neoparamoeba pemaquidensis* DNA. Specific *N. pemaquidensis* PCR primers (now known to cross-react with *Neoparamoeba branchiphila*) were received from Phil Crosbie- these primers were based on sequence information of the cultured cell line, PA027. The primers were designed to produce a shorter amplification product and an oligonucleotide detection probe was designed to detect the amplified product. PCR conditions for quantitation of *N. pemaquidensis* DNA were optimised, and excellent quantitation was achieved (Fig. 1). This was done using DNA extracted from PA027.



2. Detection of *N. pemaquidensis* in spiked sediments. Sediments were spiked with PA027 cells, ranging from 10^3 to 10^6 cells/ 200 g sediment. Detection of 10^6 cells/200 g was reliable but below this level, detection was not reliable, indicating that detection in sediments is not very sensitive for this organism (Fig. 2).



3. Detection of *N. pemaquidensis* DNA on netting and in water. Detection of AGD cells on netting and in water (by collection on filters) was determined by addition of PA027 cells to netting pieces (2-3 cm net pieces) by incubation for 48 hours, drying, and extraction of DNA. Detection of AGD cells in seawater was determined by addition of PA027 cells to 500 ml seawater filtration and extraction of DNA from filters DNA extraction was followed by quantitative PCR, and *N. pemaquidensis* DNA determined (Table 1).

Table 1. Detection of *N. pemaquidensis* (PA027 cells) in netting and seawater.

Sample	Sample Detail	Cells Added	*N. pemaquidensis* DNA Reading
1	Net pieces, dried O/N @ 40C (1)	$10e^6$	293492
2	Net pieces, dried O/N @ 40C (2)	$5x10e^5$	301358
3	Net pieces, dried O/N @ 40C (3)	$2.5x10e^5$	290257
4	Net pieces, dried O/N @ 40C (4)	0	475
5	Filter (0.45um), dried O/N @ 40C (1)	$5x10e^5$	295665
6	Filter (0.45um), dried O/N @ 40C (2)	$2.5x10e^5$	301593
7	Filter (0.45um), dried O/N @ 40C (3)	$10e^4$	77548
8	Filter (0.45um), dried O/N @ 40C (4)	$10e^3$	7422
9	Frozen net (sample No 1)	$10e^6$	80439
10	Frozen net (sample No 2)	$5x10e^5$	82816
11	Frozen net (sample No 3)	$2.5x10e^5$	3177
12	Frozen net (sample No 4)	0	987
13	Frozen Filter (Sample No 1)	$5x10e^5$	20097
14	Frozen Filter (Sample No 2)	$2.5x10e^5$	139902
15	Frozen Filter (Sample No 3)	$10e^4$	19870
16	Frozen Filter (Sample No 4)	$10e^3$	3043

Results show that detection of PA027 cells was possible on both netting and in seawater, with good sensitivity - 10^3 cells on filter were readily detected. Drying the filters or netting prior to DNA extraction was a more effective method of detection than freezing prior to extraction and detection.

5. Detection of 'wild' *N. pemaquidensis* cells

N. pemaquidensis DNA was not reliably detected when the same techniques were applied to detection of AGD in naturally collected sediments, netting and seawater. Initially, it was thought that this was due to insufficient sensitivity in the DNA detection. However when amoebae were freshly collected from infected gills, they were not detected by the DNA detection technique (Table 2). The preparation of cells (frozen or dry prior to DNA extraction, did not affect detection.

Table 2. Detection of wild and cultured *N. pemaquidensis* cells

Sample Detail	*Treatment*	N. pemaquidensis *DNA Reading*
Wild Isolate (host-derived amoebae)	Frozen	200
Reference strain PA027	Frozen	32059
Host-derived (dried)	Dried (40C x 14 h)	215
PA027 (dried)	Dried (40C x 14 h)	384583

Conclusions: Detection of cultured *N. pemaquidensis* cells by DNA detection is possible in sediments, on netting and in filtered water. Detection in sediments is less sensitive and may not be sufficient to detect environmental levels of the pathogen, but this remains to be fully tested. The DNA primers do not appear to adequately detect wild strains of *N. pemaquidensis*. Further sequencing is underway at CSIRO to improve primer design but it is not clear how easy this will be to achieve.

Appendix 6

Flow cytometric analysis of
Neoparamoeba pemaquidensis

Based on report written by
John D. Hayball and A. Bruce Lyons
Dame Roma Mitchell Cancer Research Laboratories
Hanson Institute
Institute of Medical and Veterinary Science
Adelaide, South Australia.

Summary

Amoebic gill disease (AGD) is the most serious health problem of cultured Atlantic salmon in Tasmania. Evidence suggests that the pathogen responsible for this disease is *Neoparamoeba pemaquidensis (N. pemaquidensis)*. However, at this stage there is a paucity of diagnostic reagents and techniques available for the detection and quantification of viable *N. pemaquidensis* on infected fish and in the environment.
To this end, the aims of this project were to determine whether flow cytometric techniques utilising propidium iodide (PI) exclusion and polyclonal antisera raised against a laboratory cultured strain of *N. pemaquidensis*, PA027, would prove useful for the simultaneous identification and viability assessment of *N. pemaquidensis*.
PI staining followed by flow cytometric analysis proved to be as effective as trypan blue staining with light microscopy, when used to differentiate between live and dead *N. pemaquidensis*. However, when used in conjunction with anti-PA027 antisera, it was possible to specifically identify viable versus dead *N. pemaquidensis,* as compared to other microorganisms found on the gills. The practical level of sensitivity of flow cytometry to detect *N. pemaquidensis* was shown to be approximately 2 organisms per ml. This was based on concentrating 5l water samples. However, in the field, no viable *N. pemaquidensis* could be detected in such samples. If flow cytometry is to be used to *N. pemaquidensis* in the water column, then other technologies must be developed to process larger volumes of water is to be detected Finally, various water treatments were tested for their effect on PA027 viability. Low ionic strength (currently used in the industry for the treatment of AGD) and a variety of chemical agents proposed as possible chemotherapeutic agents (Chloramine T, hydrogen peroxide and copper sulphate), proved to be surprisingly ineffective at killing PA027, whereas bleach and formaldehyde were both very effective. The results are discussed in the context of the practical application and usefulness of flow cytometry to the development of treatment regimes for AGD.

Introduction

Amoebic gill disease (AGD) is the most serious health problem of cultured Atlantic salmon in Tasmania. The only effective treatment currently available to industry involves bathing fish in fresh water. This is a time consuming, expensive and possibly ecologically unsustainable practise, which is having a detrimental impact on the viability of the industry. Clearly there is impetus to develop new treatment regimes for AGD.

There is a large body of evidence that suggests *Neoparamoeba pemaquidensis* is the pathogen responsible for the disease. Most convincing is the induction of AGD-like symptoms in healthy fish after exposure to fish infected with *N. pemaquidensis.* Fresh water treatment of fish with AGD offers temporary relief of symptoms as determined by reduced total microbial gill load followed by recovery of the lesions. The most likely explanation for this is that fresh water treatment results in a physiological shock to the fish leading to sloughing off of the mucus on the gills, and thus gill-associated microorganisms. Whilst it is clear that some of the gill-associated, amoebic-like microbes are killed by this treatment, at this stage it is not possible to say unequivocally that *N. pemaquidensis* is. Indeed, reinfection rates increase dramatically following freshwater treatment, suggesting that the bathing techniques used may be leading to the generation of an even larger environmental pool of the pathogen. That is, *N. pemaquidensis* is not being killed by the fresh water, but simply shed from the fish in a viable state back into the water column, following the reintroduction of the bathing water into the lease site. Flow cytometry is a powerful technique which, when used with the appropriate specific antibody and chemical stains, can be used to simultaneously determine many phenotypic characteristics of individual cells in mixed populations. With specific antisera against *N. pemaquidensis* available, it now becomes possible to measure the viability of this organism within a mixed population of other microorganisms. This is a critical first step towards developing effective regimes to control of fish-associated *N. pemaquidensis* populations and possibly environmental reservoirs of the pathogen, thus ultimately the incidence of AGD in cultured Atlantic salmon

Materials and Methods

Culture and recovery of PA027:

PA027 is a laboratory strain of *N. pemaquidensis* which was isolated from infected fish and has been maintained in culture for several years. PA027 was recovered from existing culture plates by washing gently with filtered seawater (FSW). 100 μL of the suspension was used to inoculate fresh agar plates (0.01% malt extract, 0.01% yeast extract (both Oxoid) and 2% agar (Difco) in 75% FSW). These plates had been seeded with 100 μL of a live suspension of *Stenotrophomonas maltophilia* as a food bacterium, which had been allowed to absorb into the agar for 30min prior to inoculating with PA027. After 2-3d culture, PA027 was recovered from the plates by washing gently with FSW. The supernatant was passed through a 70 μM nylon filter (Falcon). The mixture of bacteria and PA027 were centrifuged (1500g for 5min) and washed in FSW, centrifuged and recovered into phosphate buffered saline containing 0.1% bovine serum albumin (PBS/0.1% BSA). The number of viable PA027 was determined by trypan blue staining and counting in a

haemocytometer using phase contrast microscopy. Typical recovery rates were in the order of 2 x 10^6 viable PA027 per plate.

Recovery of gill-associated N. pemaquidensis and other amoeba:
Gill arches were removed from infected fish and associated microorganisms removed by "end over end" agitation for 10 min in 25 mL of FSW in 50 mL tubes. The supernatant was passed through a 70 μ nylon filter (Falcon) and the cells recovered by centrifugation (1500 x *g* for 5 min). The cell pellet, which by inspection contained significant red blood cell contamination, was treated with red blood cell alkaline lysis buffer (ALB, 150 mM NH_4Cl, 1 mM $KHCO_3$, 100 μM Na_2EDTA, pH 7.3), for 5min at room temperature (RT), and the cells recovered by centrifugation. The pellet was resuspended and washed once in FSW, centrifuged, then resuspended in PBS/0.1% BSA. The number of viable recovered microorganisms was determined by trypan blue exclusion staining and counting in a haemocytometer using phase contrast microscopy.

Viability staining of cultured PA027 and gill-recovered amoeba using trypan blue and propidium iodide:
Aliquots of either cultured PA027 or gill-recovered microorganisms were mixed with an equal volume of filtered trypan blue solution (0.25% in PBS), applied to a haemocytometer slide and examined using phase contrast microscopy. The larger amoebic-like organisms were quite distinct from very small bacteria. Viable from non-viable amoebic-like organisms were identified by the absence of blue intracellular staining.
In parallel, samples were also stained with PI (10 μg/mL final concentration) and analysed by flow cytometry (FACScan, Becton Dickinson). Amoebic-like microorganisms were selectively gated on, based on their relatively high forward and intermediate side scatter characteristics, and then the proportion of viable microorganisms quantified by intracellular PI staining. This was done by measuring the proportion of cells positive and negative for PI staining in the FL2 channel of the FACScan flow cytometer.

Antibody staining of cultured PA027 and gill-associated N. pemaquidensis:
Previously, antisera had been raised against PA027 in sheep and rabbit, both of which have specificity for PA027 as well as gill-associated and environmental samples of *N. pemaquidensis.* Both of these serum stocks have been pre-adsorbed against the food source for cultured PA027 (*Stenotrophomonas maltophilia)*, to eliminate antibodies against these bacteria.
To determine whether these antisera worked in flow cytometry, samples of both PA027 and gill associated *N. pemaquidensis* (5 x 10^5 in 200 μL PBS/0.1%BSA) were stained with sheep anti-PA027 (50 μL of a 50% serum/glycerol stock) or rabbit anti-PA027 (50 μL of a 50% serum/glycerol stock) on ice for 30 min. The samples were washed twice in PBS/0.1%BSA and incubated with FITC labelled donkey anti-sheep or FITC labelled goat anti-rabbit respectively (1μL in 50 μL PBS/0.1%BSA, both from Dako) and incubated on ice for 30min. The cells were washed twice in PBS/0.1%BSA in resuspended in 400 μL PBS/0.1%BSA containing propidium iodide (10 μg/mL). The cells were then analysed by flow

cytometry by firstly gating on amoeba-like microorganisms based on forward scatter and side scatter properties, then analysed in the FL1 channel for antisera binding and the FL2 channel for viability. In every experiment, control samples consisting of cells incubated with either normal donkey or normal rabbit serum followed by FITC labelled donkey anti-sheep or FITC labelled goat anti-rabbit respectively, or simply by FITC labelled donkey anti-sheep or FITC labelled goat anti-rabbit were included to account for background staining.

Flow cytometric detection limits of PA027 recovered from spiked water samples using centrifugation:
Aliquots of seawater (50 mL) were spiked with various concentrations of cultured PA027. The samples of PA027 were recovered by centrifugation (1500 x *g* for 5 min), resuspended in PBS/0.1% BSA and stained with rabbit anti-PA027 as described earlier. Samples were washed in PBS/0.1% BSA and resuspended in PBS/0.1% BSA and analysed by flow cytometry. The number of recovered PA027 was quantified by the addition of a known number of FlowCount beads (Becton Dickinson) to each tube before analysis (50,000 bead per sample). These beads are highly fluorescent and have very high side scatter characteristics and thus do not appear on the forward scatter versus side scatter density plots used to identify PA027. Nevertheless they can be quantified by setting an extended gate to capture these events. The absolute number of recovered PA027 was quantified by dividing the number of beads added to each sample (50000) with the number of beads collected upon flow cytometric analysis. This figure was then multiplied by the number of PA027 found in the same analysis file, resulting in the absolute number of PA027 recovered from each sample.

Results
Cultured and gill-isolated N. pemaquidensis can be characterised by their forward scatter and side scatter properties in flow cytometric analyses.
When analysed by flow cytometry, PA027 had quite characteristic pattern of forward and side light scattering, reminiscent of macrophages (Fig. 1a). They were easily distinguished from contaminating bacteria, the food source for PA027, which are far smaller and less transparent, resulting in relatively low forward light scattering. A more heterogenous light scattering profile was observed in the sample isolated from infected fish gills (Fig. 1b), with red blood cell contamination quite evident as very transparent population (high forward and low side scatter) which was eliminated when the sample was treated in such a way to specifically lyse red blood cells (Fig. 1c). The light scattering heterogeneity observed in the putative "*N. pemaquidensis*" population most likely resulted from there being a diverse mixture of other microorganisms as well as *N. pemaquidensis* on these gill preparations.

Polyclonal antibodies raised against PA027 recognise PA027 and gill isolated N. pemaquidensis.
Perhaps not surprisingly, sheep antiserum raised against PA027 was quite effective at staining PA027 in flow cytometry with almost all the cells staining positive (Fig. 2c). This staining was specific as no fluorescence was observed

when either PA027 was incubated with the secondary labelled antibody by itself, nor when incubated with normal sheep serum and then secondary labelled antibody. The antiserum was also able to identify *N. pemaquidensis* in the gill isolates. In samples where red blood cells had not been lysed, approximately 75% of cells stained with the antisera (Fig. 2a), increasing to 84% of cells after red blood cell lysis treatment (Fig. 2b). As these samples were gated around the putative "*N. pemaquidensis*" population, based on forward and side scatter characteristics, this would suggest the red blood cell lysis treatment was also lysing some of the other cells in the "*N. pemaquidensis*" population. Nevertheless, it was evident that the flora on the gills of fish with symptoms of AGD contained a high proportion of microorganisms, which stained positive with the anti-PA027 antisera.

Rabbit anti-PA027 antiserum is a more effective antibody stain in flow cytometry than sheep anti-PA027 antiserum.

Although the sheep anti-PA027 antiserum was reasonably effective in flow cytometry, there was still room for an increase in binding specificity and avidity. To this end, polyclonal antibodies against PA027 were also raised in rabbits. When directly compared to the sheep antiserum (Fig 3a), the rabbit antiserum had higher avidity and lower non-specific binding (Fig 3b) and was therefore used in all subsequent experiments.

Propidium iodide staining is equally effective as trypan blue exclusion to differentiate live and dead PA027.

The strength of flow cytometry is its ability to analyse multiple parameters on individual cells within heterogeneous populations. To this end we were interested to determine whether flow cytometry could be used to identify *N. pemaquidensis* and at the same time, be used to assess cell viability. Propidium iodide (PI) is a fluorescent DNA stain commonly used to assess the membrane integrity and hence viability of eukaryotic cells. We found that it could also be used to measure the viability of PA027. When fresh PA027 were stained with PI, there appeared to be few if any FL2 positive cells (1.58% positive, Fig. 4a(iv)). However, when incubated at room temperature in PBS for 1h, a small distinct FL2 positive population became evident. Fig. 4b(iii). This population of putatively dead and/or dying PA027 was located approximately within the forward and side scatter parameters previously identified for viable PA027 (Fig. 4b(iv)). These figures correlated exactly with those observed when the same samples were tested for viability by trypan blue exclusion (data not shown). To determine whether PI staining could also be used in conjunction with staining specific for PA027, samples were stained with PI and rabbit-anti-PA027 and analysed by two colour flow cytometry immediately or after heat treatment to kill a majority of PA027 (Fig. 5a). In these samples, viable PA027 are clearly identifiable by their forward scatter and side scatter characteristics (Fig 5a(i)), their high FL1 and low FL2 staining (Fig. 5a(iii, v, vii and x)). In comparison, heat-killed PA027 displayed slightly higher side scatter characteristics, typical of head and dying eukaryotic cells (Fig. 5a(ii)), and high FL1 as well as high FL2, indicative of inclusion of the PI stain (Fig. 5a(iv, vi, ix and xi)).

Next we wished to demonstrate that PI staining was comparable to trypan blue as a technique to accurately differentiate live versus dead PA027 in conjunction with PA027-specific staining. Rabbit anti-PA027 and PI stained samples of PA027 were heat treated for various periods of time and analysed by two colour flow cytometry as described above. The same samples were then stained with trypan blue and analysed by phase contrast light microscopy (Fig. 5b). It was found that the proportion of dead PA027 exactly correlated with the number of dead PA027 as determined by trypan blue exclusion.
Interestingly however, was the low level of PI staining of dead and dying PA027 as compared to, that which is typically seen with mammalian cells. This may be due to the fact that PA027 is somewhat autofluorescent in the FL2 channel (and for that matter, the FL1 channel), or may be due to a lower per cell DNA content as compared to typical mammalian cells.

Environmental sampling suggests the concentration N. pemaquidensis in the water column around salmon farms is less than 2/ml-centrifugation may not be the best approach to concentrate dilute environmental samples of N. pemaquidensis for analysis by flow cytometry
To assess the practicality and effectiveness of centrifugation as a technique to concentrate *N. pemaquidensis* for analysis by flow cytometry, laboratory trial experiments were performed using seawater samples spiked with various concentrations of cultured PA027. The organisms were recovered by centrifugation of the different samples and they were stained with rabbit anti-PA027 and analysed by flow cytometry. The dilution and recovery via centrifugation did not affect antibody staining of PA027 (Fig 6a). The total number of recovered PA027 was determined by including a known number of calibration beads in each sample. This also made it possible to determine the percentage recovery of PA027 at the various concentrations tested, and thus establish practical detection limits for environmental samples.
It was found that there was a direct correlation between the number of, and the proportion of recoverable and detectable PA027 in each of the samples. When added at a relatively high concentration ($2x10^4$/mL), almost 90% of PA027 could be detected by flow cytometry (Fig. 6b). However, this was drastically reduced to less than 40% of the original number of PA027 when the sample was spiked to a concentration of $2x10^4$/mL. Further reductions were noted in subsequent dilutions, where at the most dilute (2/mL), only 12% (12 events) were recovered following centrifugation.
Whilst the power of flow cytometry is its ability to detect and count individual cells in mixed samples, there are limits of confidence when the number of specific events is less that 1000. Therefore, if for instance *N. pemaquidensis* were to be found in environmental samples at a concentration of in the order of 2/mL, and centrifugation were to be used to concentrate samples for flow cytometric analysis, then at least 5 L of water sample would need to be processed to generate 1000 events.
Accordingly, preliminary field experiments were performed where 5 L samples of water were collected from within and around sea cages containing salmon in an area with a know history of AGD. These samples were concentrated by

centrifugation, stained with rabbit anti-PA027, but no organisms could be detected in flow cytometric analyses. This would suggest that the concentration of *N. pemaquidensis* in these water samples was less that 2/mL.

PA027 viability is not affected by low ionic strength

Given that it was possible to differentiate between live and dead and/or dying PA027 using flow cytometry, and that fresh water bathing is used to treat fish affected with AGD, we were interested to test whether low ionic strength was sufficient to kill the microorganism. Samples of PA027 were incubated for 10, 30 and 60 min in various concentrations of seawater diluted with DI water and it was found that even when diluted to very low ionic strength, there was no effect on PA027 viability (Fig. 7a). Similarly, various concentrations of magnesium chloride and calcium chloride had no effect on PA027 viability (Fig 7b and 7c)

Discussion

There is little doubt that *N. pemaquidensis* is the causative agent of amoebic gill disease in Atlantic salmon. This disease is causing significant difficulties for the industry in Tasmania, where the only current form of treatment available is the expensive and time consuming practice of freshwater bathing. To this end, it is of vital importance to either develop novel, or modify existing technologies to counter *N. pemaquidensis* infection and reinfection rates.

However, to achieve this goal, as with attempts to control any infectious pathogenic disease, it is an absolute requirement that assays be developed to reliably assess rates of infection, the extent of infection and identify reservoirs of the pathogen. Furthermore, it is vital to advance technologies to quantify the numbers of pathogenic organisms associated with the host as well as identify and quantify the number of pathogens within environmental reservoirs. These last issues are critical if it is planned to test the efficacy of any strategies designed to control pathogen numbers both on the host and within their environmental reservoirs.

The power of flow cytometry lays in its ability to analyse multiple parameters on individual cells within heterogeneous populations. However, this is dependent on the availability of antibody stains specific for the cell population of interest. Using antisera against PA027, we have shown that not only does the antibodies tested bind to PA027 but also to gill-associated *N. pemaquidensis.* When used in conjunction with the fluorescent viability stain, propidium iodide, we have shown that it is possible to simultaneously identify *N. pemaquidensis* within a mixed population and at the same time accurately assess cell viability. This has allowed us to test a number of candidate treatments for AGD by testing their ability to kill PA027 *in vitro*. However, the results were somewhat disappointing in that the high concentrations of any of these compounds required to kill PA027 would most likely preclude them from further field studies. Nevertheless, it still remains to be determined how effective these treatments might be against gill-associated *N. pemaquidensis*.

We have attempted to use centrifugation and flow cytometry to analyse environmental samples for the presence of *N. pemaquidensis*. Trial laboratory experiments indicated that flow cytometry could be used to identify and count *N. pemaquidensis* when at a concentration of at least two organisms per ml, in a

sample size of no less than 5l. This is the largest sample size that can be processed with existing equipment. However, when applied to a field setting, no viable *N. pemaquidensis* could be detected in individual 5 L environmental samples. This would suggest that if it does exist in the water column, around and within AGD-affected farms, then high throughput technologies for concentrating large sample volumes would be requisite. Such technologies might include ultrafiltration or continuous flow centrifugation. These will most likely have to be performed *in situ* on the farms, and thus will require effective collaboration between the different parties involved. However, in a single experiment, a sample of the fresh water used to bath AGD-affected fish was analysed by flow cytometry. In this sample we found a very high concentration of viable N*. pemaquidensis*. This is a very important observation as this bath water is routinely released back into the lease site and may be having the effect of simply increasing the environmental pool of the pathogen. There is already anecdotal evidence for this where the rates of infection are increasing dramatically in some lease sites. It is paramount these experiments be carefully repeated to confirm this initial observation and if they prove to be true, this strongly suggests that the bath water should be sterilised in some way before release into the environment.
In conclusion, we have shown that antisera raised against a laboratory strain of *N. pemaquidensis* can be used to stain this strain in flow cytometry and also *N. pemaquidensis* isolated from AGD affected Atlantic salmon. Furthermore, PI staining can be used in conjunction with these antisera facilitating simultaneous identification of *N. pemaquidensis* within mixed populations and viability assessment. By utilising these techniques we have tested a number of candidate control agents for toxicity against the laboratory strain of *N. pemaquidensis* and found none to be outstandingly efficacious. Nevertheless, these findings should form the basis of future field experiments on *N. pemaquidensis*, to assess the efficacy of current AGD control methodologies, and facilitate the development of new treatment regimes.

FACS analysis of gill-associated *N. pemaquidensis:*
-gills washed “end over end” in 25 mL filtered sea water (FSW).
-cells in supernatant passed through 70 μ cell strainer
-cells recovered by centrifugation (1500 x *g*/5min)
-red blood cells lysed by addition of 10 mL alkaline lysis buffer (ALB), RT/5min
-cells recovered by centrifugation (1500 x *g*/5min)
-cells washed in PBS/0.1%BSA and recovered by centrifugation (1500 x *g*/5min)
-cells resuspended in PBS/0.1%BSA (200 μL at $2x10^6$/ml)
-rabbit anti-PA027 serum added (50 μL of 50% glycerol stock), ice/30min
-cells washed in PBS/0.1%BSA and recovered by centrifugation (1500xg/5min)
-cells resuspended in PBS/0.1%BSA (200 μL) with FITC anti-rabbit (1 μL), ice/30min
-cells washed in PBS/0.1%BSA and recovered by centrifugation (1500 x *g*/5min)
-cells resuspended in PBS/0.1%BSA (200 μL) with PI (10 μg/mL)
-cells analysed by flow cytometry

FACS analysis of cultured *N. pemaquidensis:*
-plates washed with 25 mL filtered sea water (FSW).
-cells in supernatant passed through 70 μ cell strainer
-cells recovered by centrifugation (1500 x *g*/5min)
-cells washed in PBS/0.1%BSA and recovered by centrifugation (1500 x *g*/5min)
-cells resuspended in PBS/0.1%BSA (200 μL at $2x10^6$/ml)
-rabbit anti-PA027 serum added (50 μL of 50% glycerol stock), ice/30min
-cells washed in PBS/0.1%BSA and recovered by centrifugation (1500 x *g*/5min)
-cells resuspended in PBS/0.1%BSA (200 μL) with FITC anti-rabbit Ig (1 μL), ice/30min
-cells washed in PBS/0.1%BSA and recovered by centrifugation (1500 x *g*/5min)
-cells resuspended in PBS/0.1%BSA (200 μL) with PI (10 μg/mL)
-cells analysed by flow

Quantification of *N. pemaquidensis:*
-cells prepared and stained as described above
-50000 FlowCount beads added to sample
-sample analysed by flow (noting that beads will be off scale on side scatter)
-beads gated (using off-scale gate) and *N. pemaquidensis* gated separately
-number of *N. pemaquidensis* determined by dividing 50000 by the number of beads collected, then multiplying the number of *N. pemaquidensis* collected to get the absolute number of *N. pemaquidensis* in the sample

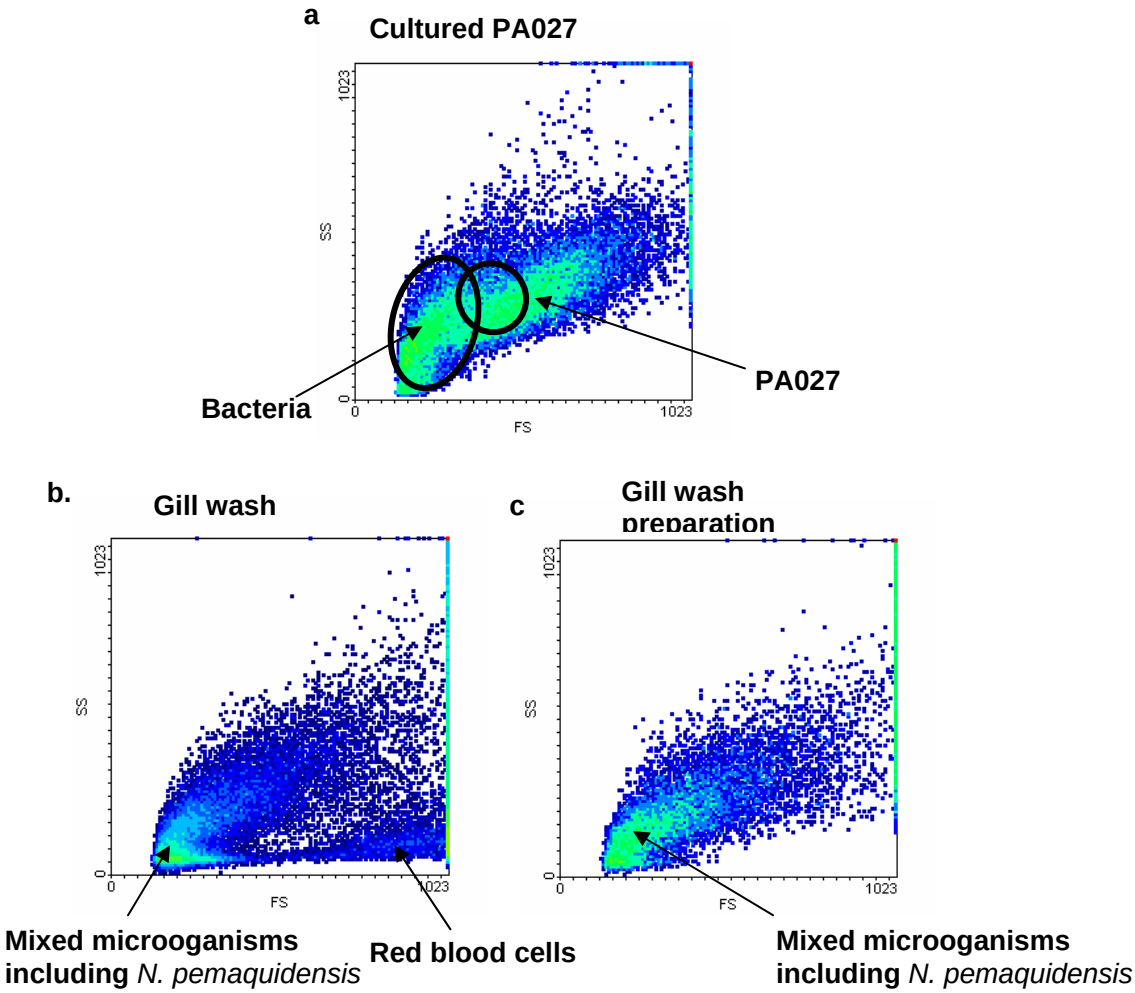
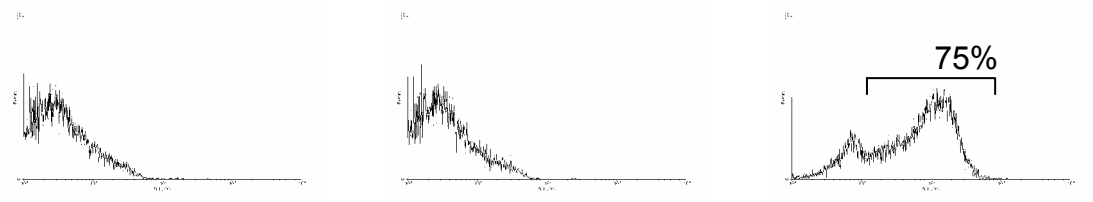


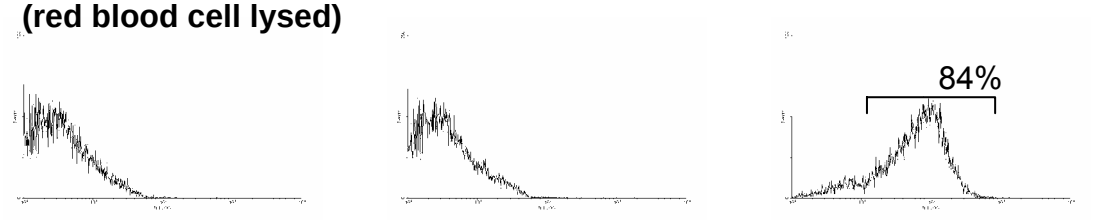
Figure 1. Comparison of light scattering properties of cultured and gill isolated *N.pemaquidensis*.

Cultured *N. pemaquidensis* (PA027) were isolated by washing plates in PBS/0.1%BSA (a) and compared to *N. pemaquidensis,* in a mixed population of other microorganisms, recovered from infected fish by washing the gill arches in PBS/0.1%BSA (b), or by subsequently treating these preparations with alkaline lysis buffer to lyse the red blood cell contamination (c). The cells were analysed by flow cytometry. The results shown are the ungated forward and side scattering profiles of these different *N.pemaquidensis* preparations.

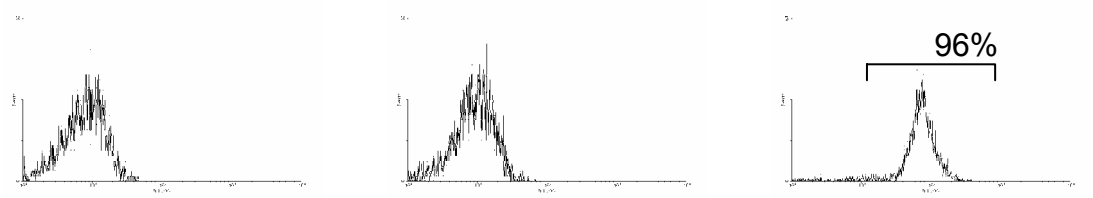
a. Gill wash preparation



b. Gill wash preparation (red blood cell lysed)



c. Cultured PA027 preparation



No primary antibody +FITC donkey anti-sheep

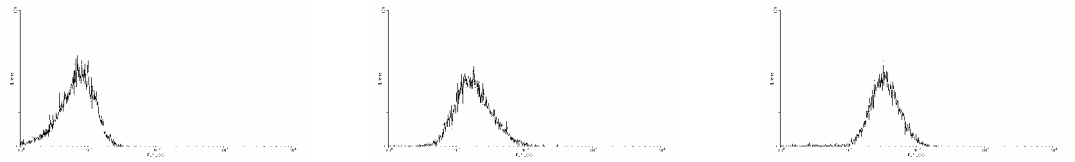
Normal sheep serum +FITC donkey anti-sheep

Sheep anti-PA027 serum +FITC donkey anti-sheep

Figure 2. Comparison of antibody staining of gill isolate and cultured *N.pemaquidensis*.

N.pemaquidensis were isolated from infected fish in (a), by washing the gill arches in PBS/0.1%BSA), (b) by subsequently treating these preparations with alkaline lysis buffer to lyse the red blood cell contamination. These were compared to (c), cultured *N. pemaquidensis* (PA027). Cells were resuspended ($5x10^5$ in 200 µL PBS/0.1%BSA) and stained with sheep anti-PA027 (50 µL of a 50% serum/glycerol solution) on ice/30min. Cells were washed and stained with FITC donkey anti-sheep (50 µL of 0.005% solution) on ice/30min. Control samples were also incubated with no primary antibody or normal sheep or rabbit serum. Cells were resuspended in PBS/0.1%BSA and analysed by flow cytometry. *N. pemaquidensis* were gated on using previously determined forward scatter and side scatter parameters and the results are expressed as the percentage of FL1 positive cells in single parameter histograms.

a. Sheep anti-PA027 serum

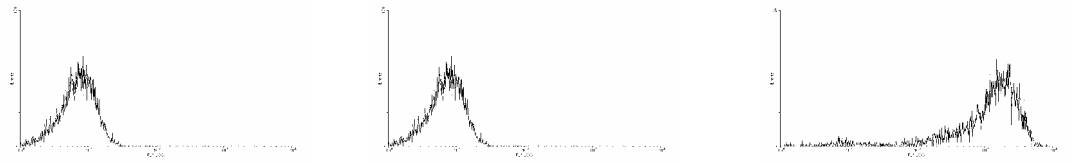


No primary antibody +FITC donkey anti-sheep

Normal sheep serum +FITC donkey anti-sheep

Sheep anti-PA027 serum +FITC donkey anti-sheep

b. Rabbit anti-PA027 serum



No primary antibody +FITC donkey anti-rabbit

Normal rabbit serum +FITC goat anti-rabbit

Rabbit anti-PA027 serum +FITC goat anti-rabbit

Figure 3. Comparison of sheep anti-PA027 and rabbit anti-PA027 serum staining on cultured *N.pemaquidensis*.

Cultured *N.pemaquidensis* (PA027, $5x10^5$ in 200 µL SW/0.1%BSA) were stained with (a) sheep anti-PA027 or (b) rabbit anti-PA027 (50 µL of a 50% serum/glycerol solution) on ice/30min. Cells were washed and stained with FITC donkey anti-sheep or FITC goat anti-rabbit (50 µL of 0.005% solution) on ice/30min. Control samples were also incubated with no primary antibody or normal sheep or rabbit serum. Cells were resuspended in SW/0.1% BSA and analysed by flow cytometry. *N.pemaquidensis* were gated on using previously determined forward scatter and side scatter parameters and the results are expressed as single parameter FL1 histograms.

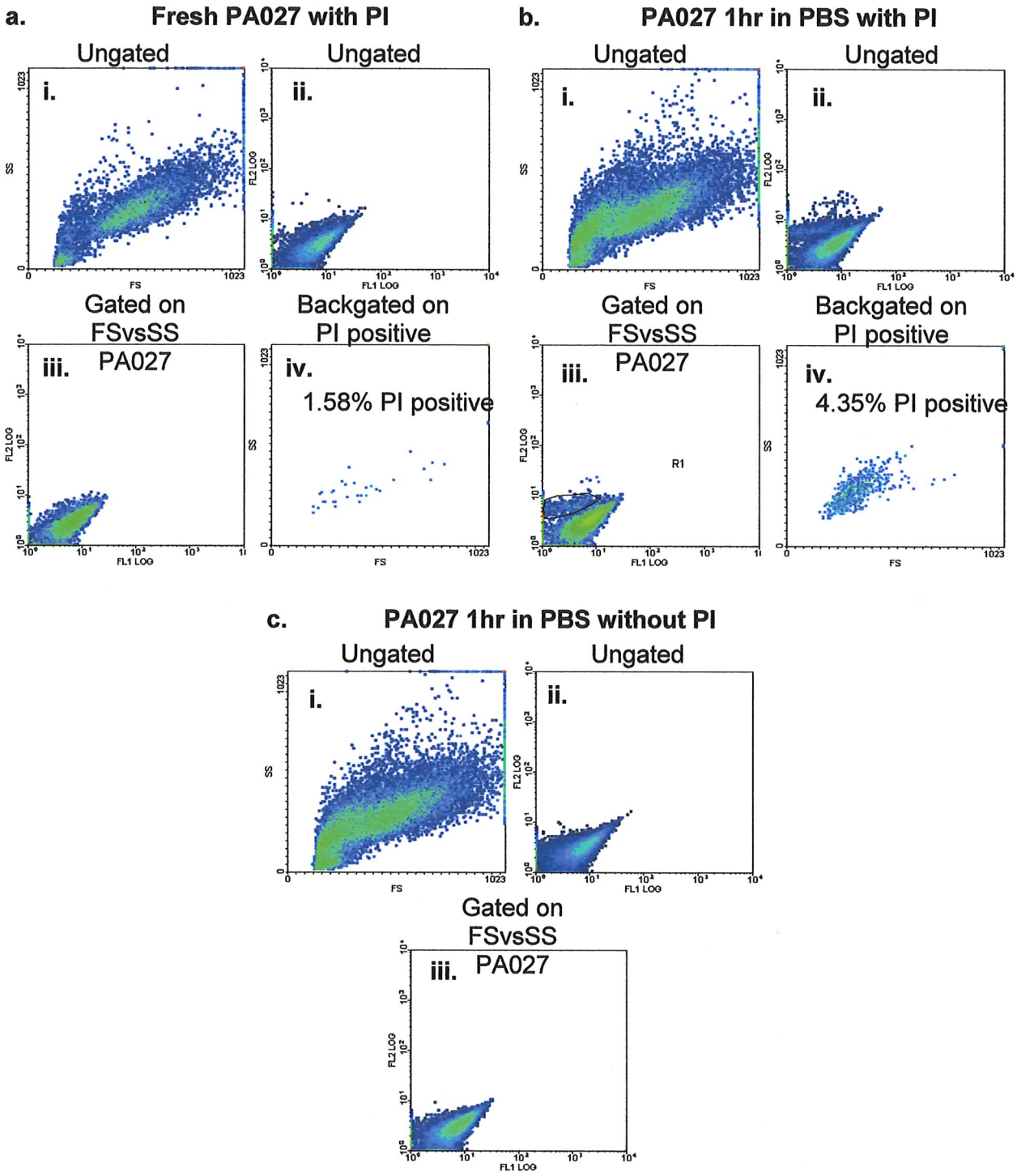


Figure 4. Determination of the effectiveness of propidium iodide staining to identify viable N.pemaquidensis.

Cultured N.pemaquidensis (PA027, 5x105 in 200 µL PBS) were stained with propidium iodide (10 µg/ml) and analysed immediately (a), or in (b), incubated at room temperature in PBS for 1hr prior to analysis. In (c), cells were incubated in PBS for 1hr and then analysed in the absence of PI staining. Panel (i) shows ungated forward scatter versus side scatter whereas panel (ii) shows ungated FLI versus FL2. In panel (iii), the FL1 versus FL2 profile of PA027 is shown, gated using previously determined forward scatter and side scatter parameters, whereas panel (iv) shows the forward scatter versus side scatter characteristics of PI-positive PA027

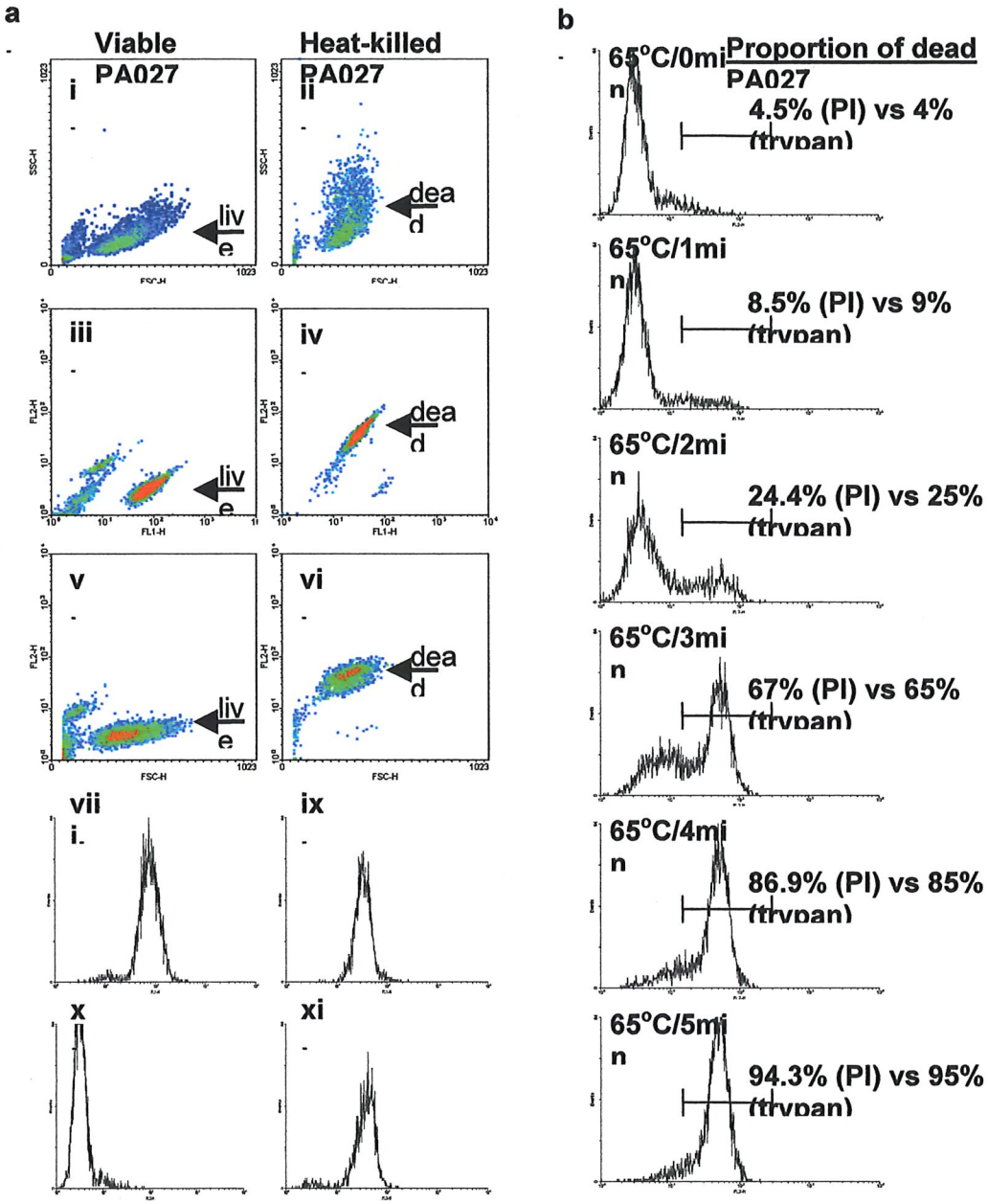


Figure 5. Comparison of viability assessment of cultured N.pemaquidensis using propidium iodide staining and trypan blue exclusion.

Cultured N.pemaquidensis (PA027, 5x105 in 200 µL SW/0.1%BSA) were stained with rabbit anti-PA027 (50 µL of a 50% serum/glycerol solution) on ice/30min. Cells were washed and stained with FITC goat anti-rabbit (50 µL of 0.005% solution) on ice/30min. Control samples were also incubated with no primary antibody or normal sheep or rabbit serum. In (a), the forward scatter versus side scatter profiles of live and dead (65oC heat treatment/10min) PA027 are shown, along with the FL1 versus FL2 profiles, the forward scatter and FL2 profiles (all ungated, but PA027 indicated by the arrows), and the FL2 histograms (gated on PA027) after the addition of propidium iodide (10 µg/ml final concentration). In (b), samples of PA027 (5x105 in 200 µL SW/0.1%BSA) were stained as described above and heat treated (65oC) for the times indicated. The samples were then divided and either stained with PI (10 µg/ml final concentration) and or trypan blue (0.1% final concentration) and analysed by flow cytometry or in a haemocytometer in phase contrast light microscopy, respectively. Live versus dead PA027 were quantified by flow cytometry by firstly gating on PA027 using previously determined forward scatter and side scatter parameters, and then determining the proportion of PI-positive cells in the FL2 channel. This was compared to the number of trypan blue-positive PA027.

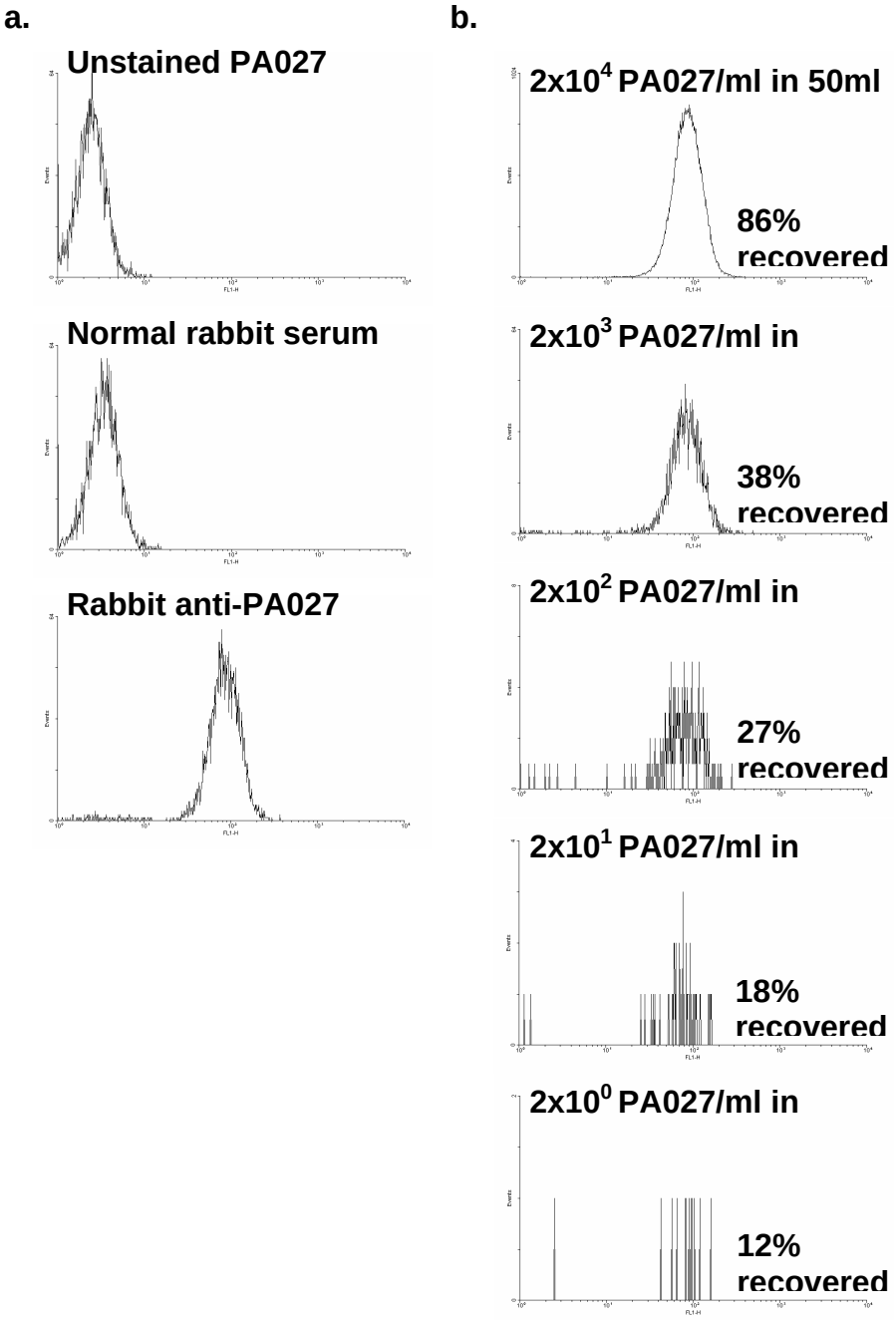


Figure 6. Recovery rates of cultured *N.pemaquidensis* from water samples by centrifugation.

Cultured *N.pemaquidensis* (PA027, $5x10^5$ in 200 μL SW/0.1%BSA) were stained with rabbit anti-PA027 (50 μL of a 50% serum/glycerol solution) on ice/30min. Cells were washed and stained with FITC goat anti-rabbit (50 μL of 0.005% solution) on ice/30min. Control samples were also incubated with no primary antibody or normal sheep or rabbit serum (a). In (b), various numbers of cultured PA027 were diluted into 50 mL SW and then recovered by centrifugation (1500 x *g* for 5min) and stained with rabbit anti-PA027 as described above. Cells were resuspended in SW/0.1% BSA and analysed by flow cytometry. *N.pemaquidensis* were gated on using previously determined forward scatter and side scatter parameters and the results are expressed as single parameter FL1 histograms.

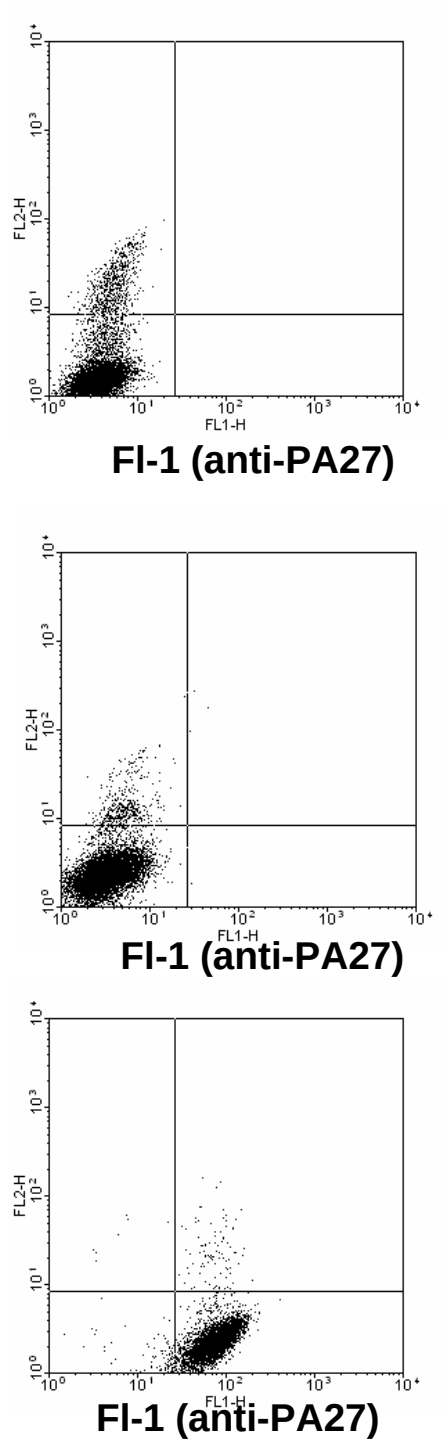


Figure 7. Flow cytometry can specifically detect viable *N. pemaquidensis*.
Cross- reactivity of biotinylated rabbit anti PA027. FL1 (cells positive to anti-PA027) versus FL2 (cells positive to propidium iodide showing cell viability).

Appendix 7

PRESENTATIONS MADE IN RELATION TO THIS REPORT

The Second Scientific Conference of the Atlantic Salmon Aquaculture Subprogram 8th July 2002 – Hobart, Tasmania
Detection of *Neoparamoeba pemaquidensis* in marine sediments in Tasmania. (Crosbie, Carson & Nowak, B.)
Amoebic gill disease – research highlights. (Nowak, Douglas-Helders, Gross, Bridle, Morrison, Crosbie, Bagley, Adams, Butler & Carson)

Aquafest September 2002, Hobart, Tasmania
Aquafin CRC Health Program – research highlights. (Nowak)

Aquafin CRC Conference 22-24 September 2002 – Hobart, Tasmania
Detection of *Neoparamoeba pemaquidensis* in marine sediments in Tasmania. (Crosbie, Carson, and Nowak)
Risk factors in Amoebic Gill Disease. (Bagley, Douglas-Helders, and Nowak)
Epidemiology in the future (Douglas-Helders)

International Symposium on Aquatic Animal Health, New Orleans, Louisiana, USA, September 2002
Amoebic Gill Disease in cultured salmonids (Nowak, Powell, Douglas-Helders, Adams, Crosbie & Carson)

Annual Scientific Meeting of the Australian Society for Parasitology, Hobart, Tasmania, 2002
Amoebae – fish parasitologists challenge? – invited keynote address (Nowak, Dykovà, Carson, Wong & Elliott)
Detection of *Neoparamoeba pemaquidensis* in marine sediments in Tasmania (Crosbie, Carson & Nowak)
11th International Conference of EAFP, Malta, Putting the Spotlight on Amoebic Gill Disease in Atlantic Salmon, C. Bagley, B. Nowak, M. Douglas-Helders and R. Morrison, poster presentation.

11th International Conference of EAFP, Malta, September 2003
Characterisation of Host-and Environment-Isolated Strains of *Neoparamoeba pemaquidensis*, the Causative Organism of Amoebic Gill Disease P.B.B. Crosbie, I. Dykova, B.F. Nowak and J. Carson, poster presentation.

International Symposium on Veterinary Epidemiology and Economics, Chile, November 2003
Putting the Spotlight on Amoebic Gill Disease in Atlantic Salmon, C. Bagley, B. Nowak, M. Douglas-Helders and R. Morrison, oral presentation.

Aquatic Animal Health Subprogram Scientific Conference, Geelong, October 2003
Amoebic Gill Disease research progress. B.Nowak., oral presentation.

Aquafin CRC Conference, Adelaide, October 2003
Characterisation of Host-and Environment-Isolated Strains of *Neoparamoeba pemaquidensis*, the Causative Organism of Amoebic Gill Disease P.B.B. Crosbie, I. Dykova, B.F. Nowak and J. Carson, poster presentation.
Putting the Spotlight on Amoebic Gill Disease in Atlantic Salmon, C. Bagley, B. Nowak, M. Douglas-Helders and R. Morrison, poster presentation.

TAFI Annual Review, Hobart, June 2004
Overview of AGD research, B.Nowak, oral presentation.

Salmon Industry Research Seminar, Hobart, July 2004
Overview of AGD research, B.Nowak, oral presentation.
AGD management and husbandry, B.Nowak, oral presentation

Appendix 8
PUBLICATIONS ARISING FROM THIS REPORT

Adams, ,M.B., Ellard, K., Nowak, B. (2004) Gross pathology and its relationship with histopathology of amoebic gill disease (AGD) in farmed Atlantic salmon *Salmo salar* L. Journal of Fish Diseases, 27, 151-161.

Crosbie, P.B.B., Nowak, B.F. and Carson, J. (2003) Isolation of *Neoparamoeba pemaquidensis* Page, 1987 from marine and estuarine sediments in Tasmania. Bulletin of European Association of Fish Pathologists, 23, 241-244.

Douglas-Helders, M., O'Brien, DP, McCorkell BE, Zilberg D, Gross A, Carson J and Nowak B (2003) Temporal and spatial distribution of paramoebae in the water column - a pilot study. Journal of Fish Diseases, 26, 231-240.

Douglas-Helders G.M., Weir I.J., O'Brien D.P., Carson J., Nowak B.F. (2004) Effects of husbandry on prevalence of amoebic gill disease and performance of reared Atlantic salmon (*Salmo salar* L.). Aquaculture, 241, 21-30.

Douglas-Helders, M, Saksida, S, Nowak, B.F (2004) Questionnaire-based risk assessment for amoebic gill disease (AGD) and evaluation of freshwater bathing efficacy of reared Atlantic salmon (*Salmo salar* L.) Diseases of Aquatic Organisms, in press.

Douglas-Helders, M., Nowak, B.F., Butler, R. (2004) The effect of environmental factors on the distribution of *Neoparamoeba pemaquidensis* in Tasmania. Journal of Fish Diseases, submitted.