Final Report

Atlantic Salmon Aquaculture Subprogram: Culture and cryopreservation of *Neoparamoeba perurans* (AGD)

Philip B. B. Crosbie, Andrew R. Bridle and Barbara F. Nowak

FRDC PROJECT NUMBER: 2012/048

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Atlantic Salmon Aquaculture Subprogram: Culture and cryopreservation of *Neoparamoeba perurans* (AGD)

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Atlantic Salmon Aquaculture Subprogram: Culture and cryopreservation of *Neoparamoeba perurans* (AGD)

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OBJECTIVES:

1 To improve culture and preservation techniques of *Neoparamoeba perurans*

2 To determine the effect of time in culture on viability and virulence of clones of *Neoparamoeba perurans*

Non-Technical Summary

OUTCOMES ACHIEVED

This project has refined the culture techniques for *Neoparamoeba perurans* and has confirmed the retention of virulence in culture for up to 2.5 years, although there is some evidence of reduced virulence. Amoebae have been supplied to other researchers undertaking FRDC projects, and also training provided in amoebae culture and isolation techniques. Techniques for *in vitro* manipulation of amoebae have been developed which will have wider applications.

This project has shown that the culture of *N. perurans* can be improved by incubating culture plates at 10ºC. At lower temperatures the growth of contaminating organisms can be slowed which allows longer periods between sub-culturing. Cryopreservation techniques have been attempted however viability of cells post thaw has not been demonstrated. The toxicity of some

cryoprotectant compounds used on *N. perurans* has been assessed and concentrations not detrimental to the amoebae were identified. The results from the infectivity trials where clonal cultures of *N. perurans* were assessed for their ability to cause AGD were the most intriguing. Although the cultures were shown to cause AGD there is some evidence of a loss of virulence of the clones in culture over 2.5 years. This observation requires verification but if true could open up opportunities to do comparative studies between virulent and non-virulent *N. perurans*.

KEYWORDS:

Atlantic salmon, AGD, parasitic disease, amoebae, culture

Acknowledgments

This study formed part of salmon industry research, and was supported by the Fisheries R&D Corporation. We would like to thank the salmon industry for their collaboration and support.

Background

Amoebic Gill Disease remains the most important health issue for the Atlantic salmon industry in Tasmania. Significant progress was made during project 2008/218, including proving Koch's postulates and development of a PCR based detection method. While a culture method for virulent *Neoparamoeba perurans* has been developed reducing the need for infection tank and thus improving fish welfare, the current technique still requires frequent passaging of the cultures and is laborious and time consuming.

Cryopreservation methods for the virulent pathogen should be developed both to reduce the need for continuous passage and the likelihood of any genetic variation leading to attenuation of virulence. Improved culture techniques and cryopreservation will increase the scope but at a reduced cost for all AGD research. If successful they will remove the need for maintaining infection tank and reduce the number of fish needed for AGD research.

The project was developed on request from salmon industry and the development included extensive consultations with the Tasmanian Salmonid Growers Association who has discussed the proposal at different stages with salmon industry technical group.

Need

Amoebic Gill Disease (AGD) research requires access to *N. perurans*, which is the causative organism for this disease. The use of an infection tank is still a reasonably expensive option for obtaining amoebae. If the culture and cryopreservation are successful AGD researchers, including industry, will have access to cheaper amoebae. Culture of clones will allow research which is currently impossible with wild isolates.

Objectives

1 To improve culture and preservation techniques of *Neoparamoeba perurans*

2 To determine the effect of time in culture on viability and virulence of clones of *Neoparamoeba perurans*

Methods

Objective 1: To improve culture and preservation techniques of *Neoparamoeba perurans*.

Initially experiments were carried out to test methods of extending the sub-culture period for *N. perurans* and reduce the labour involved. Prior to this project the amoebae required sub-culturing every 5-7 days to avoid cells lifting from the agar and being overgrown by contaminating bacteria and protozoans. Previous observations of the growth of *N. perurans* on malt yeast agar (MYA) show that the amoebae initially settle onto the agar and then tend to float in the overlaying liquid after a period of some days. Small motile contaminating organisms then become apparent in the cultures and the *N. perurans* cells tend to look smaller at this time. Generally after 7-9 days the motile organisms predominate the plate and the amoebae tend to disappear. Hence the sub-culturing occurs before the plates get to this stage. At the beginning of the project there were 2 clonal cultures of *N. perurans* designated clone 4 and clone 6. Unfortunately clone 6 was lost soon after an infectivity trial in December 2012, however a new clonal culture was established by July 2013 and designated clone 8.

Previous attempts to cryopreserve the amoeba using the American Type Culture Collection (ATCC) protocol and reported in FRDC project 2008/218 have been unsuccessful. This procedure used dimethyl sulphoxide (DMSO) at 7.5% as a cryoprotectant and controlled cooling to limit cell damage. However, the amoebae numbers used previously in cryopreservation experiments have been less than 2 x 106 cells per mL which is the suggested cell density for optimal recovery post thaw. Additionally the use of alternate concentrations of DMSO or other compounds as cryoprotectants has not been examined, nor has the potential toxicity of these compounds on the amoeba prior to freezing attempts.

Experiment 1: *Neoparamoeba perurans* culture at different temperatures

A total of 6 MYA plates were seeded with *N. perurans* (collected by gentle washing from the surface of culture plates and pooled) and cells allowed to settle on the agar for 30 mins. Amoebae cells were then counted in 5 fields of view at 200x magnification on an inverted microscope. Duplicate plates were then incubated at 10, 14 and 18°C and counts performed on the amoebae cells settled onto the agar as above every 24 hours for 9 days. A replicate second trial was carried out concurrently using amoebae which were harvested as described above but from a different culture plate, therefore a total of 4 agar plates were incubated at each temperature.

Experiment 2: Cryopreservation attempts for *Neoparamoeba perurans*

The first cryopreservation attempt used the ATCC method and cultured *N. perurans*. Amoebae were washed form the culture plates and then placed into several clean petri dishes and allowed to adhere so that excess bacteria could be removed by washing. The plates were left at room temperature overnight and the bacteria were washed off. Attached amoebae were removed by placing the petri dishes on ice for 5 mins and by adding a 1-5 mL of 0.5 % trypsin EDTA. Detached cells were collected in 50 mL tubes and then concentrated by centrifuge at 600 x g for 5 mins at 4ºC and the numbers determined to be 2.0 x 106 cells per mL. A 15% (v/v) DMSO solution was prepared when 1.5 mL DMSO was placed into a tube on ice and allowed to solidify before the addition of chilled seawater (8.5 mL) to the tube and then the DMSO dissolved by inverting the tube. The amoebae suspension (0.5mL) and the DMSO (0.5 mL) were then mixed into cryotubes and allowed to equilibrate for 20 mins at room temperature, before being placed into a -80ºC freezer in a cooling unit suspended in isopropanol which allows controlled cooling at 1ºC per min for 2h. The cryotubes were then immersed in liquid nitrogen contained in a dewar. The final DMSO concentration was 7.5% and the net quantity of amoebae was 1 x 106 per tube. After 24h one of the cryotubes was removed from the dewar and rapidly thawed for 2 mins at 37ºC. Portions of the thawed amoebae were then either:

-tested for viability,

-inoculated to fresh MYA culture plates,

-or used to challenge Atlantic salmon.

For viability a neutral red inclusion assay was used where 20 µL of the amoebae suspension was mixed with 20 µL of neutral solution (50 µg mL-1 in phosphate buffered saline) in a 1 mL microcentrifuge tube and incubated at room temperature for at least 25 mins. Excess dye was removed when the tube was filled with filtered seawater and centrifuged at 14000 x g for 10 secs, most of the supernatant was removed apart from 20-30 µL which contained the amoebae. The percentage of viable cells was then determined for the amoebae on microscopic examination (using a stage microscope with a x100 or 200 objective) using a haemocytometer. Cells were judged to be viable if they showed the dye internalised (see Figure 1). For culture 100 uL of the thawed cell suspension was added to single MYA plate and immediately overlayed with 15-20 mL of filtered seawater. For challenge, 880 µL of the amoebae suspension was immediately added to 500 mL of filtered seawater and added to a 300 L recirculating tank containing 15 seawater acclimated Atlantic salmon smolt (see second objective).

The second attempt used amoebae from the AGD infection tank with a combination of DMSO and glycerol at 6.5% solutions in seawater as a cryoprotectant which has been successfully applied to free-living amoebae (Seo et al., 1992). Amoebae were sourced from the AGD-affected salmon using the adherence method of Morrison et al. (2004). The cells were

concentrated and washed in 0.2 µm filtered seawater by centrifugation at 550 x g for 5 mins and numbers determined to be 1.4 x 106 cells per mL. A 15% stock solution of DMSO was prepared as described above and then 1.5 mL of glycerol was added so that the final concentrations of both the DMSO and glycerol were 13% v/v. The DMSO/glycerol and the amoeba suspension were then mixed in equal volumes (0.5mL) in a cryotube to give a final concentration of 6.5% DMSO/glycerol and a net number of 7 x 105 amoebae and then allowed to equilibrate at room temperature for 25 mins. The cryotubes were then placed into a controlled cooling container in a -80ºC freezer for 90 min before being immersed in a dewar containing liquid nitrogen.

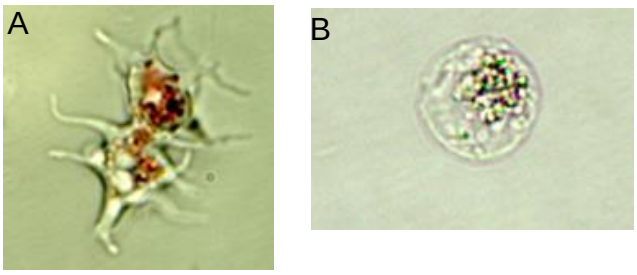

Figure 1. Amoebae after the neutral red viability assay. A. Live cells showing pseudopodia and the neutral red dye internalised into vacuoles. B. Dead cell with spherical shape, no pseudopodia or dye inclusions.

The same cryopreservation protocol was used in a third attempt using cultured amoebae (clone 4) with a final concentration of 10% glycerol as a cryoprotectant. Prior to all cryopreservation attempts the viability of amoebae was assessed using the neutral red inclusion assay.

Experiment 3: Toxicity testing of cryoprotectants on *Neoparamoeba perurans*

As initial attempts to cryopreserve *N. perurans* using the protocol of the ATCC, which has been successfully used to cryopreserve *N. pemaquidensis* strains, were not successful experiments were carried out investigating the toxicity of cryoprotectants on the amoeba. Cryopreservation attempts have used DMSO (7.5%) or combined with glycerol (both at 6.5%) and just glycerol (10%) in seawater as cryoprotectants. To test whether these concentration was toxic for the amoeba a series of exposures to various concentrations of DMSO and glycerol were carried out.

Amoebae, isolated from the AGD-affected salmon using the adherence method of Morrison et al. (2004), were exposed to DMSO at concentrations of 7.5, 10 and 15% and glycerol at 10, 20 and 30%. Stock solutions of DMSO, prepared on ice as previously described, and glycerol solutions, prepared at

room temperature, were made at 2x required concentrations to allow for dilution when mixed with equal volumes of the amoebae suspension. Cryoprotectants and amoebae were mixed at 0.5 mL volumes each in microcentrifuge tubes and incubated at room temperature. After 20, 30 and 40 mins samples of 100 µL were removed from each tube and placed into fresh microcentrifuge tubes with the addition of 500 µL of filtered seawater to dilute the cryoprotectant. The tubes were then microcentrifuged at full speed for 10 secs to pellet the cells and 550 µL was removed and discarded. Finally a neutral red viability assay was carried out on the pelleted cells as described previously.

Objective 2: To determine the effect of time in culture on viability and virulence of clones of *Neoparamoeba perurans*

Experiment 4: Infection trials with cultured *Neoparamoeba perurans*

Initially two infection trials were carried out to test the virulence of *N. perurans* cultures, clone 4 and clone 6 which have been in continuous culture since May 2011 and August 2012 respectively. Although the clonal cultures (i.e. cultures started from a single cell) were established on the dates above, both had been originally isolated and cultured non-clonally since February 2011 (parent culture of clone 4) and August 2011 (parent culture of clone 6)

For the first trial trophozoites of clone 6 were harvested from several agar plates by gentle flushing with filtered seawater then decanting the amoebae suspension onto clean Petri dishes and allowing the cells to attach to the plastic over 45 mins. Extraneous material including some agar and bacterial mats was then washed off the Petri dishes leaving behind the attached amoebae. Cells were then removed by the addition of trypsin and then counted after washing in filtered seawater. A control group of *N. perurans* which had been isolated from fish with AGD and housed in the infection tank at the University were collected using the attachment technique of Morrison et al (2004). For the experiment 3 groups of 15 salmon were exposed to clone 6, freshly isolated *N. perurans* (as a positive control) or no amoebae (a negative control). The exposures occurred in 3 x 45L tubs (5 fish per tub) at a concentration of approximately 6000 cells per litre for both the clone 6 group and the positive control whilst the negative control group was treated the same way but was not exposed to any amoebae. After 6 hours all of the fish were moved to 3 separate 500 L recirculating tanks containing seawater. One fish from each exposure group was euthanased at 6 days post infection for gill assessment and gill samples were taken; half the gills were placed into filtered seawater for amoebae isolation and the other half fixed in seawater Davidson's fixative for histology. Similar samples were taken from dead or moribund fish for the duration of the trial. At 38 days post infection all remaining fish were euthanased and samples taken for AGD diagnosis.

The second trial was run in a similar fashion using positive and negative control groups and exposure to clone 4 as a treatment group with12 salmon per group. The amoebae concentrations for this trial were approximately 5000 cells per litre for both groups and the exposure occurred in the 500L tanks which had been filled to 150L for 2 hours before the tanks were filled to their 500L capacity. As with the first trial samples were taken from moribund or dead fish during the trial for histology. The trial was terminated after 34 days and all remaining fish euthanased and sampled for amoebae isolations and histology.

The third, fourth and fifth infection trials were conducted in September and October 2013. The third trial was confirming clone 4 virulence (now in culture for 30 months), the fourth was assessing clone 8 (5 months in culture) and the fifth tested virulence of clone 4 after attempts at cryopreservation. All cultured amoebae were collected by gently flushing cells from agar plates with filtered seawater, then concentrating cells by centrifugation (450 x g for 5 mins) then cells were added to tanks of salmon at a concentration of 3100 cells per L. Control groups included salmon exposed to freshly isolated and non-cultured *N. perurans* (also at a concentration of approximately 3100 cells /L) and salmon exposed to no amoebae. The amoebae (clone 4) which had been through the cryopreservation process were added to tanks at a concentration of 3100 cells per L, however as only 5% of these cells were viable based on neutral red inclusion the dose was 155 viable cell per L.

All trials were carried out concurrently in upgraded systems consisting of independent 280L recirculating tanks with external biofilters and protein skimmers. Each tank was stocked with 15 seawater acclimated Atlantic salmon and for initial exposure the water levels were dropped to approximately 80L and pumps turned off, after 2 hours water levels were returned to capacity and pumps turned on. As with previous trials any moribund or dead fish observed before the trials were terminated were removed and gills fixed for histology.

For all the infection trials described above the water was at 35 ppt salinity and the temperature maintained between 16-17ºC, water quality was monitored every 2-3 days and water exchanges performed as required.

Results/Discussion

Objective 1: To improve culture and preservation techniques of *Neoparamoeba perurans.*

Experiment 1: *Neoparamoeba perurans* culture at different temperatures

Data from the 2 growth trials are presented separately. In the first trial growth data show that the mean numbers of settled cells increased in all plates for the first 3 days (Figure 1A) but then began to decline in plates incubated at 18°C. The cell numbers generally stabilised at the lower temperatures, before the numbers declined in plates incubated at all temperatures after 8 days.

There was more variation in growth in trial 2 with growth rates initially higher at 14 and 18°C however cell numbers declined again after 8 days. Slight differences in growth seen between trials may relate to the different origin of inoculating cells used for each trial, but in both trials the same decline in cells numbers after 8 days was evident at all the incubation temperatures.

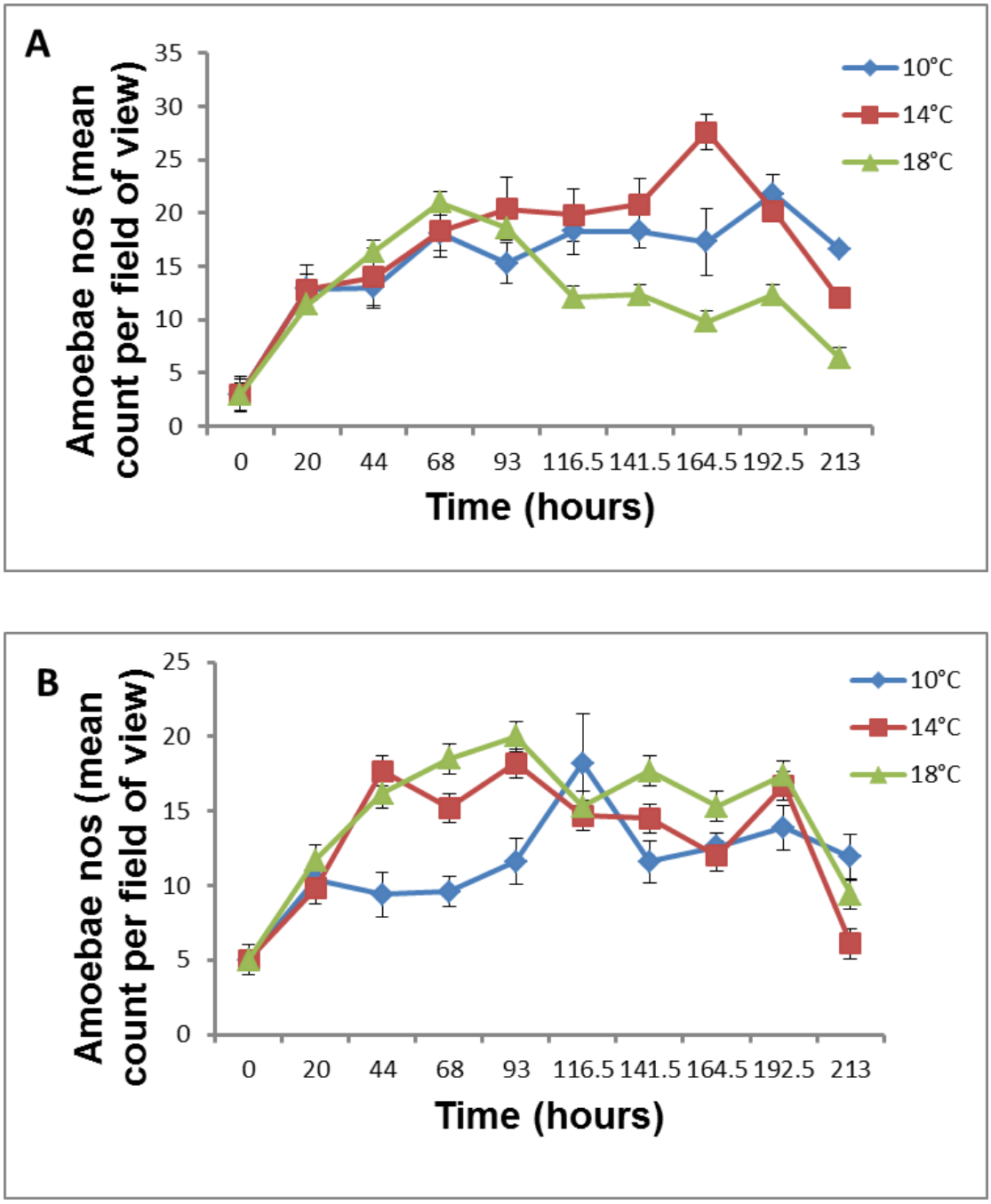

Figure 2. Growth data for *Neoparamoeba perurans* incubated at various temperatures during 2 trials (A and B). Count data are the means numbers (± S.E.) of settled cells from 5 fields of view from each of 2 agar plates at each time point.

However, aside from an increase in growth after 5 days in trial 2 it was noted that cell numbers were relatively more stable with fewer free floating cells and less contaminating organisms when incubated at 10°C compared to those incubated at the higher temperatures. These results suggest that incubating the amoebae at 10°C should extend the sub-culturing period to at least 10 days and possibly longer which in turn reduces the culture maintenance time

Experiment 2: Cryopreservation attempts for *Neoparamoeba perurans*

The majority of amoebae used in all cryopreservation attempts were viable at the beginning of cryopreservation procedures. Neutral red assays showed that viability varied between 88% and 95.8% for all batches used.

The first cryopreservation attempt using 7.5% DMSO as a cryoprotectant and 1 x 106 cells per mL was not successful. Cell viability post thaw was estimated at 5% based on the neutral red assay and these cells were used to challenge salmon in an infection experiment however, there was no histological evidence of AGD in these fish when the experiment was terminated (see following objective). While the cells inoculated onto MYA culture plates initially appeared to be normal in that the cell shape was irregular and pseudopods were evident, after 2- 3 days many of the cells had lysed or appeared to be round indicating that membrane integrity was compromised and that the cells had not survived the freezing or thawing process.

The next two attempts where either a combination of DMSO and glycerol (both at 6.5%) or glycerol solely at 10% were used as cryoprotectants were similarly unsuccessful with no viable cells seen after the neutral red assay or on MYA culture plates.

Experiment 3: Toxicity testing of cryoprotectants on *Neoparamoeba perurans*

Viability testing of amoebae after exposure to DMSO at 7.5% showed no loss of viability after 40 mins with amoebae survival ranging from 84 to 85.7% (Figure 3). However, amoebae were far less tolerant of the higher concentrations of DMSO with no survival seen after exposure to 15% DMSO and survival ranging from 8.5 to 11.7% at 10% DMSO. Exposure to glycerol at 10% showed diminishing viability the amoebae with survival dropping from 66-72% after 20-30 mins to 48% after 40 mins exposure. Also there was very low viability at the higher glycerol concentrations of 20 and 30% with only 5-6% survival after 20 mins exposure and no survival after 30 mins.

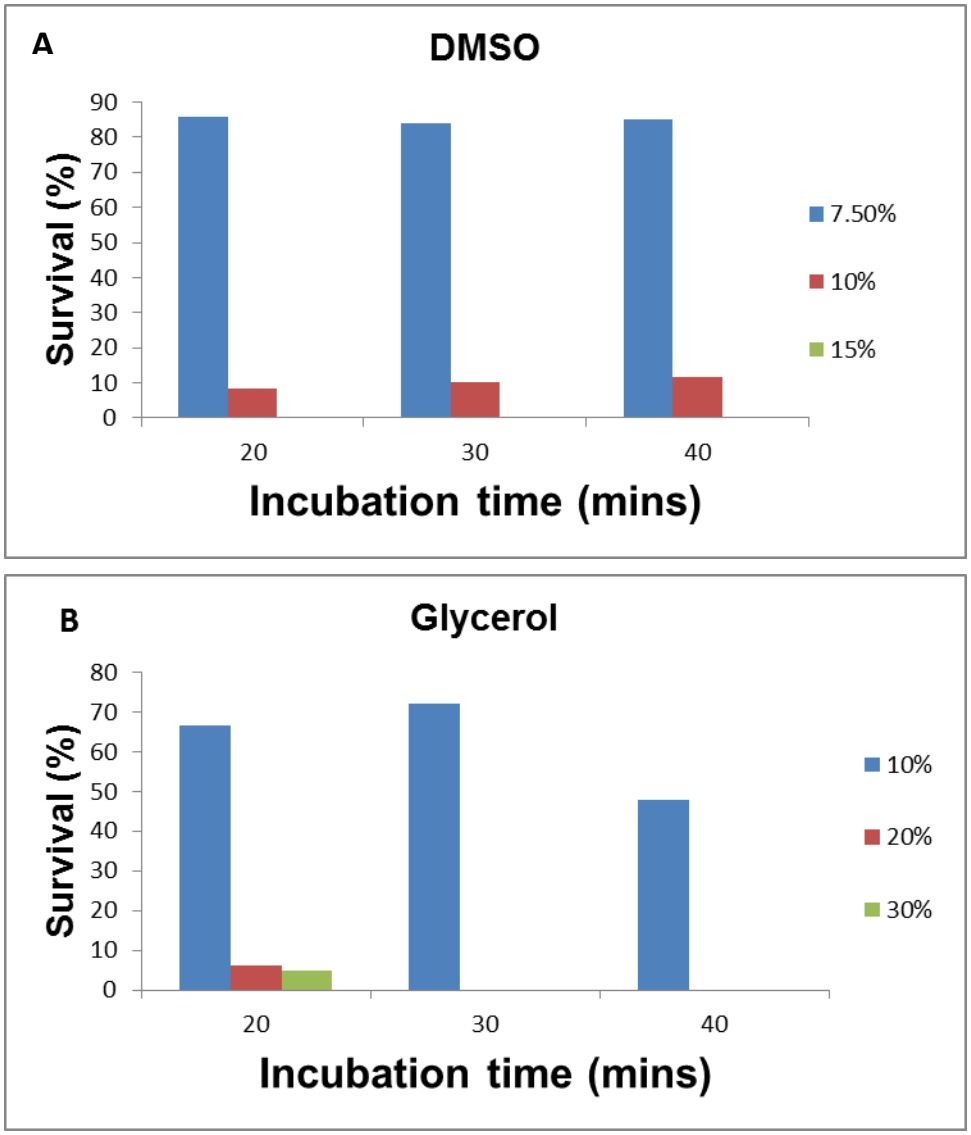

Figure 3. Survival of *Neoparamoeba perurans* based on a neutral red viability assay after exposure to various concentrations of either dimethyl sulphoxide (DMSO) (A) or glycerol (B) for 20, 30 and 40 mins.

Objective 2: To determine the effect of time in culture on viability and virulence of clones of *Neoparamoeba perurans*

Experiment 4: Infection trials with cultured *Neoparamoeba perurans*

In the first infection trial using clone 6 one fish was sampled 6 days post infection and although one attached amoeba was seen on an isolation plate there were no gross or histological signs of AGD. Fish sampled during the experiment and at the end of the trial, 38 days post infection when 9 of the 15 fish remained, similarly showed no histological signs of AGD nor were any amoebae seen on isolation plates. However, the positive control fish, which

were exposed to amoebae isolated from AGD-affected salmon had all succumbed to AGD within 14 days of being infected. The positive control fish had obvious severe gross signs of AGD and histology performed on a moribund fish confirmed the diagnosis. All the negative control fish not exposed to any amoebae showed no gross or histological evidence of AGD.

The results from this trial suggest that clone 6 had lost virulence. Although there was no neutral red viability assay performed on clone 6 prior to exposure to salmon the active attachment of the cells to Petri dishes during the harvesting process was also an indication of viability. So the trial was undertaken with viable amoebae. The fact that a single amoebae was recovered on a Petri dish from a salmon gill exposed to clone 6 after 6 days could be an incidental finding as it could expected that even non-virulent free-living cells would be present in the systems given that around 6000 per L were added to initiate the challenge. The rapid impact of AGD in the positive control group was not surprising given the infective dose used, which was much higher than the usual experimental dose of between 250-500 cells per L currently used (see Crosbie et al., 2010; Villavedra et. al., 2010; Adams et al., 2012). The same infective dose was used for each treatment group to allow for comparisons between onset of AGD and the dose was high because the trial was addressing the question of whether clone 6 can cause AGD within a reasonable time. Unfortunately soon after the trial this clone culture was lost and it was not possible to repeat the challenge to confirm loss of virulence in clone 6.

In the second infection trial where 12 salmon were exposed to clone 4 there were 6 fish remaining at the end of the trial (35 dpi) and small numbers of amoebae were isolated from all but 1 of these fish. However, histology showed that only 2 of these 6 fish were positive for AGD based on small numbers of lesions with associated amoebae (Figure 4).

Of the 6 fish that died and were sampled during the trial, 2 had amoebae associated with some pathology but some slides were difficult to interpret due to tissue degradation occurring between the time of death and gill fixation. Gill histology of the positive control group, exposed to amoebae isolated from the gills of AGD-affected fish, showed that the numbers of amoebae and severity of lesions appeared to be higher compared to those seen in clone 4 exposed fish. However, it is difficult to draw conclusions on comparative virulence due to the low numbers of fish that could be examined by histology because of the tissue degradation prior to fixation. Nevertheless from this trial it can be concluded that clone 4 was still virulent at the time of testing.

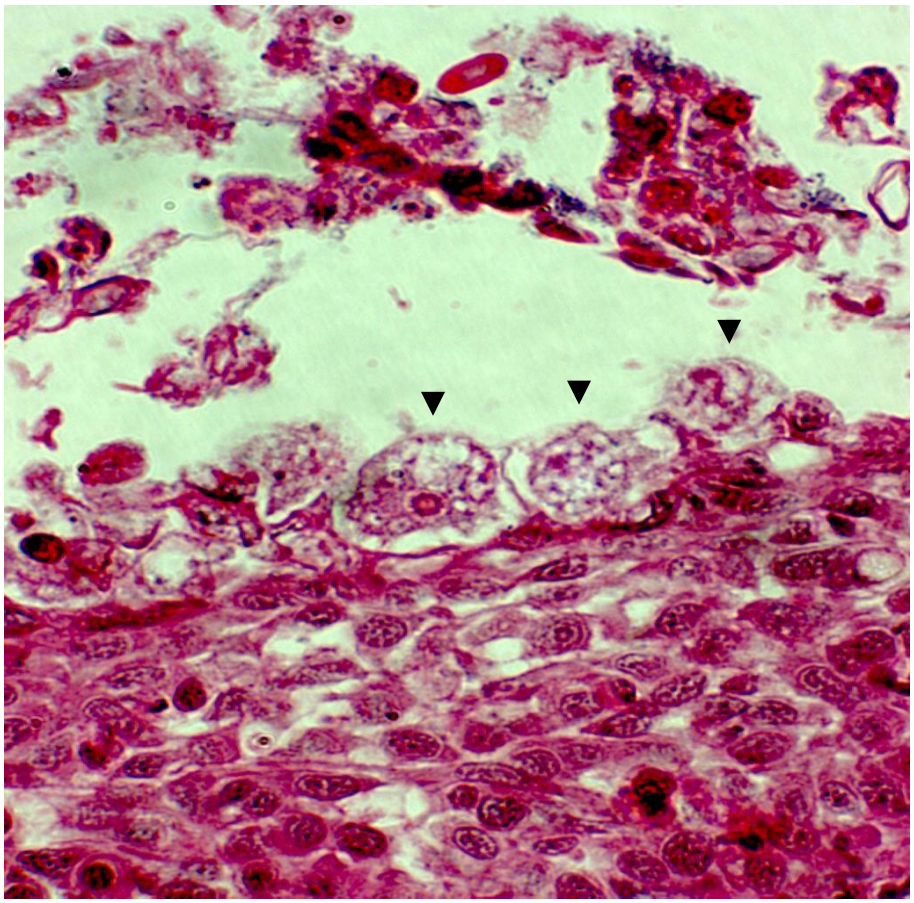

Figure 4: Typical AGD gill lesions produced from cultured *N. perurans* (clone 4). Arrows indicate amoebae trophozoites

The third trial assessed virulence of clone 4 after more than 2.5 years in culture and the results suggest there may be a loss of virulence. Of the 6 fish remaining at the end of the trial (47 dpi) there were no obvious gross lesions seen nor were any amoebae isolated from gill tissue. Similarly histological examination revealed no AGD lesions or associated amoebae. As with previous trials many of the samples taken from fish that died during the trial were difficult to interpret due to tissue degradation occurring before the gill could be fixed, however there was one fish that presented with AGD lesions and associated amoebae. The majority of positive control group of fish exposed to non-cultured amoebae isolated from AGD-affected fish succumbed to AGD, which was confirmed by histology, within 18 dpi. There were no lesions or amoebae seen on the negative control fish. The results from this trial suggest that there may be some loss of virulence of clone 4, although the results should be treated with caution because of the lack of fresh gill samples to examine histologically from the clone 4 exposed fish.

The infection trial with the most recently cultured isolate (clone 8), which has been in culture for 5 months, revealed that only 2 of 8 fish sampled showed histological evidence of AGD. These 2 fish were sampled at the end of the

trial at 33 dpi. Many fish had been dead for too long to be processed for histology and 4 fish that were examined 3 to 9 dpi were negative for AGD.

The final infection trial used clone 4 which been frozen and which appeared still to be viable after the cryopreservation process albeit at only 5%. None of these salmon showed any signs of AGD. This result suggests that either 5% viability of the infecting population was too low or that the viability test was misleading in that the cells died soon after being thawed as indicated by the culture results reported for Experiment 2(see above).

Benefits and Adoptions

This project directly benefits the industry by development of methods for culture of virulent *N.perurans*, and supply of virulent *N.perurans* for other AGD research during the project. In particular if the attenuation of virulence of *N. perurans* can be unequivocally demonstrated after 2.5 years in culture opportunities will exist to look specifically for virulence factors. Further research is needed before these results can be adopted by the industry.

Further Development

Results of this project have been widely disseminated throughout the salmon industry through industry meetings and email communications. Given that there was an indication that virulence may be at least partially attenuated in the cultured amoeba it is a future imperative to further investigate this observation. If shown to be avirulent cultured *N. perurans* will be a valuable resource that can be exploited for comparative studies with virulent members of the same species.

Planned Outcomes

This project is proactive and directly benefited salmon industry and salmon researchers. Amoebae were supplied for other projects such as:

- FRDC project 2011/070 “Atlantic Salmon Aquaculture Subprogram: Comparative susceptibility and host responses of endemic fishes and salmonids affected by amoebic gill disease in Tasmania”

- FRDC project 2011/071 Atlantic Salmon Aquaculture Subprogram: AGD resistance - learning from other species to bolster the natural Atlantic salmon response

- Seafood CRC project 2008/749 “Using the mucosal antibody response to recombinant Neoparamoeba perurans attachment proteins to design an experimental vaccine for amoebic gill disease”

Training in amoebae isolation, culture techniques and *in vitro* manipulation was provided to PhD students and Honours students who were working on AGD. Some of the results of this project were disseminated to other researchers and industry via an Australian Society for Parasitology workshop on parasitic diseases in fish mariculture hosted at the NCMCRS, University of Tasmania in February 2013.

Aspects of the culture of amoebae were also discussed with visitors from the Tasmanian and Scottish salmon industries in 2012 and 2013.

Conclusions

1. This project has shown that the culture of *N. perurans* can be improved by incubating culture plates at 10ºC. Training in amoebae culture techniques and isolation of amoebae from salmon gills has been provided to other researchers working other AGD-related project.

2. Although cryopreservation techniques have not resulted in viable cells post thaw, the toxicity of some cryoprotectant compounds used on *N. perurans* has been assessed and concentrations not detrimental to the amoebae identified. The cryopreservation process still requires further refinement.

3. Infectivity trials using clonal cultures of *N. perurans* were assessed for their ability to cause AGD and indicated some evidence of a loss of virulence after being in culture over 2.5 years. This requires verification but potentially provides an opportunity for comparative studies between infective and non-infective amoebae of the same species

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Appendix 1

INTELLECTUAL PROPERTY

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

1. Copyright in this report

Appendix 2

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