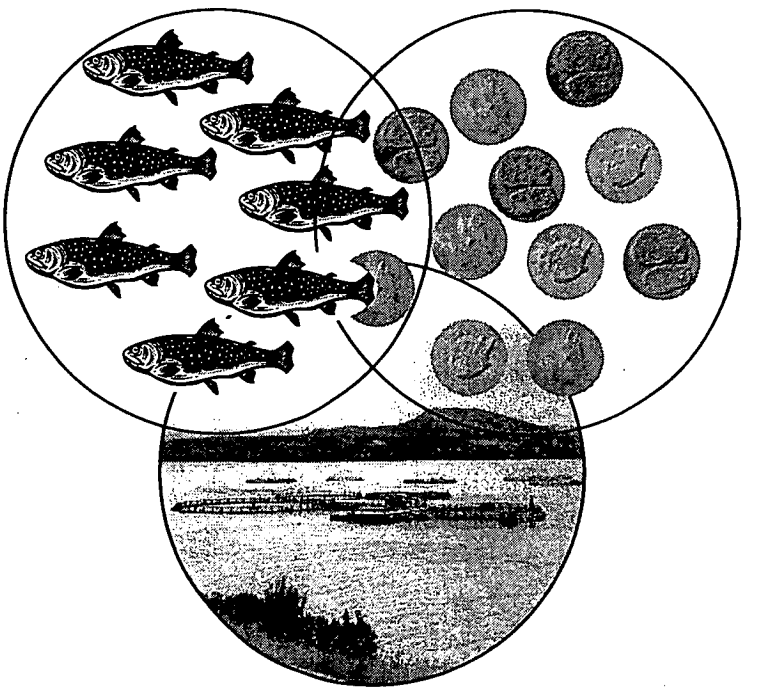
# Epidemiology of amoebic gill disease



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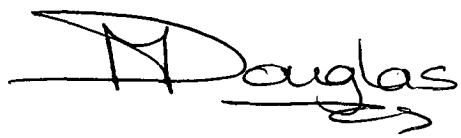

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## Abstract

Amoebic gill disease (AGD) is the main disease affecting the salmon industry in Australia, however inadequate information is available on the epidemiology of amoebic gill disease (AGD) and the biology of the pathogen, *Neoparamoeba pemaquidensis* (Page, 1987). Thus far no convenient mass screening test was available. In this project a pathogen specific and non-lethal dot blot test was developed and validated against indirect fluorescence antibody testing (IFAT), the 'gold standard'. The agreement between the 300 paired gill mucus samples that were analysed using both tests was high, with a corrected kappa value of 0.88. The overall aim of this project was to investigate distributions and seasonal patterns of the pathogen, identify risk factors for the disease and reservoirs of *N. pemaquidensis*, and develop and review husbandry methods in order to reduce AGD prevalence.

Results of an infection trial implied that transmission of AGD infections in the field do not only occur from fish to fish, but also from water to fish. Therefore distribution of paramoebae in the water column and seasonal patterns were investigated. The spatial and temporal distribution of paramoebae was determined using the dot blot test and most probable number techniques for the identification and quantification respectively. Associations between paramoebae densities and environmental conditions were also explored.

Potential reservoirs were investigated in both field and laboratory trials. In a laboratory study it was determined that dead AGD infected fish may be a reservoir of *N. pemaquidensis* when left in sea cages. In the laboratory trial, *N. pemaquidensis* remained on infected gills for at least 30 hours after death of the

host, and these protozoa from dead infected fish could colonise gills of previously uninfected dead fish. This would potentially increase the bio-burden of *N. pemaquidensis* on infected farms. AGD was not detected in wild fish and wild fish did not seem to be a reservoir of the pathogen.

Five different husbandry options were evaluated in extensive field trials with the aim to minimise the impact of AGD. Three of the husbandry options seemed beneficial in reducing either cost due to the disease and/or AGD prevalence on Tasmanian salmon farms. All three options could easily be incorporated into existing management plans.

## Acknowledgments

This epidemiological project was a steep learning curve, especially since my previous studies concentrated on immunology and parasitology. Many people, whom had a direct or indirect effect on the course of this project, promoted the steepness of the learning curve. First of all I would like to express my gratitude towards my two supervisors, Barbara Nowak, University of Tasmania, School of Aquaculture and Jeremy Carson, DPIWE, Fish Health Unit. The revision of the papers must have driven you to despair at times, but without your input I would not have been able to produce this report now. I also would like to thank Margaret Williams, DPIWE, manager and Jeremy Carson, DPIWE, Fish Health Unit for allowing me to use the facilities at Mount Pleasant Laboratories, even when offices and computers were scare. Also many thanks to Chris Baldock (Ausvet), whom offered his expertise on epidemiology through courses and farm visits. Thanks also to Mart de Jong and Klaas Frankena of the University of Wageningen, Department of Veterinary Epidemiology for taking the time to listen and the advice given. Most of the field trials were done at Huon Aquaculture Company. Lot of thanks go to those people who not only offered me accommodation on the regular farm visits, but also had their boats and gear ready every time. Special thanks to Innes Weir and Dominic O'Brien for taking care of the logistics, and giving me a helping hand with sampling. I definitely want to mention Adrian Steenholdt, whose organised and efficient way of working made the trips so much easier and quicker, and Bernadette Potter for getting data organised in excel sheets and answering many of my questions. Also thanks to many people of Tassal, Aquatas, Nortas, van Diemen Aquaculture, Sevrup, and SouthEast Atlantic (SA) for providing me with the

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# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 SALMON AQUACULTURE

Salmonid sea cage aquaculture in Tasmania emerged in 1984 with rainbow trout (*Oncorhynchus mykiss*), marketed as sea run trout, and Atlantic salmon (*Salmo salar*) as the culture species (Dix, 1986, Munday *et al.*, 1990). The first harvest yielded a modest 55 tonnes (Stanley, 1993), which increased to 11,742 tonnes with a value of AUD $ 92,847 million by 2000 (O'Sullivan & Roberts, 2001). Globally this is a relatively small yield compared to countries such as Norway, which produced 440,000 tonnes. Soon after the introduction of salmonid aquaculture in Tasmania amoebic gill disease or AGD was detected and described (Munday *et al.*, 1990). This disease is caused by a protozoan *Neoparamoeba pemaquidensis*, and has become the predominant disease affecting the profitability of the salmon industry in Tasmania at present (Munday *et al.*, 1990). Of the total production cost, 10-20% is spend on the management of AGD (Munday *et al.*, 2001), resulting in a less favourable position for competition ion with overseas salmonid markets. It is therefore of vital importance for Tasmanian salmonid growers to gain a better understanding of the epidemiology of AGD, so that efficient control procedures can be developed and executed.

## 1.2 EPIDEMIOLOGY IN AQUACULTURE

Epidemiology is the study of disease in populations and of factors that determine its occurrence (Thrusfield, 1995), so that transmission or expression of the disease can be minimized (Hammell, 1999). Epidemiological studies can be observational,

in which the natural occurrence of diseases is studied (Thrusfield, 1995), and include cross-sectional, case-control, and cohort studies (Thrusfield, 1995, Frankena & Thrusfield, 1997). Other epidemiological studies are experimental, in which the efficacy of treatments by intervention are tested (Thrusfield, 1995). The determination of the unit of concern is of great importance, and sampling should occur accordingly. In salmon aquaculture the sea cage should be the unit of concern, so that bias can be minimized (Thrusfield, 1995, Hammell, 1999). Bias is the average of errors of the estimate (Hammell, 1999). The preferred method of sampling is random, for obtaining an accurate estimate of the disease status in a population, unless random sampling is not feasible or extremely expensive (Snedecor & Cochran, 1980). In the Atlantic salmon production it is rare that all individual fish can be sampled randomly (Hammell, 1999). The most common sampling method used in sea rearing facilities are crowding followed by dip netting (Hammell, 1999). This is a non-random or convenient sampling method (Martin *et al.*, 1987), and had the potential for bias (Hammell, 1999). However, Thorburn (1992) found that the number of times fish were caught using crowd and dip netting did not significantly differ from random sampling in a tank situation.

Diagnostic tests are used to enable an estimation of the prevalence or amount of disease in a known population, at a designated time, without distinction between old and new cases (Thrusfield, 1995). For epidemiological studies the diagnostic test should be non-lethal, specific, sensitive, and have a high repeatability, so it can be used to conveniently analyze large numbers of samples. However, diagnostic tests are imperfect, and test results can be prone to false positive and false negative interpretation of the true situation (Henken *et al.*, 1997, Hammell, 1999). The

usefulness of any diagnostic test will depend on the disease prevalence in a population (Baldock, 1990), and the validity of the test, which is measured by its sensitivity and specificity (Henken *et al.*, 1997). Tests with high sensitivity are useful when no false negative results are allowed (eg. zoonotic diseases), or when the probability of the disease is low (Henken *et al.*, 1997). Tests with high specificity are useful if false positives are undesired (eg. when positive animals require slaughtering). The precision of the sensitivity and specificity can be obtained by calculating the confidence interval (CI, Thrusfield, 1995, Henken *et al.*, 1997).

## 1.3 WHY DO DISEASES SUCH AS AGD OCCUR?

Diseases occur as an interaction between pathogen, susceptible host(s) and the environment, called the "triad" (Martin *et al.*, 1987, Thrusfield, 1995, Callinan, 1999). For AGD the pathogen is *N. pemaquidensis*, susceptible hosts are certain fish species, and the environment is the sea. The interactions between these factors can be complex (Thrusfield, 1995, Frankena & Thrusfield, 1997, Menzies *et al.*, 1998), which makes it often difficult to determine the cause(s) of a disease. Those elements that increase the risk of a disease are called risk factors (Thrusfield, 1995). A factor that is associated with the independent factor and the dependent factor under study is called a confounding factor (Martin *et al.*, 1987, Frankena & Thrusfield, 1997, Hammell, 1999). Confounding is a common phenomenon, and many host variables such as sex and age may be confounding factors, and can mask a real association between a causal factor and disease (Martin *et al.*, 1987). Risk factors can be attributed to pathogenic risk factors, host risk factors, and

environmental risk factors (Davidson, 1999), and interaction of these factors can result in diseases such as AGD.

## 1.4 AMOEBIC GILL DISEASE

Presently AGD is the main disease that affects the salmonid industry in Tasmania (Nowak, 2001). *Neoparamoeba pemaquidensis*, the disease causing protozoan, is not confined to Tasmanian waters (Munday *et al.*, 1990, 1993), with outbreaks recorded in Ireland (Rodger & McArdle, 1996, Palmer *et al.*, 1997), France (Findlay & Munday, 1998), Spain (Dyková *et al.*, 2000), New Zealand (Clark & Nowak, 1999), Washington State and California, USA (Kent *et al.*, 1988), and in Chile (D.Groman & P.Bustos, pers. comm.). Fish species affected by *N. pemaquidensis* include Atlantic salmon (*Salmo salar*), chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*), turbot (*Scophthalmus maximus*), European sea bass (*Dicentrarchus labrax*), and sharpsnout seabream (*Diplodus puntazzo*) (Kent *et al.*, 1988, Clark & Nowak, 1999, Dyková *et al.*, 2000, Munday *et al.*, 2001, Dyková & Novoa, 2001).

All parasites use energy of the host, which otherwise would be available for host growth and survival (Bakke & Harris, 1998). The losses due to AGD outbreaks in Tasmania can be high, with mortalities as high as 50% reported when the disease was left untreated (Munday *et al.*, 1990). Other factors adding to the costs of AGD is high treatment costs (Munday, *et al.*, 1990, Parsons *et al.*, 2001), and retarded growth in infected fish (Rodger & McArdle, 1996, Dyková, *et al.*, 1998).

Freshwater bathing is the main and, thus far, most successful treatment method for AGD (Munday *et al.*, 1990, Parsons *et al.*, 2001). In a freshwater bath fish are immersed into oxygenated fresh water for a duration of up to four hours (Munday *et al.*, 1990, Parsons *et al.*, 2001). Early *in vitro* studies showed that 100% of cultured *N. pemaquidensis* died after exposure to fresh water (Howard & Carson, 1993). In the field AGD prevalence was reduced for up to 21 days post freshwater bath (Clark & Nowak, 1999), but a total removal of the parasite has yet not been achieved (Parsons *et al.*, 2001). The addition of the immuno-stimulant levamisole in the freshwater bath was trialed to optimise of the removal of *N. pemaquidensis*, but success of the laboratory trials did not transfer to the field. A significant decrease in mortalities (Zilberg *et al.*, 2000), an indication of stimulation of the nonspecific immune system (Findlay & Munday, 2000), and enhanced resistance to reinfection of fish with *N. pemaquidensis* (Findlay *et al.*, 2000) were reported in laboratory studies. However, in the field the addition of levamisole in the freshwater bath did not affect the bathing efficiency and reinfections are common (Clark & Nowak, 1999). Alternative methods to reduce the impact of AGD for farmers are necessary for the salmonid industry in Tasmania.

## 1.5 POSSIBLE RISK FACTORS AND AGD

It is not until the disease and its causal factors are known that effective control and/or prevention measurements can be implemented (Thrusfield, 1995). Pathogenic risk factors of *N. pemaquidensis* are its behavior in the environment, its virulence, and reservoirs of infection. Within sea cages, pathogen dispersal does not take place efficiently, which allows for reinfection (Bakke & Harris, 1998). Pathogens are often not spread homogeneously through its environment, but are

aggregated in reservoirs (Bakke & Harris, 1998). Suggested reservoirs of *N. pemaquidensis* were AGD infected fish (Munday *et al.*, 2001), the water column (Tan *et al.*, 2002), nets of sea cages (Tan *et al.*, 2002), and biofouling (Tan *et al.*, 2002). An increase in virulence was noted with continuous passage through naïve hosts (Findlay *et al.*, 2000), but why the protozoan colonised the gills of these hosts, remains unknown (Nowak, 2001). It was suggested that *N. pemaquidensis* is an amphizoic protozoan (Dyková *et al.*, 1998, 1999, Leiro *et al.*, 1998), and that under certain conditions the normally free-living protozoan becomes parasitic (Lom & Dyková, 1992). Freshwater resistant strains could have developed over time, as a result of selection of paramoebae that survived the freshwater bath (Parsons *et al.*, 2001).

Host risk factors are factors that affect the host's susceptibility to infections, or when infected, determine the outcome of the infection (Davidson, 1999). The host susceptibility is determined by species, age, and general health status at infection, as well as genetic make up of the host (Davidson, 1999, Munday *et al.*, 2001, Nowak, 2001). Susceptibility to AGD may differ between fish species reported to be affected, and it has been suggested that Atlantic salmon and rainbow trout were more susceptible to AGD compared to chinook salmon (Munday *et al.*, 2001). Older fish, but not very large fish, were reported to be less sensitive to AGD (Nowak, 2001), while sexually mature fish appeared to be more susceptible (Munday *et al.*, 2001). Poor gill health, mainly due to the presence of lesions, may predispose fish to AGD (Nowak, 2001), and gills with severe lesions due to jellyfish attacks were rapidly colonised by the protozoan (Munday *et al.*, 2001). The susceptibility of the host to AGD may be affected by its ploidy status, with

triploids thought to be more sensitive to AGD compared to diploids (Nowak, 2001). Acquired resistance to AGD has been reported on several occasions (Findlay *et al.*, 1995, Clark & Nowak, 1999) suggesting that vaccine strategy may be useful, but the extent of this protection remains unclear. The effects of general farm practices that are able to cause stress on fish, such as fish handling, cage movement, and stocking densities remain unknown (Nowak, 2001), as is the effect of the quality of the smolt (Nowak, 2001). In general, stresses, related to captive rearing in aquaculture, reduce the immuno-competence and predispose salmonids to diseases (Bakke & Harris, 1998).

Environmental risk factors can be divided into the physical environment, biological climate, and the socioeconomic environment (Davidson, 1999). The physical environment, such as salinity and temperature, is well known to influence AGD outbreaks (Rodger & McArdle, 1996, Clark & Nowak, 1999, Dyková *et al.*, 1998, Munday *et al.*, 2001, Nowak, 2001). Low rainfall, influencing salinity, was associated with AGD outbreaks in Tasmania and Ireland (Clark & Nowak, 1999, Munday *et al.*, 1993, Palmer *et al.*, 1997). Seasons was determined to be a risk factor for AGD, with outbreaks occurring in months with high temperature and salinity (Kent *et al.*, 1988, Munday *et al.*, 1990, Dyková *et al.*, 1998, Clark & Nowak, 1999). A bimodal pattern of AGD prevalence peaks was detected in Tasmania, with the first and highest peak in summer (December/January), followed by a smaller peak in autumn (March/April) (Clark& Nowak, 1999). Strong water currents were suggested to be negatively correlated with the prevalence of AGD (Nowak, 2001), as was the dissolved oxygen level at one of the four farms studied in the field (Clark & Nowak, 1999). *Neoparamoeba pemaquidensis* did not seem to

be very much affected by pollutants and was found in heavily polluted water, including those contaminated by heavy metals (Corpe, 1976; Sawyer, 1980). The effects of other physical environmental factors, such as chemical properties of the water column or amount of suspended matter in the water column are unknown.

The effect of biological environmental factors, such as number of bacteria on gills, presence of jellyfish, occurrence of algal blooms, and interaction with wild fish, have been studied. However, the effect of some of these factors on AGD prevalence remained unclear. Field observations showed that algal blooms did not affect the gill health of farmed Atlantic salmon (Cameron, 1993, Clark & Nowak, 1999), though results of experimental studies on the effect of blooms and AGD prevalence have not been reported. Jellyfish can cause severe gill lesions, causing massive fish mortalities (Munday *et al.*, 2001, Nowak, 2001), but the effect on AGD prevalence remains uncertain (Nowak, 2001). Excessive numbers of bacteria on gills often result in poor gill health (Cameron, 1993), but its effect on AGD remains unclear. Interaction with wild fish is likely to occur, but only would be a problem if wild fish were a carrier or reservoir for the pathogen.

The effects of the socioeconomic environment, such as management variables and economical conditions (Davidson, 1999), form an important part in AGD research. Effects of husbandry, such as cage density on site, stocking densities in cages, freshwater bathing strategies, frequency of fish handling, number of net changes, multi-age class profiles on site, cage movement and site fallowing periods, maintenance of high standards for cage and farm hygiene, control of contact between different farms, feeding rate and type, usage of food additives, and the

effect of an established health monitoring surveying program, remain largely unstudied in AGD research. The effect of the release of bath water after freshwater bathing on AGD prevalence, or the increase of organic matter due to intensive sea cage culture is unknown. A reduced AGD prevalence could be achieved by reducing fish biomass (Munday *et al.*, 2001, Nowak, 2001), and increasing the number of net changes (Clark & Nowak, 1999). Economical factors such as commodity prices, interest rates, and legislation (Davidson, 1999) could also be factors influencing the viability of salmonid farming in Tasmania.

## 1.6 PATHOLOGICAL EFFECTS OF AGD ON FISH

Fish with AGD seemingly suffer from respiratory distress evident by sluggish behaviour and by swimming to the surface with increased rate of opercular movement (Kent *et al.*, 1988, Munday *et al.*, 1990, 2001). Lack of appetite has also been reported (Munday *et al.*, 1990, Rodger & McArdle, 1996, Dyková *et al.*, 1998). It was suggested that fish died of AGD due to respiratory failure (Munday *et al.*, 1990, Bryant *et al.*, 1995, Dyková *et al.*, 1995), but in later studies this has been disputed (Powell *et al.*, 2000). It remains unclear what causes death of AGD infected fish.

Macroscopically, lesions are visible as slightly raised white mucoid patches on one or more gill filaments (Munday *et al.*, 1990, Alexander, 1991, Dyková *et al.*, 1998, Adams & Nowak, 2001). These patches were mostly found in the dorsal region of the gill arch (Adams & Nowak, 2001). In laboratory infection trials *N. pemaquidensis* was detected on histological sections of the gills as early as one day after exposure to the protozoan, and lesions were seen after two days post exposure

(Zilberg & Munday, 2000). These lesions are described as hypertrophy and hyperplasia of the lamellar epithelium, eventually resulting in fusion of the lamellae and the formation of crypts (Kent *et al.*, 1988, Roubal *et al.*, 1989, Rodger & McArdle, 1996, Dyková, *et al.*, 1998; Munday *et al.*, 1990; Clark & Nowak, 1999, Adams & Nowak, 2001). Though the severity of infection was proportional to the number of paramoebae administered in a laboratory infection trial (Zilberg *et al.*, 2001), severely AGD affected turbot gills did not necessarily harbor the pathogen in large numbers (Dyková *et al.*, 2001).

## 1.7 *Neoparamoeba pemaquidensis*

*Neoparamoeba pemaquidensis* is a naked and lobose protozoan (Page, 1976), and was formerly known as *Paramoeba pemaquidensis* (Page, 1987). The protozoan belongs to the subclass Gymnamoebia (Hackel, 1862), phylum of Rhizopoda (von Siebold, 1845), order of Amoebida (Ehrenberg, 1830), and genus Neoparamoeba (Page, 1987). With the inability to infect fish with cultured *N. pemaquidensis* and induce AGD in naïve fish, Koch's postulates have not been totally fulfilled (Zilberg *et al.*, 2001). However, it is generally accepted that *N. pemaquidensis* is the primary disease causing organism (e.g. Dyková *et al.*, 2000, Nowak, 2001). Dyková *et al.* (2001) suggested that the near related *Neoparamoeba aestuarina* should be taken into consideration as the agent for AGD as well.

*Neoparamoeba pemaquidensis* was isolated for the first time from marine water of Maine, USA (Page, 1970), and is now know to have a worldwide spread (Cann & Page, 1982). This free-living organism lives only in marine environments (Page, 1983), and is often found in coastal waters and the lower reaches of estuaries (Page,

1973). The success of survival of *N. pemaquidensis in vitro* is dependent on the temperature, with an optimum of 15°C reported by Kent *et al.* (1988), and temperatures never in excess of 22°C in studies by Howard (2001).

For reproduction and predation, the protozoan requires attachment to solid surfaces (Martin, 1985, Dyková *et al.*, 1995). Reproduction occurs asexually by binary or multiple fission (Page, 1970, Howard, 2001). The protozoan was detected in the environment on several occasions in the past, but significant reservoirs remain largely unknown. *N. pemaquidensis* was detected on nets of sea cages (Tan, *et al.*, 2002), biofouling organisms on nets (Tan, *et al.*, 2002), a parasitic isopod found on Atlantic salmon (Howard, 2001), sea water column (Elliott, *et al.*, 2001, Tan *et al.*, 2002), and sediments (Cann & Page, 1982).

## 1.8 METHODS FOR DETECTION OF *N. pemaquidensis*

On Tasmanian farms fish are crowded and dip netted from cages on regular time intervals, and the gills checked for the presence, size and number of white patches (Alexander, 1991). The number of affected fish and the severity of each infection result in a score, with each farm in Tasmania using their own specific scoring system (A. Steenholdt, pers. comm, Clark & Nowak, 1999). The score is commonly used by Tasmanian farmers to determine the need of freshwater bathing for a cage. However, this method was found to be an unreliable indication for AGD on Tasmanian salmon farms (Clark & Nowak, 1999). In addition, Dyková *et al.* (2001) found that gross lesions on gills were not always present when the protozoan had colonised gill of turbot. Diagnosis by biopsy, offering the advantage of non-lethal testing, underestimated the apparent prevalence and was not

considered a reliable means of confirming the presence of AGD in a population (Nowak & Lucas, 1997).

Detection of the presence of *N. pemaquidensis* can be achieved by using pathogen non-specific or specific tests, and samples can be obtained either by lethal or non-lethal sampling. An example of non-lethal sampling and non-specific testing is the wet mount preparation, where gill mucus is smeared onto a microscope slide and examined microscopically. The gill mucus smear can also be stained with non-specific dyes such as Quick Dip® (Zilberg *et al.*, 1999), to enable easier distinguishing between the different cells. In both cases, diagnosis is mainly based on the morphology of the pathogen. Histology is a reliable and commonly used diagnostic method for AGD (Adams & Nowak, 2001), but requires lethal sampling. Histological gill sections are often stained with non-specific stains, including haematoxylin and eosin (H&E) and less commonly reported a combined Alcian blue (AB, pH = 2.5) and periodic acid-Schiff (PAS) stain (Zilberg & Munday, 2000). Diagnosis, when using histology, is based on the morphology of the pathogen as well as the presence of AGD characteristic gill lesions. Specific stains involve pathogen specific labeling, so that *N. pemaquidensis* is visualised through attachment to a detectable label. These stains include indirect fluorescent antibody test or IFAT (Howard & Carson, 1993) and immuno-cytochemistry (Zilberg & Munday, 2000, Howard, 2001), and can be performed on histological sections as well as on gill mucus smears. IFAT is routinely used on Tasmanian farms as a reliable diagnostic method (Howard, 2001). While IFAT has proven to be an essential monitoring tool, it is not suitable for processing very large numbers of samples because of microscopy fatigue. Recently a specific PCR has been

developed, where diagnosis is based on the presence of an unique section of DNA in the conservative 18S rDNA gene region (Elliott *et al.*, 2001). However, at this stage this test is not optimised for use on gill samples. In this project AGD positive fish were defined as fish that tested positive for the presence of *N. pemaquidensis*, using IFAT, immuno-dot blot, or immuno-cytochemsitry.

# CHAPTER 2

# DETECTION OF NEOPARAMOEBA PEMAQUIDENSIS USING PATHOGEN SPECIFIC TESTS

## 2.1 DEVELOPMENT AND VALIDATION OF A NEW DOT BLOT TEST FOR THE DETECTION OF *Paramoeba pemaquidensis* (PAGE) IN FISH

M. Douglas-Helders, J. Carson, T. Howard and B. Nowak

### 2.1.1 Abstract

In this study, the development of a dot blot assay to assess amoebic gill disease (AGD) using non-lethal gill mucus samples is described and its performance validated by comparing the assay with indirect fluorescent test (IFAT), the 'gold standard' test. The agreement between the two tests was high, with a positive predictive value of 95% and negative predictive value of 93%, with a corrected *kappa* value of 0.88. The sensitivity and specificity of the test were 97% and 91%, respectively. The immuno-dot blot is both sensitive and specific for *Paramoeba pemaquidensis* and is formatted so that large numbers of samples can be conveniently analysed.

### 2.1.2 Introduction

Amoebic gill disease (AGD) is a major production limiting disease of farmed Atlantic salmon, *Salmo salar*, L., in Tasmania (Munday, Foster, Roubal & Lester,

1990; Clark & Nowak, 1999). The disease-causing agent has been identified as *Paramoeba pemaquidensis* (Page) (Kent, Sawyer, Hedrick, 1988; Howard & Carson, 1993). Amoebic gill disease became a major problem in Tasmania once salmon production became intensive and more full strength salinity rearing sites came into use (Munday *et al.*, 1990). Mortalities appear to be related to elevated water temperatures and salinity (Munday, Lange, Foster, Lester & Handlinger, 1993; Clark and Nowak, 1999). Problems with AGD in farmed fish are not limited to Tasmanian waters. Disease outbreaks have been reported in the USA (Kent *et al.*, 1988), Ireland (Rodger & McArdle, 1996), Spain (Dyková, Figueras & Novoa, 1995) and Chile (P. Buston, D. Groman and T. Wagner, personal communication). Clinical signs of disease are often seen at water temperatures above 12°C and when salinity approaches 35 ppt (Munday *et al.*, 1990; Clark & Nowak, 1999). Little is known about the epidemiology of the disease, largely because there has been no convenient test for mass screening of fish. Such a test must be non-lethal, have a format suitable for analysing large numbers of samples, be pathogen specific, sensitive, have a very high repeatability and should be convenient to perform.

Currently several tests are available to detect *P. pemaquidensis*. Histological examination of gills is considered to be the most reliable means of confirming AGD. *Paramoeba pemaquidensis* attaching to gills cause a characteristic cytopathology. The gills firstly produce excess mucus followed by thickening of the secondary lamellae. Hypertrophy and hyperplasia of the lamellar epithelium will occur, eventually resulting in fusion of the lamellae and the formation of crypts (Roubal, Lester & Foster, 1989; Munday *et al.*, 1990; Dyková, Figueras, Novoa & Casal, 1998; Clark & Nowak, 1999). Although a reliable means of confirming the

disease, it has the disadvantage that fish are killed to reach a diagnosis. Non-lethal testing by gill biopsy underestimates the apparent prevalence and is not considered a reliable means of confirming the presence of AGD in a population (Nowak & Lucas, 1997).

Farms also monitor Atlantic salmon for the severity of infection by gross gill checks. White mucoid patches or excessive mucus are an indication of AGD infection. These gross signs, however, are not a reliable indication of AGD (Clark & Nowak, 1999). In Tasmania, farms routinely confirm gross signs of disease by preparing smears of gill mucus for the detection of *P. pemaquidensis* by indirect fluorescent antibody test (IFAT) (Howard & Carson, 1993). The antibody is specific for *P. pemaquidensis* and can also be used for immuno-cytochemistry staining in histological sections (Howard & Carson, 1993). While IFAT has proved to be an essential monitoring tool, it is not suitable for processing very large numbers of samples because of slide reading fatigue.

For epidemiological studies, the need has arisen for the development of a specific and sensitive test that can be used to conveniently analyse large numbers of samples. The immuno-dot blot format is ideally suited for the detection of particulate as well as soluble antigens in a 96 well format. The test has wide application and has been used to detect antigens such as malarial proteins (Noya & Noya, 1998) as well as antibodies to verototoxin produced by *Escherichia coli* (EC; Chart & Rowe, 1997).

This study describes the development and validation of an immuno-dot blot test for the detection of *P. pemaquidensis* antigen in gill mucus.

## 2.1.3 Materials and Methods

### 2.1.3.1 Antigen preparation

*Paramoeba pemaquidensis*, clone Department of Primary Industries Water and Environment (DPIWE) PA027, the bacterial substrate *Stenotrophomonas maltophilia* (Hugh) (DPIWE PA1 strain) and *Escherichia coli* (ATCC 25922) were obtained from the culture collection of the Fish Health Unit of the DPIWE, Launceston, Australia. *Paramoeba pemaquidensis* was grown on malt east (MY) extract agar plates with 75% natural seawater, to which 500 µL pimaracin (P0440 Sigma-Aldrich Chemicals, sterile suspension 25 mg $mL^{-1}$) was added as an antifungal agent. Just prior to inoculation with paramoebae, the plates were seeded with a live suspension of either *S. maltophilia* (SM) or EC. Purity of the bacterial suspensions was tested by subculture on blood agar (Oxoid Blood Agar Base No.2, enriched with 7% defibrinated sheep's blood) and incubated at 37°C for 48 hours.

At harvest, MY plates were flooded with sterile seawater and the paramoebae gently removed from the plates by scraping. The cell suspensions were washed three times with sterile seawater at 1,000 *g* for 20 min to reduce the bacterial load. After washing, the pellet was resuspended in 6ml of sterile seawater and the density of paramoebae determined using a haemocytometer with Neubauer rulings.

*Paramoeba pemaquidensis* grown on SM were sonicated (Branson B-15, Danbury, CT, USA) on ice using five pulses of 60 Watt for 3 min each with 5 min cooling

between pulses (Catty & Raykundalia, 1989). Cells were checked for cell lysis by microscopy after each round of sonication. The suspension was then centrifuged at 2,000 *g* for 20 min and the supernatant removed. The pellet was resuspended in 6 mL of sterile seawater. The paramoebae grown on EC were not sonicated. The protein content of the prepared paramoebae suspension was measured by microanalysis using the bicinchroninic acid protein assay kit, Sigma procedure number TPRO-562 (Sigma, B-9643). The suspension was inactivated by the addition of formalin to a final concentration of 0.5% v/v and incubated at 4°C overnight before storing at –20°C. The sterility of the inactivated paramoebae suspension was checked by inoculation of the suspension onto blood agar plates and incubating at 37°C for 48 h. The SM preparation was used for raising antisera in rabbits for serum production while the EC preparation was used to titrate the rabbit antisera, and as positive controls for cross-reaction tests by IFAT and immuno-dot blot assay.

2.1.3.2 Serum production and characterisation

Antisera to *P. pemaquidensis* were prepared in six rabbits by two subcutaneous injections of 1.0 mg protein, equivalent to 9.1 X $10^6$ cells of sonicated *P. pemaquidensis*. A booster dose of 1.0 mg protein equivalent of 9.1 X $10^6$ *P. pemaquidensis* cells was given by subcutaneous injection 1 week later. After 3 weeks the rabbits were bled twice over a period of 2 weeks and the serum collected. Before use of the serum, it was adsorbed with a surplus amount of SM. For adsorption, 1.5 mL of 2 X $10^{10}$ cells $mL^{-1}$ of SM was centrifuged for 1 min at 2,300 *g* to pellet the cells. One millilitre of the antiserum was added to the pellet and vortexed to prepare a homogenous suspension, agitated for 2 h at room

temperature, and stored at 4°C overnight. The suspension was centrifuged at 2,300 *g* for 90 s and the adsorbed serum (SAS) collected and stored at -20°C (Poole, 1989).

The specificity of the primary antibody was assessed by IFAT on a known positive slide where either *P. pemaquidensis* PA027 adsorbed primary antibody or pre-bleed negative serum was used. As a control, non-PA027 absorbed positive serum was used on a known positive slide. The method of adsorption used was as previously described for SM adsorption. The titre of the serum was determined by IFAT (Howard and Carson, 1993) using an Olympus BX40F-3 epi-fluorescence microscope and FITC filter set. In addition, specificity of the serum was determined by IFAT for near related paramoebae using the following reference strains: *P. pemaquidensis* (American Type Culture Collection-ATCC 50172 and 30735), *Neoparamoeba pemaquidensis* (Page) (Culture collection of Algae and Protozoa-CCAP 560/4 and 1560/5), *Pseudoparamoeba pagei* (Sawyer) (CCAP 1566/1), *Paramoeba eilhardi* (Schaudinn) (CCAP 1560/2) and *Neoparamoeba aestuarina* (Page) (CCAP 1560/7). The prepared serum (SAS) was used as primary antibody in both IFAT and dot blot tests.

2.1.3.3 Sample collection and preparation

The fish were caught by crowd and dip netting and anaesthetised in 0.5% Aqui-S®, Lower Hutt, New Zealand. Mucus was scraped off the second gill arch on the left hand side of the fish, using a wooden (white birch) toothpick (Alpen, China), suspended in a 1.5 mL microfuge tube containing 400 µL, 0.22 µm filtered and autoclaved (121°C, 15 min) natural seawater and kept on ice during sampling. The

mucus was digested by adding 400 µl of 1% w/v of the mucolytic agent N-acetyl-L-cysteine (BDH, Melbourne, Australia) in distilled water (Desjardin, Perkins, Teixeira, Cave & Eisenach, 1996) and incubated at 37°C for 1 h. The digested mucus was decolorized and cells lysed by adding 40 µl of 0.21% v/v sodium hypochlorite and 0.045% v/v sodium hydroxide, vortexed, and incubated on a shaker at room temperature for 8 min. The samples were further treated by adding 10 µL of 2 N hydrochloride, vortexed, and incubated on a shaker at room temperature for 30 min. Finally, the samples were frozen at –20°C, thawed rapidly at 37°C and re-frozen. Just prior to use, the samples were centrifuged for 20 s at 15,600 *g*, the supernatant collected and used for dot blotting.

2.1.3.4 Assay protocol

Immobilon-P$^{TM}$ PVDF membrane (Bedford, MA 01730, USA) with 0.45 µm pore size (Millipore, Bedford, MA 0.1730, USA) was soaked in 100% ethanol for 15 s, then reagent grade water (<2µ Sm) for 2 min, followed by phosphate buffered saline (PBS; 0.1 M, pH 7.4) for 5 min. Digested mucus samples were applied in duplicate to the membrane in a 96 well vacuum dot-blotter (Millipore) and incubated for 18 min at room temperature. The samples were then drawn through the membrane by applying 15mmHg of vacuum. The membrane was removed from the blotter and washed in a four step protocol with the following buffers, each for 5 min: once in PBS, twice in PBS-0.05% Tween 20 (PBS-T), and once more in PBS. The membrane was blocked by incubating for 1 hour in 2.5% w/v casein (BDH, cat. no. 44016) in PBS-T followed by the four step wash protocol.

Prepared membranes were probed with the SAS primary antibody (rabbit) to *P. pemaquidensis* PA027 diluted 1:600 in PBST, incubated for 25 min at room temperature and washed as described. A secondary antibody, anti-rabbit alkaline phosphatase conjugate (Silenus, Melbourne, Australia) diluted 1:4000 in PBS-T was applied to the membrane and incubated for 20 min at room temperature. The membrane was then washed for 5 min with each of the following: once in Tris buffered saline (TBS; 0.1 M, pH 7.4), twice in TBS-0.05% v/v Tween 20 (TBS-T), and once more in TBS.

The blots were visualised using fast BCIP/NBT (Moss Inc., MD, USA) as the alkaline phosphatase substrate; colour development was stopped by washing the membrane twice for 5 min in reagent grade water. Best visualisation was obtained when the membrane was still wet and all tests were read at this stage. Each assay included a positive control of *P. pemaquidensis* (PA027) grown on EC and negative controls of gill mucus from freshwater Atlantic salmon and PBS. The immuno-dot blot assay was optimised by determining the best possible concentration of primary antibody required to maximize the blot signal with the least amount of non-specific background coloration essential for obtaining the best possible signal-to-noise ratio with good test sensitivity.

2.1.3.5 Test validation

The capacity of the immuno-dot blot to detect both soluble as well as particulate antigens of *P. pemaquidensis* was assessed by immuno-dot blot, using whole cell PA027 antigen as well as particulate cell fragments of cultured PA027 and particulate cell fragments of amoebae collected from the gill.

In total, 300 Atlantic salmon were sampled from various sources (Table 1). The immuno-dot blot was validated using gill mucus samples from several populations of Atlantic salmon with AGD of varying severity. Each gill mucus sample was used for both dot blot and IFAT analysis. One of the paired gill mucus samples was tested for presence/absence of *P. pemaquidensis* by IFAT, the reference 'gold standard' test. The IFAT was performed as described and the entire mucus area on the slide scanned for the presence of fluorescent labelled paramoebae cells at 100X final magnification using an Olympus BX40F-3 UV epi-fluorescence microscope and FITC filter set. The other paired mucus sample was tested by the optimized immuno-dot blot assay. Samples were all tested in duplicate dot blots. Repeatability of the blots was determined by selecting 22 mucus samples at random and re-analysing them at a later date.

Thirty-six of the Atlantic salmon samples were analysed blind (12%) for which paired gill samples were taken, and analysis was carried out for dot blot and IFAT by different people.

For comparison of the IFAT test with the immuno-dot blot test, IFAT validation data of Howard & Carson was used (Table 2) and a corrected *kappa* coefficient, an index of concordance, was calculated (Cicchetti & Feinstein, 1990). Data of immuno-dot blot and IFAT comparison were calculated in the same manner.

**Table 1**: Sources of samples for immuno-dot blot test validation

| Source | # Fish sampled | # cages | Location | Type of samples |
|---|---|---|---|---|
| Commercial farms | 183 | 6 cages | Farms B,C,D | Routine gill checks |
| Experimental infection trials | 33 | 9 & 6 tanks | University of Tasmania | Infectivity experiment |
| Known AGD positive stock | 17 | 1 tank | University of Tasmania | Validation test |
| Known AGD negative stock | 24 | 1 tank | University of Tasmania | Validation test |
| Commercial farm | 29 | 1 cage | Farm A | Harvest samples |
| Commercial farm | 14 | 1 cage | Farm A | Post freshwater bathing samples |

**Table 2**: Histopathology and IFAT comparison matrix, based on data from Howard and Carson (1993)

| | Histopathology | | |
|---|---|---|---|
| | + | _ | Total |
| IFAT | | | |
| + | 50 (31.1) | 4 (2.5) | 54 (33.6) |
| _ | 2 (1.2) | 105 (65.2) | 107 (66.4) |
| Total | 52 (32.3) | 109 (67.7) | 161 (100) |

Data as number of test in agreement or not in agreement. Figures in parentheses are percentages.

## 2.1.4 Results

The yields of *P. pemaquidensis* PA027 for antiserum production in two lots were 19.2 X $10^6$ cells $mL^{-1}$ and 6.75 X $10^6$ cells $mL^{-1}$, with protein concentrations of 2.11 and 1.67 g $L^{-1}$ respectively. The optimum dilution of the SAS for IFAT was 1:150 using homologous antigen of whole cells of *P. pemaquidensis* PA027. The reactivity of the antibody was assessed by IFAT with other strains of *P. pemaquidensis* and near related species and genera (Table 3); with some species, other than *P. pemaquidensis*, cross-reaction was detected.

**Table 3**: Reactivity of *Paramoeba pemaquidensis* DPIWE PA027 antibody with closely related species of paramoebae

| Species | IFAT | Source |
|---|---|---|
| *P. pemaquidensis* (ATCC 30735) | Positive | Seawater, USA |
| *P. pemaquidensis* (ATCC 50172) | Positive | Coho salmon, USA |
| *P. eilhardi* (CCAP 1560/2) | Negative | Seawater, France |
| *Neoparamoeba pemaquidensis* (CCAP 1560/4 and 5) | Positive | Seawater, Wales, UK |
| *Neoparamoeba aestuarina* (CCAP 1560/7) | Positive | Seawater, Portugal |
| *Pseudoparamoeba pagei* (CCAP 1566/1) | Positive | Seawater, England |

The specificity of the antibody was further assessed using gill smears prepared from fish with clinical signs of AGD and testing in duplicate by IFAT using SAS adsorbed with PA027, non-PA027 adsorbed SAS and normal serum (pre-bleed). *Paramoeba pemaquidensis* were detected only with the non-PA027 adsorbed SAS; no positive cells were detected with the PA027 adsorbed SAS and normal serum.

Significant reduction in background colour was achieved when SM adsorbed antiserum was used in the tests.

For the immuno-dot blot assay, the primary adsorbed antiserum (SAS) for the detection of *P. pemaquidensis* gave optimum performance at a dilution of 1:600 and the secondary antibody, anti-rabbit alkaline phosphatase conjugate, at a dilution of 1:4,000. Colour reactions were not detected using pre-bleed with positive samples, nor was colour detected using gill mucus samples collected from specific pathogen free fish (Atlantic salmon hatchery pre-smolts) or PBS negative controls.

All gill mucus samples were tested in duplicate, and no difference in colour intensity could be detected between either replicate. Twenty-two mucus samples were chosen at random and re-tested; in 20 cases there was complete agreement between the first and second tests.

The minimum detection level for the immuno-dot blot assay was determined by titration of a suspension of *P. pemaquidensis* PA027. The cut off value, determined as the last blot with clearly evident colour and the negative controls still colourless, was determined at a dilution of 1:40,960 equivalent to 16 paramoebae or 4.1 g of protein in 100 µL when using untreated whole cells of PA027. When the PA027 cell suspension was digested as described in the protocol, the cut off value was 4 paramoebae or 1.4 ng of protein (Figure 1). By testing whole cell as well as soluble PA027 antigens, it was established that both soluble and whole cell paramoebae antigens could be detected successfully by the immuno-dot blot assay.

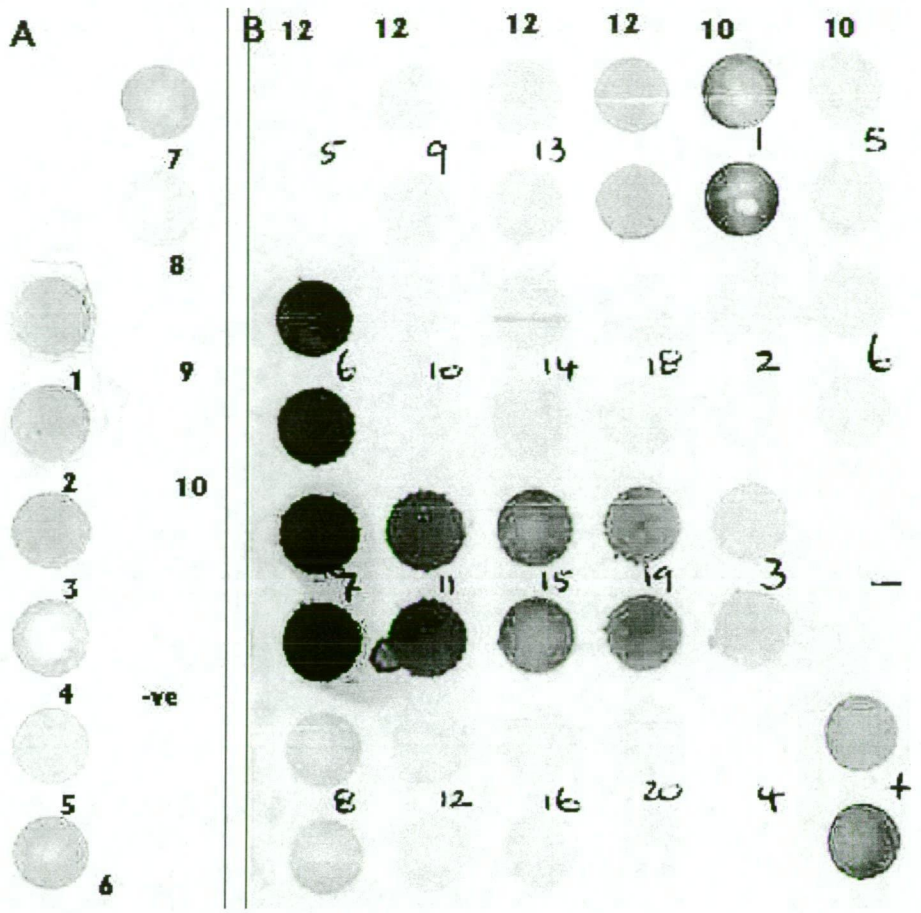


**Figure 1**: Immuno-dot blot test for *Paramoeba pemaquidensis*. Antigen titration of *P. pemaquidensis* PA027 (A). Dilutions 1-10 were equal to 527 cells (1), 264 cells (2), 132 cells (3), 66 cells (4), 33 cells (5), 16 cells (6), 8 cells (7), 4 cells (8), 2 cells (9), 1 cell (10); -ve: negative control sample (PBS).

*Paramoeba* extracted from gills of farmed Atlantic salmon in sea cages 10 and 12 (B). All samples in duplicate; -: negative control (PA027). Samples 5 and 10 from sea cage 12 and samples 2 and 4 from sea cage 10 were considered negative in this test.

Performance of the immuno-dot blot assay was compared with IFAT (Table 4). There was no significant difference between the two tests ($p < 0.05$) when a likelihood $\chi^2$ analysis was used for comparison of the numbers of positives recorded for each of the tests. In other words, the results of this chi-square analysis indicated that the result obtained (positive or negative) is independent of the test used (IFAT or immuno-dot blot).

**Table 4**: IFAT and immuno-dot blot comparison matrix

| | | IFAT | | |
|---|---|---|---|---|
| | | + | − | Total |
| Immuno-dot blot | | | | |
| | + | 165 (55%) | 14 (4.6%) | 179 (59.7%) |
| | − | 5 (1.7%) | 116 (38.7%) | 121 (40.3%) |
| Total | | 170 (56.7%) | 130 (43.3%) | 300 (100%) |

Data as number of tests in agreement or not in agreement. Figures in parentheses are percentages.

Analysing the data, using the method of Cicchetti & Feinstein (1990), showed that the immuno-dot blot assay has a sensitivity of 97% and a specificity of 91%; positive and negative predictive values were 95% and 93% respectively. Prevalence at the time of sampling was 57% and the calculated predictive values are valid for this level of prevalence only. Positive and negative predictive values for prevalences from 0 to 100% are given in Fig. 2 (Baldock, 1990) to enable estimation of positive and negative predictive values at any given prevalence. The corrected *kappa* coefficient of test concordance for the immuno-dot blot test when

compared with IFAT was 0.88 (SE 0.057), indicating a high level of agreement between the two tests. In 14 of the 300 samples tested (Table 4) the immuno-dot blot was positive while the IFAT assay was negative which translate to a 4.6% non-agreement, but overall there was a very good agreement between IFAT and the immuno-dot blot test.

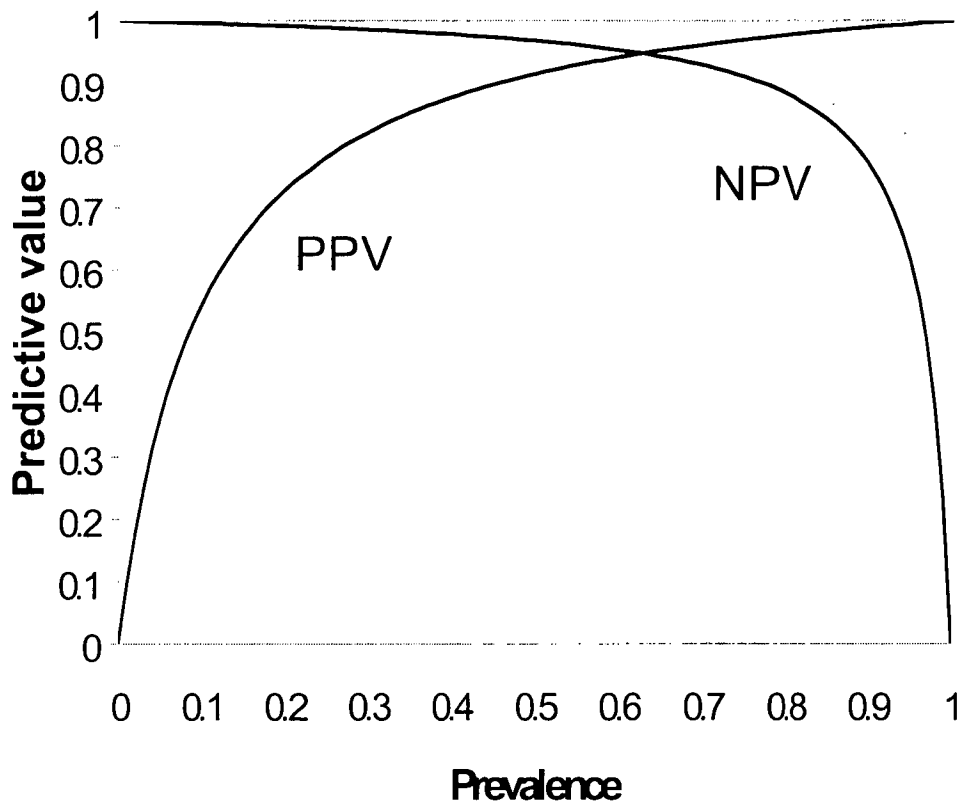


**Figure 2**: Positive and negative predictive values for the immuno-dot blot test at prevalences ranging from zero to 100%. PPV: Positive predictive values; NPV: Negative predictive values

## 2.1.5 Discussion

Signs of clinical disease in Atlantic salmon are assessed on farms by examining the gills of fish for the presence of pathognomonic mucous patches (Munday *et al.*, 1990). Gill examination however, as a means of assessing presence or absence of the disease or in determining the severity of infection has been found to be unreliable (Clark & Nowak, 1999). Confirmation of the disease can be achieved by histological examination of the gills or by the use of a non-lethal test, IFAT using antiserum specific for *P. pemaquidensis* (Howard & Carson, 1993). This method is now used widely in Tasmania as a rapid and convenient confirmation tool and is the *de facto* 'gold standard'.

Specificity of polyclonal antisera to *P. pemaquidensis* DPIWE PA027 has been assessed using a range of amoebae commonly found on gills of fish with AGD (Howard & Carson, 1993). No cross reactivity was detected with *Platyamoeba plurinucleolus* (Page), *Platyamoeba/Vanella* (Page) or *Flabellula* (Schmoller) (DPIWE FLB 004) (Howard and Carson, 1993) and specificity of the antiserum was considered high for the purpose of confirming presence or absence of *P. pemaquidensis* in gill mucus. Our results demonstrate that antiserum to *P. pemaquidensis* PA027 react with *P. pemaquidensis* ATCC 50172, isolated from coho salmon, *Oncorhynchus kisutch* (Walbaum), in Washington State, USA (Kent *et al.*, 1988) as well as the near related species *N. aestuarina* and *Pseudoparamoeba pagei* but not *Paramoeba eilhardi*. When these species were tested with antiserum adsorbed with PA027, or pre-bleed, negative serum, the IFAT tests were negative and are an indication not only of specificity but also the presence of a common antigen amongst these near related species of paramoebae.

*P. pemaquidensis* is reported as the predominant species on the gill of fish with AGD; other amoebae are known to be present but only in very low numbers (Howard & Carson, 1993). The near related species of paramoebae found to cross-react with *P. pemaquidensis* antiserum have not been isolated from the gills of fish with AGD (Howard & Carson, 1993). On this evidence, the antiserum developed for the immuno-dot blot is considered to be highly predictive of *P. pemaquidensis* in the gills of fish.

Further evidence of the antiserum specificity and utility of the immuno-dot blot assay in detecting *P. pemaquidensis* on the gills of fish was obtained by validation with IFAT and co-validation with histopathology. In calculating the index of concordance between IFAT and histopathology for the data of Howard & Carson (1993) (Table2), the marginal totals were found to be symmetrical but unbalanced and the high value of the observed proportion of agreement, Po, can as a result be drastically reduced (Feinstein & Cicchetti, 1990). This anomaly was assessed by calculating the positive predictive value ($P_{pos}$) and negative predictive value ($P_{neg}$), which are analogous to sensitivity and specificity when comparing two diagnostic tests (Cicchetti & Feinstein, 1990). The calculated positive predictive value for the IFAT test was 94% and the negative predictive value was 97%, resulting in a corrected *kappa* coefficient of 0.91 indicating a very high level of agreement between the IFAT assay and histopathology. As the agreement between histopathology and IFAT and the agreement between immuno-dot blot and IFAT are both very high, we can infer that a good correlation exists between immuno-dot blot and histopathology.

The agreement between immuno-dot blot and IFAT was high and the number of dot blot positive/IFAT negative results was surprisingly high as well. This does not necessarily indicate false positive reactions with the immuno-dot blot assay but is more likely to be the result of better test sensitivity. For example, improved detection of influenza A virus has been found using dot blot compared with direct immuno-fluorescent assay (Reina *et al.*, 1996) and with *Vibrio anguillarum*, dot blot was 100 times more sensitive than IFAT in detecting bacterial antigen (Cipriano, Pyle, Starliper & Pyle, 1985). Given that immuno-dot blot assays are inherently more sensitive than IFAT, it is likely that the 14 discrepant results that were IFAT negative/dot blot positive are true positives. Assessment of these samples by independent means such as histopathology was not possible as gill mucus was obtained by non-lethal sampling. As discrepant samples were identified *a posteriori*, it was not possible to re-sample these fish held in commercial production.

Evidence of test sensitivity, inherent in dot blots, was found when determining minimum detection levels. It was found that as little as 4.1 ng of PA027 paramoebae protein could be detected in whole cell suspensions, but when using the digestion process, as little as 1.4 ng of protein could be detected, equivalent to 4 paramoebae. This sensitivity is comparable to the assay for *V. anguillarum*, which can detect 2.3ng of protein (Cipriano *et al.*, 1985). Our results indicate that both particulate and soluble antigens are detected by the immuno-dot blot and are factors that contribute to the overall sensitivity of the assay.

This study describes the development of a new immuno-dot blot test for the detection of *P. pemaquidensis* on the gills of fish with AGD. The test appears to be sensitive and specific and is well suited for mass screening of fish in future epidemiological studies.

## 2.1.6 Acknowledgments

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## 2.1.7 References

Baldock, F.C. (1990) Making decisions based on results from imperfect diagnostic tests. In: *Epidemiology at Work, Proceedings of the 144. Postgraduate Committee in Veterinary Science* (Ed. by D.I. Bryden), pp. 136-138, University of Sydney

Catty, D., Raykundalia, C. (1989) Production and quality control of polyclonal antibodies. In: *Antibodies: A Practical Approach* Vol. I (ed. by D.Catty), IRL Press, Oxford, Washington DC.

Chart, H., Rowe, B. (1997) A simple dot immunoassay for detecting antibodies to the lipopolysaccharide of verocytotoxin-producing *Escherichia coli* in patients with haemolytic uraemic syndrome. *Journal of Microbiological Methods* 28(1), 85-88.

Cicchetti, D.V., Feinstein, A.R. (1990) High agreement but low kappa: II. Resolving the paradoxes. *Journal of Clinical Epidemiology* 43(6), 551-558.

Cipriano, R.C., Pyle, J.B., Starliper, C.E. and Pyle, S.W. (1985) Detection of *Vibrio anguillarum* antigen by the dot blot assay. *Journal of Wildlife Diseases* 21(3), 211-218.

Clark, A., 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.

Desjardin L.E., Perkins M.D., Teixeira L., Cave M.D., Eisenach K.D. (1996) Alkaline decontamination of sputum specimens adversely affects stability of mycobacterial mRNA. *Journal of Clinical Microbiology* 34, 2435-2439

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

Dyková, L., Figueras, A., Novoa, B., Casal, J.F. (1998) *Paramoeba* sp., an agent of amoebic gill disease of turbot *Scophthalmus maximus*. *Diseases of Aquatic Organisms* 33, 137-141

Feinstein, A.R., Cicchetti, D.V. (1990) High agreement but low kappa: I. The problems of two paradoxes. *Journal of Clinical Epidemiology* 43(6), 543-549

Howard, T., Carson, J. (1993) Verification that Paramoeba species are consistently associated with gill damage in fish affected with amoebic gill disease. In: *Proceedings of the Saltas research and development review seminar, Tasmania 1993*, pp. 69-80. Salmon Enterprises of Tasmania Pty Ltd Dover, Tasmania.

Kent, M.L., Sawyer, T.K., Hedrick, R.P. (1988) *Paramoeba pemaquidensis* (Saromastigophora: Paramoebidae) infestation of the gills of coho salmon, *Oncorhynchus kisutch*, reared in seawater. *Diseases of Aquatic Organisms* 5, 163-169.

Munday, B.L., Foster, C.K., Roubal, F.R., Lester, R.J.G. (1990) Paramoebic gill infection and associated pathology of Atlantic salmon, *Salmo salar*, and rainbow

trout, *Salmo gairdeneri*, in Tasmania. In: *Pathology in marine Science* (ed. by F.O. Perkins & T.C. Cheng), pp 215-222. Academic Press, San Diego, Ca.

Munday, B.L., Lange, K., Foster, C., Lester, R.J.G., and Handlinger, J. (1993) Amoebic gill disease of sea-caged salmonids in Tasmanian waters. *Tasmanian Marine Fisheries Research* 28, 14-19

Nowak, B.F., Lucas, C.T. (1997) Diagnosis of structural changes in fish gills- can biopsy replace necropsy? *Aquaculture* 159, 1-10

Noya, O., Noya de B.A. (1998) The multiple antigen blot assay (MABA): a simple immunoenzymatic technique for simultaneous screening of multiple antigens. *Immunology letters* 63(1), 53-56

Poole, G.D. (1989) Antibodies in blood group serology. In: *Antibodies: A Practical Approach*, Vol. II. (Ed. by D.Catty). IRL Press, Oxford, Washington DC

Reina, J., Munar, M., Blanco, I. (1996) Evaluation of a Direct Immunofluorescent Assay, Dot-Blot Enzyme Immunoassay, and Shell Vial Culture in the Diagnosis of Lower Respiratory Tract Infections Caused by Influenza A Virus. *Diagnostic Microbiology and Infectious Disease* 25, 143-145

Rodger, H.D., McArdle, J.F. (1996) An outbreak of amoebic gill disease in Ireland. *Veterinary record* 139, 348-349

Roubal, F.R, Lester, R.J.G., Foster, C. (1989) Studies on cultured and gill-attached *Paramoeba* sp. (Gymnamoebae: Paramoebidae) and the cytopathology of paramoebic gill disease in Atlantic salmon, *Salmo salar* L., from Tasmania. *Journal of Fish Diseases* 12, 481-493

# CHAPTER 3

# DISTRIBUTION AND BIOLOGY OF *Neoparamoeba pemaquidensis*

## 3.1 ASSESSMENT OVER TIME OF THE INFECTIVITY OF GILL-DERIVED AND SEAWATER DISPERSED *Neoparamoeba pemaquidensis* (PAGE 1987)

Douglas-Helders, G.M., Handlinger, J., Carson, J., Nowak, B.

### 3.1.1 Abstract

A laboratory infection trial tested if *Neoparamoeba pemaquidensis*, the protozoan responsible for AGD, remained infectious when out of contact with host tissues for up to 14 days. Atlantic salmon (*Salmo salar*) were exposed to gill-derived paramoebae, which had been out of contact with hosts for up to 14 days. At the conclusion of the trial infection was established in most fish. This implies that zone of infection around salmon farms may be very extensive.

### 3.1.2 Introduction

Sea farming of Atlantic salmon was established in Tasmania in 1984. Not long after, amoebic gill disease (AGD) was seen (Roubal *et al.*, 1989, Munday *et al.*, 1990). Presently AGD is the main disease affecting the salmon industry in Australia (Clark & Nowak, 1999, Nowak, 2001). *Neoparamoeba pemaquidensis*, the disease causing protozoan of AGD, is not confined to Tasmanian waters

(Munday *et al.*, 1990, 1993) with outbreaks recorded in Ireland (Rodger & McArdle, 1996, Palmer *et al.*, 1997), France (Findlay & Munday, 1998), Spain (Dyková *et al.*, 2000), New Zealand (Clark & Nowak, 1999), Washington State and California, USA (Kent *et al.*, 1988), and in Chile (D.Groman & P.Bustos, pers. comm.). Fish species affected by *N. pemaquidensis* are Atlantic salmon (*Salmo salar*), chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*), turbot (*Scophthalmus maximus*), European sea bass (*Dicentrarchus labrax*), and sharpsnout seabream (*Diplodus puntazzo*) (Kent *et al.*, 1988, Clark & Nowak, 1999, Dyková *et al.*, 2000, Munday *et al.*, 2001, Dyková & Novoa, 2001).

Transmission of AGD has been successfully achieved through co-habitation of salmon with naive salmon (Akhlagi *et al.*, 1996, Zilberg *et al.*, 2000). Infection could also be established by exposure of fish to paramoebae freshly harvested from gills of fish known to have AGD (Zilberg *et al.*, 2001). AGD could not however be achieved when fish were exposed to cultured paramoebae (Kent *et al.*, 1988, Howard *et al.*, 1993). To date it remains unclear why the cultured protozoan loses its ability to induce AGD in fish, and it is not known if *N. pemaquidensis* requires regular contact with host gill tissues to remain infective. This study described an infectivity trial in which specific pathogen free (SPF) Atlantic salmon were exposed to *N. pemaquidensis* that were out of contact with fish tissues for up to 14 days.

### 3.1.3 Materials and Methods

The Aquatic Key Centre, University of Tasmania, Launceston, Tasmania donated fourteen seawater adapted and SPF Atlantic salmon. The trial was performed in

three re-circulation systems, each consisting of three tanks with a working volume of 50 L each and a common bio-filter, which was positioned in a 30 L sump. The systems were filled with 5 µm filtered seawater. One of the three recirculation systems (S1) was inoculated with gill-harvested paramoebae at the same time as four Atlantic salmon were placed into this system; and this group was used as a positive control for the trial. The second recirculation system (S2) was inoculated with gill-harvested paramoebae three days before six SPF salmon were added to this system. The third system (S3) was inoculated with gill-harvested paramoebae, followed by the placement of four SPF Atlantic salmon into this system 14 days later. The average fork length of the fish was 28.5cm (SE 0.26) for the S1 group, 25.4cm (SE 0.83) for the S2 group, and 30.1cm (SE 1.05) for the S3 group. All fish in this trial were exposed to paramoebae for seven days, after which the fish were killed by anaesthetic overdose using 100 mg/L benzocaine.

The gill harvested paramoebae, which were used for inoculation of the systems, were obtained from known AGD infected donor fish, which were held in an experimental tank at 37 ppt salinity and a temperature of 13°C. Paramoebae harvesting was carried out as described by Zilberg *at al.* (2001). In short, gills arches were dissected and the arches placed in a sterile 2.5% w/v ammonium chloride solution at 4°C overnight. After discarding the ammonium chloride, mucus was collected by carefully scraping it off the lamellae for each gill arch. The suspension was washed twice with sterile (121°C, 15 minutes) and 0.1µm filtered seawater, and a paramoebae cell count performed using a haemocytometer and 0.5% trypan blue as an indicator of cell viability. Each system was seeded with 1.5 million freshly harvested paramoebae by direct addition of the cell suspension to

the challenge tanks of each re-circulation system. The average salinity during the trial was 35.0 ppt (SE 0.00), 38.3 ppt (SE 0.33), and 35.2 ppt (SE 0.17) for S1, S2, and S3 respectively. The average temperature during the trial was 17.8°C (SE 0.20) for S1, 18.7°C (SE 0.10) for S2, and 18.4°C (SE 0.23) for S3. The ammonia level remained below 0.1 ppm at all times during the trial.

At the conclusion of the trial, gill mucus smears were taken from the third gill arch on the left hand side of each fish for detection of the presence of *N. pemaquidensis* using immuno-dot blot (Douglas-Helders *et al.*, 2001). All gill arches from the right hand side of the fish were dissected, fixed in seawater Davidson's fixative, standard processed, and haematoxylin and eosin (H&E) stained for histological examination of the gills. Infection in this trial was defined as the presence of *N. pemaquidensis* in a mucus smear or on gills of a histological section. If any mortalities occurred during the trial, fish were removed from the system and gill samples were tested with the previously mentioned techniques.

When analysing the samples using histology, it became apparent that a co-existing *Flavobacterium* (flexibacter-like, J. Handlinger pers. comm.) infection had taken place. Therefore the number of bacterial populations was roughly estimated and ranged from scare, few, common, and heavily loaded, corresponding with a score of 1, 2, 3, and 4 respectively. An average bacterial load score was calculated to estimate the bacterial infection load for each treatment group.

### 3.1.4 Results

At the conclusion of the trial, salmon of all treatment groups tested positive for *N. pemaquidensis*, determined by dot blot and histology. Percentages of AGD positive fish for each treatment group and testing method are shown in table 1.

**Table 1**: Percentages of *N. pemaquidensis* positive fish, tested with immuno-dot blot, and histology, for the three different treatment groups S1, S2 and S3. Tanks were inoculated with gill harvested paramoebae, followed by placement of Atlantic salmon to these tanks at zero (S1), three (S2) and fourteen (S3) days post-paramoebae inoculation.

| Treatment group | Number of fish | Immuno-dot blot | Histology |
|---|---|---|---|
| S1 | 4 | 100% | 100% |
| S2 | 6 | 83.3% | NA* |
| S3 | 4 | 75% | 75% |

*NA: Not available due to autolysis of the gills and loss of gill integrity

Histologically, early signs of infection with *N. pemaquidensis* were visible in both the S1 and S3 groups, with marked thickening and focal fusion of the secondary lamellae and evidence of excessive mucus production. However, classical AGD lesions as seen in fish with prolonged AGD were not detected in any of the fish. The histological gill sections of the S2 group could not be read due to autolysis resulting in severe deterioration of the gill structure and cell disintegration.

Mortalities occurred from as early as one day post-exposure, which happened in the S2 group, and continued onwards in all three treatments. All fish in the S2 group died before the conclusion of the trial, with maximum exposure duration to

paramoebae of three days. A fish exposed to paramoebae for one and two days from the S2 group tested negative and positive for dot blot respectively. One fish (25%) of the S3 group and three fish (75%) of the S1 group died before the termination of the trial. The S3 group showed the least number of mortalities, compared to the other two treatment groups. The average bacterial score was 2.75 for S1, 2.67 for S2, and 1.00 for S3, indicating a lower bacterial load in the S3 group.

### 3.1.5 Discussion

Results showed that infection (presence of *N. pemaquidensis*) occurred, even when paramoebae were out of contact with a host for 14 days. This suggests that the gill-derived *N. pemaquidensis* remains infective for at least 14 days after dispersal into the water column. Water currents form the main transport mechanism for free-floating protozoans like *N. pemaquidensis* (Rodriguezzaragoza, 1994). Thus the protozoan would be able to infect other hosts away from the point of origin when carried by water currents.

The percentage of paramoebae positive fish, determined by immuno-dot blot and histology, increased with decreasing time between paramoebae inoculation and fish introduction to the system. Also, the lowest number of mortalities was seen in the group where the paramoebae lacked contact with host tissues for the longest duration (S3). This might indicate a slight reduction in virulence of *N. pemaquidensis* over time when no contact with a host is made. The decrease in virulence in this S3 group was however minor, with 75% of the fish positive for immuno-dot blot and histology, compared to 100% in the S1 group.

The infection in this trial proved to be very aggressive, resulting in many mortalities. Though the paramoebae dose was high with usage of about 6,500 times the minimal infectious dose (Zilberg & Munday, 2000), it was successfully used in a previous study in which the same number of paramoebae was used (Douglas-Helders *et al.*, 2002). Ammonia concentrations in the tanks remained below considered as dangerous levels, and were similar in all groups, suggesting that water quality did not interfere with the infection trial. Especially the S2 group showed a very high mortality rate, with a maximum life span of the fish of three days post paramoebae exposure. This group also showed a high bacterial score, similar to the S1 group, and experienced the highest temperatures and salinities compared to S1 and S3. The results suggest that bacterial presence, in combination with higher temperatures and salinities, increased the severity of paramoebae infection and had a negative effect on fish health, resulting in death. It remains unclear however, if the bacterial infection were a pre-disposing factor for the paramoebae infection.

This study showed that *N. pemaquidensis* remains infective, despite lack of contact with hosts for up to 14 days. This implies that transmission of AGD infections in the field do not only occur from fish to fish, but also from water to fish and suggests that the zone of infection around a farm may be very extensive. The role of the water column as carrier or reservoir may be significant.

### 3.1.6 Acknowledgments

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### 3.1.7 References

Akhlaghi, M., Munday, B.L., Rough, K., Whittington, R.J. (1996) Immunological aspects of amoebic gill disease in salmonids. Diseases of Aquatic Organisms 25, 23-31

Clark, A., 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

Douglas-Helders, G.M., Carson, J., Howard, T., Nowak, B.F (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, G.M., Dawson, D.R., Carson, J., Nowak, B.F (2002) Wild fish are not a significant reservoir for *Neoparamoeba pemaquidensis* (Page, 1987). *Journal of Fish Diseases*, In press

Dyková, I., Figueras, A., Peric, Z. (2000) *Neoparamoeba* Page 1987: light and electron microscopic observations on six strains of different origin. Diseases of Aquatic Organisms 43, 217-223

Dyková, I., Novoa, B. (2001) Comments on diagnosis of amoebic gill disease (AGD) in turbot, *Scophthalmus maximus*. Bulletin of the European Association of Fish Pathologists 21, 40-44

Findlay, V.L., Munday, B.L. (1998) Further studies on acquired resistance to amoebic gill disease (AGD) in Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 21, 121-125

Howard, T.S., Carson, J., Lewis, T. (1993) Development of a model of infection for amoebic gill disease. In: Proceedings of the Saltas Research and Development Seminar, Salmon Enterprises of Tasmania, Hobart, Tasmania pp. 103-112

Kent, M.L., Sawyer, T.K., Hedrick, R.P. (1988). *Paramoeba pemaquidensis* (Saromastigophora: Paramoebidae) infestation of the gills of coho salmon *Oncorhynchus kisutch* reared in seawater. Diseases of Aquatic Organisms 5, 163-169

Munday, B.L., Foster, C.K., Roubal, F.R., Lester, R.J.G. (1990) Paramoebic gill infection and associated pathology of Atlantic salmon, *Salmo salar*, and rainbow trout, *Salmo gairdeneri*, in Tasmania. In: Pathology in Marine Science. Eds: F.O. Perkins, T.C. Cheng. Academic Press, pp. 215-222

Munday, B.L., Lange, K., Foster, C., Lester, R.J.G., and Handlinger, J. (1993) Amoebic gill disease of sea-caged salmonids in Tasmanian waters. Tasmanian Marine Fisheries Research 28, 14-19

Munday, B.L., Zilberg, D., Findlay, V. (2001) Gill disease of marine fish caused by infection with *Neoparamoeba pemaquidensis*. Journal of Fish Diseases 24, 497-507

Nowak, B.F. (2001) Qualitative evaluation of risk factors for Amoebic Gill Disease in Atlantic salmon. 18pp. 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 (OIE), 148-155

Page F.C. (1987). The classification of 'naked' amoebae of phylum Rhizopoda. Archive fur Protistenkunde 133:199-217

Palmer, R., Carson, J., Ruttledge, M., Drinan, E., Wagner, T. (1997) Gill disease associated with *Paramoeba*, in sea-reared Atlantic salmon in Ireland. Bulletin of the European Association of Fish Pathologists 3-4, 112-114

Rodger, H.D., McArdle, J.F. (1996) An outbreak of amoebic gill disease in Ireland. Veterinary Record 139, 348-349

Rodriguezzaragoza, S. (1994) Ecology of free-living amoebae. Critical Reviews in Microbiology 20, 225-241

Roubal, F.R., Lester, R.J.G., Foster, C.K. (1989) Studies on cultured and gill-attached *Paramoeba* sp. (Gymnamoebae: Paramoebidae) and the cytopathology of paramoebic gill disease in Atlantic salmon, *Salmo salar* L., from Tasmania. Journal of Fish Diseases 12, 481-492

Zilberg, D., Munday, B.L. (2000) Pathology of experimental amoebic gill disease in Atlantic salmon, *Salmo salar* L., and the effect of pre-maintenance of fish in sea water on the infection. *Journal of Fish Diseases* 23, 401-407

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, 15-19

Zilberg, D., Gross, A., Munday, B.L. (2001) Production of salmonid amoebic gill disease by exposure to *Paramoeba* sp. harvested from the gills of infected fish. Journal of Fish Diseases 24, 79-82

## 3.2 TEMPORAL AND SPATIAL DISTRIBUTION OF *Paramoeba* sp. IN THE WATER COLUMN – A PILOT STUDY

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### 3.2.1 Abstract

Amoebic gill disease (AGD) is the main disease affecting the salmonid industry in Tasmania, but no information on the distribution of the pathogen, *Neoparamoeba pemaquidensis* in the aquatic environment is available. This pilot study aimed to determine temporal and spatial distributions of paramoebae in the water column, using the immuno-dot blot technique. Water samples were collected from inside cages at various depths (0.5, 5.5, and 11.0 m) in both summer and winter, and at various distances (0, 0.5, 240, 280, 750, and 1100 m) away from the sea cages and farming site. Paramoebae densities were estimated using the most probable number technique (MPN). Temperature, salinity, dissolved oxygen, turbidity, nitrite and nitrates, and bacterial counts were measured for each water sample. Data were analysed using a residual maximum likelihood (REML) test, and significant associations between paramoebae densities and environmental factors were analysed. Results showed that densities were significantly higher in summer (P=0.017), at 5.5 metres depth (P=0.029), and reduced to the lowest density at 1100 metres away from the cage sites (P=0.008). Bacterial counts, turbidity, and temperature were found to be significantly associated with paramoebae densities.

## 3.2.2 Introduction

Amebas are extremely abundant in the marine environment (Sawyer, 1980) and have been collected from inshore areas, throughout the oceanic water column, as well as from sediments (Bovee & Sawyer, 1979; Sawyer, 1980). For example, Bovee & Sawyer described 76 species of marine amoebae from the waters of northeastern Unites States. Most marine amoebae are bactivorous (Bovee & Sawyer, 1979, Anderson, 1988, Paniagua, Parama, Iglesias, Sanmartin & Leiro, 2001), although some are also known to feed on other protozoans, algae or organic detritus (Bovee & Sawyer, 1979, Sawyer, 1980, Page, 1983). Amoebae were shown to be affected by season, which in turn was correlated to water temperature, dominance and competition among different marine amoebae species (Anderson, 1988). Water temperature, salinity, and the availability of food were suggested to be major factors affecting amoeba distributions (Bovee & Sawyer, 1979). Aquatic organisms show highest growth and survival at optimum growth conditions (Rheinheimer, 1974). For example, growth *in vitro* of *Neoparamoeba pemaquidensis* (Page, 1987) was enhanced at temperatures above 5°C (Kent, Sawyer & Hedrick, 1988), with an upper limit of temperatures above 22°C (Howard, 2001). Also, optimum growth of this protozoan was seen at 15‰ salinity, with little decline in growth rate up to 30‰ salinity (Kent *et al.*, 1988).

Six species from the genus *Paramoeba* were described by Kent *et al.* (1988), including *P. aestuarina* Page, *P. pemaquidensis* Page, now know as *Neoparamoeba pemaquidensis* Page, *P. eilhardi* Schaudinn, *P. schaudinni* de Faria, *P. perniciosa* Sprague, and *P. invadens* Jones. Of these, *P. perniciosa* was pathogenic for the blue crab, *Callinectes sapidus*, Rathbun (Sparague, Beckett &

Sawyer, 1969) and *P. invadens* was pathogenic for the sea urchin, *Strongylocentrotus droebachiensis*, Müller (Jones, 1985). *Neoparamoeba pemaquidensis* was found to be pathogenic for salmonids, turbot, *Scophthalmus maximus* L., European sea bass, *Dicentrarchus labrax* L., and sharpsnout seabream, *Diplodus puntazzo* Cuvier (Kent *et al.*, 1988, Munday, Foster, Roubal & Lester, 1990; Roubal, Lester & Foster, 1989; Clark & Nowak, 1999, Kent, 2000; Dyková, Figueras & Peric, 2000, Dyková & Novoa, 2001). *Neoparamoeba pemaquidensis* is thought to be an amphizoic (Scholz, 1999) or opportunistic protozoan (Kent *et al.*, 1988), which means that the normally free-living protozoan becomes pathogenic under certain conditions (Scholz, 1999).

Amoebic gill disease (AGD) is the main disease affecting the salmonid industry in Tasmania. AGD is caused by the naked and lobose protozoan *Neoparamoeba pemaquidensis* (Page, 1983). It is unable to form cysts and does not have flagella (Bovee & Sawyer, 1979). Though some epidemiological studies have been reported (Douglas-Helders, Nowak, Zilberg & Carson, 2000; Douglas-Helders, Saksida, Raverty & Nowak, 2001a; Douglas-Helders, Dawson, Carson & Nowak, 2002a, Douglas-Helders, Weir, O'Brien, Carson & Nowak, 2002b), to date no information on the spatial or temporal distribution of the pathogen in the water column is available. *Neoparamoeba pemaquidensis* was first detected in the water column from the marine waters off Maine, USA (Page, 1970), and is the most common marine amoebae (Page, 1983), known to have a worldwide distribution (Cann & Page, 1982). The protozoan is often found in coastal waters and the lower reaches of estuaries (Page, 1983). *Neoparamoeba pemaquidensis* has also been

detected in heavily polluted waters, including those contaminated by heavy metals (Sawyer, 1980).

This pilot study is aimed at both; providing the first estimation of the spatial and temporal distribution of *Neoparamoeba pemaquidensis* in and around a Tasmanian salmon farm, and provisionally relating these distribution to environmental conditions. This would provide with an insight into the ecology of this pathogenic protozoan, which will help to determine future AGD research, control, and, monitoring programs.

## 3.2.3 Materials and Methods

### 3.2.3.1 Validation and sensitivity for the testing of paramoebae in water

Gill isolate in 0.45 μm filtered and sterile (121°C, 15 min) seawater was used to determine the sensitivity of the immuno-dot blot for testing crude natural seawater samples. The gill isolate was obtained from a known infected AGD Atlantic salmon donor fish, which originated from AGD infected stocks, held in an experimental tank at 13 °C and at a salinity of 37‰. The donor fish was anaesthetised using 100 mg $L^{-1}$ of benzocaine and the paramoebae isolated as described by Zilberg, Gross & Munday (2001). The isolate was washed twice with 0.45 μm sterile (121°C, 15 min) and filtered sea water (SFS) by centrifugation at 2,600 *g* for 15 min. The pellet was resuspended in 10 mL SFS, and a viable cell count performed using 0.5% trypan blue and haemocytometer (Zilberg *et al.*, 2001). Triplicate dilutions were made, with final paramoebae cell numbers of 1000, 100, 10, and one cells in 1 mL of SFS. One mL of SFS was used as a negative control. From all tubes an 80 μL aliquot was used for testing with immuno-dot blot

technique as described by Douglas-Helders, Carson, Howard & Nowak (2001b), including the digestion and cell lysis steps.

Water samples were taken from various locations in Tasmania (Table 1), to determine if paramoebae could be detected in the aquatic environment, and to validate testing using the immuno-dot blot technique. Samples were taken from two salmon farms in the Huon Estuary, southeast Tasmania, at three different sites. Two of these sites contained infected salmon, while the other site was being fallowed. Water samples were also taken from the East coast of Tasmania, more than 100 km away from any salmon farming sites, and from the mouth of the Tamar river in the north of Tasmania, with one salmon farm approximately 20 km away. This farm was known to be free from AGD. Turbid fresh water samples were taken downstream from the Tamar river to assess the effect of organic particles in the sample. Water samples were stored on ice until processed in the laboratory. Sample volumes of 100, 50, 0.240, and 0.08 mL were concentrated to 800 µL to determine the minimal required sample volume needed to provide a positive detection signal. Volumes of 80, 160, 200, 240, and 320 µL were inoculated onto the test membrane to determine the minimal test volume to enable a positive dot blot result. All water samples were processed as described by Douglas-Helders *et al.* (2001b), with the exclusion of the mucus digestion step. SFS and PBS enriched with *N. pemaquidensis* PA027 (DPIWE) were used as positive controls, while un-lysed natural seawater samples were used as a control for the lysis process, and SFS as well as PBS were used for negative controls. Nine of the water samples were tested for presence of *N. pemaquidensis*, using nested PCR (Elliott, Wong & Carson, 2001).

**Table 1**: Sources, replicates, and number of samples taken from each destination for the field validation samples, taken at different depths and in multiple volumes

| Region | Source | n | Sampling depth (m) | Total volume sampled (mL) | Test volume (μL) |
|---|---|---|---|---|---|
| East coast Tasmania | Bicheno 1 | 3 | 0 - 0.5 | 100 | 80, 200, 400 |
| | Bicheno 2 | 6 | 0 - 0.5 | 50 | 80, 200, 400 |
| North coast Tasmania | Tamar freshwater | 2 | 0 – 0.5 | 50 | 800 |
| | Tamar mouth | 32 | 0 – 0.5 | 50 | 800 |
| southeast coast Tasmania | Hideaway Bay | 36 | 0, 5, 10 | 2000 | 80, 240, 320 |
| | Garden Island | 36 | 0, 5, 10 | 2000 | 80, 240, 320 |
| | Tinderbox | 4 | 0 - 0.5 | 2000 | 80, 240 |

### 3.2.3.2 Distributions

The AGD prevalence status on the lease sites at the times of sampling was estimated using the farms gross gill lesions scoring system. White mucoid patches or excessive mucus are an indication of AGD infection, and can range from: small, light spot-like discolouration affecting one or two gill lamellae, to more visible mucus build up, only very small area of the gill affected, to larger part of the gill affected by mucus build up and white patches can be clearly seen. The severity of AGD infection in a cage was based on the number of fish examined, usually between 20 and 30 fish, and the degree of infection for each fish. This resulted

either in a light, medium, or heavy score for the cage. Water sampling took place on two different sites at one farm, in the Huon Estuary, southeast Tasmania, Australia. The water samples were taken using a five litres Niskin bottle, connected to a rope with clearly marked one-metre intervals. The paramoebae distribution in sea cages at different depths and seasons was determined by duplicate water sampling of two cages from three different depths, both in summer and winter. Duplicate water samples were also taken from inside sea cages and at 0.5, 240, 280, 750 and 1100 metres away from these sea cages at 5.5 metres depth for determining the spatial distribution. Paramoebae densities in water samples were quantified using the most probable number technique (MPN, Oblinger & Koburger, 1975, Gonzalez, 1996). For this, five sub-samples of 400 mL, 200 mL, 100 mL, 50 mL, and 25 mL each, for each water sample, were concentrated to 800 µL by centrifugation. The resulting 25 sub-samples for each water sample were tested for the presence of paramoebae, using the immuno-dot blot technique as described by Douglas-Helders *et al.* (2002b). The number of dot blot positive sub-samples for each of the five concentrations resulted in five numbers, each between zero and five. These numbers represented the most probable number (MPN) of paramoebae per L, using the custom made MPN table based on the program described by Gonzalez (1996).

3.2.3.2 1 *Seasonal and depth distribution in cages*

Duplicate water samples were taken from two sea cages, from slack tide to an outgoing tide, both in summer (February 2002) and winter (August 2001). Samples were taken from 0.5, 5.5, and 11.0 metres depths, which represented the surface, middle, and bottom the sea cage. The winter sampling took place on the Hidaway

Bay lease site in the Huon Estuary, southeast Tasmania. In summer, no cages were present at this lease site, and water samples were collected from the Garden Island lease site, in close proximity to the Hidaway Bay site, southeast Tasmania.

3.2.3.2.2 *Spatial distribution*

Duplicate water samples were taken in summer (February 2002) from two sea cages at the Garden Island site. Sampling took place on two consecutive days when the greatest difference between high and low tides occurred, and at the start of the outgoing tide. All water samples were taken from 5.5 metres or mid-sea cage depth, where paramoebae densities were presumed to be high. Firstly, water was sampled from the centre of the sea cage (0 m sample), and from just outside the sea cage (0.5 m sample). In an attempt to follow the same water mass, two current measuring devices (CMD) or drogues were placed into the water column (Figure 1) at 3 m ($CMD_3$) and 6 m ($CMD_6$) depth respectively. The CMDs were constructed from a two litre, empty, and sealed plastic bottle as the floating device, to which a three ($CMD_3$) or six metres ($CMD_6$) length rope (3 mm polyethylene) was attached. At the end of the rope, two circular pieces of plastic (4 mm polyvinyl sheet) were attached at 90 degrees to each other (Figure 1). The CMD was weighed using a 0.5 kg lead weight. Once the CMDs were placed into the water column, their position was regularly monitored using a global positioning system (Differential GPS, Garmin GPSMAP 135 receiver/sounder with attached Ausnav Aztec RXMAR 1). The GPS monitored distance from point of origin, total travel distance, and current position. At 240 and 280 metres from origin, water samples were taken following the $CMD_6$, while at 750 and 1100 metres from origin samples were taken following

the $CMD_3$. This was necessary to enable sampling at greater distances from the site before turn of the tide, which would not have been possible using the $CMD_6$ only.

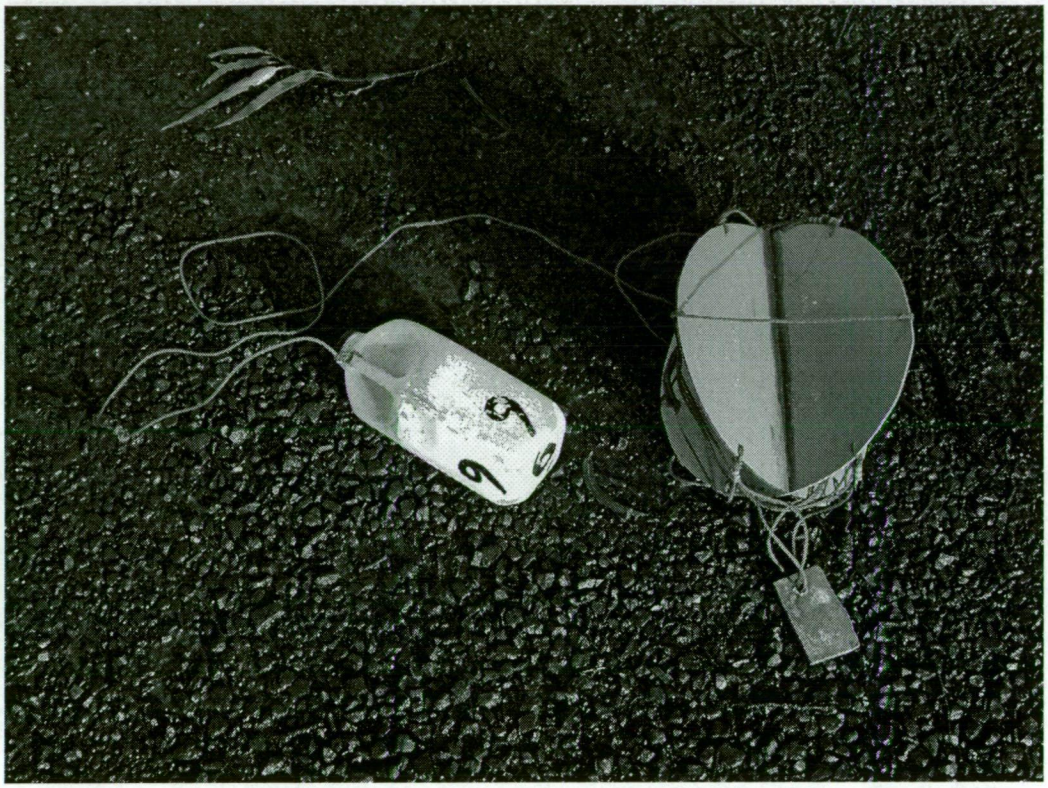

**Figure 1**: A current measuring device (CMD) or drogue, which were used to track a water mass for determining the paramoebae distribution in the water column

3.2.3.3 Environmental measurements

Environmental factors such as temperature, salinity, and dissolved oxygen were measured for each water sample. The number of bacteria in the sea water samples was estimated using the protocol developed by the Fish Health Unit, Department of Primary Industries, Water and Environment, Launceston, Tasmania. A sterile 30 mL container was submerged into the water sample, the screw cap removed and the cap replaced underwater to avoid contact with the surface layer. Five replicate Johnson's marine agar (JMA, Johnson, 1968) plates per water sample were inoculated with 50 µL of the 30 mL containers, spread with sterile hockey stick spreaders (Oxoid, Australia), using a different spreader for each plate. The plates were stored on ice during transport and placed into a 20°C incubator (Kelvinator 380) for 24 h, then incubated at 15°C (Thermoline, Selby, Australia) for 48 h, after which the number of colony forming units was counted. The viable bacterial count per mL was determined only for plates with counts of 10 to 300 colonies, to avoid an unacceptably large degree of error. Turbidity was determined in triplicate for each water sample, using a 2100P turbidity meter (Hach Company, P.O. Box 389, Colorado, USA) set at auto range and signal averaging. The turbidity was expressed in nephelometric turbidity units (NTU). The comparison of turbidity measurements and total microbial counts were used to draw some conclusions on the kind of substances responsible for turbidity (Rheinheimer, 1974). A positive correlation between turbidity and bacterial counts meant that the turbidity was due to an increase in the amount of suspended organic matter (Rheinheimer, 1974). Dissolved nutrients (nitrites and nitrates in N-µg $L^{-1}$) were measured by APHA Method 4500, and performed by the NATA accredited Analytical Services, Tasmania Laboratories in Hobart, Tasmania.

3.2.3.4 Statistical analysis

Distribution data were analysed using residual maximum likelihood (REML) technique (Patterson & Thompson, 1971), using the software package Genstat version 4.2, fifth edition (VSN International Ltd., Oxford OX2 8DR, UK). This test estimates the treatment effects and variance components in a linear mixed model. This technique was used instead of ANOVA in this situation because the data were unbalanced. The REML analysis produces a Wald statistic, which is analogous to the F-statistic in ANOVA. Wald statistics have an approximately chi-squared distribution and are evaluated in terms of chi-squared probabilities for the degrees of freedom associated with particular fixed effects. The response variate was the MPN or the estimate of paramoebae numbers; the fixed factors were depth, season, distance and the interaction of depth and season. Replicates (cage depth and seasonal distribution), sample, and sample.replicates (spatial distribution) were fitted into the random model. Correlation coefficients of the assessment for association between environmental factors and MPN estimates were calculated using Genstat version 4.2.

## 3.2.4 Results

3.2.4.1 Validation and sensitivity

Ten paramoebae cells per mL of SFS was the consistent sensitivity of the immuno-dot blot when testing water samples, while all SFS samples tested dot blot negative. Of the nine seawater samples tested with both immuno-dot blot and nested PCR, four samples were positive for dot blot while no *N. pemaquidensis* could be detected using PCR (Elliott *et al.*, 2001). The results of the field samples showed

that all East coast samples and Tamar river fresh and seawater samples were negative for the presence of paramoebae, while samples from salmonid farms in the Huon Estuary tested positive, depending on the sample volume used (Table 2). Presence of paramoebae could be detected when a sample volume of 100 mL and occasionally when 50 mL samples were used, but not in volumes of 240 and 80 µL. The volume applied to the dot blot membrane did not affect the test result.

3.2.4.2 Distributions

The cages sampled to determine the cage depth and seasonal distribution were all heavily infected with AGD, according to farm records. The site from which the spatial distribution was determined was medium to heavily infected with AGD at the time of sampling. Within sea cages, the highest paramoebae density was found at 5.5 metres depth (P=0.029, df2, Wald stat 7.06, Figure 2), while densities were significantly higher in February compared to these in August (P=0.017, df1, Wald stat. 5.69, Figure 2). Significantly lower paramoebae densities were found at 240 and 1100 metres from the sea cage (P=0.008, df5, Wald stat. 15.75, Figure 3). At 1100 metres the CMD was outside the lease site area, at approximately 750 metres from the last sea cage of this site. The CMDs took 259 minutes to travel 240 metres, 94 minutes for 280 metres, 71 minutes for 750 metres, and 276 for 1100m. This represented travel speeds of 92.7 ($CMD_6$, day 1), 297.8 ($CMD_6$, day 2), 1056.3 ($CMD_3$, day 2), and 398.5 ($CMD_3$, day 1) cm $s^{-1}$. Averages of the environmental conditions during sampling are shown in Table 3

**Table 2**: Results of testing field water samples from Atlantic salmon farming sites for different sample volumes and different test volumes

| Region | Sample volume (mL) | Test volume (μL) | Dot blot results for each sample and replicate |
|---|---|---|---|
| Hideaway Bay 0 meter | 100, 50, 0.240, 0.08 | 80 and 240 | 100: 100% weak pos.<br>50, 0.24, 0.08: 100% neg |
| Hideaway Bay 5 meters | 100, 50, 0.240, 0.08 | 80 and 240 | 100, 50: 100% pos.<br>0.24, 0.08: 100% neg. |
| Hideaway Bay 10 meters | 100, 50, 0.240, 0.08 | 80 and 240 | 100: 100% weak pos.<br>50, 0.24, 0.08: 100% neg. |
| Garden Island 0 meter | 100, 50, 0.240, 0.08 | 80 and 240 | 100: 100% pos.<br>50: 100% weak pos.<br>0.24, 0.08: 100% neg. |
| Garden Island 5 meters | 100, 50, 0.240, 0.08 | 80 and 240 | 100: 100% pos.<br>50: 100% weak pos.<br>0.24, 0.08: 100% neg. |
| Garden Island 10 meters | 100, 50, 0.240, 0.08 | 80 and 240 | 100: 75% weak pos.<br>50: 100% weak pos.<br>0.24, 0.08: 100% neg. |
| Tinderbox 1 | 100, 50 | 160 | 100: 100% pos.<br>50: 100% neg. |
| Tinderbox 2 | 100, 50 | 160 | 100: 100% pos.<br>50: 100% neg. |

Neg: negative, weak pos.: weak positive, pos.: positive

**Table 3**: Averages of the environmental conditions (SD) during water sampling for the spatial and temporal studies

| Environmental variable | | Spatial study | Cage depth and temporal study | |
|---|---|---|---|---|
| | | Summer | Summer | Winter |
| Salinity | 0.5 m | | 28.7 (0.3) | 34.4 (0.1) |
| (ppt) | 5.5 m | 34.1 (0.5) | 33.7 (0.5) | 34.5 (0.1) |
| | 11.0m | | 34.6 (0.2) | 34.6 (0.1) |
| Temperature | 0.5 m | | 14.3 (0.2) | 12.5 (0.1) |
| (°C) | 5.5 m | 16.2 (0.3) | 15.3 (0.1) | 12.5 (0.0) |
| | 11.0 m | | 15.5 (0.2) | 12.5 (0.0) |
| Bacterial counts | 0.5 m | | 459.3 (111.2) | 445.8 (319.8) |
| (cfu) | 5.5 m | 1239.9 (1386.6) | 740.3 (268.9) | 692.0 (272.8) |
| | 11.0 m | | 1910.0 (1118.5) | 1015.0 (373.4) |
| Nitrite & nitrate | 0.5 m | | 8.0 (1.41) | 33.5 (2.9) |
| (µg/L) | 5.5 m | NM* | 5.75 (1.3) | 33.0 (2.3) |
| | 11.0 m | | 11.5 (3.4) | 34.5 (0.6) |
| Turbidity | 0.5 m | | 1.57 (0.11) | 1.80 (0.41) |
| (NTU) | 5.5 m | 1.37 (0.4) | 1.02 (0.10) | 3.00 (0.43) |
| | 11.0 m | | 1.00 (0.14) | 2.48 (1.34) |

* NM: not measured

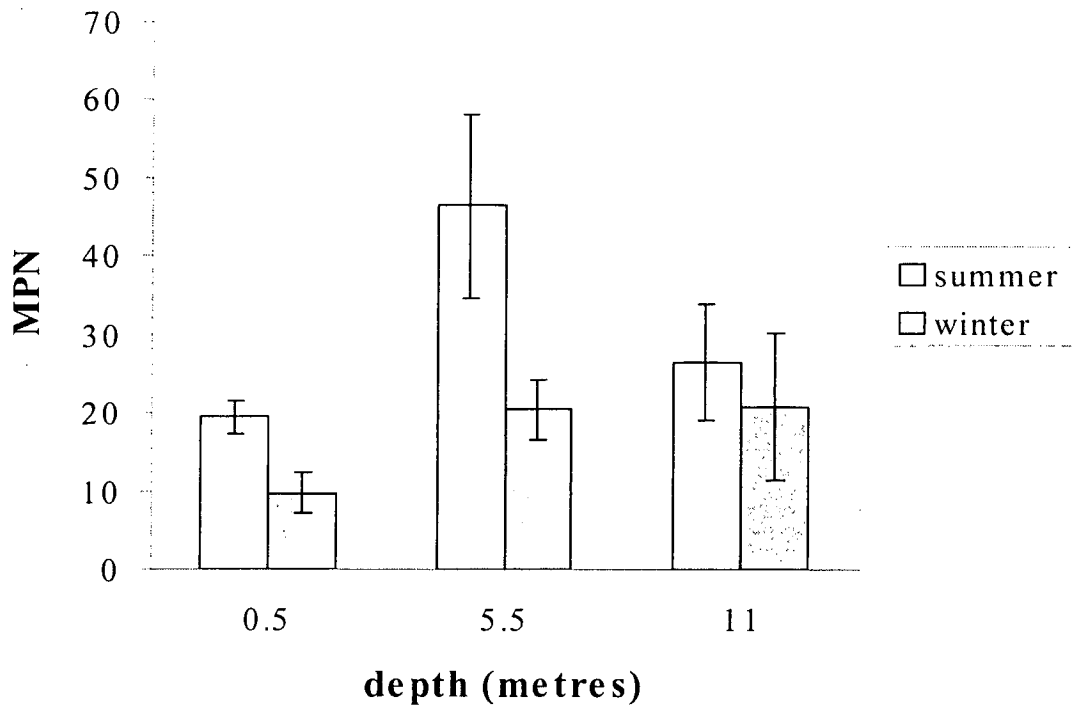


**Figure 2**: Temporal and spatial paramoebae distributions in sea cages on medium to heavily AGD infected farming site

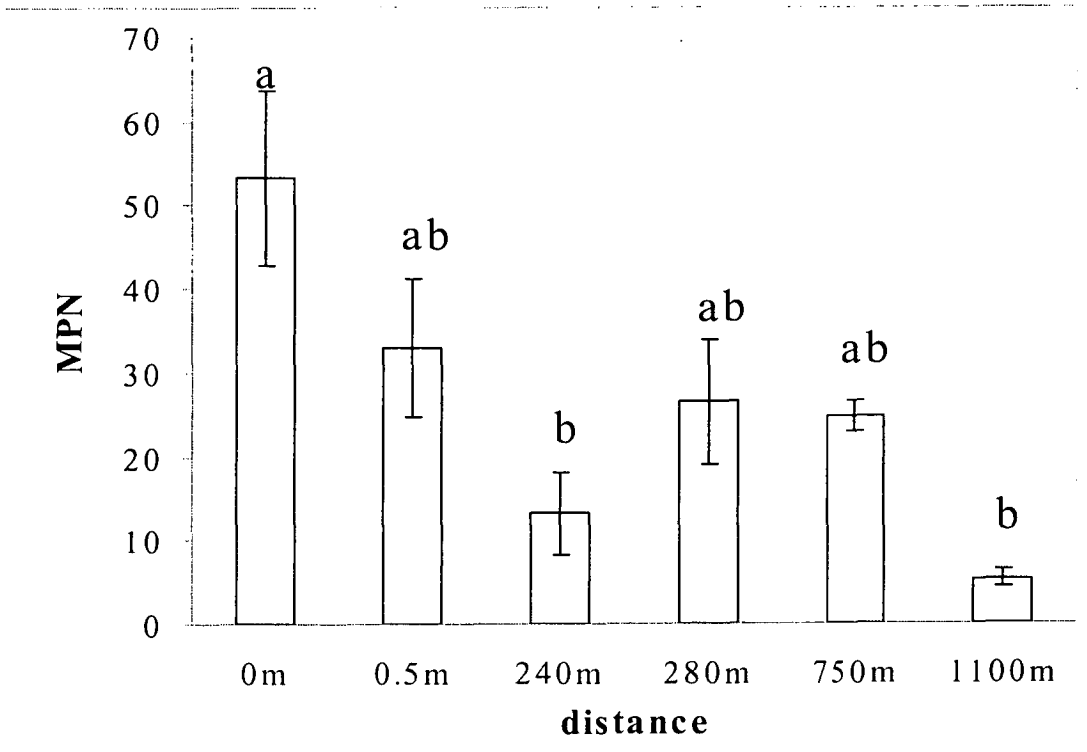


**Figure 3**: Spatial distribution of paramoebae at 5.5 m depth from sea cages up to 1100 m away from the cages

A significant association was found between paramoebae densities and the number of bacteria, both for the cage depth and temporal study ($r=0.841$, $P<0.01$) and the spatial study ($r=0.807$, $P<0.01$). Temperature was positively correlated with paramoebae densities ($r=0.431$, $P<0.05$) in the cage depth and temporal study, while turbidity was positively correlated in the spatial study ($r=0.549$, $P<0.05$). In addition, there was a positive correlation between temperature and numbers of bacteria in the water column in the cage depth and temporal distribution study ($r=0.439$, $P<0.05$), as well as between bacterial numbers and turbidity in the spatial study ($r=0.695$, $P<0.01$). When correlation analysis was performed for the different depths, significant correlations were found between paramoebae densities and temperature ($r=0.784$, $P<0.05$), salinity ($r=-0.792$, $P<0.05$), and dissolved nutrients ($r=-0.807$, $P<0.05$) at the surface. At 5.5 metres and 11 metres the only significant correlation found was between paramoebae densities and bacterial numbers in the water column.

### 3.2.5 Discussion

Paramoebae densities were highest in summer, at 5.5 metres depth inside sea cages, and densities reduced away from the farming site. Average seawater temperatures in the summer are higher than in winter, affecting a range of biological factors in the water column. Increasing temperatures, within the organism's viable range, promotes biological reactions, such as increase in bacterial population due to seasonal temperature fluctuations (Rheinheimer, 1974). In the spring and summer algal blooms are more likely to occur due to increased temperatures and longer day light hours. Jelly fish, including possibly harmful species, can be more numerous at this time of the year. In addition the dissolved oxygen level in the water column

decreases at higher temperatures. Thus, due to complexity, causal relationships between environmental factors, pathogen presence and AGD can be difficult to determine without laboratory experiments. The significant positive correlation between paramoebae densities and temperature in this study suggests that temperature may be a causal factor in AGD, with similar conclusions drawn in previous studies (Clark & Nowak, 1999, Nowak, 2001, Munday, Zilberg & Findlay, 2001, Douglas-Helders *et al.*, 2001a). A significant correlation between paramoebae and the number of bacteria in the water column was also found in this study, as well as a significant correlation between temperature and number of bacteria. While both temperature and bacterial counts are possible risk factors, either or both of these factors might be confounding (Thrusfield, 1995). However, at 5.5 and 11 metres depth, where paramoebae densities were higher compared with 0.5 metres depth, bacterial count was the only variable significantly correlated to paramoebae densities, but not temperature. This suggests that bacterial counts could be a causal risk factor for AGD infections. Controlled trials are required to resolve these issues of interrelation.

In this study the highest paramoebae densities were found at 5.5 metres inside sea cages. The fluctuations of environmental factors such as salinity, temperature and dissolved oxygen are greatest at the surface layer (pers. observation), and may negatively affect paramoebae densities at this depth. At deeper levels such as 5.5 and 11 metres, the physical environment is more stable and fluctuations are narrower (pers. observation). This may be a more suitable environment for a naked and lobose protozoan (Cann & Page, 1982) that is known to be unable to form cysts (Bovee & Sawyer, 1979) for surviving unfavourable conditions. Perhaps the

highest paramoebae density at 5.5 metres was due to the high abundance of available hosts at that depth. The vertical distribution of Atlantic salmon varies with seasonal and diurnal rhythms (Fernö, Huse, Juell & Bjordal, 1995). Fish tend to avoid the surface due to light intensity, as well as the bottom of sea cages (Fernö *et al.*, 1995). Extensive studies are needed to fully understand which factors determine paramoebae density at mid sea cage depth.

Paramoebae densities generally decreased with increasing distance from the cages, The lowest density was found furthest from the sea cages and outside the farming site. This could be due to paramoebae attaching to solid surfaces such as nets or hosts while in transport with the water flow, or merely through dilution, thus creating a washout effect of protozoan numbers with distance travelled from the cage. In previous studies *N. pemaquidensis* has been found on nets (Tan, Nowak & Hodson, 2002) and attachment to fish gills is well known (Roubal *et al*, 1989, Munday *et al.*, 1990, Adams & Nowak, 2001). The significant correlation of paramoebae densities with turbidity and bacterial numbers and the significant correlation between bacterial numbers and turbidity suggest that these factors were interrelated. A strong association between bacterial numbers and turbidity was also found in a study of the water column in the Western Baltic (Rheinheimer, 1974). The relationship between paramoebae densities and turbidity were not found in the cage depth and temporal study, but sampling always took place within a sea cage, and turbidity was likely to be cage-dependant. Since a positive correlation was found between turbidity and bacterial numbers, it was concluded that the turbidity was due to the amount of suspended organic matter (Rheinheimer, 1974). It is not unlikely that particles in the water column play an important part as a vector in the

transmission process of paramoebae to fish. Controlled laboratory experiments are needed to determine the causality of bacterial densities, turbidity, and total suspended organic particles, for AGD occurrence.

Detection of paramoebae using immuno-dot blot was successful, even though no PCR positive water samples could be detected. The test sensitivities of immuno-dot blot and nested PCR are different, with the dot blot being able to detect 10 cells in one mL (Douglas-Helders *et al.*, 2001b), while PCR only detects 16 cells in 100 µL (Elliott *et al.*, 2001). While the PCR specifically test for the presence of *N. pemaquidensis*, the immuno-dot blot may also react with closely related *Paramoeba* species, due to the cross-reactivity of the polyclonal primary antibody (Douglas-Helders *et al.*, 2001b), and thus possibly producing false positives. The antibody cross-reacted with *Neoparamoeba aestuarina* Page and *Pseudoparamoeba pagei* Sawyer but not *Paramoeba eilhardi* Schaudinn (Douglas-Helders *et al.*, 2001a). No cross reactivity was detected with *Platyamoeba plurinucleolus* Page, *Platyamoeba/Vanella* Page or *Flabellula* Schmoller (DPIWE FLB 004) (Howard and Carson, 1993). Paramoebae densities were possibly overestimated in this study, but all control samples that were taken away from AGD positive farms tested dot blot negative, suggesting that overestimation was minimal. Production of a monoclonal antibody would be required to detect *N. pemaquidensis* only. However, Dyková *et al.* (2000) suggested that more refined diagnostic methods would be needed before *N. aestuarina*, one of the cross-reactive species of the immuno-dot blot test, can be excluded as a possible agent of AGD next to *N. pemaquidensis*.

## 3.2.6 Acknowledgments

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## 3.2.7 References

Adams, M.B., Nowak, B.F. (2001) Distribution and structure of lesions in the gills of Atlantic salmon (*Salmo salar* L.) affected with amoebic gill disease. *Journal of Fish Diseases* 24, 535-542

Anderson, O.R. (1988) Densities and diversity of Gymnamoebae in relation to some inshore aquatic habitats at Bermuda. *Journal of Eukaryotic Microbiology* 45, 151-155

Bovee, E.C., Sawyer, T.K. (1979) Marine flora and fauna of the Northeastern United States. Protozoa: Sarcodina: Amoebae. NOAA Technical *Report National Marine Fisheries Service Circular* 419: 1-56

Cann, J.P., Page, F.C. (1982) Fine structure of small free-living *Paramoeba* (Paramoebida) and taxonomy of the genus. *Journal of Marine Biology Association* 62, 25-43

Clark, A., 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

Douglas-Helders, M. Nowak, B., Zilberg, D., Carson, J. (2000) Survival of *Paramoeba pemaquidensis* on dead salmon: implications for management of cage hygiene. *Bulletin of the European Association of Fish Pathologists* 20(3), 167-169

Douglas-Helders, G. M., Saksida, S. Raverty, S., Nowak, B.F. (2001a) Temperature as a risk factor for outbreaks of amoebic gill disease in farmed Atlantic salmon. *Bulletin of European Association of Fish Pathologists* 21, 114-115

Douglas-Helders, G.M., Carson, J., Howard, T., Nowak, B.F. (2001b) 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, G.M., Dawson, D.R., Carson, J., Nowak, B.F. (2002a) Wild fish are not a significant reservoir for *Neoparamoeba pemaquidensis* (Page, 1987). *Journal of Fish Diseases*, In press

Douglas-Helders, G.M., Weir, I. J., O'Brien, D.P., Carson, J., Nowak, B.F. (2002b) Effects of husbandry on prevalence of amoebic gill disease and performance of reared Atlantic salmon (*Salmo salar* L.). *Journal of Fish Diseases*. Submitted

Dyková, I., Figueras, A., Peric, Z. (2000) *Neoparamoeba* Page, 1987: light and electron microscopic observations on six strains of different origin. *Diseases of Aquatic Organisms* 43, 217-223

Dyková, I., Novoa, B. (2001) Comments on diagnosis of amoebic gill disease (AGD) in turbot, *Scophthalmus maximus*. *Bulletin of the European Association of Fish Pathologists* 21, 40-44

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

Fernö, A., Huse, I., Juell, J.E., Bjordal, Å. (1995) Vertical distribution of Atlantic salmon (*Salmo salar* L.) in net pens; trade-off between surface light avoidance and food attraction. *Aquaculture* 132, 285-296

Gonzalez, J.M. (1996) A general purpose program for obtaining most probably tables *Journal of Microbiological Methods* 26, 215-218

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

Johnson, P.T. (1968) A new medium for maintenance of marine bacteria. *Journal of Invertebrate Pathology* 11, 114

Jones, G.M. (1985) *Paramoeba invadens* n. sp. (Amoebidae, Paramoebidae), a pathogenic amoeba from the sea urchin, *Strongyloccentrotus droebachiensis*, in Eastern Canada. *Journal of Protozoology* 32, 564-569

Kent, M.L., Sawyer, T.K., Hedrick, R.P. (1988) *Paramoeba pemaquidensis* (Saromastigophora: Paramoebidae) infestation of the gills of coho salmon, *Oncorhynchus kisutch*, reared in seawater. *Diseases of Aquatic Organisms* 5, 163-169

Kent, M.L. (2000) Marine netpen farming leads to infections with some unusual parasites. *International Journal for Parasitology* 30, 321-326

Munday, B.L., Foster, C.K., Roubal, F.R., Lester, R.J.G. (1990) Paramoebic gill infection and associated pathology of Atlantic salmon, *Salmo salar*, and rainbow trout, *Salmo gairdeneri*, in Tasmania. In: *Pathology in Marine Science* (ed. By F.O. Perkins & T.C. Cheng), pp 215-222. Academic Press, San Diego, Ca.

Munday, B.L., Zilberg, D., Findlay, V. (2001) Gill disease of marine fish caused by infection with *Neoparamoeba pemaquidensis*. *Journal of Diseases* 24, 497-507

Nowak, B.F. (2001) Qualitative evaluation of risk factors for Amoebic Gill Disease in Atlantic salmon. 18pp. In: *Proceedings of the OIE International Conference on Risk Analysis in Aquatic Animal Health*, Paris, France, 8-10 February 2000. (Ed: Rodgers, C.J.), pp 148-155. Office International des Epizooties (OIE), 148-155

Oblinger, J.L., Koburger, J.A. (1975) Understanding and teaching the most probably number technique. *Journal of Milk and Food Technology* 38, 540-545

Page, F.C. (1970) Two new species of *Paramoeba* from Maine. *Journal of Protozoology* 17, 421-427

Page, F.C. (1983) Marine Gymnamoebae. *Institute of Terrestrial Ecology, Culture Centre of algae and protozoa*, Cambridge, UK, 1-54

Page, F.C. (1987) The classification of 'naked' amoebae of phylum Rhizopoda. *Archive fur Protistenkunde* 133, 199-217

Paniagua, E., Parama, A., Iglesias, R., Sanmartin, M.L., Leiro, J. (2001) Effects of bacteria on the growth of an amoeba infecting the gills of turbot. *Diseases of Aquatic Organisms* 45, 73-76

Patterson, H.D, Thompson, R. (1971) Recovery of inter-block information when block sizes are unequal. *Biometrica* 58, 545-554

Rheinheimer, G. (1974) The influence of physical and chemical factors on aquatic micro-organisms. In: *Aquatic microbiology*, pp 79-100. Wiley-Interscience Publication, John Wiley & sons, London, New York, Sydney, Toronto

Roubal, F.R, Lester, R.J.G., Foster, C. (1989) Studies on cultured and gill-attached *Paramoeba* sp. (Gymnamoebae: Paramoebidae) and the cytopathology of paramoebic gill disease in Atlantic salmon, *Salmo salar* L., from Tasmania. *Journal of Fish Diseases* 12, 481-493

Sawyer, T.K. (1980) Marine ameba from clean and tressed bottom sediments of the Atlantic Ocean and the Gulf of Mexico. *Journal of Protozoology* 27, 13-32

Scholz, T. (1999) Parasites in cultured and feral fish. *Veterinary Parasitology* 84, 317-335

Sprague, V., Beckett, R. L., Sawyer, T.K. (1969) A new species of *Paramoeba* (Amoebidae, Paramoebaediae) parasitic in the crab *Callinectes sapidus. Journal of Invertebrate Pathology* 2, 167-174

Tan, C.K.F., Nowak, B.F., Hodson, S.L. (2002) Biofouling as a reservoir of *Neoparamoeba pemaquidensis* (Page 1970), the causative agent of AGD in Atlantic salmon. *Aquaculture.* In press

Thrusfield, M. (1995) *Veterinary epidemiology.* Blackwell Science, Oxford, 479 pp.

Zilberg, D., Gross, A., Munday, B.L. (2001) Production of salmonid amoebic gill disease by exposure to *Paramoeba* sp. harvested from the gills of infected fish. *Journal of Fish Diseases* 24, 79-82

# CHAPTER 4

# POSSIBLE RISK FACTORS FOR AGD OUTBREAKS

## 4.1 SURVIVAL OF *Paramoeba pemaquidensis* ON DEAD SALMON: IMPLICATIONS FOR MANAGEMENT OF CAGE HYGIENE

M. Douglas-Helders, B. Nowak, D. Zilberg and J. Carson

### 4.1.1 Abstract

Amoebic gill disease (AGD) is the most serious disease problem in Atlantic salmon aquaculture in Tasmania at present. Little is known however, about the sources or reservoirs of *Paramoeba pemaquidensis*, the causative agent of AGD. This study evaluated the possibility of mortalities being a reservoir of *P. pemaquidensis* that could infect live naive fish as well as uninfected dead fish. Using Immuno-fluorescent antibody test (IFAT) for *P. pemaquidensis* on gill mucus smears it was determined that paramoebae remain on infected gills for at least 30 hours after death of the host and that during this time the number of paramoebae appear to increase. In addition it was established that paramoebae from dead infected fish can colonise the gills of previously uninfected dead fish thereby potentially increasing the bio-burden of paramoebae on infected farms.

## 4.1.2 Introduction

Amoebic Gill Disease, caused by the protozoan pathogen *Paramoeba pemaquidensis* is the main disease affecting Atlantic salmon culture in Tasmania (Munday *et al* 1990, 1993; Clark and Nowak, 1999). Salmon farms have different time frames for removing mortalities from net-pens and range from anywhere between one and fourteen days, the frequency of removal dependant on the number of dead fish in the pens. While it is known that infected dead fish present in pens can be a reservoir for some pathogens, for example infectious salmon anaemia virus (ISAV) (Jarp and Karlsen 1997), it is not known if this is true for *P. pemaquidensis*.

The main aim of this study was to determine if paramoebae remain on infected fish after death and if paramoebae from such fish can colonise dead uninfected fish. Evidence of dead fish as reservoirs of AGD infection would have significant implications for the management of cage hygiene.

## 4.1.3 Materials and Methods

Seawater-adapted Atlantic salmon ranging in fork length from 27 to 35 cm were obtained from the Aquatic Key Centre, University of Tasmania, Launceston. Three uninfected fish were taken from specific pathogen free stocks of Atlantic salmon kept in brackish seawater of 30 ppt. Three *P. pemaquidensis* donor fish were taken from stocks known to have AGD that were held in an experimental tank with seawater at a salinity of 37 ppt. Both tank systems were kept at a constant temperature of 13°C. The fish in the two groups were killed by anaesthetic overdose in a bath containing 100 mg $L^{-1}$ of benzocaine.

A 450 l bin was filled with 175 l of filtered seawater at a salinity of 38 ppt and used as the exposure tank for the trial. The water temperature was measured every hour during the experiment and ranged from 20 to 21°C. Immediately after euthanasia, the fish were tagged individually, and a baseline gross gill score as an index of infection was determined for each fish (Clark & Nowak, 1999). The score was represented as clear (no signs of infection), light, medium or heavy infection. A gill smear to detect *P. pemaquidensis* by IFAT (Howard & Carson, 1993) was also made for each fish to determine parasite load at time zero. The two groups of fish were placed randomly into the 450 l bin. The bottom of the bin was large enough for the fish to rarely have physical contact. The water in the bin was gently stirred at each sample interval to prevent localised concentrations of paramoebae from occurring. Gill mucus smears were taken from each fish at times t= 1, 2, 3, 4, 5, 6, 7, 24 and 30 hours, and the number of paramoebae semi-quantified by means of IFAT. All fish were sampled on the following gill arches: first left (t=0 and 1), second left (t=2), third left (t=3), fourth left (t=4), first right (t=5), second right (t=6), third right (t=7). At t=24 and 30 hours no distinct gill arches could be distinguished and a mucus samples was taken from the whole left and right side gill respectively.

The IFAT to detect paramoebae in gill mucus smears followed the protocol developed by Howard & Carson (1993) using a primary rabbit antibody prepared to *P. pemaquidensis* strain PA027. Cells of paramoebae were counted in 10 random fields of view, at a 100X final magnification using an Olympus BX40F-3 UV epi-fluorescence microscope and FITC filter set.

### 4.1.4 Results and Discussion

The infected group had a gross gill score respectively of 'light' (18 paramoebae by IFAT), 'medium' (79 paramoebae) and 'heavy' (193 paramoebae). Although the mean number of paramoebae on the infected fish varied over time (Table 1), there was an overall increase in paramoebae numbers compared to time zero samples, with the maximal number of paramoebae seen on the infected gills after six hours. Paramoebae nevertheless were still very abundant on the gills of infected fish at termination of the experiment after 30 hours.

The uninfected group had a gross gill score of 'clean', which was confirmed by negative IFAT tests at time zero. These fish remained uncolonised during the first 4 hours, after which time paramoebae were detected in 1 of the 3 fish tested. After 24 hours, all 3 initially negative fish were colonised by paramoebae, and by termination of the experiment after 30 hours (Table 1) the number of paramoebae evident on the gills had increased. Sampling after 30 hours was not attempted because of the degree of autolysis and tissue deterioration that had occurred in both groups of fish.

**Table 1.** Mean number of paramoebae/10 fields (± SD) determined by IFAT

| Time (hour) | Infected fish<br>Mean numbers Paramoebae | Control fish<br>Mean number Paramoebae |
|---|---|---|
| 0 | 96.7 (72.5) | 0.0 (0) |
| 1 | 222.6 (159.6) | 0.0 (0) |
| 2 | 351.3 (231.2) | 0.0 (0) |
| 3 | 356.3 (250.8) | 0.0 (0) |
| 4 | 299.0 (230.4) | 0.3 (0.5) |
| 5 | 229.0 (126.9) | 0.0 (0) |
| 6 | 423.3 (226.7) | 0.7 (0.9) |
| 7 | 297.7 (209.5) | 0.0 (0) |
| 24 | 103.3 (53.8) | 3.3 (1.2) |
| 30 | 272.0 (162.6) | 6.3 (5.4) |

The fact that paramoebae not only remained on the dead infected fish, but also seemed to increase in numbers suggests that AGD mortalities are a potentially important reservoir of infection. Additionally, the experimental evidence suggests that paramoebae can colonise dead naive fish leading to an increase in their number over time. This apparent amplification of paramoebae has important implications in the management of cage hygiene by farms. It can be argued that prompt removal of carcases from cages may reduce the bio-burden of paramoebae and so reduce the likelihood of infecting naive fish and potentially limit the severity of AGD outbreaks.

Currently, disease diagnosis is undertaken by combinations of gross gill pathology, detection of *P. pemaquidensis* by IFAT of gill mucus smears, or gill histopathology. IFAT is currently limited to collecting gill mucus smears from live fish. The evidence from the work reported here indicates that dead fish can also be used for reaching a diagnosis of AGD using IFAT. Clearly some caution would

need to be exercised with this approach since dead fish without AGD could be colonised with paramoebae thereby exaggerating the severity of an outbreak.

The experimentation was carried out at 20-21°C and was chosen to reflect conditions that occur during the main outbreaks of AGD on Tasmanian salmon farms during the warm summer months (Clark and Nowak 1999). At cooler temperatures, the survival times of paramoebae on the gills of the already infected fish as well as colonisation of dead uninfected fish is unknown. It is most likely however, that the colonisation and reproduction times of paramoebae would be temperature dependent and slower at lower temperatures.

## 4.1.5 Acknowledgments

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## 4.1.6 References

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.

Howard, T. and Carson, J. (1993) Verification that Paramoeba species are consistently associated with gill damage in fish affected with amoebic gill disease. In: *Seeking and solving, Proceedings of the Saltas research and development review seminar, Tasmania 1993*, pp. 69-80. Salmon Enterprises of Tasmania Pty Ltd Dover, Tasmania.

Jarp, J. and Karlsen, E. (1997) Infectious salmon anaemia (ISA) risk factors in sea-cultured Atlantic salmon *Salmo salar*. *Diseases of Aquatic Organisms* **28**, 79-86.

Munday, B.L., Foster, C.K., Roubal, F.R. and Lester, R.G.J. (1990) Paramoebic gill infection and associated pathology of Atlantic salmon, *Salmo salar*, and rainbow trout, *Salmo gairdneri*, in Tasmania. In: *Pathology in Marine Science* (Ed. By F.O. Perkins & T.C. Cheng), pp. 215-222. Academic Press, San Diego, CA.

Munday, B.L., Lange, K., Foster, C, Lester, R.J.G. and Handlinger, J. (1993) Amoebic gill disease of sea-caged salmonids in Tasmanian waters. *Tasmanian Fisheries Research* **28**, 14-19.

# 4.2 EFFECTS OF COPPER-BASED ANTIFOULING TREATMENT ON THE PRESENCE OF *Neoparamoeba pemaquidensis* PAGE 1987 ON NETS AND GILLS OF REARED ATLANTIC SALMON (*Salmo salar*)

Douglas-Helders, G.M., Tan, C., Carson, J., Nowak, B.F.

## 4.2.1 Abstract

Amoebic gill disease (AGD) is the main disease affecting the salmon industry in Australia. Little information is available on the epidemiology of AGD and the biology of *Neoparamoeba pemaquidensis* (Page, 1987), the disease causing organism of AGD. In previous studies *N. pemaquidensis* was found on biofouled netting of sea cages, and a reduction in AGD prevalence was achieved with increasing number of net changes. How important the source of *N. pemaquidensis* on netting is for inducing AGD is unknown. To reduce biofouling on nets, the use of antifouling paints is a common practice on Tasmanian salmon farms. This study investigated the effects of a copper-based antifouling paint on the prevalence of *N. pemaquidensis* on nets and the AGD prevalence of Atlantic salmon within these nets. Four sea cages stocked with 5 to 9 kg $m^{-3}$, year 2000 stock, Atlantic salmon were used for this trial. Two nets were coated with a copper-based antifouling paint and two nets were not treated and used as a control. Fish were sampled every two weeks for ten weeks. A gross gill score was determined and gill mucus samples were taken for dot blot analysis to determine the presence of *N. pemaquidensis* for each fish. Biofouling samples from netting were inoculated onto 75% malt yeast

(MY) agar culture plates, and presence of *N. pemaquidensis* confirmed using culture techniques, followed by indirect immuno-fluorescent antibody test (IFAT). Culture enriched biofouling samples from week two and eight were tested using a nested PCR to reconfirm presence of *N. pemaquidensis*. Prevalence at the conclusion of the trial was corrected for baseline prevalence, and the corrected prevalence used to determine any significant treatment effects. Results showed that copper paint treated cages showed a significantly higher paramoebae (P=0.002) and AGD (P=0.014) prevalence compared to the control cages. No treatment effect was found on the prevalence of positive scored fish (P=0.243). At the conclusion of the trial the paramoebae prevalence of net samples was 58.5% (SE 1.5) and AGD prevalence was 42.5% (SE 2.5) for copper treated nets, while no paramoebae were found on control nets and AGD prevalence was 35.0% (SE 5.0). Nets could be a source of *N. pemaquidensis* for infection of fish with AGD, and therefore copper paint treated nets could be a risk factor for AGD.

## 4.2.2 Introduction

Amoebic gill disease (AGD) is a major constraint on the marine farming of salmonids in Tasmania, Australia. Presently little information is available on the epidemiology of amoebic gill disease (AGD) in farmed Atlantic salmon (*Salmo salar* L.) in Tasmania. The disease is caused by *Neoparamoeba pemaquidensis*, an endemic, free-living, and amphizoic protozoan (Page, 1987, Dykova *et al.*, 2000) with a worldwide spread (Cann & Page, 1982). Factors known to contribute to the disease are salinity, temperature, poor water quality (Kent *et al.*, 1988; Nowak, 2001), and number of net changes performed on marine farms (Clark & Nowak,

1999). In order to manage AGD in an economical way, risk factors and reservoirs of *N. pemaquidensis* need to be better understood.

Biofouling on nets of sea cages is a great concern for the salmon growers in Tasmania. It can cause reduced water flow through nets, resulting in a lowered dissolved oxygen and increased ammonia content of the water body within the net (Hodson & Burke, 1994). When a net of a sea cage is first immersed, there will be a succession of organisms that colonise the net. Bacteria that colonise the net initially are known to produce a polysaccharide layer, which may act as a protective barrier from the treated surface of the net (Marszalek *et al.*, 1979; Dempsey, 1981). Some organisms such as the protozoan *N. pemaquidensis*, can only multiply or capture its prey when attached to a particulate surface (Martin, 1985) and nets could form an excellent substrate for paramoebae. Less chemically resistant fouling organisms are now provided with an opportunity to colonise the net, due to the protective polysaccharide layer, as a successional community. Such organisms range from bacteria, diatoms, protozoa and choanoflagellates (Milne, 1975; Dempsey, 1981; Hodson & Burke, 1994) to algae, barnacles, bivalves, marine worms, and ascidians (Milne, 1975). There is a possibility that nets are a reservoir for disease causing organisms such as paramoebae. In biofouling samples collected from nets from an AGD infected salmon farm in Tasmania *N. pemaquidensis* was indeed identified, using culture enriched IFAT and PCR (Tan *et al.*, 2002).

To reduce the amount of fouling, and hence increase the water quality and decrease the chance of diseases, nets can be coated with antifouling paints (Balls, 1987;

Hodson & Burke, 1994). Antifouling paint releases a biocide into a thin layer of water that closely surrounds the net (Balls, 1987), preventing survival of any susceptible organism that tries to attach to the net. However, the antifouling paint could act as a selective medium for organisms that are either less susceptible or even tolerant to the biocide, or are able to attach to the treated surface despite the coating. This creates a noncompetitive environment for such species in which they flourish (Dempsey, 1981).

This study explored the effects of a commercial copper oxide based antifouling paint on the presence of *N. pemaquidensis* in biofouling on nets and their effect on the AGD prevalence in reared fish.

### 4.2.3 Materials and Methods

This trial used Atlantic salmon of the year 2000 stock, originating from two Tasmanian hatcheries. Fish were introduced from June to October 2000 to a marine lease site on a salmon farm in the Huon Estuary, Tasmania, Australia. The salmon remained at the same lease site and had the same treatment history until commencement of the trial. Prior to the trial the experimental fish were treated for AGD infection with freshwater bathing, transferred to four cages with new nets of 120 m circumference, and all trial cages (treated and control) moved to a different lease site. The trial cages resided next to each other on the lease site, but were at significant distance from non-trial cages. Two of the 4 nets were treated with a copper based antifouling paint (Hempel paint, NSW, Australia) according to manufacturer instructions, and soaked in seawater for 72 hours before stocking. The other two nets were washed with fresh water in a netwasher and also soaked

for 72 hours in seawater before stocking. The four cages were stocked with similar densities, ranging from 5 to 9 kg $m^{-3}$. Environmental measurements of salinity, temperature and dissolved oxygen were taken during the trial.

Sampling took place on day 0 (before freshwater bathing and introduction to the treated/untreated net) and on days 15, 30, 45, 58, and 71 (week 10), when freshwater bath treatment was required. Initial gill mucus samples for immuno-dot blot were taken and gross gill scores determined prior to freshwater bathing and were used as a baseline score for the rest of the experiment for these cages. Biofilm samples from the nets were taken prior to stocking. Twenty fish per cage were sampled for dot blot and 20 to 27 fish per cage assessed for gross gill scores on each of the sampling days. Fish were caught by crowd and dip netting and anaesthetised in 0.5% Aqui-S (Lower Hutt, New Zealand). All gill arches of each fish were carefully examined and a gross gill score determined, followed by taking a mucus sample for immuno-dot blot analysis. Gross gill scores are routinely used by the Tasmanian salmon industry for the detection of AGD (Munday *et al.*, 1990). Gross gill scores were noted as: clear (no mucus build up present), faint spot (small, light spot-like discolouration affecting one or two gill lamellae), spot (more visible mucus build up, only very small area of the gill affected) and patch (larger part of the gill affected by mucus build up and white patches can be clearly seen). The data for gross gill scores were recorded as positive (faint spot, spot or patch) or negative (clear). The gill mucus sample was taken from one of the visible AGD infected area(s) on the gill if any were present. If the gill did not show any signs of AGD, the mucus sample was taken from the second gill arch on the left side of the fish. The dot blot samples were processed and analysed as described in Douglas-

Helders *et al.* (2001). The immuno-dot blot results were recorded as positive (presence of *N. pemaquidensis*) or negative (absence of *N. pemaquidensis*). The experiment was completed when freshwater bathing was required for AGD treatment, which occurred after 10 weeks (71 days).

Concurrent to sampling of fish, five replicate microbial biofilm samples were taken from each net by lifting the first 10-50 cm of submerged netting and swabbing sections of netting onto plates as described by Tan *et al.* (2002). In short: the samples were inoculated onto autoclaved (121°C, 15 min) seawater MY extract (Oxoid, Victoria, Australia) agar plates with 75% natural seawater of 35‰ salinity. Pimaracin (500 µL, Sigma, NSW, Australia, sterile suspension 25 mg $mL^{-1}$) was added to the plates as an antifungal agent. After inoculation the plates were incubated for 7 to10 days at 20°C and a swab of the growth area was taken and smeared onto a glass microscope slide to confirm *N. pemaquidensis* presence, using indirect fluorescent antibody test (IFAT) (Howard & Carson, 1993). Samples taken in week two and eight were tested for the presence of *N. pemaquidensis* using a nested PCR, developed by Elliott *et al.* (2001). The IFAT to detect paramoebae in gill mucus smears followed the protocol developed by Howard & Carson (1993) using a polyclonal primary rabbit antibody prepared to *N. pemaquidensis* strain PA027 (Douglas-Helders *et al.*, 2001) and a anti-rabbit alkaline phosphatase conjugated secondary antibody (Silenus, Melbourne, Australia). Positive samples were characterised by the presence of fluorescent cells, viewed at a 100X final magnification using an Olympus BX40F-3 UV epi-fluorescence microscope and FITC filter set.

The AGD prevalence and gross gill score prevalence between the two treatment groups at time zero (used as the baseline data in this trial) were analysed for treatment differences, using a two-tailed Student's t-test. All prevalence data at the conclusion of the trial, in week 10, were corrected for the baseline prevalence at time zero. For this, the baseline prevalence was divided by "final prevalence + 1" for each cage. The addition of the value one was to avoid division by zero when calculating the corrected prevalence. Netting of cages were sampled for paramoebae culture prior to stocking and only 1 of 5 samples from one of the copper paint treated cages was positive for *N. pemaquidensis*. This prevalence value was subtracted from the final prevalence for the same cage. Any significant difference in the corrected prevalence due to treatment was determined using a two-tailed Student's t-test. Results of all statistical analysis were considered significant when $P \leq 0.05$.

A corrected *kappa* coefficient, an index of concordance, was calculated (Cicchetti & Feinstein, 1990) for testing with PCR versus IFAT and dot blot AGD prevalence versus gross gill score prevalence.

### 4.2.4 Results

At time zero no significant difference in paramoebae prevalence (P=0.500), AGD prevalence (P=0.063), and gross gill score prevalence (P=0.177) existed between the copper paint treated cages and the control cages. The corrected prevalence values for paramoebae, AGD and gross gill scores are shown in Table 1. The average paramoebae prevalence on nets in week 10, the conclusion of the trial, was 48.5% (SE 8.5) for the copper paint treated cages, while no paramoebae were

detected on the nets of the control cages at this point in time. The corrected paramoebae prevalence on the copper paint treated nets was significantly higher compared to the control nets (P=0.002). The average paramoebae prevalence over time was 57.6% (SE 8.3) for the copper paint treated cages, and 5.5% (SE 3.9) for the control cages. Paramoebae prevalence over time for both treatments is shown in Figure 1. The paramoebae prevalence on control nets was lower than on the copper paint treated nets at all sampling times, except for week eight when the prevalence was equal. Salinity ranged from 17.6 to 35.4‰ and temperature ranged from 7.3 to 10.8°C and was the same for all trial cages.

**Table 1**: Average corrected paramoebae, AGD and gross gill score prevalence values for the two treatments

| Average (±SE) corrected prevalence (%) | Treatment | |
|---|---|---|
| | Control | Copper |
| Paramoebae | 1.000 (0.000) | 0.021 (0.004) |
| AGD | 1.208 (0.042) | 1.708 (0.042) |
| Gross gill score | 1.057 (0.274) | 0.395 (0.299) |

The average AGD prevalence in week 10 in cages with copper paint treated nets was 42.5% (SE 2.4), while the control cages showed a lower prevalence of 35.0% (SE 5.0). The corrected AGD prevalence in the copper treated cages was significantly higher than the control cages (P=0.014), this was due to a greater reduction in AGD prevalence in the copper treated cages. The average AGD prevalence over time was 59.5% (SE 4.5) for copper treatment and 40.0% (SE 5.7) for control treatment. The AGD prevalence for each treatment over time is shown

in Figure 2. The time trend was the same for both treatments with a peak for AGD prevalence in week six. AGD prevalence in the control cages was lower than the prevalence in the copper paint treated cages at all times.

At the conclusion of the trial the average prevalence of positive scored fish at gross level was 63.5% (SE 1.6) for the copper treatment and 47.8% (SE 0.19) for the control cages. The corrected prevalence in the copper treatment group was similar to this in the control group (P=0.243). The average prevalence over time was 52.5% (SE 7.7) percent for the copper and 30.1% (SE 6.0) for the control treatment. Figure 3 shows the trend in prevalence of positive scored fish over time for both treatments. The prevalence for fish in the control cages was below the prevalence for fish in the copper treatment cages at all times but week eight, where the prevalence was slightly higher in the control cages.

*N. pemaquidensis* was detected by PCR in the biofouling from samples taken in week 2 and 8. A good correlation was found between PCR and IFAT testing (Elliott *et al.*, 2001). The agreement between the two tests was good with a corrected *kappa* value (Cicchetti & Feinstein, 1990) of 0.89 (SE 0.036), positive predictive value of 0.92 and negative predictive value of 0.96. The dot blot prevalence data and the gross gill score prevalence data showed a low agreement between the two tests, with a corrected *kappa* value of 0.55 (SE 0.09), positive predictive value of 0.73 and negative predictive value of 0.79

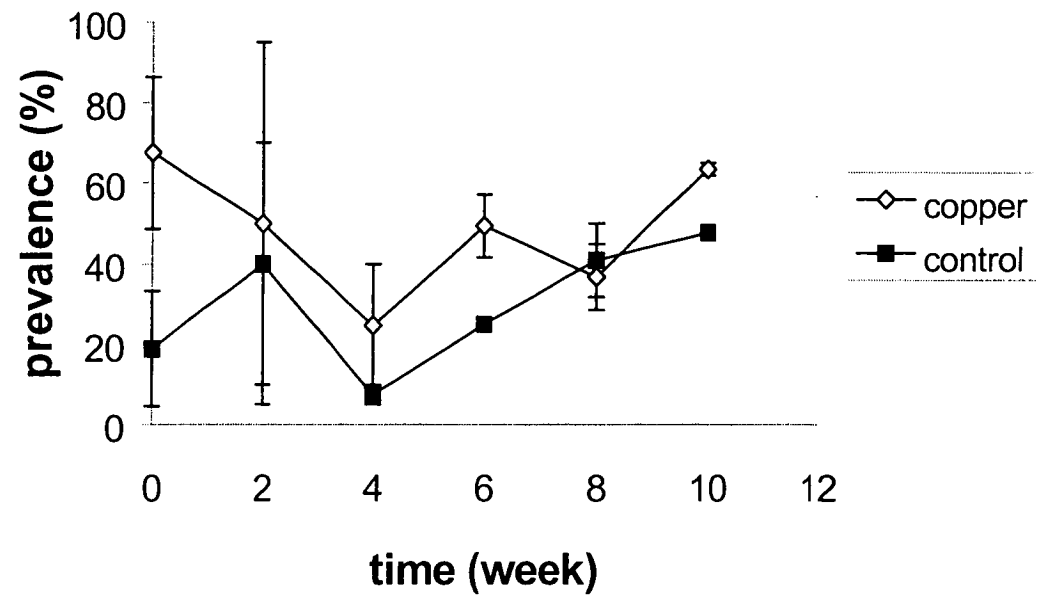


**Figure 1**: Average *N. pemaquidensis* prevalence (%), for the control and copper (± SE) treatments over a period of ten weeks

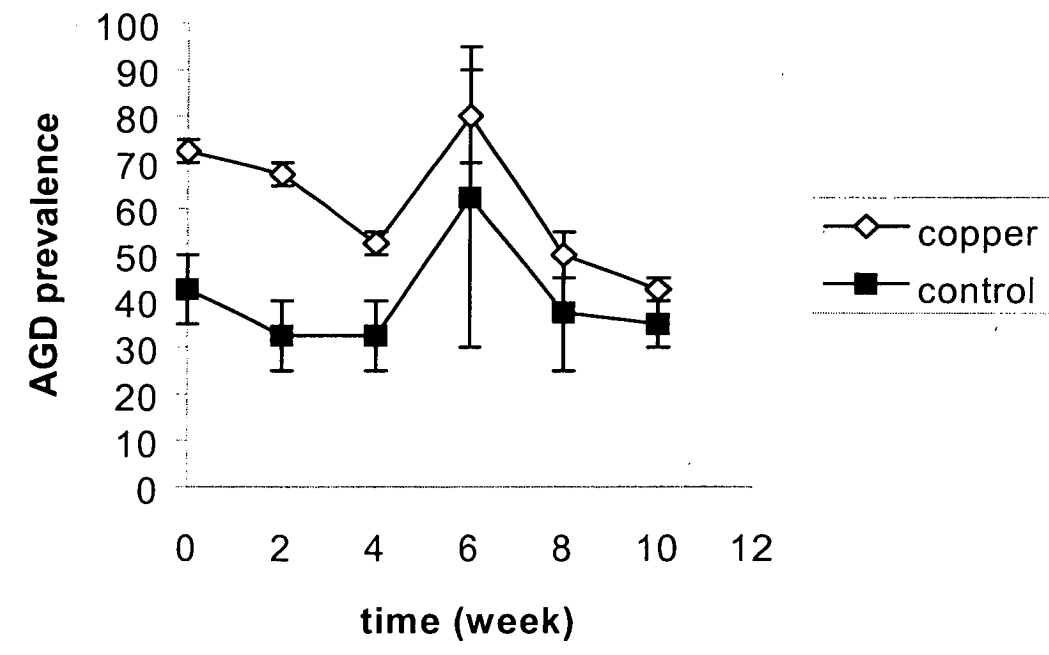


**Figure 2**: Average AGD prevalence for control and copper (± SE) treatments over a period of ten weeks

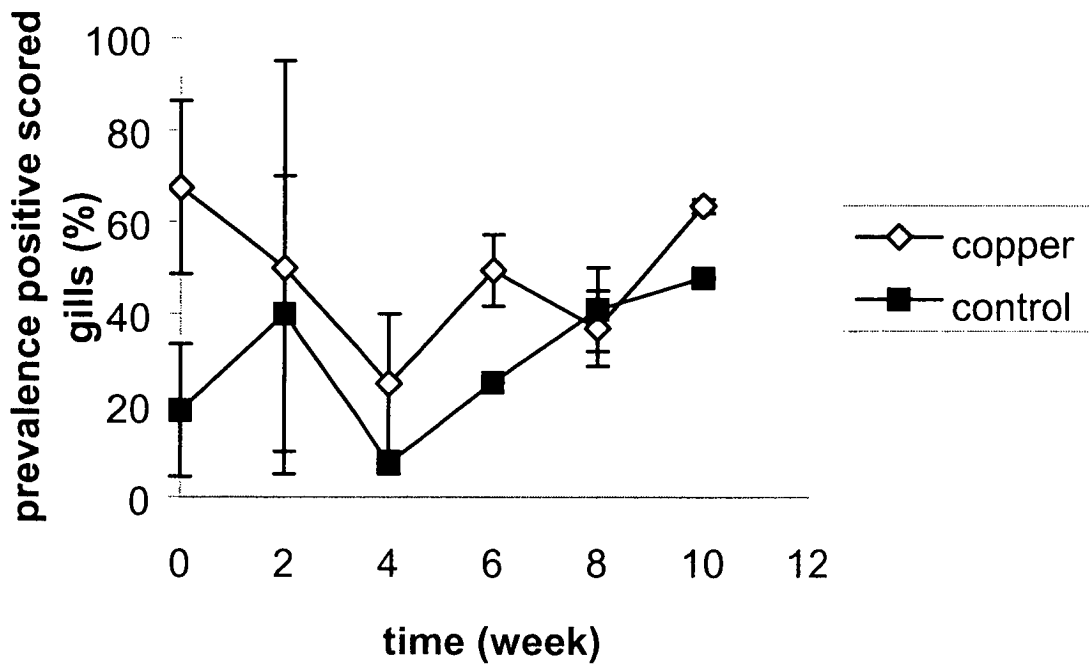


**Figure 3**: Average prevalence of grossly scored positive fish for control and copper (± SE) treatments over a period of ten weeks

## 4.2.5 Discussion

Results from this study showed that *N. pemaquidensis* prevalence was significantly higher on copper paint treated nets compared to control nets. Presence of *N. pemaquidensis* on biofouled netting of sea cages has been reported in previous studies of Tan *et al.* (2002). Copper antifouling paint did not preclude the presence of *N. pemaquidensis* on the treated nets in this trial. Several explanations are possible; first, the existence of cuprous resistant bacteria is well known (Marszalek *et al.*, 1979; Dempsey, 1981), and it has been shown that cages treated with cuprous oxide paint harbour four times more bacteria compared to nets treated with other antifouling paints or untreated nets (Dempsey, 1981). Protozoans, such as *N. pemaquidensis*, are known to feed on bacteria (Paniagua *et al.*, 2001), and the high bacterial load on copper paint treated nets might attract bacterivorous protozoans. To enable predation and replication *N. pemaquidensis* requires attachment to a solid substrate (Marszalek *et al.*, 1979, Martin, 1985), such as treated nets. Also, *N. pemaquidensis* does not seem to be very much affected by pollutants and have been found in heavily polluted water, including those contaminated by heavy metals (Corpe, 1976; Sawyer, 1980). Therefore, *N. pemaquidensis* might not be deterred or even prefer attaching to copper paint treated surfaces. Biofouling samples were taken from 10-50 cm depth. No information on the distribution of paramoebae on nets at different depths is available.

The impact of the positioning of the trial cages on the lease site in this trial was not studied and therefore cannot be estimated. If *N. pemaquidensis* is attracted or has a preference for copper anti-fouling paint treated nets, as shown in this study with a significantly higher *Neoparamoeba* prevalence on the treated nets), these nets

might have had a "magnet" effect for the protozoan and thus relatively lowered levels on the control nets compared to the natural base-level that would be expected. As a consequence, this could have led to a significant difference between the two treatments (treated and untreated nets). However, presently a mix of anti-fouling paint treated and untreated nets co-exist on one lease site in the field, and the results of this study are therefore more applicable to the industry than when the trial cages were kept seperated.

The primary anti-paramoeba antibody used in this trial to assess presence of *N. pemaquidensis* in biofouling using IFAT is known to cross react with *N. aestuarina* and *Pseudoparamoeba pagei* (Douglas-Helders *et al.*, 2001). Though the cross-reactive nature of this antibody proved not to be a problem for testing gill isolates (Douglas-Helders *et al.*, 2001), this is not known for environmental samples. When samples were analysed by both IFAT and PCR a good correlation was found (Elliott *et al.*, 2001). The *kappa* value showed a good agreement between the two tests, suggesting that the used primary antibody is reliable for testing presence of *N. pemaquidensis* in biofouling samples from nets.

Besides a significantly higher paramoebae presence on the copper paint treated nets, the corrected AGD prevalence within these nets was significantly higher as well. This suggests that copper paint treated cages are a major source or reservoir of *N. pemaquidensis* for infection of fish with AGD. Other suggested reservoirs for *N. pemaquidensis* are water column, or dead infected fish left inside nets (Douglas-Helders *et al.*, 2000). Future epidemiological studies need to be undertaken to fully understand the significance of these reservoirs.

A low agreement was found between the dot blot test and gross gill score assessment. White mucoid patches or excessive mucus, markers for a positive gill score, is commonly used as an indication of AGD infection for salmon growers. These gross signs however have been found to be unreliable as indicator of paramoebae presence, especially in the lower infection range (Clark & Nowak, 1999). Studies on gill pathology have shown that mucus is produced as a host reaction to physical (eg gill parasites) and/or chemical irritants in the water column (Mallatt, 1985; Laurent & Perry, 1991; Nowak & Munday, 1994). In this study copper released from the treated nets could have been an irritant, explaining the significant higher gross gill score prevalence in these cages.

The results of this study show that copper based antifouling paint increases the paramoebae prevalence on netting of sea cages, and induces a higher AGD prevalence of the population within these cages. Future large-scale studies are needed to fully establish these relationships.

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## 4.2.7 References

Balls, P.W. (1987) Tributylin (TBT) in the waters of a Scottish Sea Loch arising from the use of antifoulant treated netting by salmon farms. *Aquaculture* 65, 227-237

Cann, J.P., Page, F.C. (1982) Fine structure of small free-living *Paramoeba* (Paramoebida) and taxonomy of the genus. *Journal of the Marine Biology Association* 62, 25-43

Cicchetti, D.V., Feinstein, A.R. (1990) High agreement but low kappa: II. Resolving the paradoxes. *Journal of Clinical Epidemiology* 43(6), 551-558

Clark, A., 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

Corpe, W.A. (1976) Primary bacterial films and marine microfouling. In: *proceedings 4th International Congress on Marine Corrosion and Fouling*. (Ed. V. Romanovsky), pp. 97-100 Juan-les-Pins, Antibes, France

Decostere, A., Haesebrouck, F., Turnbull, J.F., Charlier, G. (1999) Influence of water quality and temperature on adhesion of high and low virulence *Flavobacterium columnare* strains to isolated gill arches. *Journal of Fish Diseases* 22(1), 1-11

Dempsey, M.J. (1981) Colonisation of antifouling paints by marine bacteria. *Botanica Marina* XXIV, 185-191

Douglas-Helders, G.M., Nowak, B.F., Zilberg, D., Carson, J. (2000) Survival of *Paramoeba pemaquidensis* on dead salmon: implications for management of cage hygiene. *Bulletin of the European Association of Fish Pathologists* 20(3), 167-169

Douglas-Helders, G.M., Carson, J., Howard, T., Nowak, B.F. (2001) Development and validation of a new dot blot test for the detection of *Paramoeba pemaquidensis* (Page) in fish. *Journal of Fish Diseases* 24(5), 273-280

Douglas-Helders, G.M., Saksida, S., Raverty, S., Nowak, B.F. (2001) Temperature as a risk factor for outbreaks of Amoebic Gill Disease in farmed Atlantic salmon (*Salmo salar*). *Bulletin of the European Association of Fish Pathologists* 21(3), 114-116

Dykova, I., Figueras, A., Novoa, B., Casal, J.F. (1998) *Paramoeba* sp., an agent of AGD of turbot, *Scophthalmus maximus* L. *Diseases of Aquatic Organisms* 33, 137-141

Dykova, I., Figueras, A., Peric, Z. (2000) *Neoparamoeba* Page 1987: light and electron microscopic observations on six strains of different origin. *Diseases of Aquatic Organisms* 43, 217-223

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

Hodson, S.L, Burke, C. (1994) Microfouling of salmon-cage netting: a preliminary investigation. *Biofouling* 8, 93-105

Hodson, S.L., Lewis, T.E., Burke, C.M. (1997) Biofouling of fish-cage netting: efficacy and problems of in situ cleaning. *Aquaculture* 152, 77-90

Howard, T., Carson, J. (1993) Verification that paramoeba species are consistently associated with gill damage in fish affected with amoebic gill disease. In: *Proceedings of the Saltas Research and Development Review Seminar*, Tasmania, pp. 69-80, Salmon Enterprises of Tasmania Pty Ltd Dover, Tasmania

Kent, M.L., Sawyer, T.K., Hedrick, R.P. (1988) *Paramoeba pemaquidensis* (Saromastigophora: Paramoebidae) infestation of the gills of coho salmon *Oncorhynchus kisutch* reared in seawater. *Diseases of Aquatic Organisms* 5, 163-169

Laurent, P., Perry, S.F. (1991) Environmental effects on fish gill morphology. *Physiolgical Zoology* 64(1), 4-25

Mallatt, J. (1985) Fish gill structural changes induced by toxicants and other irritants: A statistical review. *Canadian Journal of Fisheries and Aquatic Science* 42, 630-648

Marszalek, D.S., Gerchakov, S.M., Udey, L.R. (1979) Influence of substrate composition on marine biofouling. *Applied Environmental Microbiology* 38(5), 987-995

Martin, R.E. (1985) Population growth in stationary and suspension culture of *Paramoeba pemaquidensis* Page (Amoebida, Paramoebidae). *Journal of Protozoology* 32, 738-739

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

Milne, P.H. (1975) Fouling of marine cages, part 1. *Fish Farming International* 2,15-19

Munday, B.L., Foster, C.K., Roubal, F.R., Lester, R.J.G. (1990) Paramoebic gill infection and associated pathology of Atlantic salmon, *Salmo salar*, and rainbow trout, *Salmo gairdeneri*, in Tasmania. In: *Pathology in Marine Science*, Perkins, F.O., Cheng, T.C. (Eds), pp 215-222, Academic Press

Munday, B.L., Lange, K., Foster, C., Lester, R.J.G., and Handlinger, J. (1993) Amoebic gill disease of sea-caged salmonids in Tasmanian waters. *Tasmanian Marine Fisheries Research* 28, 14-19

Nowak, B.F., Munday, B.L. (1994) Histology of gills of Atlantic salmon during the first few months following transfer to seawater. *Buletin of the European Association of Fish Pathologists* 14, 77-81

Nowak, B.F. (2001) Qualitative evaluation of risk factors for Amoebic Gill Disease in Atlantic salmon. 18pp. In: *Proceedings of the OIE International Conference on Risk Analysis in Aquatic Animal Health*, Paris, France, 8-10 February 2000. (Ed Rodgers, C.J.), pp. 148-155, Office International des Epizooties (OIE)

Paniagua, E., Parama, A., Iglesias, R., Sanmartin, M.L., Leiro, J. (2001) Effects of bacteria on the growth of an amoeba infecting the gills of turbot. *Diseases of Aquatic Organisms* 45, 73-76

Page F.C. (1987) The classification of 'naked' amoebae of phylum Rhizopoda. *Archive Protistenkunde* 133:199-217

Palmer, R., Carson, J., Ruttledge, M., Drinan, E., Wagner, T. (1997) Gill disease associated with *Paramoeba*, in sea-reared Atlantic salmon in Ireland. *Bulletin of the European Association of Fish Pathologists* 3-4, 112-114

Rodger, H.D., McArdle, J.F. (1996) An outbreak of amoebic gill disease in Ireland. *Veterinary Record* 139, 348-349

Sawyer, T.K. (1980) Marine amoebae from clean and stressed bottom sediments of the Atlantic Ocean and Gulf of Mexico. *Journal of Protozoology* 27(1), 13-32

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

# 4.3 TEMPERATURE AS A RISK FACTOR FOR OUTBREAKS OF AMOEBIC GILL DISEASE IN FARMED ATLANTIC SALMON (*Salmo salar*)

Marianne Douglas-Helders, Sonja Saksida, Stephen Raverty and Barbara, F. Nowak

## 4.3.1 Abstract

Amoebic Gill Disease outbreaks in Atlantic salmon have recently occurred below the lower temperature limit previously recognised for *Neoparamoeba pemaquidensis*. This observation challenges the role of ambient water temperatures as one of the prime risk factors for AGD

## 4.3.2 Review

Amoebic Gill Disease affects cultured salmonids in Australia (Munday *et al.*, 1990, Clark & Nowak, 1999), Ireland (Rodger & McArdle, 1996, Palmer *et al.*, 1997), France (Carson pers. comm.), Spain (Carson pers. comm.), Chile (Groman & Buston pers. comm) and the USA (Kent *et al.*, 1988). In Tasmania, Australia, AGD is the prime health concern affecting Atlantic salmon culture (Roubal *et al.*, 1989, Munday *et al.*, 1990, Nowak, 2001). Temperature has been identified previously as one of the main risk factors for AGD (Nowak, 2001) and in Tasmania, this environmental factor is considered second only to salinity as a significant environmental factor affecting AGD outbreaks (Clark & Nowak, 1999). Clinical AGD was documented in Atlantic salmon at temperature ranges from 15 to 20°C in Tasmania (Munday *et al.*, 1990) and from 12 to 21°C in Ireland (Rodger & McArdle, 1996, Palmer *et al.*, 1997). During a histological survey of salmon

cultured in Tasmania AGD lesions were recorded at the minimum temperature of 10.6°C (Clark & Nowak, 1999). Amoebae were observed on the gills of cultured Atlantic salmon in winter in Tasmania (Munday *et al.*, 1990, Howard & Carson, 1993), with no attendant clinical disease or histological lesion. Experimental tank infections suggested that temperatures above 16°C drastically increased fish mortalities and that temperature below 13°C precluded mortalities (D. Zilberg pers. comm.). The optimum temperature for *in vitro* culture of *Neoparamoeba pemaquidensis* was 15°C (Kent *et al.*, 1988). This indicated that temperature played an important role in AGD outbreaks, which were reported to occur only at higher water temperatures.

Recently we observed AGD outbreaks and AGD associated mortalities requiring treatment at lower temperatures. This was in contrast to the general belief that outbreaks only occurred when average temperatures were 13°C or higher (Munday *et al.*, 1993, Clark & Nowak, 1999). These outbreaks at lower than expected temperatures took place both in the Northern (USA) and Southern (Australia) hemispheres. Mortalities of Atlantic salmon at a net pen facility in Puget Sound, Washington, USA were observed at temperatures sustained below 10°C. The mortalities occurred from September through to November with peak mortality of 21.85% in October when the mean water temperature was 9.2°C (Table 1). The presence of *Neoparamoeba pemaquidensis* was confirmed by species-specific polyclonal antibodies employed in an indirect IFAT (Howard & Carson, 1993) of branchial sections of the gills from moribund fish.

**Table 1**: Mortalities and water temperature at affected salmon farm in Washington state, USA.

| Month | Mortality (%) | Cumulative mortality (%) | Water temperature (°C) |
|---|---|---|---|
| August | 0.02 | 0.02 | 10.3 |
| September | 10.87 | 10.89 | 9.9 |
| October | 21.85 | 32.75 | 9.2 |
| November | 5.31 | 38.05 | 9.1 |
| December | 0.15 | 38.21 | 8.9 |

While AGD is seen as a summer problem in Atlantic salmon cultured in Tasmania (Clark & Nowak, 1999), recent winter outbreaks of clinical disease have been observed. Fifty percent out of season smolt (100-220 g) exhibited variable (light or greater) AGD infection (as determined by gross gill checks and confirmed by IFAT on gill smears) approximately 3 months after transfer to sea water. This outbreak occurred when the average water temperatures were about 10°C (maximum 13°C) and the stock required freshwater treatment to limit mortality (M. Hortle pers comm.).

Isolates of *Neoparamoeba pemaquidensis* from outbreaks in Tasmania, Ireland and USA were shown to have near identical DNA sequence for the 18S-rDNA gene (F.Wong & N. Elliott pers comm.). This finding supports previous morphological and immunological observations that the same species (*Neoparamoeba pemaquidensis*) is responsible for AGD outbreaks worldwide (Wong & Elliott pers. comm.). Thus, temperature differences during outbreaks between Washington State

and Tasmania or Ireland cannot be explained simply by inherent species-specific differences and potential alterations in optimal temperature for isolation of this pathogen from disparate geographical locations should be further examined. Furthermore, Kent *et al.* (1988) suggested that factors other than temperature such as abundance of food organism in the water may cause bloom of *Neoparamoeba pemaquidensis* and result in disease outbreak.

The epizootiological observations described here question the role of high temperatures as one of the main environmental risk factors with epizootics and reveal that clinical disease can occur at temperatures below 10°C. Although we cannot and did not attempt to conclude that low temperature is a risk factor for AGD outbreaks in this manuscript, it does point out that outbreaks occur despite low temperatures. The potential role of other stessors predisposing the fish to AGD outbreaks and environmental factors favouring the pathogen should be further investigated.

## 4.3.3 Acknowledgments

We would like to thank salmon farms for providing information, in particular Mr. M. Hortle, Tassal Ltd. We are grateful to Dr. F. Wong and Dr. N. Elliott, CSIRO Marine Research for the information on DNA sequencing of different *Neoparamoeba pemaquidensis* isolates. MH was supported by funding from Cooperative Research Centre for Aquaculture

## 4.3.4 References

Clark, A., 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

Howard, T., Carson, J. (1993) Verification that *Paramoeba* species are consistently associated with gill damage in fish affected with amoebic gill disease. In: *Proceedings of the Saltas research and development review seminar*, Tasmania 1993, Salmon Enterprises of Tasmania Pty Ltd Dover, Tasmania pp. 69-80

Kent, M.L., Sawyer, T.K., Hedrick, R.P. (1988) *Paramoeba pemaquidensis* (Saromastigophora: Paramoebidae) infestation of the gills of coho salmon, *Oncorhynchus kisutch,* reared in seawater. *Diseases of Aquatic Organisms* 5, 163-169.

Munday, B.L., Foster, C.K., Roubal, F.R., Lester, R.J.G. (1990) Paramoebic gill infection and associated pathology of Atlantic salmon, *Salmo salar*, and rainbow trout, *Salmo gairdeneri*, in Tasmania. In: *Pathology in marine Science* (ed. By F.O. Perkins & T.C. Cheng), pp 215-222. Academic Press, San Diego, Ca.

Nowak, B.F. (2001) Qualitative evaluation of risk factors for Amoebic Gill Disease in Atlantic salmon. 18pp. 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 (OIE), 148-155

Palmer, R., Carson, J., Ruttledge, M., Drinan, E., Wagner, T. (1997) Gill disease associated with *Paramoeba*, in sea-reared Atlantic salmon in Ireland. *Bulletin of the European Association of Fish Pathologists* 3-4, 112-114

Parsons, H., Nowak, B., Fisk, D., Powell, M. (2001) Effectiveness of commercial freshwater bathing as a treatment against amoebic gill disease in Atlantic salmon. *Aquaculture* 195, 205-210

Rodger, H.D., McArdle, J.F. (1996) An outbreak of amoebic gill disease in Ireland. *Veterinary record* 139, 348-349

Roubal, F.R, Lester, R.J.G., Foster, C. (1989) Studies on cultured and gill-attached *Paramoeba* sp. (Gymnamoebae: Paramoebidae) and the cytopathology of paramoebic gill disease in Atlantic salmon, *Salmo salar* L., from Tasmania. *Journal of Fish Diseases* 12, 481-493

# 4.4 WILD FISH ARE NOT A SIGNIFICANT RESERVOIR FOR *Neoparamoeba pemaquidensis* (PAGE, 1987)

Douglas-Helders, G.M., Dawson, D.R., Carson, J., Nowak, B.F.

## 4.4.1 Abstract

Amoebic gill disease (AGD), caused by the protozoan *Neoparamoeba pemaquidensis* (Page, 1987) is the most important disease affecting salmon farms in Tasmania. Reservoirs for this protozoan parasite are largely unknown. This study investigated wild fish as a potential reservoir of *N. pemaquidensis*. A total of 325 wild fish, comprising 12 different fish species, were caught from and around salmon farms and examined for the presence of AGD. None of the wild fish were infected with AGD. In a laboratory trial, seahorse, *Hippocampus abdominalis*, greenback flounder, *Rhombosolea tapirina*, and Atlantic salmon, *Salmo salar* were challenged with *N. pemaquidensis*. *Neoparamoeba pemaquidensis* was detected on the gills on 10 of 15 (66.7%) flounder, nine of 24 (37.5%) seahorses, and six of six (100%) Atlantic salmon. However, paramoebae positive flounder and seahorse lacked the characteristic AGD gill pathology. It is concluded that AGD does not appear in wild fish and wild fish do not seem to be a reservoir of the pathogen.

## 4.4.2 Introduction

Exposure to pathogens, coupled with stresses associated with captive rearing, creates opportunities for disease outbreaks in cultured fish. Infections with gill amoebae have become an important factor in cost-efficiency of marine-based Atlantic salmon, *Salmo salar* L., and rainbow trout, *Ocorhynchus mykiss* (Walbaum), farming in Tasmania (Munday, Lange, Foster, Lester & Handlinger, 1993). Amoebic gill disease (AGD), which is caused by the protozoan parasite *Neoparamoeba pemaquidensis* (Kent, Sawyer & Hedrick, 1988; Howard & Carson, 1993; Dykova, Figueras & Peric, 2000), has been an ongoing problem for

the Tasmanian industry since its first recognition in 1984 (Munday *et al.*, 1993). Mortalities may reach 2% a day, and up to 50% in total, in untreated caged fish (Munday, Foster, Roubal & Lester, 1990). Presently, the only effective treatment of AGD in Tasmania is freshwater bathing of the affected fish (Munday *et al.*, 1990, Parsons, Nowak, Fisk & Powell, 2001). As multiple freshwater baths are required through the warmer months of the grow-out season, it has become a substantial cost for the salmon industry in Tasmania.

Presence of *N. pemaquidensis* on fish gills is associated with characteristic gill pathology. The first signs of the disease are the production of excess gill mucus followed by thickening of the secondary lamellae. Hypertrophy and hyperplasia of the lamellar epithelium is followed by fusion of the lamellae and formation of crypts (Roubal, Lester & Foster, 1989, Munday *et al.*, 1990; Dykova, Figueras, Novoa, Casal, 1998; Clark & Nowak, 1999, Adams & Nowak, 2001). Initially it was considered that AGD gill lesions caused respiratory distress and anorexia (Bryant, Lester & Whittington, 1995), but this was recently disputed by findings that impaired gas transfer did not contribute to respiratory failure during hypoxia (Powell, Fisk & Nowak, 2000).

Generally, pathogens are not distributed homogeneously in the environment, but have aggregated distributions, where reproduction takes place (Bakke & Harris, 1998). Suggested reservoirs for *N. pemaquidensis* are dead AGD infected fish (Douglas-Helders, Nowak, Zilberg & Carson, 2000), the nets of sea cages (Roubal *et al.*, 1989, Clark & Nowak, 1999, Douglas-Helders, Tan, Carson & Nowak, 2002, Tan, Nowak & Hodson, 2002), biofouling on nets and sediment (Tan, *et al.*, 2002), the water column (Elliott, Wong, Carson, 2001, Tan *et al.*, 2002, Douglas-Helders *et al.*, 2002), and sediments (Cann & Page, 1982).

Fish that share the same water body, as cultured and wild fish do, are likely to share diseases. Success of transmission will depend on the host specificity of the pathogen (Hammell, 1999), susceptibility of the host, and environmental factors influencing pathogen numbers, movement and survival (Bakke & Harris, 1998) in seawater, and are often affected by biological co-factors. Horizontal transmission of the pathogen could occur through direct contact or through release of the parasite into the water column adjacent to farmed fish (Stephen & Iwama, 1997; Hammell, 1999). The chance for interaction of pathogens between wild and cultured fish is increased by the attraction of wild fish to farm sites (Sterud, Mo & Poppe, 1998). It is likely that such interactions occur between salmonids and wild fish populations in Tasmania.

For the development of a disease risk factor management system across Tasmanian salmon farms, attention should be directed to determine reservoirs, transmission and infection processes of AGD. In this study, the potential of wild fish as carrier or reservoir for *N. pemaquidensis*, the causative agent of AGD, was investigated.

## 4.4.3 Materials and Methods

### 4.4.3.1 Field trial

In total, 325 wild fish of 12 different species were caught during the field study. The fish were caught using line and reel or were dip netted from Atlantic salmon cages during crowding of the salmon for harvesting. The three most common species caught were jack mackerel, *Trachurus declivus*, (Jenys), sand flathead, *Platycephalus basensis* (Cuvier), and red cod, *Pseudophycis bachus* (Forster). The wild fish were captured from three different farm sites, and from three control sites, which were at least 10 km away from the farm sites. Those fish caught at farm sites were captured from inside and outside of nets of the sea cages. All fish were anaesthetised in 1.0% Aqui-S (Fish Transport Systems Ltd, Lower Hutt, New Zealand) and the gills dissected and transferred to seawater Davidson's fixative.

After 24 h the gills were transferred to 70% ethanol for standard histological processing. Duplicate sections of 5 μm thickness were cut; one of the duplicates was stained with haematoxylin and eosin (H&E), and examined using a Leitz Biomed light microscope at 400X final magnification. As no paramoebae were observed on any of the H&E stained gill sections, 10 samples were selected which displayed significant hyperplasia of the secondary lamellae, similar to that seen in Atlantic salmon with AGD. A combined Alcian blue (AB, pH= 2.5) and periodic acid-Schiff (PAS) stain was then used to stain the duplicate section of these samples (Bancroft & Cook, 1994). This stain makes paramoebae more visible compared with standard H&E stain, allowing fragments of paramoebae to be distinguished from debris (Zilberg & Munday, 2000).

To estimate the AGD prevalence on the farm, gill mucus samples were taken from 20 Atlantic salmon per cage. Gill mucus was taken from the second gill arch on the left hand side of each fish. A total of 21 cages were sampled, and the presence of *N. pemaquidensis* determined using indirect fluorescent antibody test (IFAT; Howard & Carson, 1993). The salmon were caught using crowd and dip netting and anaesthetized in 0.5% Aqui-S during regular gill checks. The mucous smears were air-dried at room temperature and heat fixed before processing. IFATs were conducted at the Department of Primary Industries Water and Environment, Fish Health Laboratories, Tasmania, using a primary rabbit antibody prepared to *N. pemaquidensis* strain PA027. Presence of paramoebae cells were determined at a 100X final magnification using an Olympus BX40F-3 UV epi-fluorescence microscope and FITC filter set.

4.4.3.2 Laboratory trial

Twenty-four seahorses, *Hippocampus abdominalis* Lesson, 15 greenback flounder, *Rhombosolea tapirina* Günter, and six AGD naïve Atlantic salmon were used for the laboratory trial. These fish species were not caught in the field studies because

of the capture techniques used. All fish for the laboratory trial originated from the Aquatic Key Centre, University of Tasmania, Launceston, and were known to be free from AGD infection. Fork lengths ranged from 7.9 to 11.0 cm for seahorse, 14.8 to 18.6 cm for greenback flounder and from 27.2 to 31.0 cm for salmon. Three re-circulation systems each with three 50 L tanks, a biofilter and a sump of 30 L were used. Each system contained one group of the above mentioned species, with eight seahorses in the first tank, five flounder in the second, and two Atlantic salmon in the third tank. The salmon originated from freshwater tanks and were adjusted to full-strenght 5 µm filtered seawater just prior to the infection trial. The systems were filled with 5 µm filtered seawater prior to commencement of the trial. Paramoebae for inoculation of the systems were obtained from the gills of an Atlantic salmon which originated from AGD infected stocks, which was held in an experimental tank at 13°C and at a salinity of 37‰. The donor fish was anaesthetized using 100 mg $L^{-1}$ of benzocaine and the paramoebae isolated as described by Zilberg, Gross & Munday (2001). In short: gill arches were dissected and placed into 2.5% ammonium chloride overnight. The gill mucus was then carefully removed, collected and washed twice by centrifugation at 2,600 *g* for 15 min, using 0.45 µm filtered and sterile (15 min at 121°C) natural seawater (FS). The pellet was re-suspended in 15 mL FS and the number of viable paramoebae cells determined using a haemocytometer and 0.5% trypan blue (Zilberg *et al.*, 2001). Each system was inoculated with 1,500,000 paramoebae cells, about 6500 times the minimum effective dose required to infect Atlantic salmon (Zilberg & Munday, 2000). Each trial tank was inoculated directly into the water column with approximately 500,000 viable paramoebae cells.

External conditions such as water flow and light periods were kept a constant. Temperatures in the tank systems ranged from 17.0 to 19.1°C, and salinities from 35 to 38‰. Nitrite and ammonia levels remained below detectable limits during the trial.

Any dead fish were removed from the tank and gill mucus sampled for immuno-dot blot analysis (Douglas-Helders, Carson, Howard & Nowak, 2001), followed by dissection of the gills and fixation in Seawater Davidson's fixative for histological processing. Immuno-dot blot samples were taken from the second gill arch on the left and histology samples by dissecting all gill arches from the right side of the fish. The dot blot samples were immediately frozen after sampling and processed later as described by Douglas-Helders *et al.* (2001). Gill samples for histology were processed as previously described.

The trial was terminated 7 days post-exposure, which was found to be sufficient for experimentally inducing AGD in Atlantic salmon (Zilberg & Munday, 2000). After 7 days the surviving fish were sacrificed using an overdose of 100 mg $L^{-1}$ benzocaine. The fish were bled to avoid blood contamination of the gills and samples taken as described previously. The seahorse gills proved too small for dot blot samples and only histology samples could be taken from these animals. Duplicate slides were prepared for each fish, one for non-specific H&E staining and one for pathogen specific immuno-histochemistry (Howard & Carson, 1992). Each slide was examined for paramoebae presence at 400X final magnification.

## 4.4.4 Results

### 4.4.4.1 Field survey

No *N. pemaquidensis* or AGD lesions were detected in any of the gill samples from wild fish in the field, even those caught within cages containing heavily infected salmon. The AGD infection levels of Atlantic salmon were heavy at the time of sampling, with all salmon positive for *N. pemaquidensis*.

4.4.4.2 Laboratory trial

Asymptomatic carriage was achieved in flounder and seahorse by laboratory challenge (Table 1). All Atlantic salmon were infected with AGD. Paramoebae cells were detected on the gills of nine of 24 seahorses (37.5%). Gills from 10 of 15 (66.7%) flounder were found infected with paramoebae, using immuno-dot blot and histochemistry techniques for detection. When H&E staining was used, gills of nine of 15 (60.0%) flounder were found to be positive (Table 1).

**Table 1**: Numbers of fish that were infected with paramoebae after 7 days of exposure to the pathogen, and tested with immuno-dot blot, histology (H&E) and immuno-histochemistry techniques

| | Species | | |
|---|---|---|---|
| Diagnostic test | Flounder (n=15) | Seahorse (n=24) | Salmon (n=6) |
| Immuno-dot blot | 10 | NT* | 6 |
| Histology -H&E | 9 | 9 | 6 |
| Immuno-cytochemistry | 10 | 9 | 6 |

*NT- not tested

Temperature and salinity ranges did not affect the infection process in salmon. During the experiment, three mortalities occurred, all among the Atlantic salmon. One Atlantic salmon died as early as 3 days after exposure to the gill isolate, and the other two died 4 days after exposure. Although the exposure time for these fish was very short, all mortalities were affected by AGD.

Histologically, all Atlantic salmon showed characteristic pathological signs of AGD. Signs observed were thickening of the secondary gill lamellae, hyperplasia, fusion and the formation of crypts (Figure 1). Although paramoebae were present on some of the gills of seahorse and flounder, none of the characteristic pathological signs were found in the gills (Figure 2 and Figure 3).

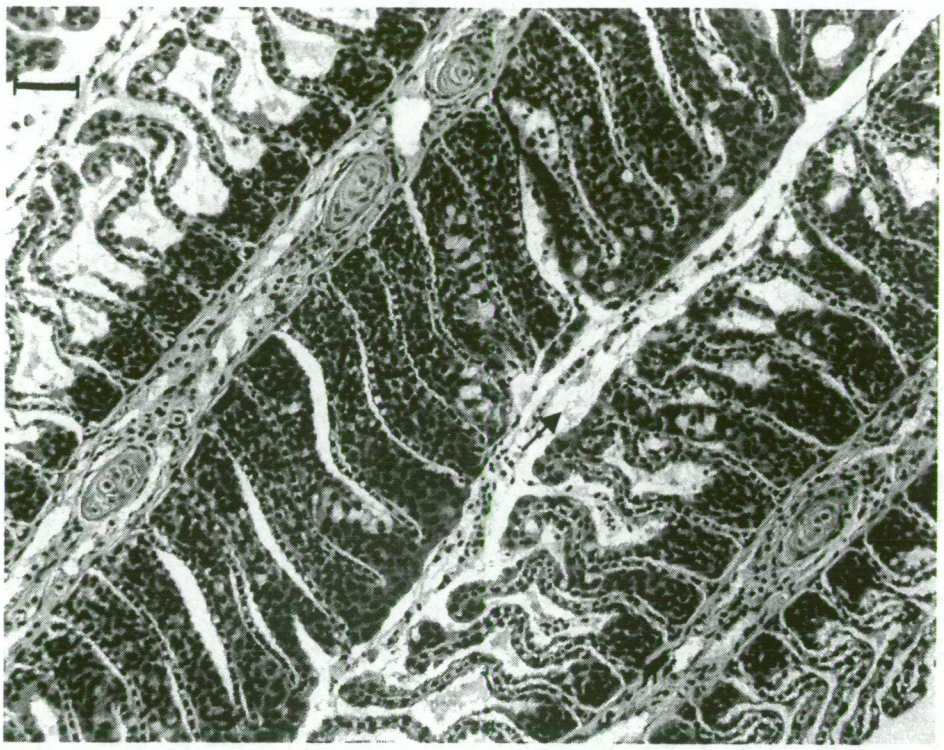

**Figure 1**: Amoebic gill disease affected gills of Atlantic salmon, showing characteristic lesions as hyperplasia and formation of crypts (H&E, bar= 40 μm)

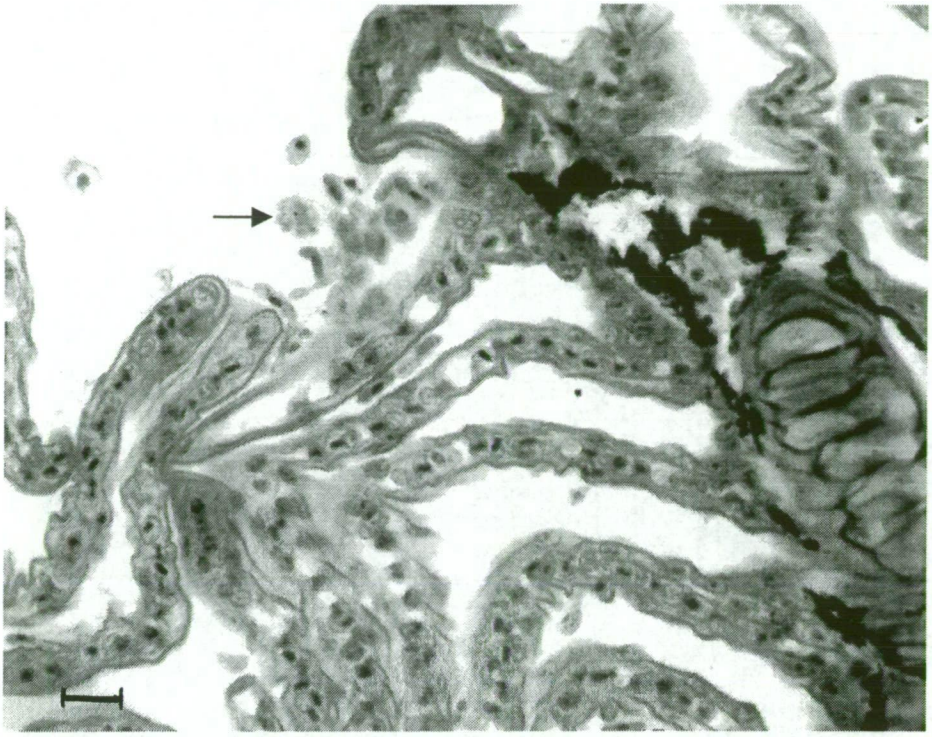

**Figure 2**: Seahorse gills with presence of *Neoparamoeba pemaquidensis*, is indicated by arrow. Characteristic gill pathology caused by amoebic gill disease is lacking (H&E, bar= 25 μm)

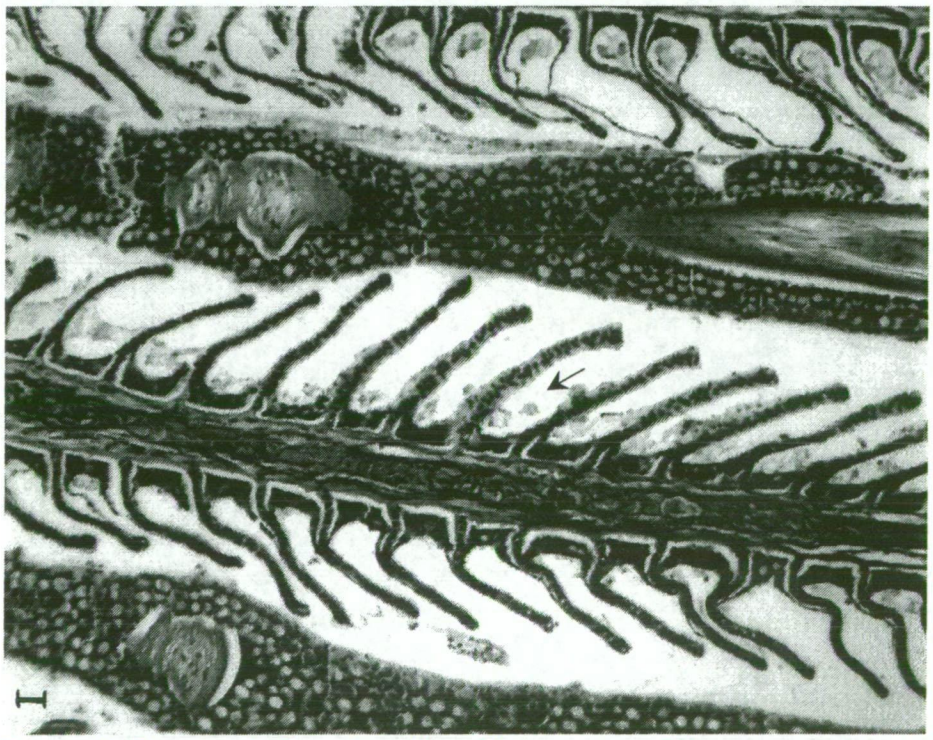

**Figure 3**: Greenback flounder gills with presence of *Neoparamoeba pemaquidensis*, is indicated by the arrow. Characteristic gill lesions caused by amoebic gill disease are lacking (H&E, bar= 40 μm)

### 4.4.5 Discussion

Paramoebae were not found on the gills of wild fish sampled from salmon farms, despite 100% infection of the farmed salmon tested at the same time. These results were further supported by the laboratory infection, during which paramoebae did not attach to the gills of seahorse or flounder in large numbers. The paramoebae did not cause AGD lesions in seahorse and flounder, despite causing AGD and resulting mortality in Atlantic salmon. This suggests that wild fish are not an important reservoir of AGD.

The diagnostic method used for wild fish was histology. This method is commonly used in AGD diagnosis (Munday *et al.*, 1990, Dykova *et al.*, 1998, Clark & Nowak, 1999, Adams & Nowak, 2001). While it is not pathogen specific, it allows the observation of host responses and the presence the pathogen. This method is used whenever it is possible to dissect whole gill arches from the fish. It could be advantageous to combine histology with more pathogen-specific methods, such as the immuno-dot blot. However, this method was only being developed at the time of the field survey. The results of our field study agree with previous results of a survey of parasites in marine fish from southeastern Tasmania (Su, 1994), in which no paramoebae were found. The absence of paramoebae in both surveys, on two different occasions, is a strong indication that wild fish do not carry paramoebae under natural conditions.

Co-factors, eg. From bacterial or viral origin, might have played a part in the infection process. However, since the Atlantic salmon used for this trial were adjusted to higher salinities only just prior to the exposure to *N. pemaquidensis*, using filtered seawater. It is likely that common marine pathogens would not have been present, and would not have played a significant role in the development of AGD in this trial. The filtered seawater used in this trial originated from the same source, and if co-factors were to be present in this water, all trial fish would have

been exposed to these same factors. We used the Atlantic salmon as a control for the disease process, and despite having identical external factors for all fish species, it was only the Atlantic salmon that showed clinical AGD. This suggests that co-factors might not have affected this infection trial, and that the presence of *N. pemaquidensis* cells on gills of the wild fish species was merely through entrapment of this protozoan due to respiration, combined with a very high *Neoparamoeba* load in the infection tanks.

The salmon that had been exposed to the protozoan for as little as 3 days in the infection trial were found to be AGD positive and gill lesions could be clearly seen. In a previous study, paramoebae were detected on gills of Atlantic salmon 1 day after exposure and characteristic gill lesions appeared between 2 and 4 days after exposure to the pathogen (Zilberg & Munday, 2000).

Wild species might be able to carry *N. pemaquidensis*, but are unlikely to be a reservoir for *N. pemaquidensis* and thus not a risk factor. Further attention should be directed towards identifying the reservoirs of *N. pemaquidensis* and factors involved in the transmission and infection process in order to manage AGD in an economically and environmentally sustainable way.

## 4.4.6 Acknowledgments

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## 4.4.7 References

Adams, M.B., Nowak, B.F. (2001) Distribution and structure of lesions in the gills of Atlantic salmon (*Salmo salar* L.) affected with amoebic gill disease. *Journal of Fish Diseases* 24, 535-542

Bakke, T.A., Harris, P.D. (1998) Diseases and parasites in wild Atlantic salmon (*Salmo salar*) populations. Canadian *Journal of Fisheries and Aquatic Science* 55(1), 247-266

Bancroft, J.D., Cook, H.C. (1994) *Manual of histological techniques and their diagnostic application.* Churchill Livingstone Inc., New York, USA pp.457

Bryant, M.S., Lester, R.J.G., Whittington, R.J. (1995) Immunogenicity of amoebic antigens in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* 18, 9-19

Cann, J.P., Page, F.C. (1982) Fine structure of small free-living *Paramoeba* (Paramoebida) and taxonomy of the genus. Journal of Marine Biology Association 62, 25-43

Clark, A., 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

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

Douglas-Helders, G.M., Carson, J., Howard, T., Nowak, B.F. (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, G.M., Tan, C.K.F., Carson, J., Nowak, B.F. (2002) Effects of copper-based antifouling treatment on the presence of *Neoparamoeba pemaquidensis* (Page, 1987) on nets and gills of reared Atlantic salmon (*Salmo salar*) gills. *Aquaculture*, Submitted

Dykova, L., Figueras, A., Novoa, B., Casal, J.F. (1998) *Paramoebae* sp., an agent of amoebic gill disease of turbot *Scophthalmus maximus*. *Diseases of Aquatic Organisms* 33, 137-141

Dykova, L., Figueras, A., Peric, Z (2000) *Neoparamoeba* Page, 1987: light and electron microscopic observations on six strains of different origin. *Diseases of Aquatic Organisms* 43, 217-223

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

Hammell, K.L. (1999) Methods of disease transmission in aquaculture, In: *Aquaculture Epidemiology*. Canadian Aquaculture Institute, International Education in Aquaculture medicine and management, Sydney April 25-26, 10pp.

Howard, T., Carson, J. (1992) Studies of Amoeba Associated with Amoebic Gill Disease in Atlantic salmon, In: *Barriers & Breakthroughs, Proceedings of the Saltas research and development review seminar, Tasmania 1992*. Salmon Enterprises of Tasmania Pty Ltd Dover, Tasmania pp. 101-122

Howard, T., Carson, J. (1993) Verification that *Paramoeba* species are consistently associated with gill damage in fish affected with amoebic gill disease. In: *Proceedings of the Saltas research and development review seminar, Tasmania 1993*. Salmon Enterprises of Tasmania Pty Ltd Dover, Tasmania pp. 69-80

Kent, M.L., Sawyer, T.K., Hedrick, R.P. (1988) *Paramoeba pemaquidensis* (Saromastigophora: Paramoebidae) infestation of the gills of coho salmon *Oncorhynchus kisutch* reared in seawater. *Diseases of Aquatic Organisms* 5, 163-169

Munday, B.L., Foster, C.K., Roubal, F.R., Lester, R.J.G. (1990) Paramoebic gill infection and associated pathology of Atlantic salmon, *Salmo salar*, and rainbow trout, *Salmo gairdeneri*, in Tasmania. In: *Pathology in marine Science* (ed. By F.O. Perkins & T.C. Cheng), Academic Press, San Diego, Ca., pp 215-222

Munday, B.L., Lange, K., Foster, C., Lester, R.J.G., and Handlinger, J. (1993) Amoebic gill disease of sea-caged salmonids in Tasmanian waters. *Tasmanian Marine Fisheries Research* 28, 14-19

Page F.C. (1987) The classification of 'naked' amoebae of phylum Rhizopoda. *Archive Protistenkunde* 133, 199-217

Parsons, H., Nowak, B., Fisk, D., Powell, M. (2001) Effectiveness of commercial freshwater bathing as a treatment against amoebic gill disease in Atlantic salmon. *Aquaculture* 195, 205-210

Powell, M.D., Fisk, D., Nowak, B.F. (2000) Effects of graded hypoxia on Atlantic salmon (*Salmo salar* L.) infected with amoebic gill disease (AGD). *Journal of Fish Biology* 57, 1047-1057

Roubal, F.R, Lester, R.J.G., Foster, C. (1989) Studies on cultured and gill-attached *Paramoeba* sp. (Gymnamoebae: Paramoebidae) and the cytopathology of paramoebic gill disease in Atlantic salmon, *Salmo salar* L., from Tasmania. *Journal of Fish Diseases* 12, 481-493

Stephen, C., Iwama, G. (1997) Fish Health Discussion paper. In: *Salmon Aquaculture Review*, 121pp. B.C. Environmental Assessment Office. Victoria, Province of British Colombia

Sterud, E., Mo, T.A., Poppe, T. (1998) Systemic spironucleosis in sea-farmed Atlantic salmon *Salmo salar*, caused by *Spironucleus barkhanus* transmitted from feral Arctic char *Salvelinus alpinus*? *Diseases of Aquatic Organisms* 33, 63-66

Su, X. (1994) Taxonomic and biological studies of protozoan parasites in Tasmanian marine fishes. *PhD Thesis*. University of Tasmania, Hobart, pp.1-237

Tan, C.K.F., Nowak, B.F., Hodson, S.L. (2002) Biofouling as a reservoir of *Neoparamoeba pemaquidensis* (Page 1970), the causative agent of AGD in Atlantic salmon. *Aquaculture*, In press

Zilberg, D., Munday, B.L. (2000) Pathology of experimental amoebic gill disease in Atlantic salmon, *Salmo salar* L., and the effect of pre-maintenance of fish in sea water on the infection. *Journal of Fish Diseases* 23, 401-407

Zilberg, D., Gross, A., Munday, B.L. (2001) Production of salmonid amoebic gill disease by exposure to *Paramoeba* sp. harvested from the gills of infected fish. *Journal of Fish Diseases* 24, 79-82

# CHAPTER 5

# HUSBANDRY AND AGD

## 5.1 EFFECTS OF HUSBANDRY ON PREVALENCE OF AMOEBIC GILL DISEASE AND PERFORMANCE OF REARED ATLANTIC SALMON (*Salmo salar* L.)

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### 5.1.1 Abstract

There is a need for improved husbandry methods to minimise the impact of amoebic gill disease (AGD) on Tasmanian salmon farms. This report describes three husbandry methods that aim to reduce AGD prevalence and/or minimise losses associated with AGD. All trials evaluated the effect of treatment on AGD prevalence, weight gain, and the percentage of mortalities. Water was sampled from trial cages to estimate paramoebae densities in the water column, and was related to AGD prevalence. In the first trial, cages were rotated between different sites and data compared to stationary cages that remained on a reference site. The second trial studied the effect of prophylactic bathing, while the third trial considered the effects of size of sea cages. While no significant reduction of AGD prevalence was achieved due to treatment, a reduced freshwater bathing frequency was found in the rotated cages and the un-bathed

cages, and fish grew significantly faster in these cages (P=0.038 and P=0.048 respectively). Mortalities were not affected by the treatments. An association between AGD prevalence in the cage and paramoebae densities in the water column was found. The results of these trials suggest that losses due to AGD in farmed Atlantic salmon (*Salmo salar* L.) can be managed by adjustment of husbandry methods.

## 5.1.2 Introduction

Disease is a major risk factor in commercial aquaculture, with millions of dollars lost annually (Shariff, 1998). Survival of pathogens depends on, among others, host susceptibility, and environmental factors influencing reproduction, growth and spread of the pathogen (Bakke & Harris, 1998). Husbandry is an important factor in reducing the chance of survival and spread of pathogens and hence reducing the incidence of diseases (Menzies, Crockford, McLoughlan, Wheatley, and Goodall, 1998). Salmon farms employ a range of management practices such as reducing stocking densities, frequent freshwater bathing for AGD treatment (Parsons, Nowak, Fisk, and Powell, 2001), the movement of boats and divers were restricted between farms in Norway to prevent the spread of infectious salmon anaemia (Jarp & Karlsen, 1997), and, the food additives such as glucans and vitamin C are commonly used (Verlhac, Obach, Gabaudan, Schuep, and Hole, 1998). Vaccines, antibacterial agents, and/or chemical agents are also used regularly to prevent and/or treat diseases (Alderman & Hastings, 1998), and other fish species such as wrasse (Labriadae), have been used to clean up external parasites such as sea lice (Deady, Varian, and Fives, 1995).

Amoebic gill disease (AGD) is the main disease affecting the salmon industry in Tasmania. AGD not only results in high treatment costs, but can also cause significant fish mortalities (Munday, Foster, Roubal, and Lester, 1990, Parsons *et al.*, 2001), and retard growth in infected fish (Rodger & McArdle, 1996, Dyková, Figueras, Novoa, and Casal, 1998). Freshwater bathing is the main treatment method used for AGD infections in Tasmania. In a bath treatment fish are subjected to oxygenated fresh water in a liner for two to four hours after which they are released back into the sea water (Parsons *et al.*, 2001). The effect of prophylactic bathing, or the timing of the first freshwater bath after transfer to seawater, is unknown (Nowak, 2001). Major disadvantages associated with the treatment include; the need for additional labour and bottlenecks in farm operations, the need to handle the fish causing stress, and the requirement for large volumes of fresh water (Howard & Carson, 1991). These are all factors that add to the total cost for managing AGD (Parsons *et al.*, 2001).

Though fresh water was capable of causing a 100% mortality of cultured *N. pemaquidensis* in vitro (Howard & Carson, 1993) and reduced AGD prevalence for up to 21 days post freshwater bath in a commercial situation (Clark & Nowak, 1999), a total removal of the parasite has yet not been achieved (Parsons *et al.*, 2001). The paramoebae that survived the freshwater bath could be a source of reinfection once fish and fresh water from the bath are released into the sea (Findlay & Munday, 1998). *Neoparamoeba pemaquidensis* was detected in the sea water column on several occasions (Elliott, Wong, and Carson, 2001, Tan, Nowak, and Hodson, 2002, Douglas-

Helders, Tan, Carson, and Nowak, 2002), but at this stage it remains unclear if seawater is a reservoir for this protozoan.

Since initial AGD outbreaks an increased frequency of freshwater bathing has been observed during the summer months, and the need of freshwater bathing was extended to other seasons (Parsons *et al.*, 2001). Alternative methods to minimise AGD prevalence and its related costs to salmon farms are a critical requirement for the industry in Tasmania. In this paper three husbandry methods are described which aim to reduce AGD prevalence and/or minimise the losses associated with AGD infections in salmonids.

## 5.1.3 Materials and methods

All trials were conducted at one salmon farm in southeast Tasmania. The trials tested the effects of three different husbandry options on AGD prevalence and fish performance. The first trial (rotation trial) studied the effects of moving cages of fish to recently fallowed sites A, B, and C immediately post bath. Data for these rotated cages were compared with cages that remained stationary on one lease site for the duration of the trial. The average period of fallowing of the three sites A, B, and C and the average time fish of the rotated cages spent on these sites are shown in Figure 1. Average fallowing times were 97 days, 58 days, and 4 days for site A, B and C respectively, while fish spent most time on site C (Figure 1). In order to determine if any treatment effect was due to towing of cages to different lease sites, the direct effect of towing on AGD prevalence was tested. Twenty fish from 5 towed cages were

sampled before and after a short tow. The towing speed was on average 2.8 km $h^{-1}$ for all towed cages, and the towing time never exceeded 5 h. To assess the effect of time between the two samples for each towed cage, 5 stationary control cages were sampled at the same time as the towed cages, with the same interval time between the 2 samples.

The second trial- the pre-clinical bathing trial studied the effects of freshwater bath treatment after introduction to seawater, but before any gross signs of AGD infection on the gills appeared. The third trial - the cage size trial, studied the effects of cage size when stocked with a similar biomass. Two cages with a circumference of 60 metres (60m) and three of 80 metres circumference (80m) were used for the trial. Environmental measurements of salinity, temperature and dissolved oxygen on the lease sites were taken during the trials.

5.1.3.1 Fish

Out-of-season Atlantic salmon (*Salmo salar* L.) smolt with average weight of 68.2 g (SE 2.5, rotation and cage size trials) and 87.2 g. (SE 6.1, bath trial) were introduced to a salmon farm in the Huon Estuary, Tasmania, Australia. Times of introduction, number of cages per treatment, sampling period, biomass and stocking densities per cage at the start of the trials are shown in table 1. 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.

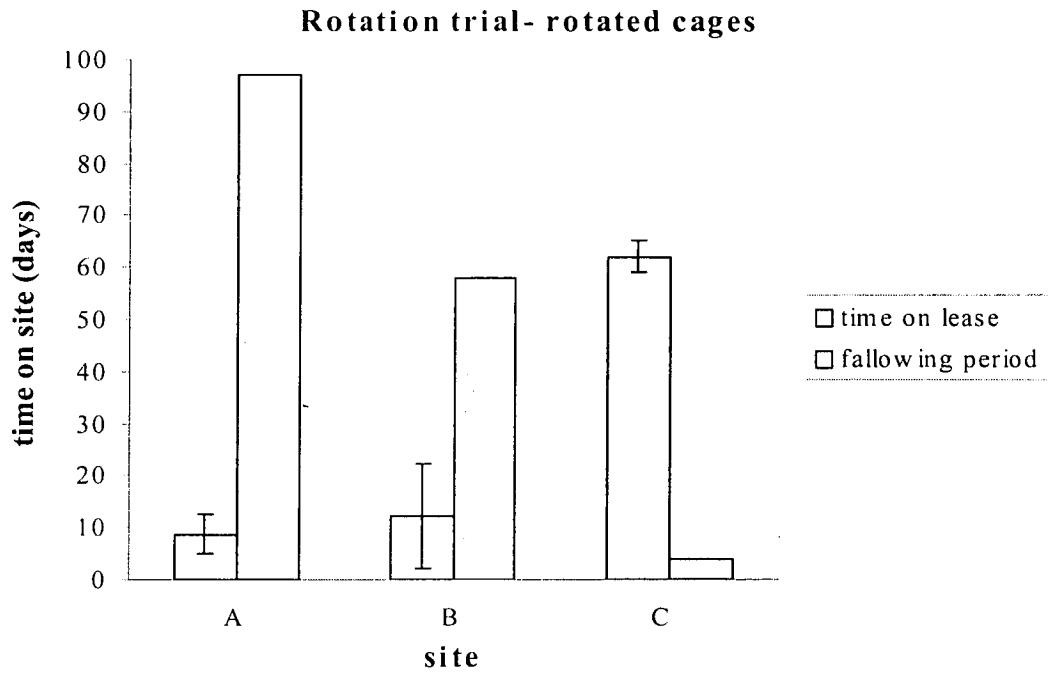


**Figure 1**: Number of days that sites A, B, and C were fallowed before fish stocking in December, January and February respectively, and the average number of days fish of the rotated cages remained on these sites

**Table 1**: Time of introductions of fish into seawater, stocking densities (± SE) and biomass (± SE) at the start of the trials, and sampling and towing (± SE) durations for each treatment groups for the three trials

| Trial | Time of introduction | Sampling period | Treatment groups | Number of trial cages | Stocking density (kg $m^{-3}$) | Biomass (kg) | Towing time (h) |
|---|---|---|---|---|---|---|---|
| Rotation | April/ May | Dec. '00-March '01 | Stationary | 4 | 3.67 (0.55) | 15026 (2195) | 1.49 (0.22) |
| | | | Rotation | 4 | 4.20 (0.87) | 17115 (3530) | 4.48 (1.71) |
| Bath | February | Oct. '00-March '01 | Bath | 3 60m cages | 4.90 (0.82) | 11663 (3255) | 3.8 (0.53) |
| | | | No bath | 3 80m cages | 5.32 (0.65) | 20929 (2409) | 4.4 (0.73) |
| Cage size | April | Aug., Oct., Nov. '00 | 60 m cages | 2 | 1.74 (0.11) | 2337 (123) | 6.5 (1.81) |
| | | | 80 m cages | 3 | 0.69 (0.03) | 2806 (119) | 6.2 (1.92) |

Signs of clinical disease were assessed monthly by examining the gills of at least 20 fish for the presence of pathognomonic mucous patches (Munday, Lange, Foster, Lester, and Handlinger, 1993). These signs range from a slight discolouration of a part of a gill filament to white mucoid patches on one or more filaments of the gill arches, depending on the severity of the AGD infection. 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. The score determined if a cage was lightly infected, moderately infected, or heavily infected (A. Steenholdt, pers. com).

This scoring system was consistently used during the trial, and determined the need of freshwater bath treatment for all cages. If a cage was heavily infected, freshwater 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. For freshwater bath treatment, cages required towing to the bathing site. Average towing durations were similar for both treatment groups of the bath and cage size trials, but were of a longer duration for the rotated cages of the rotation trial (Table 1).

5.1.3.2 Sampling

In order to determine AGD prevalence for each cage, monthly samples of gill mucus from twenty fish per cage were sampled, as described in Douglas-Helders, Carson, Howard, and Nowak (2001). The fish were anaesthetised in 5ppth clove oil and gill mucus samples taken from AGD infected site or from the second gill arch of the left-hand side of the fish. Dot blot samples were processed and analysed as previously described (Douglas-Helders *et al.*, 2001) and AGD prevalence per cage determined as percentage of fish that tested immuno-dot blot positive.

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

average weight for the sea cage estimated by dividing of the total weight by the assumed number of fish sampled and multiplying the figure by the approximate total number of fish in the cage.

When sampling fish gill mucus to determine AGD prevalence, a 50 mL water sample was taken from each of the three rotation and stationary trial cages (rotation trial) and from two bathed and un-bathed trial cages (bath trial). Water was sampled from the surface, inside the sea cage and close to the net. Water samples were kept at 4 °C until laboratory processing. The water samples were reduced to 800 µL by centrifugation at 2,900 *g* for 8 min, and treated with 40 µL of 0.21% v/v sodium hypochlorite and 0.045% v/v sodium hydroxide. After vortexing and eight minutes incubation the samples were further treated by adding 10 µL of 2 N hydrochloric acid, and incubated for 30 min. Finally, the samples were frozen at –20°C, defrosted just prior to analysis, and centrifuged for 20 s at 15,600 *g*. A volume of 200 µL of the supernatant was used for testing by means of the dot blot technique described in Douglas-Helders *et al.* (2001). A positive sample was given the value one while negative samples were given the value zero, and a percentage of paramoebae positive water samples were calculated for each treatment group. Presently, no studies on the relationship between paramoebae densities and occurrence of AGD prevalence have been reported, and though the low replication used for water sampling in the trial, and it was decided to present the exploratory results.

5.1.3.3 Statistical analysis

AGD prevalence, weight gain data and mortality data of each trial from the first and the last sampling points were analysed for treatment differences, using a two-tailed Student's t-test. Any significant difference due to treatment was determined by comparing data of the two treatments within each trial of the final sampling. 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 was analysed as the cumulative weight of each cage from which the weight of the cage at the start of the trial was subtracted. Results of all statistical analysis were considered significant when $P \leq 0.05$.

## 5.1.4 Results

The averages of AGD prevalence, average number of days between baths, and the average numbers of freshwater baths that were required for each trial are shown in Table 2. Averages of the final cage weight (adjusted for initial cage weight) and average percentages of cumulative mortalities for each trial are shown in Table 3.

**Table 2**: Average AGD prevalence (in percentages) at the start and finish of the three trials (± SE), average number of freshwater baths (± SE) required for AGD treatment during the trials, and average number of days (± SE) between freshwater baths for each treatment group

| Trial name | Treatment groups | Initial AGD prevalence | Final AGD prevalence | Number of freshwater baths | Number of days between freshwater baths in days |
|---|---|---|---|---|---|
| Site | Stationary | 51.3 (9.7) | 66.3 (3.8) | 4.8 | 28.6 (2.6) |
| Rotation | Rotation | 43.5 (4.9) | 45.0 (15.1) | 3.8 | 55.9 (26.7) |
| Bath | Bath | 20.7 (11.1) | 36.7 (6.0) | 5.3 | 53.9 (11.8) |
| | No bath | 23.3 (12.0) | 33.3 (6.7) | 4.3 | 66.9 (16.9) |
| Cage size | 60m | 40.0 (0.002) | 47.5 (17.5) | 2.0 | 46.8 (16.2) |
| | 80m | 21.7 (3.3) | 25.0 (10.4) | 2.0 | 43.8 (13.3) |

**Table 3**: Average (final-initial) weight gained (kg) and the average cumulative percentage of mortalities (%) that occurred during the trials

| Trial name | Treatment groups | Average weight per cage | Average cumulative mortalities per cage |
|---|---|---|---|
| Site rotation | Stationary | 13569.3 (1113.5) | 4.16 (0.90) |
| | Rotation | 19568.3 (1972.7) | 2.99 (1.26) |
| Bath | Bath | 18388.3 (1538.5) | 0.94 (0.16) |
| | No bath | 24996.0 (623.0) | 0.60 (0.07) |
| Cage size | 60m | 19731.5 (2536.5) | 0.77 (0.01) |
| | 80m | 24355.0 (3039.3) | 1.75 (0.68) |

5.1.4.1 Rotation trial

The effect of rotation was most visible in February and March, with AGD prevalence levels of the rotated cages below these of the stationary cages at all times. Maximum AGD prevalence levels occurred in January for both treatment groups (Figure 2A). In March the AGD prevalence of the rotated cages did not significantly differ from the stationary cages (P=0.288, Figure 2A). However, in the rotated cages the average number of days between freshwater baths was higher (P=0.076), and the average number of freshwater baths that were required during the trial was lower (P=0.190) compared to the stationary cages (see Table 2). Also, fish of the rotated cages showed a steeper growth curve, mostly from week eight onwards (Figure 2B), and fish in these cages were significantly bigger at the completion of the trial compared to the stationary cages (P=0.038, Table 3). The cumulative mortality percentage at the completion of the trial of the rotated cages was not affected by the treatment, and was similar to these of the stationary cages (P=0.467, Table 3). Towing of cages did not directly affect the AGD prevalence, and no difference in prevalence levels could be detected between towed and non-towed cages of the short towing trial (P=0.111). The average AGD prevalence at commencement and completion of the 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.

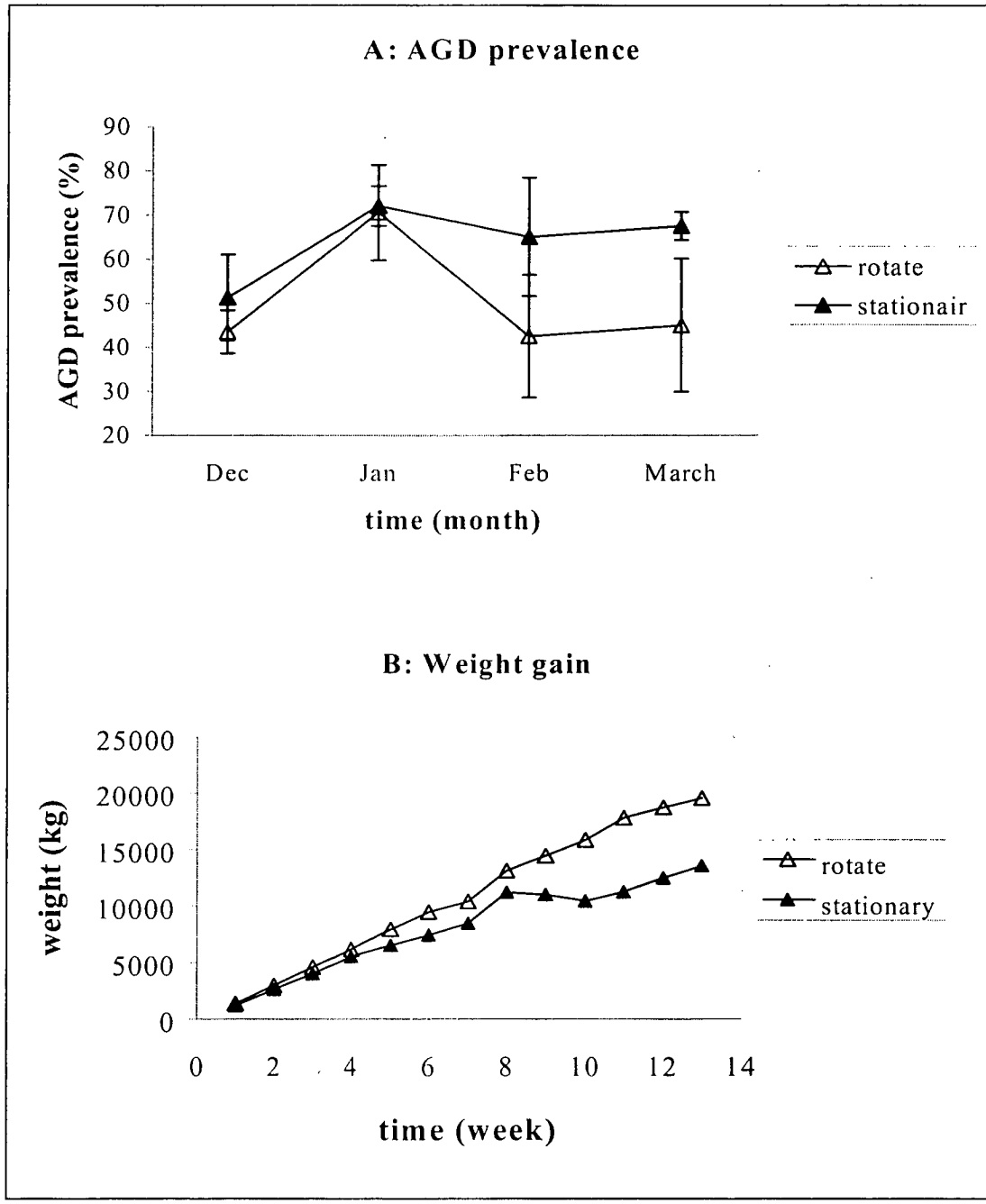


**Figure 2**: Average AGD prevalence (A) and cumulative weight gain (B) of the rotation trial cages over time

5.1.4.2 Bath trial

AGD prevalence levels increased to a maximum in December for the un-bathed cages and continued to rise until January for the bathed cages (Figure 3A). The difference in AGD prevalence levels between the two treatments was most visible between December and February, with higher levels in the bathed cages compared to the un-bathed cages (Figure 3A). At completion of the trial, AGD prevalence levels of the bathed cages did not differ significantly from these of the un-bathed cages (P=0.897). In this trial, the un-bathed cages showed an increased average number of days between baths (P=0.523) and reduced freshwater bathing frequency (P=0.101) compared to the bathed cages (Table 2). At the completion of the trial the fish in the un-bathed cages were significantly bigger compared to the fish in the bathed cages (P=0.048, Table 3). The difference between growth rates of the two treatments was greatest from early December to the end of February, with a higher growth rate of fish in the un-bathed cages (Figure 3B). Pre-clinical bathing did not affect the mortality rate, and the cumulative mortality percentage at the completion of the trial did not significantly differ between the two treatment groups (P=0.215, Table 3).

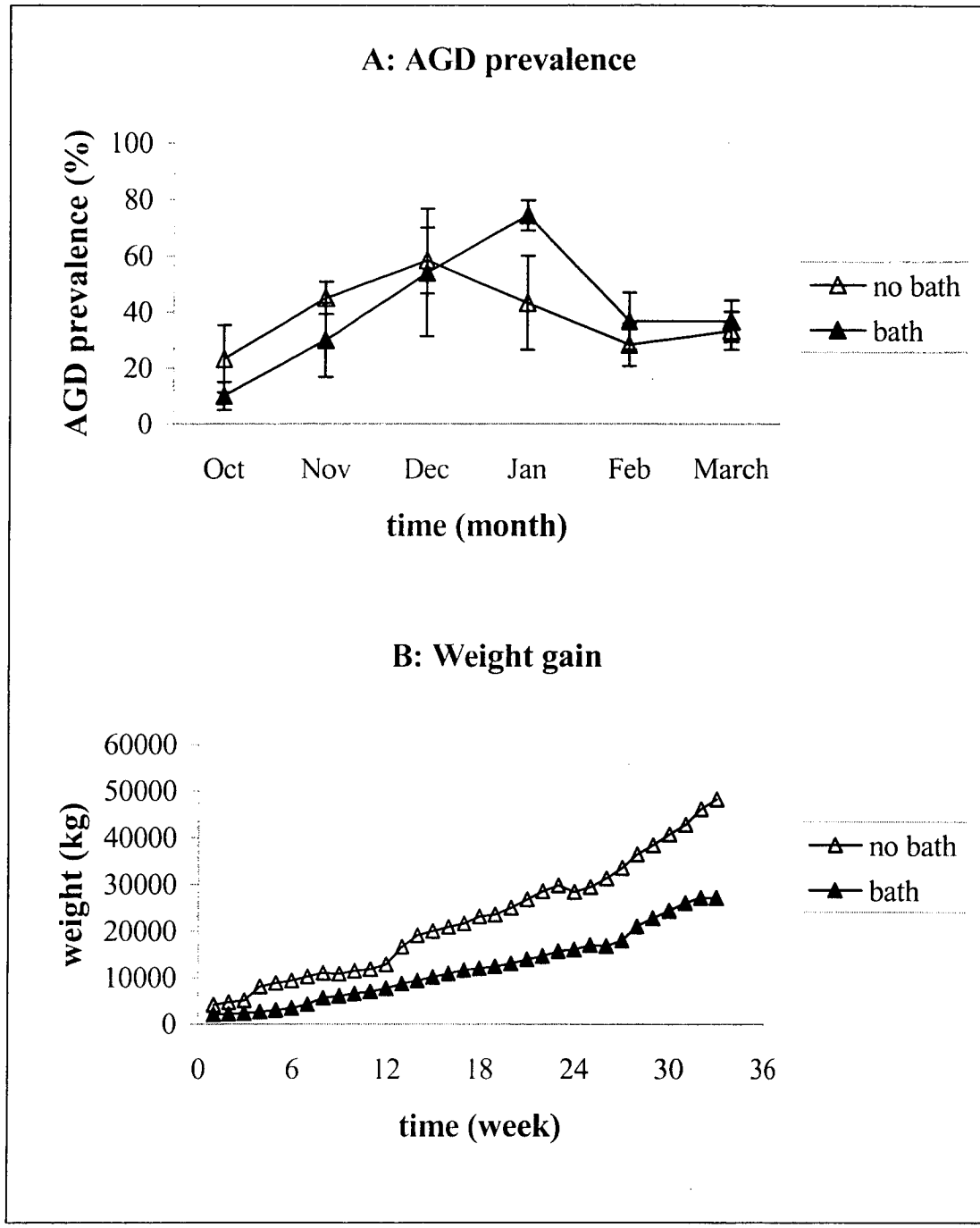


**Figure 3**: Average AGD prevalence (A) and cumulative weight gain (B) of the bath trial cages over time

5.1.4.3 Cage size trial

At the commencement of this trial a significantly higher AGD prevalence was found in the 60m cages (P=0.032). The minimum AGD prevalence level was found in October for both cage sizes. In November, AGD prevalence levels had increased, with a higher (but not significant) level in the 60m cages compared with the 80m cages (P=0.316, Figure 4). The average number of days between freshwater baths (P=0.849) and the freshwater bathing frequency (P=1.000) were similar for both cage sizes (Table 2). No significant differences in weight gain were detected due to the size of the cages (P=0.366), nor in the cumulative percentage of mortalities at the completion of the trial (P=0.286).

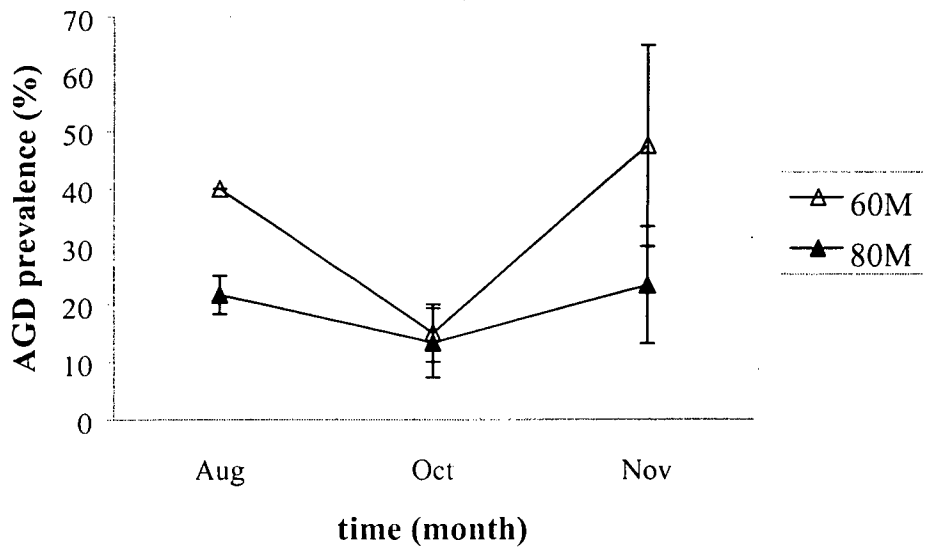


**Figure 4**: Average AGD prevalence of the cage size trial cages over time

The stationary cages showed a maximum AGD prevalence level in January, which coincided with a maximum paramoebae density at that time (Figure 5A). In the rotated cages, the maximum AGD prevalence level was found in January, while paramoebae densities in these cages were at its maximum in March (Figure 5A). The maximum density of paramoebae in water from the rotated cages corresponded with the on average longest dwell and shortest fallowing period of site C (Figure 1). Paramoebae positive water samples from the bath trial cages were only found in January and March, with paramoebae prevalence levels of the un-bathed cages below those of the bathed cages (Figure 5B). A similar patter was seen with the AGD prevalence of these cages, with a lower AGD prevalence in the un-bathed cages compared to the bathed cages in January and March (Figure 5B). With the exclusion of the rotated cages, the data suggested an association between AGD prevalence in cages and paramoebae densities in the water column. Future, more extensive, investigations are needed to confirm these findings.

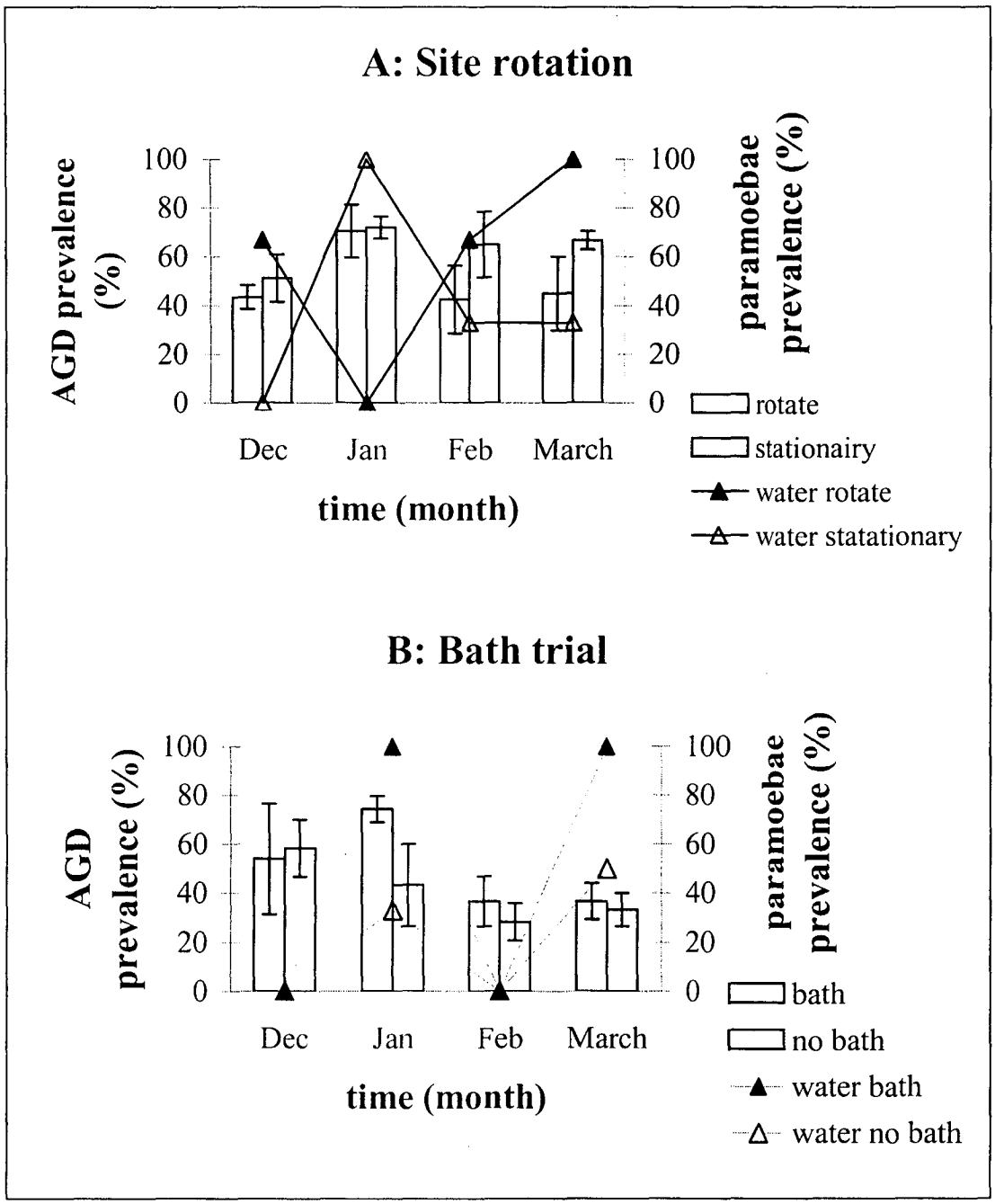


**Figure 5**: Average AGD prevalence (in bars, ± SE) and average percentage of paramoebae positive water samples (in lines) over time for the rotation trial cages (A) and the bath trial cages (B)

## 5.1.5 Discussion

The AGD prevalence at commencement of the cage size trial was significantly higher in the 60m cages compared to the 80m cages, which may be due to the fact that the 60m cages were stocked at a higher density. However, no significant difference was found at the completion of this trial, implying that the effect of cage size and/or stocking density did not affect the fish long-term. Although the treatments, which were used in the three trials, did not seem to significantly affect AGD prevalence, a reduction in the need for freshwater bathing was observed in the rotation and bath trials. The rotated cages and non pre-clinical bathed cages required fewer freshwater baths compared to the stationary and the pre-clinical bathed cages. Freshwater bathing is a costly procedure for the salmon grower (Parsons *et al.*, 2001), and a reduction in the number of freshwater bath treatments would greatly reduce the overall cost of managing AGD.

Several factors could have influenced the results of reducing the frequency of freshwater bathing in the site rotation trial. *Neoparamoeba* abundance on fallowed sites was likely to be less due to the 'flush-effect' of tides and currents, and the lack of nets which are a known reservoir of paramoebae (Tan *et al.*, 2002), and a suggested factor for AGD infection (Tan *et al.*, 2002, Douglas-Helders *et al.*, 2002). Fallowing times in the rotation trial, especially these of site C, were on average short. There is the possibility that a significantly different AGD prevalence would have been found with longer fallowing durations. The tows of the rotation cages were on average of a similar frequency, but of a longer average duration. This was not likely to influence the AGD

prevalence since no effect of towing on AGD prevalence was found in the towing trial, suggesting that the results were mostly due to regular placement onto fallowed sites.

The increased need of freshwater bathing in the pre-clinical bathed treatment group of the bath trial may have been due to the removal of mucus during freshwater bathing, and a possible change in mucus composition. During freshwater bathing excess mucus is released from the gill filaments (Parsons *et al.*, 2001), and a significant increase in the total number of mucous cells on gills was found as a result of freshwater bathing (Powell, Parsons, and Nowak, 2001). Though the total number of mucous cells on gills increased after freshwater bathing, the same authors suggested that the composition of gill mucus was likely to have changed (Powell *et al.*, 2001). Gill mucus forms a barrier against pathogens and/or chemicals in the environment (Laurent & Perry, 1991), and contains factors that strengthen this protective barrier (Alexander & Ingram, 1992, Lumsden, Ostland, Byrne, and Ferguson, 1993, Firth, Ross, Burka, and Johnson, 1998). Removal of gill mucus would pre-dispose fish to subsequent AGD infection and a change in gill mucus composition might weaken the protectiveness of the mucus barrier. The preventative freshwater bath of the bathed cages (bath trial), which occurred not long after introduction from fresh water to seawater, was likely to be a pre-disposing factor for AGD infections. Pre-disposing factors for AGD such as structural gill changes due to seawater acclimation, poor gill health, and cage hygiene, have been suggested (Nowak & Munday, 1994, Dyková *et al.*, 1998, Munday *et al.*, 1993), although these factors are not necessarily the direct cause of AGD (Nowak,

2001, Zilberg, Gross, and Munday, 2001). The significance of pre-disposing factors in AGD is a topic that needs further investigation.

Fish in the rotated cages and the non pre-clinical bathed cages were significantly larger at the conclusion of these trials, the significant weight gain being observed in those cages with a lower AGD prevalence. These cages needed to be handled less due to the lower freshwater bathing frequency, causing less stress to the fish. A decrease in feeding and/or growth rates due to AGD infections has been widely reported (Rodger & McArdle, 1996, Dyková *et al.*, 1998).

The primary polyclonal antibody used to test water samples for the presence of *N. pemaquidensis* in the two trials is known to cross-react with other near related paramoebae species (Douglas-Helders *et al.*, 2001). However, a good agreement was found between environmental biofouling samples from nets tested with PCR and indirect fluorescent antibody testing (IFAT) in previous studies (Elliott, Wong, and Carson, 2001, Douglas-Helders *et al.*, 2002). The dot blot test used the same primary rabbit-anti-*N. pemaquidensis* antibody against strain DPIWE P027 (Douglas-Helders *et al.*, 2001) as used in the previously mentioned IFAT tests for environmental biofouling samples. Possibly paramoebae densities were overestimated due to its cross-reactive nature, but the results of the trials suggested that the primary antibody used in the dot blot test was useful as an estimate of *N. pemaquidensis*.

The AGD causing protozoan has been isolated from the water column in the past (Tan *et al.*, 2002), but no attempt has been made to relate these findings to AGD prevalence in reared fish. The main method that was used to infect fish with AGD in the laboratory was by co-habitation (e.g. Findlay & Munday, 1998). This implies that the parasite was dislodged from the infected host into the water column, which carried the parasite to other hosts. It was therefore not surprising to detect paramoebae in the water column of the rotation and bath trial cages. Infection was also successfully established by using paramoebae harvested from the gills of AGD infected fish and releasing the paramoebae into the water column (Zilberg *et al.*, 2001). In this controlled method of infection, it was found that the severity of the AGD infection was proportional to the number of paramoebae harvested from the gills (Zilberg *et al.*, 2001). This was also seen in the rotation and bath trials, with increasing paramoebae densities coinciding with an increased AGD prevalence over time (stationary cages), and when AGD prevalence and paramoebae densities of the bathed and un-bathed trial cages were compared in January and March. In view of these results it is likely that the water column is a key factor for infection of fish with AGD, either as a vector or reservoir.

The results of the trials suggest that the cost of AGD management for reared Atlantic salmon can be ameliorated by adjustment of husbandry methods. Fewer freshwater baths were required and fish grew faster when cages were rotated to fallowed sites or when prophylactic bathing did not occur.

## 5.1.6 Acknowledgments

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## 5.1.7 References

Adams, M.B., Nowak, B.F. (2001) Distribution and structure of lesions in the gills of Atlantic salmon (*Salmo salar* L.) affected with amoebic gill disease. *Journal of Fish Diseases* 24, 535-542

Alderman, D.J., Hastings, T.S. (1998) Antibiotic use in aquaculture: development of antibiotic resistance- potential for consumer health risks. *International Journal of Food Science and Technology* 33, 139-155

Alexander, J.B., Ingram, G.A. (1992) Noncellular nonspecific defence mechanisms of fish. *Annual Review of Fish Diseases*, 249-279

Bakke, T.A., Harris, P.D. (1998) Diseases and parasites in wild Atlantic salmon (*Salmo salar*) populations. *Canadian Journal of Fisheries and Aquatic Science* 55(1), 247-266

Clark, A., 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

Deady, S., Varian, S.J.A., Fives, J.M. (1995) The use of cleaner-fish to control sea lice on two Irish salmon (*Salmo salar*) farms with particular reference to wrasse behaviour in salmon cages. *Aquaculture* 131, 73-90

Douglas-Helders, G.M., Carson, J., Howard, T., Nowak, B.F. (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, G.M., Tan, C.K.F., Carson, J., Nowak, B.F. (2002) 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*, Submitted

Dyková, I., Figueras, A., Novoa, B., Casal, J.F. (1998) *Paramoeba* sp., an agent of AGD of turbot, *Scophthalmus maximus* L. *Diseases of Aquatic Organisms* 33, 137-141

Elliott, N., Wong, F., Carson, J., 2001. Detection and abundance of *Paramoeba* species in the environment. *FRDC project 98/209*. Hobart, 68 pp.

Findlay, V.L., Munday, B.L. (1998) Further studies on acquired resistance to AGD in Atlantic salmon. *Journal of Fish Diseases* 21, 121-125

Firth, K.J., Ross, N.W., Burka, J.F., Johnson, S.C. (1998) Changes in protease activity in the skin of Atlantic salmon (*Salmo salar*) during sea lice *(Lepeoptheirus salmonis*) infestation. *Bulletin of the Aquaculture Association Canada* 98-2, 93-96

Howard, T., Carson, J. (1991) AGD- in vitro studies of *Paramoeba* species. In: *Proceedings of the Saltas Research Review Seminar, Tasmania. 1991*. Salmon Enterprises of Tasmania, Dover, Tasmania, pp.1-24

Howard, T., Carson, J. (1993) Verification that *Paramoeba* species are consistently associated with gill damage in fish affected with amoebic gill disease. In*: Seeking and Solving, Proceedings of the Saltas Research and Development Review seminar*, Tasmania, Salmon Enterprises of Tasmania, Dover, Tasmania, 69-80

Jarp, J. and Karlsen, E. (1997) Infectious salmon anaemia (ISA) risk factors in sea-cultured Atlantic salmon *Salmo salar*. *Diseases of Aquatic Organisms* 28, 79-86

Laurent, P., Perry, S.F. (1991) Environmental effects on fish gill morphology. *Physiological Zoology* 64(1), 4-25

Lumsden, J.S., Ostland V.E., Byrne, P.J., Ferguson, H.W. (1993) Detection of a distinct gill-surface antibody response following horizontal infection and bath challenge of brook trout *salvelinus-fontinalis* with *flavobacterium-branchiophilum*, the causative agent of bacterial gill disease. *Disease of Aquatic Organisms* 16(1), 21-27

Menzies, F.D., Crockford, T., McLoughlan, M.F., Wheatley, S.B., Goodall, E.A. (1998) An epidemiological approach to investigating disease and production losses in farmed Atlantic salmon. In: *Methodology in Fish Diseases Research* (ed. by A. Barnes, G. Davidson, M. Hiney, D. McIntosh), pp. 289-294. Fisheries Research Services, Aberdeen, Scotland

Munday, B.L., Foster, C.K., Roubal, F.R., Lester, R.J.G. (1990) Paramoebic gill infection and associated pathology of Atlantic salmon, *Salmo salar*, and rainbow trout, *Salmo gairdeneri*, in Tasmania. In: *Pathology in Marine Science* (ed. By F.O. Perkins & T.C. Cheng), pp 215-222. Academic Press, San Diego, Ca.

Munday, B.L., Lange, K., Foster, C., Lester, R.J.G., and Handlinger, J. (1993) Amoebic gill disease of sea-caged salmonids in Tasmanian waters. *Tasmanian Marine Fisheries Research* 28, 14-19

Nowak, B.F., Munday, B.L. (1994) Histology of gills of Atlantic salmon during the first few months following transfer to sea water. *Bulletin of European Association of Fish Pathologists* 14, 77-81

Nowak, B.F. (2001) Qualitative evaluation of risk factors for Amoebic Gill Disease in Atlantic salmon. 18pp. In: *Proceedings of the OIE International Conference on Risk Analysis in Aquatic Animal Health.* (Ed. Rodgers, C.J.), pp 148-155, Paris, France, 8-10 February 2000. Office International des Epizooties (OIE)

Parsons, H., Nowak, B., Fisk, D., Powell, M. (2001) Effectiveness of commercial freshwater bathing as a treatment against amoebic gill disease in Atlantic salmon. *Aquaculture* 195, 205-210

Powell, M.D., Parsons, H.J., Nowak, B.F. (2001) Physiological effects of freshwater bathing of Atlantic salmon (*Salmo salar*) as treatment for amoebic gill disease. *Aquaculture* 199, 259-266

Rodger, H.D., McArdle, J.F. (1996) An outbreak of amoebic gill disease in Ireland. *Veterinary record* 139, 348-349

Shariff, M. (1998) Impact of diseases on aquaculture in the Asia-Pacific region as exemplified by epizootic ulcerative syndrome (EUS). *Journal of Applied Ichthyology-Zeitschrift fur Angewandte Ichtyology* 14, 139-144

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

Verlhac, V., Obach, A., Gabaudan, J., Schuep, W., Hole, R. (1998) Immunomodulation by dietary vitamin C and glucan in rainbow trout (*Oncorhynchus mykiss*). *Fish and Shellfish Immunology* 8, 409-424

Zilberg, D., Gross, A., Munday, B.L. (2001). Production of salmonid amoebic gill disease by exposure to *Paramoeba* sp. harvested from the gills of infected fish. *Journal of Fish Diseases* 24, 79-82

# CHAPTER 6

# GENERAL DISCUSSION

## 6.1 DIAGNOSTIC TESTS

The conclusions made in this project are only as valid as the detection methods used. A total of 4,417 gill mucus samples and 1,649 water samples were tested with the pathogen specific immuno-dot blot in this project. The test was developed in the initial part of this project and was validated against IFAT, the *de facto* "gold standard". The agreement between the two tests was high, with a corrected kappa value of 0.88 (SE 0.057). With an increased sensitivity of the immuno-dot blot test compared with IFAT, and the possibility of processing large sample numbers it was decided that the immuno-dot blot test was useful for epidemiological studies (Douglas-Helders *et al.*, 2001a).

The immuno-dot blot in this study was used for detection of *N.pemaquidensis* in gill mucus, biofouling, and water samples. The polyclonal anti *N. pemaquidensis* antibody used in the immuno-dot blot testing was found to cross-react with near related species *Neoparamoeba aestuarina* and *Pseudoparamoeba pagei* but not *Paramoeba eilhardi* (Douglas-Helders *et al.*, 2001a). No cross-reactivity was detected with *Platyamoeba plurinucleolus* (Page), *Platyamoeba/Vanella* (Page) or *Flabellula* (Schmoller) (Howard and Carson, 1993). The cross reactive protozoan species have never been isolated from the gills of fish with AGD (Howard & Carson, 1993). Thus, the antiserum developed for the immuno-dot blot was considered to be highly predictive of *N. pemaquidensis* on fish gills.

Biofouling samples were tested with culture enriched IFAT, using the same antibody as in the immuno-dot blot tests, and findings were confirmed with the highly pathogen specific PCR test (Elliott *et al.*, 2001). A good correlation was found between the two tests (Elliott *et al.*, 2001), and with a high kappa value of 0.89 (SE 0.036), it was suggested that the antibody was reliable for testing presence of *N. pemaquidensis* in biofouling samples from nets (Douglas-Helders *et al.*, 2002a). The immuno-dot blot was also used to detect the presence of *Paramoeba* species in seawater (Douglas-Helders *et al.*, 2002b, Douglas-Helders *et al.*, 2002c). In the water sampling validation study, only samples originating from salmonid farms tested positive for *Paramoeba* species when using the immuno-dot blot. Of nine crude sea water samples tested with both immuno-dot blot and nested PCR, four were positive for dot blot while no *N. pemaquidensis* could be detected using PCR (Elliott *et al.*, 2001). This may have been due to the different sensitivities of the two tests, with the PCR being able to consistently detect 16 cultured cells in 100 µL (Elliott *et al.*, 2001), and the dot blot test 4 cells per sample of 80 µL (Douglas-Helders *et al.*, 2001a). The sensitivity results for the two tests were both based on detection of cultured *N. pemaquidensis* in 0.45 µm filtered seawater. Thus, the difference in sensitivity would have been a result of the *N.pemaquidensis* specific testing of the PCR. In case of environmental isolates, the immuno-dot blot detected a pool of near related *Paramoebae* species, and thus increased the sensitivity of the immuno-dot blot test. It is possible that paramoebae densities were overestimated using the immuno-dot blot test. However, Dyková *et al.* (2000) suggested that *N. aestuarina*, one of the cross-reactive species of the immuno-dot blot test, should also be taken into account as implicated in AGD as well. Results in this project suggested that the immuno-dot blot test was useful for detection of

*Paramoeba* species in water samples. Production of a monoclonal antibody would be required to exclude the cross-reactivity and limit the test's detection to *N. pemaquidensis*. However, due to financial and time restrictions this was not possible within this project.

## 6.2 BIOLOGICAL FACTORS

Experiments described in this thesis showed that *N. pemaquidensis* remained infective for up to 14 days, which was the longest time tested, when in no contact with fish (Douglas-Helders *et al.*, 2002d). This lead to the conclusion that transmission of AGD in the field does not only occur from infected fish to naive fish, but also from water to fish. Transmission of pathogens through the water column was described for disease agents such as infectious salmon anaemia virus to Atlantic salmon (ISA, Jarp & Karlsen, 1997, Haastein, 1997), microsporidean *Loma salmonae* to chinook salmon (Kent *et al.*, 1995), and the protozoan parasite *Perkinsus marinus*, infecting oysters (Brewster *et al.*, 2000). It was therefore important to understand the distribution of *N. pemaquidensis* in the water column. This project showed that densities of the genus *Paramoeba* were higher in February (late summer) compared with August (late winter). Paramoebae densities were highest at the middle pen depth, which in this project was at approximately 5.5 metres, possibly due to the high densities of available hosts at this depth. At this middle pen depth, paramoebae densities decreased, away from the farming site. These differences were all statistically significant. Within these variables of season, depth, and distance from the lease site, significant correlations were found between paramoebae densities, bacterial counts, and turbidity. Both bacterial counts and turbidity were significantly positively correlated with temperature. In this study it

was suggested that bacterial counts in the water column may be a risk factor for AGD (Douglas-Helders *et al.*, 2002c), while this remained unclear for temperature (Douglas-Helders *et al.*, 2001b). Although excessive numbers of bacteria on gills will often result in poor gill health, the effect of gill bacterial load on occurrence of AGD remain unclear.

## 6.3 RISK FACTORS

Identifying risk factors as a cause of disease requires the demonstration of association between an agent and a disease and consistent data (Thompson & Lawrence, 1995). This project identified several risk factors for outbreaks of AGD in farmed Atlantic salmon. Paramoebae numbers on gills of dead infected fish increased over a period of 30 hours, while the pathogen colonised gills of previously uninfected dead fish (Douglas-Helders *et al.*, 2000). It was concluded that AGD infected mortalities are a potentially important reservoir of infection. This was also seen in previous studies, where dead fish infected with ISA (Jarp & Karlsen, 1997) or furunculosis (Sangster, 1991), were a reservoir for the pathogen. This implies that when cage hygiene standards are high, and regular removal of carcasses takes place, the likelihood of infecting naive fish with some pathogens will decrease.

Biofouling on nets of sea cages may be reservoirs or vectors for fish pathogens. This was demonstrated for *Aeromonas salmonicida* in sea lice in Norway (Nese & Enger, 1993) or *Piscirickettsia salmonis* in the salmon copepod ectoparasite *Ceratothoa gaudichaudii* (Garces *et al.*, 1994). *Neoparamoeba pemaquidensis* was isolated from micro and macro-biofouling organisms in a study of Tan *et al.*

(2002), but infection could not be established after exposure of AGD naïve fish to biofouled nets in a laboratory trial (Tan *et al.*, 2002). In this study it was shown that nets that were treated with a copper-oxide containing antifouling paint, harboured significantly higher *N.pemaquidensis* densities compared to non-treated nets, as well as a significantly higher AGD prevalence in these cages (Douglas-Helders *et al.*, 2002a). Although transmission of AGD did not occur from lightly fouled netting in the laboratory trial (Tan *et al.*, 2002), the field trial results suggested that transmission was possible (Douglas-Helders *et al.*, 2002a). Munday *et al.* (2001) concluded that fouled nets did not appear to be a significant reservoir for AGD, however the studies of Tan *et al.* (2002) and this study, strongly suggest that biofouled nets are a potential reservoir for infection with AGD.

Environmental factors can often significantly contribute to disease outbreaks (Nowak, 1999). Though temperature showed the most consistent association with clinical AGD Munday *et al.* (2001), it was considered to be the second important factor affecting AGD outbreaks after salinity (Clark & Nowak, 1999). In Tasmania AGD outbreaks were associated with temperatures of 12 to 20°C (Munday *et al.*, 1990), but recently outbreaks in winter have been observed. Average temperatures of 10°C, with a maximum of 13°C, were reported in outbreaks in Tasmania (Douglas-Helders *et al.*, 2001b), and an AGD outbreak in Washington State, USA occurred with average water temperatures as low as 9.2°C (Douglas-Helders *et al.*, 2001b). The pathogen previously isolated from AGD infected fish in USA was identified as *N .pemaquidensis* (Kent *et al.*, 1988), and its similarity to the Tasmanian strain verified by Elliott *et al.* (2001). In view of these results, the role of high temperature per se as a major risk factor was evaluated. However, high

temperatures may stress cultured fish, and predispose them to AGD outbreaks. Also, increasing temperatures, remaining within the range of viability, promote biological reactions (Rheinheimer, 1974). Such are increased proliferation rate of protozoa, increased bacterial populations (Rheinheimer, 1974), increased likelihood for algal blooms and presence of jellyfish, and a general decrease of the dissolved oxygen level in the water column. The complex role of stressors as predisposing factors for AGD outbreaks, and optimum environmental conditions for the pathogen needs further investigation, and will require controlled laboratory experiments.

In view of previous studies where carriers and reservoirs of pathogenic organisms have been described (Bricknell *et al.*, 1996, Haastein, 1997), wild fish were investigated as a possible risk factor as carrier or reservoir for *N. pemaquidensis*. It was shown that wild species might be able to carry *N.pemaquidensis* as was seen by the asymptotic carriage of the protozoan in the experimental infection trial (Douglas-Helders *et al.*, 2002e). However, two field surveys failed to detect *N. pemaquidensis* in wild fish species and it was concluded that these fish species were unlikely to be a significant reservoir and therefore not a risk factor (Su, 1994, Douglas-Helders *et al.*, 2002e). Wild AGD infected fish was previously reported on one occasion by Foster & Percival (1988). These authors reported *Paramoeba* species on the gills of barracouta (*Thyrites atun*). However, pathogen specific tests such as IFAT were not used, as they became available only after this occurrence (Howard & Carson, 1993). It could therefore be questioned if the organism found on barracouta gills was *N. pemaquidensis*.

## 6.4 HUSBANDRY

Husbandry techniques offer the best intervention opportunity for farmers (Needham & Rymes, 1992) in sea cage culture of marine fish. Husbandry systems, treatment schemes, and other management aspects can and should be regulated to prevent or reduce the severity of AGD and other disease outbreaks. For example, basic hygiene methods are an excellent control measure for decreasing severity of disease outbreaks (Wheatley *et al.*, 1995). In a study of McVicar (1986) a correlation was established between severity of pancreas disease (PD) and the extent of stress factors such as presence of other diseases, rough handling, and bad husbandry practises.

Thus far not many studies focussed on improving the AGD situation in Tasmania through husbandry. In this project four different husbandry options were evaluated; the effect of cage size, towing as a treatment method, effect of prophylactic freshwater bathing, and the effect of regular cage movement to recently fallowed sites. It was shown that towing and cage size did not affect AGD prevalence. The impact of AGD was reduced when freshwater bathing occurred only after the detection of gross lesions on gills, or when cages were regularly moved onto fallowed sites (Douglas-Helders *et al.*, 2002b).

Many husbandry options remain un-researched for AGD, such as, effects of high farm densities in an area, high stocking densities within a farm lease, maturation of fish, mixed year class stocks, or type of feed and/or feeding frequency. It is not unlikely that the frequency and severity of AGD outbreaks relate to high fish densities. High densities of fish in an area or cage could facilitate transmission of

pathogens (Anderson & Norton, 1991), as was seen for the transmission of ISA among farmed Atlantic salmon along the Norwegian coast (Nylund *et al.*, 1997). Taksdal *et al.* (1998) reported that close fish to fish contact did not only lead to increased stress levels, but also increased the chance of adhesion of *Flexibacter columnaris* to external tissues of fish.

Sexual maturation was found to be a major risk factor for infections of Atlantic salmon (*Salmo salar*) with *Kudoa thyrsites*, with a 13 fold increased likelihood of infection for sexually mature salmon compared with sexually immature salmon (St-Hilaire *et al.*, 1998). Although sexual maturation appears to be a risk factor for AGD in the field, there is no scientific evidence. Multiple year class stocks were identified as a risk factor for high mortality rates in a study of Wheatley *et al.* (1995) and increased risk of ISA outbreaks in Norway (Vågsholm *et al.*, 1994). It is therefore not surprising that the Huon River Management Programme in Tasmania prevents the occurrence of mixed year class stocks on their sites (Nowak, 2001). Although risk of increased virulence with mixed year sites was mentioned (Munday *et al.*, 2001), no scientific evidence is available.

Feeding, especially excessively, results in an increased amount of suspended solids in the water column, which can damage gill filaments and reduce success of adaptation of smolt to seawater (Clarke, 1992). Taksdal *et al.* (1998) noted that *Flexibacter columnaris* grew well on particulate fish feeds, and concluded that such deposits in the water column could act as a reservoir of infection. In this study the relationship between bacteria and paramoebae was established, and suspended solids suggested as a possible vector for *N. pemaquidensis* (Douglas-Helders *et al.*,

2002c). In view of this, future research could focus on the effect of feed type and degradation rates, as well as different feeding regimes on AGD prevalence.

## 6.5 RECOMMENDATIONS

Detection of risk factors and changing trends in disease prevalence requires collection of relevant information, therefore disease monitoring systems and their related databases play an important role (Thrusfield & Noordhuizen, 1997). Such databases have been used in the past to: identify determinants of diseases for the North Sea dab in the North Sea (Wosniok *et al.*, 2000), control of production and environmental impact of aquaculture in Norway (Maroni, 2000), determine relationships between site management and mortality rates (Wheatley *et al.*, 1995) and factors affecting the profitability of Irish salmon farms (Menzies *et al.*, 1998), and study the epidemiology of pancreas disease (PD) in Ireland (Menzies *et al.*, 1996, Crockford *et al.*, 1999). In Tasmania, the Tasmanian Salmon Health Information System (www.Ausvet.com.au\salmon\index.html) is currently under trial for AGD in Tasmania. This database could be helpful and additional to existing research projects to determine risk factors and identify areas for future research in AGD outbreaks, so that the severity of AGD can be minimised. However, success of such a database will be dependent on farm participation and the development of uniform diagnostic techniques.

Another method of improving animal health is by the development of mathematical models. Disease patterns, exhibited by AGD (Munday *et al.*, 2001) may be predicted and the effects of adopted control strategies evaluated with the use of mathematical models (Graat & Frankena, 1997). Modelling has developed over the

past 200 years, increasing in complexity (Thrusfield & Noordhuizen, 1997). The reliance on modern computers is high, and several models can be linked together to produce large-scale system models (Thrusfield & Noordhuizen, 1997). Adoption of these techniques in AGD could benefit the Tasmanian salmon growers.

## 6.6 THE END

In conclusion, the work described in this thesis contributes to the understanding of behaviour, distribution, reservoirs, and provisionally touched on the optimal growth conditions of *N. pemaquidensis* in the aquatic environment. The results indicated that paramoebae are widely distributed in and around Tasmanian salmon farms, and that no host contact is required for up to 14 days for the protozoan to remain infectious. An attempt was made to identify optimum growth conditions in the field, but future laboratory trials will be required to solve or confirm causal relationships between temperature, bacterial numbers, amount of suspended particles, turbidity, paramoebae numbers in the water column, and the occurrence and severity of AGD outbreaks. It was demonstrated that losses experienced due to AGD outbreaks on Tasmanian salmon farms could be ameliorated by adopting a schedule of cage movements to fallowed sites and by avoidance of freshwater bathing before clinical signs on gills are detectable. This work is an addition to the ever-growing body of information on the ecology, aetiology and epidemiology of *N. pemaquidensis*, and will help to determine future AGD research, control, and, monitoring programs.

# REFERENCES


Adams, M.B., Nowak, B.F. (2001) Distribution and structure of lesions in the gills of Atlantic salmon (*Salmo salar* L.) affected with amoebic gill disease. *Journal of Fish Diseases* 24, 535-542

Alexander, J.M. (1991) Treatment of amoebic gill disease: field trials, 1990/91. In: *Proceedings of the Saltas Research and Development Review Seminar*, pp. 51-101. Salmon Enterprises of Tasmania Pty Ltd, Dover, Tasmania

Anderson, I.G., Norton, J.H. (1991) Diseases of barramundi in aquaculture. *Austasia Aquaculture* 5, 21-23

Baldock, F.C. (1990) Making decisions based on results from imperfect diagnostic tests. In: *Epidemiology at Work*, Proceedings of the 144 Postgraduate Committee in Veterinary Science (Ed. by D.I Bryden), pp. 136-138, University of Sydney

Bakke, T.A., Harris, P.D. (1998) Diseases and parasites in wild Atlantic salmon (*Salmo salar*) populations. *Canadian Journal of Fisheries and Aquatic Science* 55, 247-266

Brewster, J., Bushek, D., Dame, R. (2000) *Perkinsus marinus* population dynamics in North Inlet, South Carolina – An ecosystem model. *Journal of Shellfish Research* 19, 563-584

Bryant, M.S., Lester, R.J.G., Whittington, R.J. (1995) Immunogenicity of amoebic antigens in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* 18, 9-19

Callinan, R.B. (1999) Causes of disease. In: *Aquaculture Epidemiology*, 10 pp., Canadian Aquaculture Institute, International Education in Aquaculture medicine and management, Sydney April 25-26

Cameron, D.E. (1993) Amoebic gill disease research- 1992/93. In: *Seeking and solving*. Papers presented at the Saltas Research and Development Review Seminar, pp 89-102. Salmon Enterprises of Tasmania Pty Ltd, Dover, Tasmania

Cann, J.P., Page, F.C. (1982) Fine structure of small free-living *Paramoeba* (Paramoebida) and taxonomy of the genus. *Journal of Marine Biology Association* 62, 25-43

Clark, A., 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

Clarke, W.C. (1992) Environmental factors in the production of Pacific salmon smolt. *World Aquaculture* 23, 40-42

Corpe, W.A. (1976) Primary bacterial films and marine microfouling. In: *Proceedings 4$^{th}$ International Congress on Marine Corrosion and Fouling*. (Ed. by V. Romanovsky), pp. 97-100, Juan-les-Pins, Antibes, France

Crockford, T., Menzies, F.D., McLoughlin, M.F., Wheatley, S.B., Goodall, E.A. (1999) Aspects of the epizootiology of pancreas disease in farmed Atlantic salmon, *Salmo salar* in Ireland. *Diseases of Aquatic Organisms* 36, 113-119

Davidson, J. (1999) Determinants of disease. In: *Aquaculture Epidemiology*, 6 pp. Canadian Aquaculture Institute, International Education in Aquaculture medicine and management, Sydney April 25-26

Dix, T. (1986) History of Aquaculture in Tasmania. *Tasmanian Aquaculture Proceedings* 20, 9-15

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

Douglas-Helders, G.M., Carson, J., Howard, T., Nowak, B.F. (2001a) 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, G. M., Saksida, S. Raverty, S., Nowak, B.F. (2001b) Temperature as a risk factor for outbreaks of amoebic gill disease in farmed Atlantic salmon. *Bulletin of European Association of Fish Pathologists* 21, 114-115

Douglas-Helders, G.M., Tan, C.K.F., Carson, J., Nowak, B.F. (2002a) 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, In Press*

Douglas-Helders, G.M., Weir, I. J., O'Brien, D.P., Carson, J., Nowak, B.F. (2002b) Effects of husbandry on prevalence of amoebic gill disease and performance of reared Atlantic salmon (*Salmo salar* L.). *Journal of Fish Diseases*. Submitted

Douglas-Helders, G.M., O'Brien, D.P., McCorkell, B.E., Zilberg, D., Gross, A., Carson, J., Nowak, B.F. (2002c) Temporal and spatial distribution of *Paramoeba* sp. in the water column- a pilot study. *Journal of Fish Diseases*. Submitted

Douglas-Helders, G.M., Carson, J., Nowak, B.F. (2002d) Assessment over time of the infectivity of gill derived and seawater dispersed *Neoparamoeba pemaquidensis* (Page 1987) *Buletin of European Association of Fish Pathologists, In Press*

Douglas-Helders, G.M., Dawson, D.R., Carson, J., Nowak, B.F. (2002e) Wild fish are not a significant reservoir for *Neoparamoeba pemaquidensis* (Page, 1987). *Journal of Fish Diseases* 25, 569-574

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

Dyková, L., Figueras, A., Novoa, B., Casal, J.F. (1998) *Paramoeba* sp., an agent of amoebic gill disease of turbot *Scophthalmus maximus*. *Diseases of Aquatic Organisms* 33, 137-141

Dyková, I, Figueras, A., Novoa, B., (1999) Epizoic amoebae from the gills of turbot *Scophthalmus maximus*. *Diseases of Aquatic Organisms* 38, 33-38

Dyková, I., Figueras, A., Peric, Z. (2000) *Neoparamoeba* Page, 1987: light and electron microscopic observations on six strains of different origin. *Diseases of Aquatic Organisms* 43, 217-223

Dyková, I., Novoa, B. (2001) Comments on diagnosis of amoebic gill disease (AGD) in turbot, *Scophthalmus maximus*. *Bulletin of the European Association of Fish Pathologists* 21, 40-44

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

Findlay, V.L., Helders, M., Munday, B.L., Gurney, R. (1995) Demonstration of resistance to reinfection with *Paramoeba* sp. by Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* 18, 639-642

Findlay, V.L., Munday, B.L. (1998) Further studies on acquired resistance to AGD in Atlantic salmon. *Journal of Fish Diseases* 21, 121-125

Findlay, V.L., Munday, B.L. (2000) The immunomodulatory effects of levamisole on the nonspecific immune system of Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* 23, 369-378

Findlay, V.L., Zilberg, D., Munday, B.L. (2000) Evaluation of levamisole as a treatment for amoebic gill disease of Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* 23, 193-198

Frankena, K., Thrusfield, M.V. (1997) Basics of Observational studies. In: *Application of quantitative methods in veterinary epidemiology* (Eds. J.P.T.M. Noordhuizen, K. Franekna, C.M. van der Hoofd, and E.A.M. Graat), pp 99-134, Wageningen Press, The Netherlands

Garces, L.H., Corrael, P., Larenas, J., Contreras, J., Oyanedel, S., Fryer, J.L., Smith-Schuster, P.A. (1994) Findings of *Piscirickettsia salmonis* on *Cerathothoa*

*gaudichaudii. International Symposium on Aquatic Animal Health*, University of California, School of Veterinary Medicine, 4-8 September, Seattle, USA

Graat, E.A.M., Frankena, K. (1997) Introduction to theoretical epidemiology. In: *Application of quantitative methods in veterinary epidemiology* (Eds. J.P.T.M. Noordhuizen, K. Franekna, C.M. van der Hoofd, and E.A.M. Graat), pp 247-270, Wageningen Press, The Netherlands

Haastein, T. (1997) Infectious salmon anaemia (ISA): a historical and epidemiological review of the development and spread of the disease in Norwegian fish farms. In: *Workshop on Infectious Salmon Anaemia*, pp. 6-12, November 26, 1997, St. Andrews Biological Station, New Brunswick, Canada

Hammell, K.L. (1999) Methods of disease transmission in aquaculture, In: *Aquaculture Epidemiology*, 10 pp., Canadian Aquaculture Institute, International Education in Aquaculture medicine and management, Sydney April 25-26

Henken, A.M., Graat, E.A.M., Casal, J. (1997) Measurement of disease frequency. In: *Application of quantitative methods in veterinary epidemiology* (Eds. J.P.T.M. Noordhuizen, K. Franekna, C.M. van der Hoofd, and E.A.M. Graat), pp 63-97, Wageningen Press, The Netherlands

Howard, T., Carson, J. (1993) Verification that *Paramoeba* species are consistently associated with gill damage in fish affected with amoebic gill disease. In: *Proceedings of the Saltas research and development review seminar*, pp. 69-80. Tasmania 1993, Salmon Enterprises of Tasmania Pty Ltd Dover, Tasmania

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

Jarp, J., Karlsen, E. (1997) Infectious salmon anemia (ISA) risk factors in sea-cultured Atlantic salmon *Salmo salar*. *Diseases of Aquatic Organisms* 28, 79-86

Kent, M.L., Sawyer, T.K., Hendrick, R.P. (1988) *Paramoeba pemaquidensis* (Saromastigophora: Paramoebidae) infestation of the gills of coho salmon, *Oncorhynchus kisutch*, reared in seawater. *Diseases of Aquatic Organisms* 5, 163-169.

Kent, M.L., Dawe, S.C., Speare, D.J. (1995) Transmission of *Loma salmonae* (Microsporea) to chinook salmon in sea water. *Canadian Veterinary Journal* 36, 98-101

Leiro, J., Paniagua, E., Ortega, M., Parama, A., Fernandez, J., Sanmartin, M.L. (1998) An amoeba associated with gill disease in turbot, *Scophthalmus maximus* L. *Journal of Fish Diseases* 21, 281-288

Lom, J., Dyková, I. (1992) Protozoan parasites of fishes. *Developments in Aquaculture and Fisheries Science* 26, 315pp

Maroni, K. (2000) Monitoring and regulation of marine aquaculture in Norway. *Journal of Applied Ichthyology* 16, 192-195

Martin, R.E. (1985) Population growth in stationary and suspension culture of *Paramoeba pemaquidenis* Page (Amoebida, Paramoebaidae) *Journal of Protozoology* 32, 728-739

Martin, S.W., Meek, A.H., Willeberg, P. (1987) *Veterinary epidemiology: Principles and Methods*, 343 pp. Iowa State University Press, Ames, Iowa

McVicar, A.H. (1986) Observations on pancreas disease of farmed Atlantic salmon (*Salmo salar* L.). *Proceedings of the ICES Annual Service Conference*, 8 pp. Copenhagen, Denmark, 9 October

Menzies, F.D., Crockford, T., McLoughlan, M.F., Wheatley, S.B., Goodall, E.A. (1998) An epidemiological approach to investigating disease and production losses in farmed Atlantic salmon. In: *Methodology in Fish Diseases Research* (Ed. by A. Barnes, G. Davidson, M. Hiney, D. McIntosh), pp. 289-294. Fisheries Research Services, Aberdeen, Scotland.

Menzies, F.D., McLoughlin, M.F., Wheatley, S.B., Goodall, E.A. (1996) Development of a computerised information retrieval system for Atlantic salmon, *Salmo salar* L. production. *Aquaculture and Research* 27, 183-190

Munday, B.L., Foster, C.K., Roubal, F.R., Lester, R.J.G. (1990) Paramoebic gill infection and associated pathology of Atlantic salmon, *Salmo salar*, and rainbow trout, *Salmo gairdeneri*, in Tasmania. In: *Pathology in Marine Science* (Ed. by F.O. Perkins & T.C. Cheng), pp 215-222. Academic Press, San Diego, Ca.

Munday, B.L., Lange, K., Foster, C., Lester, R.J.G., and Handlinger, J. (1993) Amoebic gill disease of sea-caged salmonids in Tasmanian waters. *Tasmanian Marine Fisheries Research* 28, 14-19

Munday, B.L., Zilberg, D., Findlay, V. (2001) Gill disease of marine fish caused by infection with *Neoparamoeba pemaquidensis*. *Journal of Diseases* 24, 497-507

Nese, L., Enger, Ø. (1993) Isolation of *Aeromonas salmonicida* from sea lice *Lepeophtheirus salmonis* and marine plankton. *Diseases of Aquatic Organisms* 16, 79-81

Nowak, B.F., Lucas, C.T. (1997) Diagnosis of structural changes in fish gills- can biopsy replace necropsy? *Aquaculture* 159, 1-10

Nowak, B.F. (1999) Significance of environmental factors in aetiology of skin diseases of teleost fish. *Bulletin of the European Association of Fish Pathologists* 19, 290-292

Nowak, B.F. (2001) Qualitative evaluation of risk factors for Amoebic Gill Disease in Atlantic salmon. 18pp. In: *Proceedings of the OIE International Conference on Risk Analysis in Aquatic Animal Health*, (Ed: C.J. Rodgers), pp. 148-155, Office International des Epizooties (OIE), 8-10 February 2000, Paris, France

Nylund, A., (1997) Reservoir species for the infectious salmon anaemia virus (ISAV). In: *Workshop on Infectious Salmon Anaemia*, pp. 29-44. November 26, 1997, St. Andrews Biological Station, New Brunswick, Canada

O'Sullivan, D.,Roberts, N. (2001) Status of Australian aquaculture in 1999/2000. *Austasia Aquaculture Trade Directory*, pp. 1-19, Turtle Press, Hobart, Tasmania

Page, F.C. (1970) Two new species of *Paramoeba* from Maine. *Journal of Protozoology* 17, 421-427

Page, F.C. (1973) *Paramoeba*: a common marine genus. *Hydrobiologica* 41, 183-188

Page, F.C. (1976) A revised classification of the Gymnamoebia (Protozoa, Sarcodina). *Zool. J. Linn. Soc.* 58, 61-77

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

Page F.C. (1987) The classification of 'naked' amoebae of phylum Rhizopoda. *Archive Protistenkunde* 133, 199-217

Palmer, R., Carson, J., Ruttledge, M., Drinan, E., Wagner, T. (1997) Gill disease associated with *Paramoeba*, in sea-reared Atlantic salmon in Ireland. *Bulletin of the European Association of Fish Pathologists* 3-4, 112-114

Parsons, H., Nowak, B., Fisk, D., Powell, M. (2001) Effectiveness of commercial freshwater bathing as a treatment against amoebic gill disease in Atlantic salmon. *Aquaculture* 195, 205-210

Powell, M.D., Parsons, H.J., Nowak, B.F. (2001) Physiological effects of freshwater bathing of Atlantic salmon (*Salmo salar*) as treatment for amoebic gill disease. *Aquaculture* 199, 259-266

Rodger, H.D., McArdle, J.F. (1996) An outbreak of amoebic gill disease in Ireland. *Veterinary record* 139, 348-349

Roubal, F.R, Lester, R.J.G., Foster, C. (1989) Studies on cultured and gill-attached *Paramoeba* sp. (Gymnamoebae: Paramoebidae) and the cytopathology of paramoebic gill disease in Atlantic salmon, *Salmo salar* L., from Tasmania. *Journal of Fish Diseases* 12, 481-493

Sawyer, T.K. (1980) Marine ameba from clean and stressed bottom sediments of the Atlantic Ocean and the Gulf of Mexico. *Journal of Protozoology* 27, 13-32

Snedecor, G.W., Cochran, W.G. (1980) *Statistical Methods*, 7th Edition, Iowa State University Press, Ames, Iowa

Stanley, S.O. (1993) The Tasmanian aquaculture industry in 1993. *Marine Farming Information Series, 9.* Department of Primary Industries, Water and Environment and Fisheries, Tasmania

St-Hilaire, S., Ribble, C., Whitaker, D.J., Kent, M. (1998) Prevalence of *Kudoa thyrsites* in sexually mature and immature pen-reared Atlantic salmon (*Salmo salar*) in British Columbia, Canada. *Aquaculture* 162, 69-77

Su, X. (1994) Taxonomic and biological studies of protozoan parasites in Tasmanian marine fishes. *PhD Thesis*, pp. 1-237, University of Tasmania, Hobart

Taksdal, T., Ramstad, A., Stangeland, K., Dannevig, B.H. (1998) Introduction of infectious pancreatic necrosis (IPN) in covertly infected Atlantic salmon, *Salmo salar* L., post-smolts by stress exposure, by injection of IPN virus (IPNV) and by cohabitation. *Journal of Fish Diseases* 21, 193-204

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

Thorburn, M.A. (1992) The randomness of samples collected by dip-net methods from rainbow trout in tanks. *Aquaculture* 101, 385-390

Thrusfield, M., Noordhuizen, J.P.T.M (1997) Current areas of application of epidemiology and perspectives. In: *Application of quantitative methods in veterinary epidemiology* (Eds. J.P.T.M. Noordhuizen, K. Franekna, C.M. van der Hoofd, and E.A.M. Graat), pp 13-30, Wageningen Press, The Netherlands

Thrusfield, M. (1995) *Veterinary epidemiology*, 479 pp., Blackwell Science, Oxford, UK

Thompson, J.A., Lawrence, A.L. (1995) Concepts of herd health for shrimp. In: *UNJR Technical Report 24, Interactions between cultured species and naturally occurring species in the environment* (Eds. B.J. Keller, P.K. Park, J.P. McVey, Takayanagi, Kazufumi, Hosoya, Kazumi), pp. 91-96. U.S.-Japan Aquaculture Panel Symposium, 8-10 October, USA

Vågsholm, I., Djupvik, H.O., Willumsen, F.V., Tveit, A.M., Tangen, K. (1994) Infectious salmon anemia (ISA) epidemiology in Norway. *Preventative Veterniary Medicine* 19, 277-290

Wheatley, S.B., McLoughlin, M.F., Menzies, F.D., Goodall, E.A. (1995) Site management factors influencing mortality rates in Atlantic salmon (*Salmo salar* L.) during marine production. *Aquaculture* 136, 195-207

Wosniok, W., Lang, T., Dethlefsen, V., Feist, S.W., McVicar, A.H., Mellergaard, S., Vethaak, A.D. (2000) Analysis of ICES long-term data on diseases of North Sea dab (*Limanda limanda*) in relation to contaminants and other environmental factors. In: *Proceedings of the ICES Annual Service Conference*, 15 pp., 27-30 September, Brugge, Belgium

Zilberg, D., Nowak, B., Carson, J., Wagner, T. (1999) Simple gill smear for diagnosis of amoebic gill disease. *Bulletin of the European Association of Fish Pathologists* 19, 186-189

Zilberg, D., Findlay, V.L., Girling, P., Munday, B.L. (2000) Effects of 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, 23-27

Zilberg, D., Munday, B.L. (2000) Pathology of experimental amoebic gill disease in Atlantic salmon, *Salmo salar* L., and the effect of pre-maintenance of fish in sea water on the infection. *Journal of Fish Diseases* 23, 401-407

Zilberg, D., Gross, A., Munday, B.L. (2001) Production of salmonid amoebic gill disease by exposure to *Paramoeba* sp. harvested from the gills of infected fish. *Journal of Fish Diseases* 24, 79-82