Final Report

FRDC Salmon Subprogram - Hydrogen peroxide treatment of Atlantic salmon affected by AGD

Barbara F. Nowak, Phil Crosbie, Mark Adams

FRDC PROJECT NUMBER: 2010/218

Australian Government

Fisheries Research and Development Corporation

FRDC Salmon Subprogram - Hydrogen peroxide treatment of Atlantic salmon affected by AGD

Barbara F. Nowak, Phil Crosbie, Mark Adams

Final report

FRDC PROJECT NUMBER: 2010/218

TSGA

AMC

AUSTRALIAN MARITIME COLLEGE

UTAS

ISBN 978-1-86295-616-2

Published by University of Tasmania, 2011

Table of Contents

2010/218 FRDC Salmon Subprogram - Hydrogen peroxide treatment of Atlantic salmon affected by AGD

PRINCIPAL INVESTIGATOR: Professor Barbara Nowak
ADDRESS:
NCMCRS
University of Tasmania
Locked Bag 1370
Launceston Tasmania 7250
Telephone: 03 63243814 Fax: 03 63243804

OBJECTIVES:

1. Determine effective in vitro treatment with hydrogen peroxide against *Neoparamoeba perurans*

2. Determine effective in vivo treatment against Amoebic Gill Disease

Non-Technical Summary

OUTCOMES ACHIEVED

This project has determined effective concentrations of hydrogen peroxide for treatment of Amoebic Gill Disease (AGD) and showed that the treatment was successful under laboratory conditions.

Treatment of AGD with hydrogen peroxide in sea water was successful under laboratory conditions. The effective concentrations of hydrogen peroxide that were effective *in vitro* were 1000 mg L^{-1} for an exposure time 10-20 minutes. Salmon treated with 1250 mg L^{-1} hydrogen peroxide at both temperatures (12°C and 18°C) displayed a variable clearance of amoebae from gill lesions. In approximately half of the fish, the lesions were completely cleared, while in the majority of the remaining fish only a few amoebae remained on the gills after treatment. These results are promising and suggest that hydrogen peroxide treatment could be useful to treat AGD. Elevated blood plasma osmolality suggest that further research is needed to make sure that any physiological changes are not detrimental to long-term performance. Not all in vitro results could be easily extrapolated to in vivo results. For example, the results from gill explants study were not in agreement with the in vivo results. This suggests that caution is needed when extrapolating from in vitro (in particular from explants) results in AGD studies.

KEYWORDS:
Atlantic salmon, AGD, treatment, hydrogen peroxide, parasitic disease

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. We thank Victoria Valdenegro for doing statistical analyses and preparing graphs.

Background

Amoebic Gill Disease (AGD) is one of the most serious health issues for the Tasmanian salmonid farming industry and significantly increases production costs of Atlantic salmon in Tasmania (Nowak et al 2002). While freshwater bathing has been used successfully to control losses, the shortage of fresh water in some salmon farming areas has resulted in a search for another treatment. Hydrogen peroxide is used overseas to control sealice infections of Atlantic salmon (Bruno 1992, Thomassen 1993) and to control fluke infections in kingfish industry in Australia (Mansell et al 2005). Hydrogen peroxide was effective in vitro as a treatment against amoebae (Howard and Carson 1994), however it appeared to have low safety margin to Atlantic salmon in sea water (Cameron 1993, Cameron 1994). Toxicity of hydrogen peroxide to Atlantic salmon increases with the treatment concentration, treatment time and temperature (Johnson et al 1993). Toxicity of peroxide depends on the time of exposure (Johnson et al 1993; Treasurer et al 2000). Twenty minute treatments at 14°C with 1500 g L^{-1} hydrogen peroxide resulted in 7.7% mortality in Atlantic salmon while 100% mortality was reported at 18 °C (Johnson et al., 1993; Thomassen 1993). Treatments at lower temperature and for no longer than 20 min reduced toxicity (Treasurer and Grant 1997). It has been suggested that it may be possible to develop a successful hydrogen peroxide treatment against AGD, reducing the reliance of the industry on fresh water. However, mortalities were observed after bathing Atlantic salmon in 1000 mg L^{-1} or 1500 mg L^{-1} of hydrogen peroxide, in either freshwater or sea water bath (Powell et al 2005). These treatments were tested at 10°C and 15°C and 40 or 60 minutes bath times. Salmon industry was interested in testing efficacy of shorter bath times. Hydrogen peroxide manufacturer (Solvey Interox) recommended a concentration of 1500 mg L^{-1} for a20 min treatment at temperature range from 8 to 12 °C (Bravo et al 2011).

Need

Amoebic Gill Disease (AGD) significantly contributes to Atlantic salmon production costs in Tasmania. While freshwater bathing can control this disease, that method requires access to fresh water, which limits the sites where salmon can be farmed to those with practical access to freshwater. There is an urgent need to develop alternative treatments against AGD to allow further expansion of the Tasmanian salmon industry.

Objectives

1. Determine effective in vitro treatment with hydrogen peroxide against *Neoparamoeba perurans*

2. Determine effective in vivo treatment against Amoebic Gill Disease

Methods

Determination of effective in vitro treatment with hydrogen peroxide against *Neoparamoeba perurans*

Neoparamoeba perurans trophozoites were collected from moribund AGD-affected salmon using the method of Morrison et al. (2004). After isolation suspensions of amoebae were centrifuged at 500 x *g* for 5 min at 4°C then resuspended in 5 mL of 0.2 µm filtered sea water. Cells were then enumerated and placed into individual wells of 24-well cell culture plates at approximately 3000 cells per well in 1 mL volumes. A subsample of cells was retained for later confirmation of amoebae identity by PCR. Plates were then incubated at room temperature for 30 mins to allow amoebae to attach to the bottom of the wells then all sea water was removed and replaced with test concentrations of H_2O_2 in sea water or distilled water in 1 mL volumes for the prescribed exposure times, control wells included attached amoebae with sea water only. Hydrogen peroxide concentrations were 500, 1000 and 1500 mg L^{-1} with exposure times of 10, 20, 30 and 60 mins and incubation temperatures of 12 and 18°C. The same exposure times and incubation temperatures were used for distilled water. For each H_2O_2 concentration (or distilled water) and incubation temperature there was 1 assay plate and 4 replicate wells for each exposure time (see Fig 1).

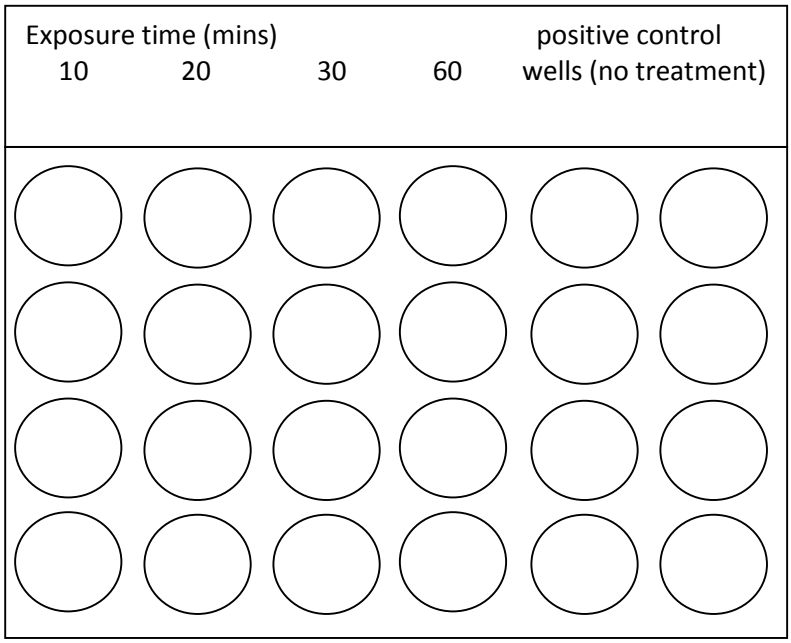

Figure 1 Plate configuration for *in vitro* testing of 3 concentrations of H_2O_2 (500, 1000, and 1500 mg L^{-1}), distilled water and positive control (sea water) in 1 mL volumes for amoebicidal activity against *Neoparamoeba perurans* with 4 replicate wells for each exposure time.

Immediately after each exposure period all the sea water, containing the H_2O_2 and many detached amoebae, was removed from all 4 replicate wells (i.e. 4 mL in total) and pooled into a single 15 mL centrifuge tube with an additional 10 mL of filtered sea water. Many amoebae remained in the wells after removal of the sea water and these wells were replenished with sea water to assess potential recovery after 24h. Collected amoebae were centrifuged as above and most of the sea water containing H_2O_2 was removed leaving cells suspended in approximately 100 µL volume of sea water. Cells were then immediately assessed for viability (see below).

Amoebae from 2 of the positive control wells were collected after the final exposure time of 60 mins, detached from the wells with trypsin/EDTA, rinsed in filtered sea water then concentrated by centrifugation at 500 x *g* for 5 min at 4°C and assessed for viability. Amoebae viability was determined by the inclusion of a vital dye (Neutral Red, Sigma). For this test 50 µL of amoebae suspension were added to 50 µL of Neutral Red solution (50 µg mL^{-1} in phosphate buffered saline) in 1 mL microcentrifuge tubes and incubated at room temperature for at least 25 mins. Excess dye was removed when tubes were filled with filtered sea water and centrifuged at 14000 x *g* for 10 seconds; most of the supernatant was removed apart from 20-30 µL which contained the amoebae. The percentage of viable cells was then determined for all groups of amoebae on microscopic examination using a haemocytometer and 10 µL of the cell suspensions. Cells were judged to be viable if they showed the dye internalised (see Fig 2).

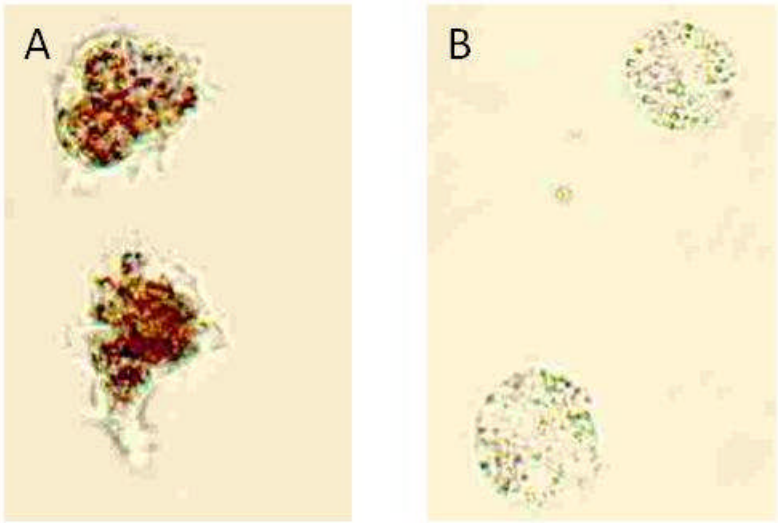

Figure 2 Amoebae after neutral red viability assay. A. Live cells showing pseudopodia and the neutral red dye internalised into vacuoles. B. Dead cells with spherical shape, no pseudopodia or dye inclusions.

To assess survival of amoebae after exposure to H_2O_2 those cells which remained in the wells were replenished with filtered sea water, together with amoebae in remaining positive control wells, were collected after a 24h recovery period and tested for viability as described above. The same methods were used to test the amoebicidal effect of distilled water on *N. perurans.*

To assess the effect of mucus on the sensitivity of amoebae, the effect of H_2O_2 on amoebae in gill explants was observed. An entire gill was removed from an AGD-affected salmon and dissected into individual arches. Four of the arches were suspended in a 1 L beaker with H_2O_2 at 500 mg L^{-1} in sea water and 4 were suspended in sea water only. At 10, 20, 30 and 60 mins 1 gill arch was removed from each beaker and placed into a 50 mL tube with filtered sea water, gently agitated then poured into a petri dish and periodic observations made to see if any amoebae attached to the surface of the petri dish.

Determination of effective in vivo treatment against Amoebic Gill Disease

Pre-treatment husbandry

During late September 2010, approximately 320 hatchery reared (Huon Aquaculture Company, Russell river) Atlantic salmon smolts (140 g) were transferred to the Aquaculture Centre at the University of Tasmania (Newnham campus). Fish were housed in a 4000 L recirculating system equipped with mechanical filtration, bio-filtration, foam fractionation and UV disinfection. Water was exchanged at approximately 10%/day. The fish were acclimated to sea water (35 g L^{-1} NaCl) over a three week period and the temperature slowly increased to 15^{o}C ± 0.5^{o}C). Following acclimation the fish were exposed to *Neoparamoeba perurans* (250 trophozoites L^{-1}). After two weeks it was noted that an ulcerative dermatitis was beginning to establish within the population. Fish were therefore treated in a freshwater bath for 24 hours and held at a salinity of 20 g L^{-1} NaCl for a further two weeks. Following this treatment the salinity was raised to 35 g L^{-1} NaCl and the fish were exposed to *N. perurans* (358 trophozoites L^{-1}) for 21 days. No further health problems were observed. Water quality during the acclimation and infection periods was maintained at > 90% dissolved oxygen, < 1 mg L^{-1} TA-N, < 5 mg L^{-1} NO_2^-, < 40 mg L^{-1} NO_3^{2-} and pH 8.0 – 8.2.

Treatment

Following gross diagnosis of AGD the population was sequentially separated into four groups of approximately 70 fish. Two temperatures (12 and 18^{o}C) were selected for hydrogen peroxide (H_2O_2) exposure for 15 minutes at a nominal concentration of 1250 mg L^{-1}. The appropriate concentration was determined from a combination of in vitro observations of H_2O_2 toxicity to *N. perurans* and a pilot experiment to confirm a safe threshold for treatment (as described in previous publications). The pilot experiment exposed three groups of 10 salmon (150g) to concentrations of 500, 1000 and 1500 mg L^1 H_2O_2 for 15 minutes and monitored recovery over a 24 hour period. Bath stocking density was 31 kg m^{-3}. Oxygen saturation in the freshwater treatments was 93% and 120% for the peroxide baths. Survival was 100% for 500 and 1000 mg L^{-1} and 90% for 1250 mg L^{-1}. To ascertain the efficacy of the H_2O_2 exposure upon AGD, each treatment was compared to a freshwater bath (using dam water obtained from Hideaway bay) at the corresponding temperatures for 3 h.

Sampling
Following the initial 21 day infection period, 10 fish were removed for gross gill inspection to confirm the gross appearance of AGD. These fish were anaesthetized, measured, assessed for any gross abnormalities, bled, and the gills removed and fixed (SW Davidson's fixative) for histology (2^{nd} left anterior hemibranch) and image analysis (2^{nd} & 3^{rd} left holobranchs). Following bath treatment, all fish were transferred to another recirculating system comprised of 10 x 300 L Reln tanks. Each treatment group was split into duplicate tanks within this system occupying 8 tanks in total. A further sentinel group of 34 smolts, naive to AGD, were introduced to the remaining two tanks. Fish from all tanks were then sampled (n=10) as above at 7, 14 and 21 days after treatment. Water quality was maintained at > 90% dissolved oxygen, < 1 mg L^{-1} TA-N, < 5 mg L^{-1} NO_2^-, < 40 mg L^{-1} NO_3^{2-}, pH 8.0 – 8.2 and temperature of 15^oC ± 0.5^oC.

Histology
Histological analysis was undertaken to determine the percentage of filaments displaying hyperplastic lesions indicative of AGD. For each lesion counted it was noted whether trophozoites of *N. perurans* were present or absent. To determine the overall severity of AGD present within the population prior to treatment the total lesion count for fish pre-bath and post-bath were averaged.

Osmolality
Blood was centrifuged and blood plasma separated and stored at -80°C until analyses. Blood plasma osmolality was obtained using a Vapro© Model 5520 vapour pressure osmometer (Wescor Inc., Logan, Utah, USA). Results were expressed as mmol.kg^{-1}.

Statistical analyses
All comparisons among treatments were performed using a one-way ANOVA analysis and a Tuckey's HSD post- hoc test with the software SPSS 17.0. Since the data violated some assumptions of the ANOVA tests (normality and homogeneity of variances), all the data were analysed after being transformed by square root, but graphs are presented with raw data.

Results/Discussion

Determination of effective in vitro treatment with hydrogen peroxide against *Neoparamoeba perurans*

Exposure to hydrogen peroxide

Observation of the wells after 10 mins showed that the majority of amoebae exposed to all concentrations of d H_2O_2 detached from the surface of all wells except those in the positive controls. Survival of some amoebae was seen immediately after exposure to all concentrations of H_2O_2 tested, although at the higher concentrations it was <10% at 18^{o}C incubation temperature and less than 20% at 12^{o}C (Fig 3). It is interesting to note that survival after exposure to H_2O_2 at 500 mg L^{-1} was zero after 30 mins at 12^{o}C but around 40% after 30 mins at 18^{o}C. It is unclear why this occurred and may be due to variation amongst the populations of amoebae used for the assay. There was also some variation noted in the higher concentrations although not as pronounced.

There was also some survival after a 24 h recovery period in the cells that remained in the wells after the H_2O_2 was removed and replaced with filtered sea water. Around 15% of cells that were initially exposed to H_2O_2 at 500 mg L^{-1} for 10 mins survived, which was reduced to around 5% after 20 mins initial exposure at 12^{o}C. There was no amoebae survival 24 h after initial exposures to the H_2O_2.

These results suggest that effective concentrations of H_2O_2 that are worth testing *in vivo* on AGD-affected fish should be greater than 1000 mg L^{-1} and that exposure times should be 10-20 minutes.

Exposure to fresh water

Observations after exposure to fresh water at both temperatures showed that many cells detached, appeared spherical and floated after 10 mins but many cells also remained attached and looked no different to the positive controls. When detached cells were tested for viability very few were observed microscopically and no cells were seen after 60 mins exposure even though there were many cells detached and collected for the viability test, therefore survival curves similar to those after H_2O_2 exposure cannot be constructed. It is thought that the process of viability testing involving 2 centrifuge steps exerted enough pressure for the cells to lyse. However some cells remained attached after all exposure times to fresh water at both 12 and 18^{o}C and after 24 h recovery period the majority of these cells were viable (Fig 4).

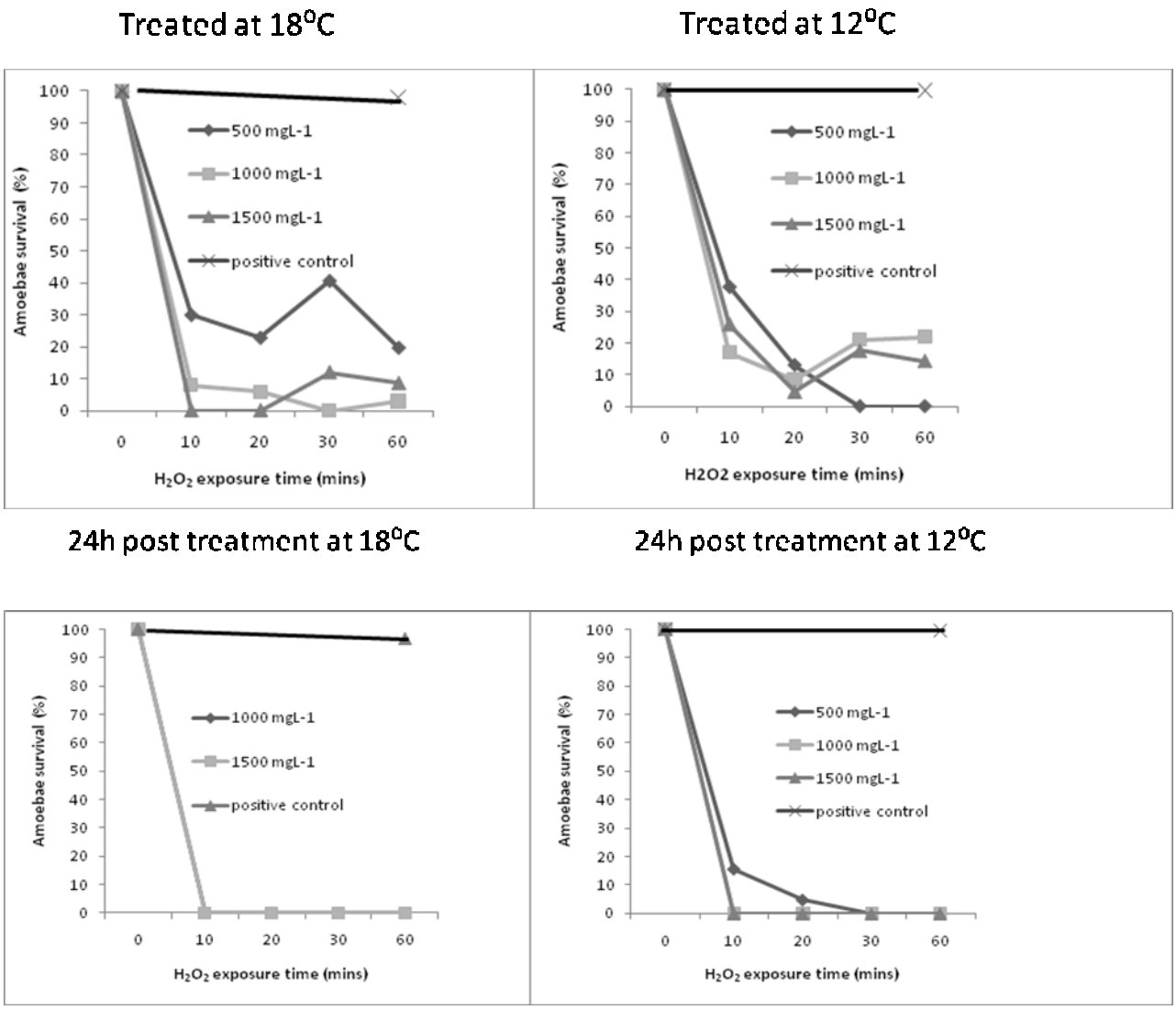

Figure 3 Survival curves for *N. perurans* exposed to H_2O_2 for various times and tested for viability immediately after exposure and 24h after exposure.

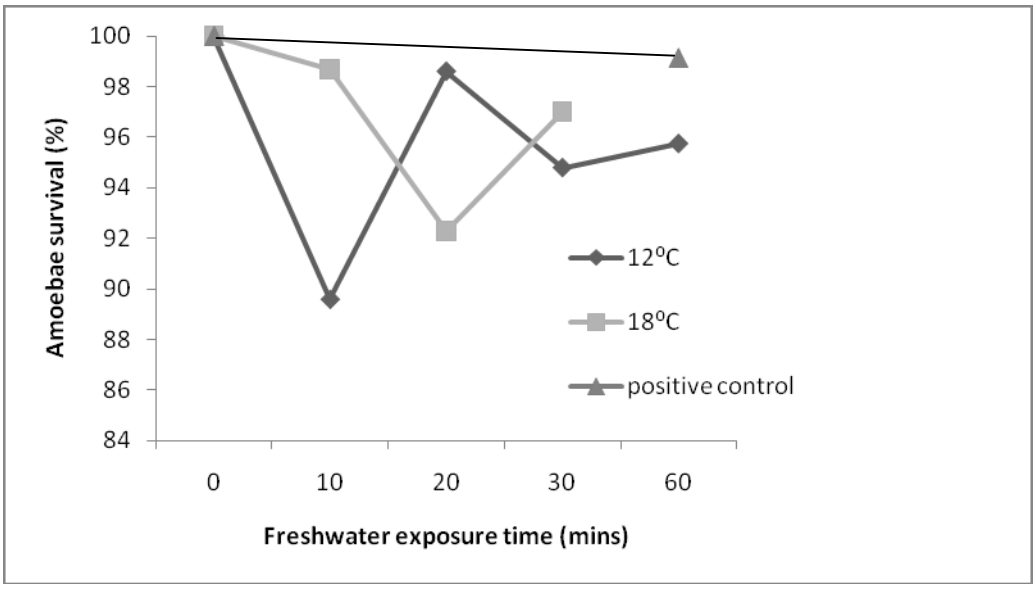

Figure 4 Survival curves for *N. perurans* exposed to freshwater at 12 and 18^0C for various times and tested for viability 24h after exposure.

Determination of effective in vivo treatment against Amoebic Gill Disease

Salmon displayed gross signs indicative of AGD prior to treatment; this was confirmed histologically (Figure 5). The intensity of disease was found to be mild in a clinical sense (18% filaments with lesions) and quite variable at the individual level (0% - 68%).

Histological observation of salmon treated with freshwater at both temperatures (pH ≈ 8, hardness ≈ 70 mg L^{-1} $CaCO_3$) showed a complete clearance of amoebae and host tissue debris from hyperplastic gill tissues (Figure 6 & 7). Resolution of AGD was evident by 7 days post-treatment (DPT) and AGD was not noted upon the gills for the duration of the re-infection period (21DPT).

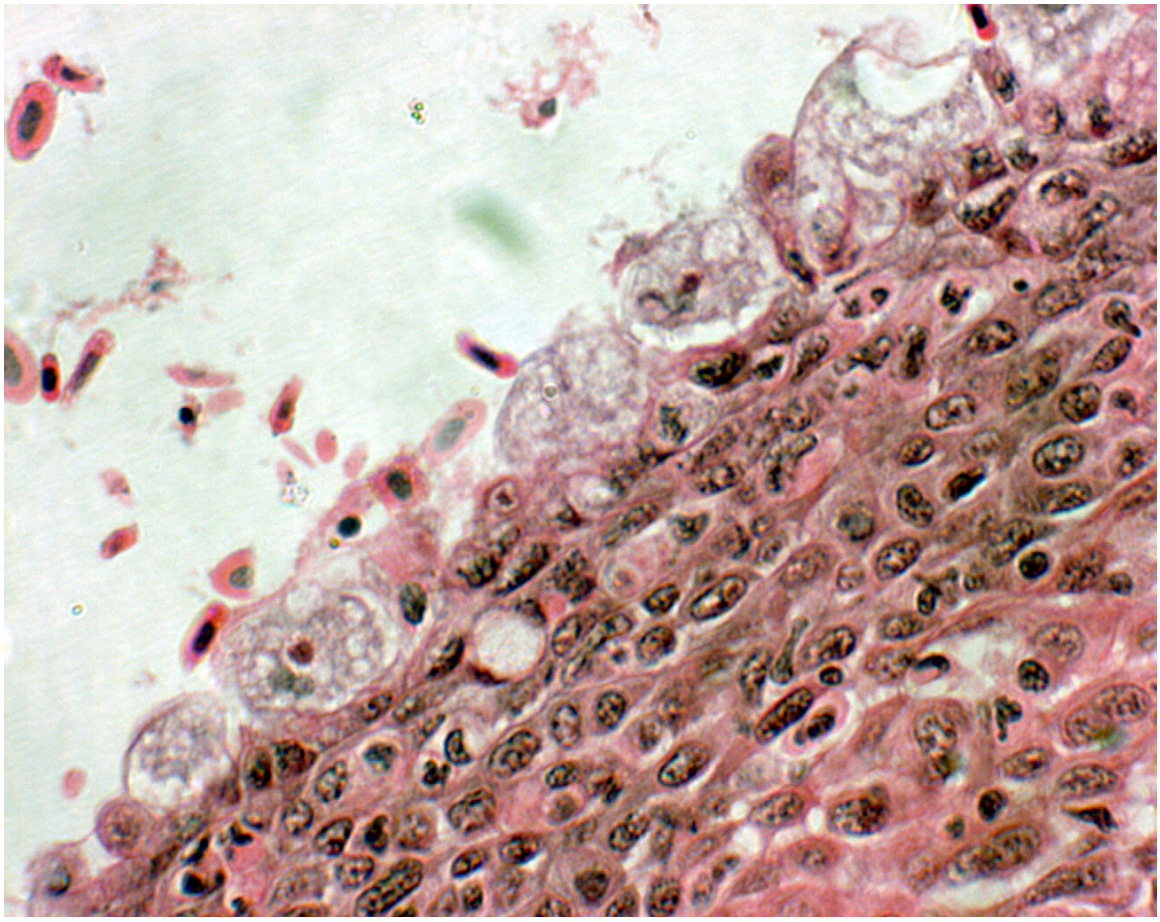

Figure 5 Amoebae present on the surface of hyperplastic lesion prior to treatment.

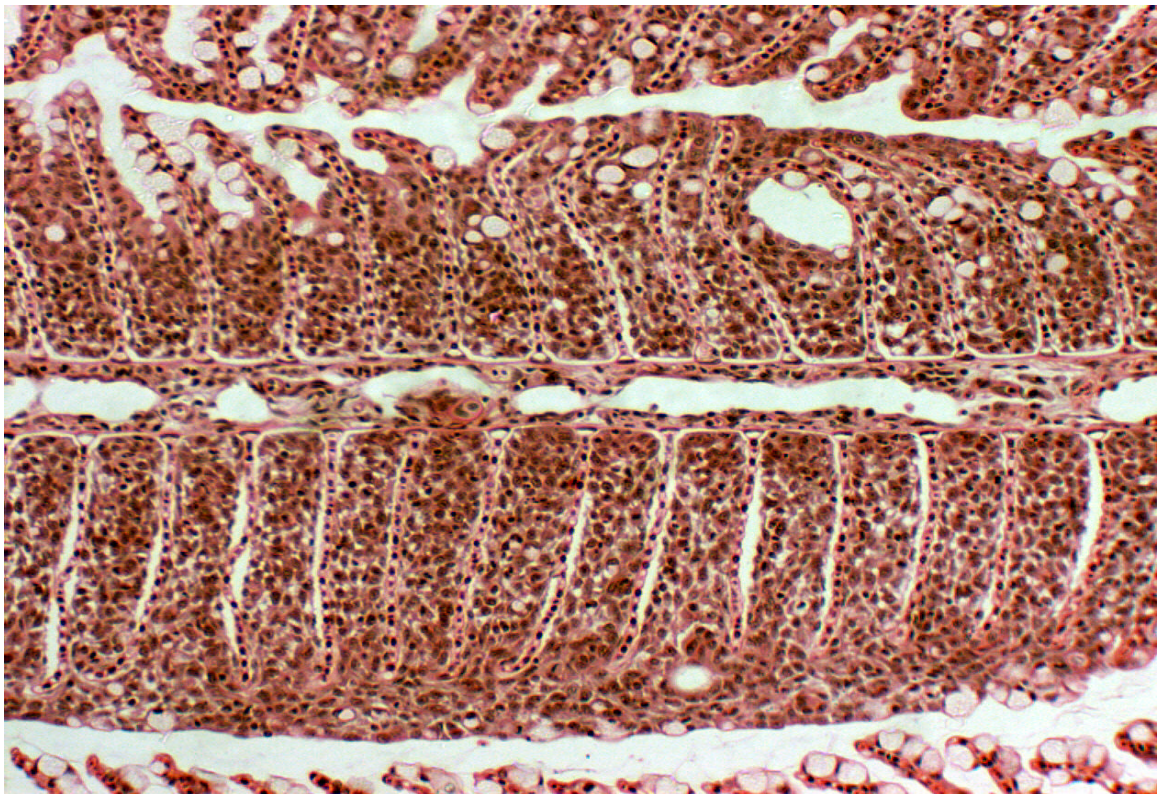

Figure 6 Hyperplastic lesions devoid of amoeboid cells after freshwater treatment.

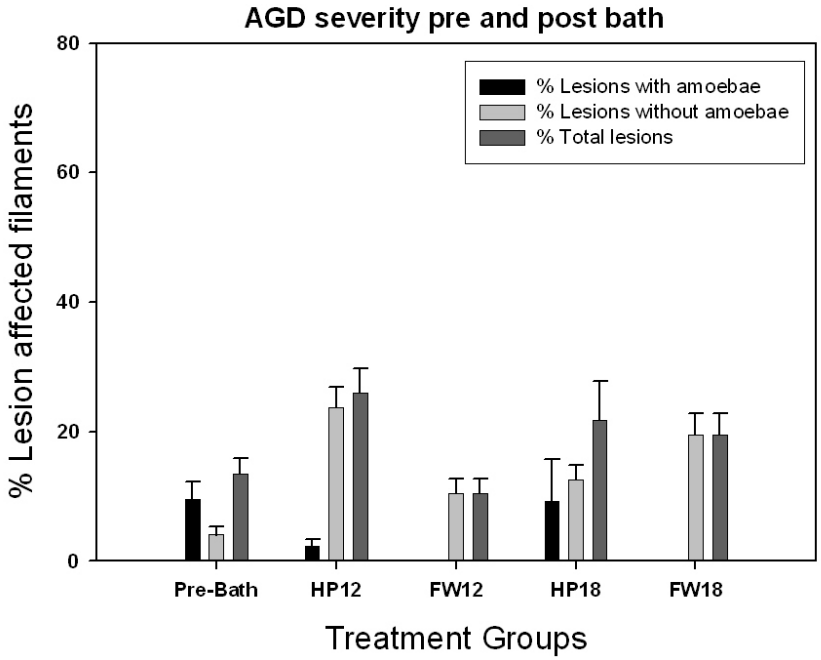

Figure 7 Comparison of hyperplastic lesions present amongst treatment groups and the proportion of lesions within each group with attached amoebae. Samples were collected immediately pre and post-bath (HP12 & HP18 correspond to hydrogen peroxide treated fish at 12°C and 18°C, FW12 & FW18 correspond to fish treated with freshwater at 12°C and 18°C).

Salmon treated with H_2O_2 at both temperatures displayed a variable clearance of amoebae from gill lesions. In approximately 50% of fish (from both H_2O_2 treatments), the lesions in view were completely cleared (Figure 7). In the majority of the remaining fish only a few amoebae remained. Such trophozoites were seen retracted/shrunken from attachment sites juxtaposed to hyperplastic tissue (Figure 8).

Two fish with the highest lesion numbers (18°C H_2O_2) showed amoebae present within inter-filament spaces and upon the face of some lesions. Often these amoebae appeared to be morphologically normal. Host cells (mainly macrophages), tissue debris and mucus were often present in association with amoeboid cells. A variable degree of oedema was noted upon approximately 50% of H_2O_2 treated fish at both temperatures an observation not as frequently noted upon the freshwater treated groups.

Although amoebae were present upon the gills of H_2O_2 treated fish immediately after bathing, gills from fish examined histologically at 7, 14 & 21 DPT were all clear of AGD. A consistent finding amongst all treatment groups (FW and H_2O_2) was the presence of resolving hyperplastic lesions at 7 -14 DPT and low numbers of lymphocytic nodules from 7 -21 DPT (Figure 9). Lamellar clubbing, thickening and fusion of lamellae tips were also common across all treatments.

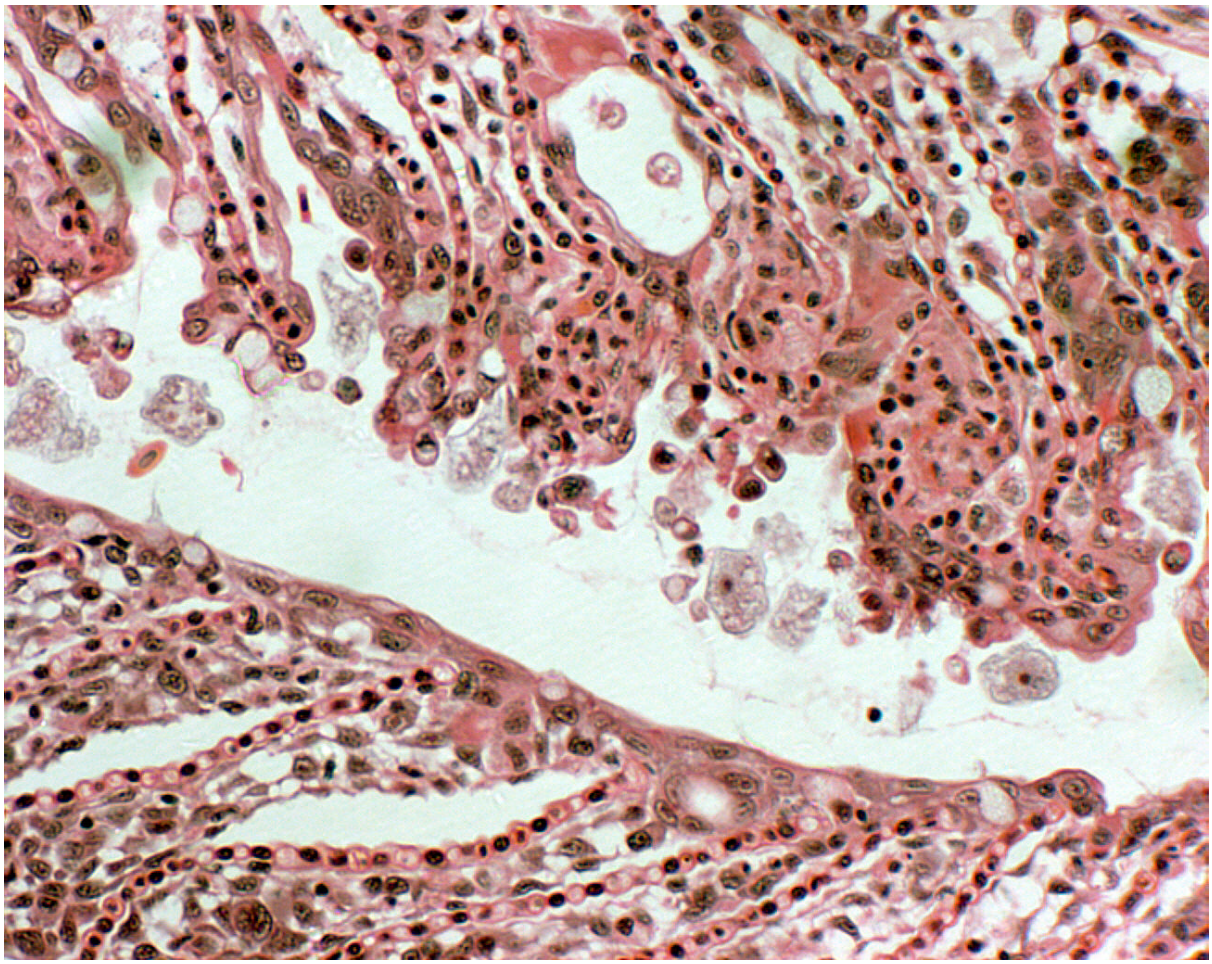

Figure 8 Hyperplastic lesion showing amoebae disassociated from nearby hyperplastic tissue from a fish treated with H_2O_2 at 12°C.

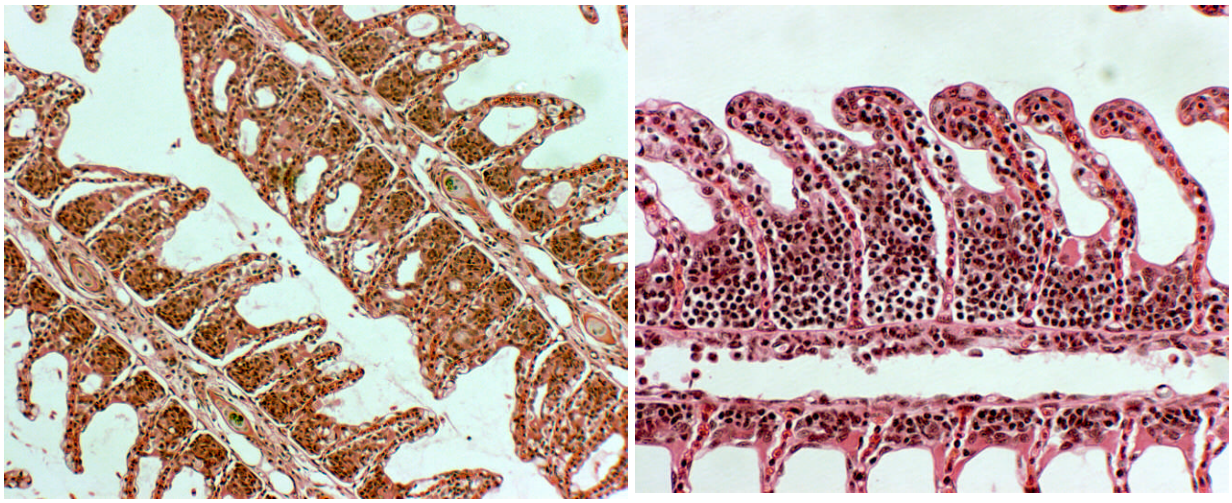

Figure 9 Residual AGD lesions seen on gills 7 days after treatment with H_2O_2 (on the left). Such lesions were common on both FW and H_2O_2 treated fish at both temperatures. Nodules containing lymphocyte-like cells were common at low intensity upon fish from all groups from 7 -21 DPT (on the right).

The average percentage of gill filaments of Atlantic salmon with lesions and amoebae was significantly different among treatments (F=6.314, df 4, 45, P<0.001). Gills of fish treated with fresh water both at 12°C and at 18°C showed no filaments with lesions that had the amoebae present and the percentage of these filaments with lesions observed was significantly lower than the percentage observed in the gills of fish before bath treatment. Fish treated with hydrogen peroxide did not showed any statistical difference with either those groups treated with freshwater or to the pre-bath time in the percentage of gill filaments with lesions positive to the amoeba (Fig 10).

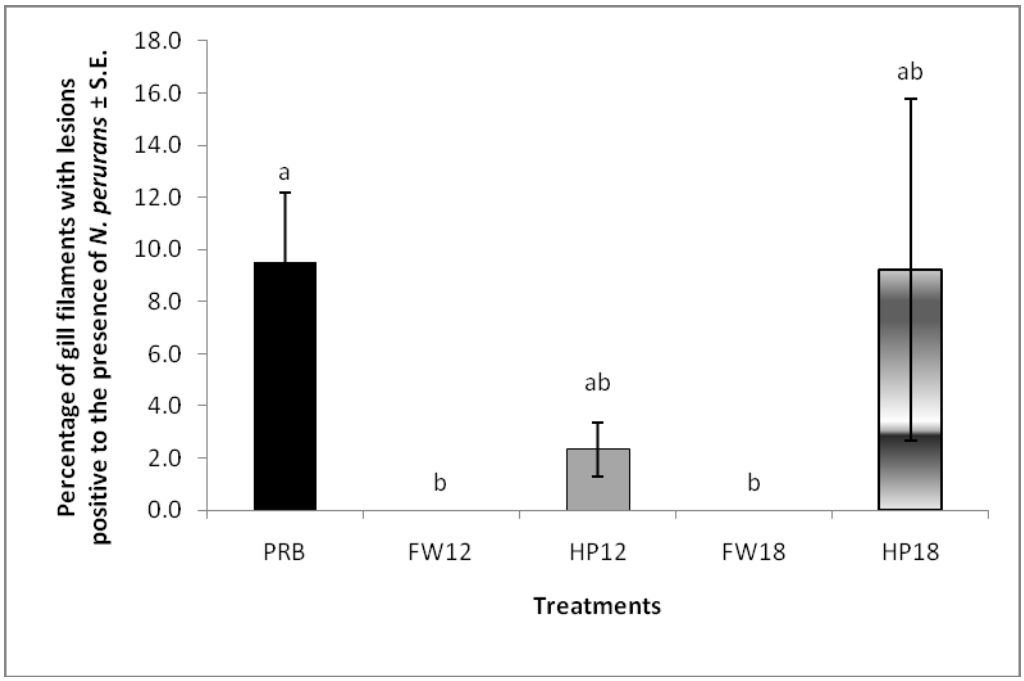

Figure 10 Average percentage ± standard errors (S.E.) of gill filaments with lesions positive to the presence of *Neoparamoeba perurans* in Atlantic salmon (*Salmo salar*). The gill filaments were assessed before bath treatment (PRB) or after treatment with either fresh water at 12°C (FW12) or at 18°C (FW18) or with hydrogen peroxide at 12°C (HP12) or at 18°C (HP18). Averages with different letters are significantly different from one another by one-way ANOVA.

There was a significant difference in the average percentage of gill filaments of Atlantic salmon that showed lesions with no *N. perurans* (F=9.804, df 4, 45, P<0.001). Salmon treated with hydrogen peroxide at 12°C showed a significantly greater percentage of gill filaments with lesions without amoebae than those salmon treated with fresh water at the same temperature. The percentage of gill filaments with lesion negative to amoebae was not different between salmon treated with fresh water and hydrogen peroxide at 18°C and these two treatments did not differ from the other two treatments – freshwater and hydrogen peroxide at 12°C- in the percentage of gill filaments affected with no amoebae. Pre-bath fish showed differences in the percentage of gill filaments with lesions without amoebae with three treatment groups, but did

not differ of the findings in those fish treated with hydrogen peroxide at 12°C (Fig 11).

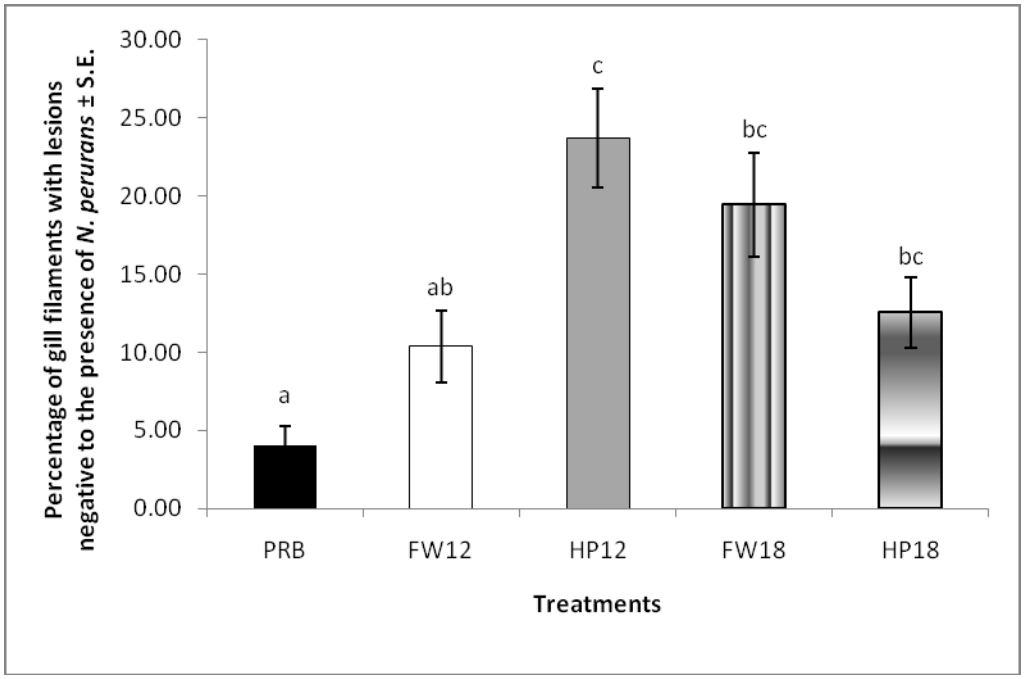

Figure 11 Average percentage ± standard errors (S.E.) of gill filaments with lesions negative to the presence of *Neoparamoeba perurans* in Atlantic salmon (*Salmo salar*). The gill filaments were assessed before bath treatment (PRB) or after treatment with either fresh water at 12°C (FW12) or at 18°C (FW18) or with hydrogen peroxide at 12°C (HP12) or at 18°C (HP18). Averages with different letters are significantly different from one another by one-way ANOVA.

The percentage of gill filaments showing lesion with or without the presence of the amoebae was significantly different between treatments ($F=3.11$, df 4, 45, $P=0.024$). The group of fish bathed with hydrogen peroxide at 12°C showed the largest percentage of gill filaments with lesions, however this percentage was not significantly different from that observed in fish before being bathed or in those salmon treated with hydrogen peroxide and fresh water at 18°C. Salmon bathed with freshwater at 12°C showed the lowest average percentage of gill filaments with lesions (Fig 12).

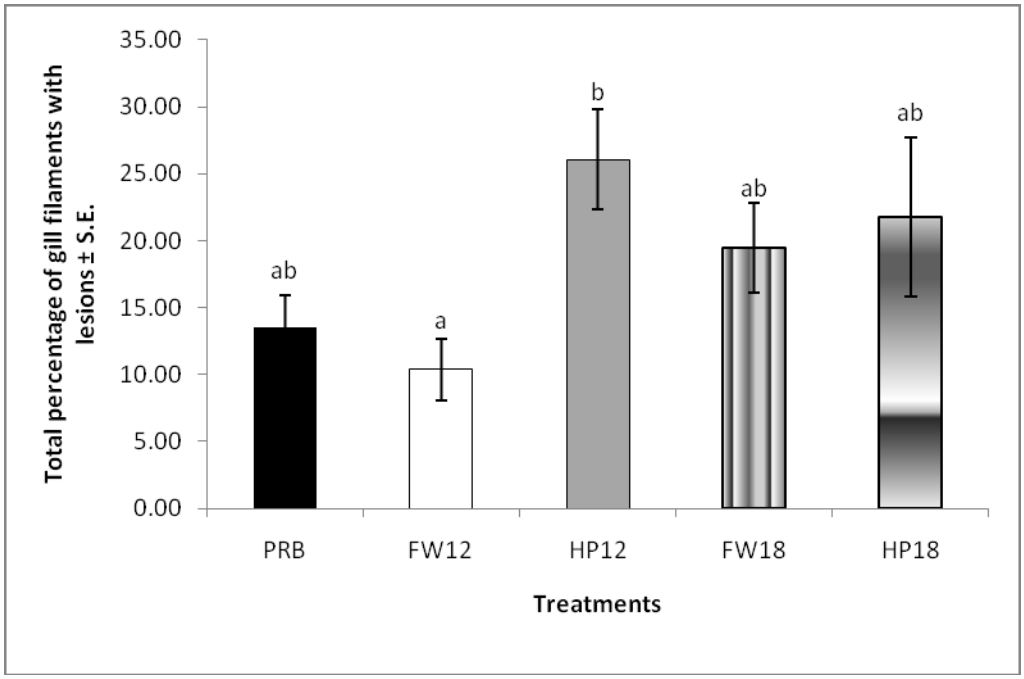

Figure 12 Average percentage ± standard errors (S.E.) of gill filaments with lesions both positive and negative to the presence of *Neoparamoeba perurans* in Atlantic salmon (*Salmo salar*). The gill filaments were assessed before bath treatment (PRB) or after treatment with either fresh water at 12^{o}C (FW12) or at 18^{o}C (FW18) or with hydrogen peroxide at 12^{o}C (HP12) or at 18^{o}C (HP18). Averages with different letters are significantly different from one another by one-way ANOVA.

The average plasma osmolality of Atlantic salmon varied significantly depending on the treatment ($F=12.850$, df 4, 45, $P<0.001$). Fish bathed with freshwater at 12^{o}C and at 18^{o}C showed the lowest mean osmolality in plasma with values ranging between 335 to 343 mmol/kg, which was not statistically different from the average plasma osmolality values observed before the fish were bath treated. Salmon treated with hydrogen peroxide both at 12^{o}C and at 18^{o}C showed mean plasma osmolality values larger than 378 mmol/kg which were significantly higher than values observed in any other group (Fig 13). This elevated blood plasma osmolality indicated osmoregulatory stress, possibly due to gill damage resulting from the exposure to hydrogen peroxide (Kiemer and Black 1997, Powell et al 2005). This potential side effect and other side effects should be evaluated further.

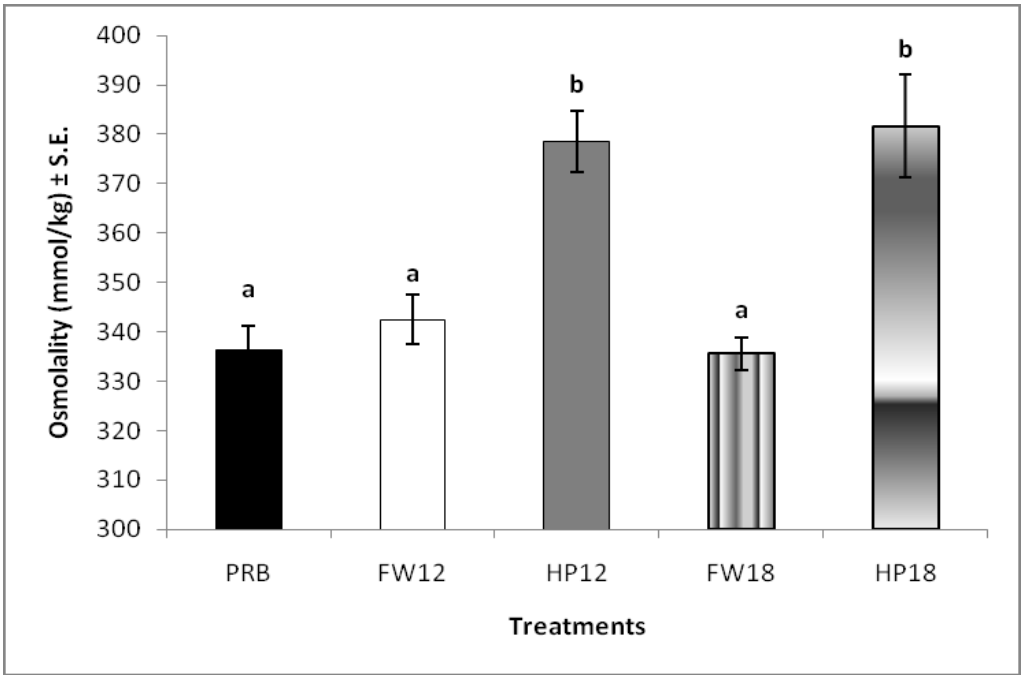

Figure 13 Average plasma osmolality ± standard errors (S.E.) of Atlantic salmon (*Salmo salar*). The plasma samples were obtained from fish before bath treatment (PRB) or after treatment with either fresh water at 12^{o}C (FW12) or at 18^{o}C (FW18) or with hydrogen peroxide at 12^{o}C (HP12) or at 18^{o}C (HP18). Averages with different letters are significantly different from one another by one-way ANOVA.

There was some morbidity post treatment (Table 1), however the mortality in the hydrogen peroxide treatment (cumulative 7.1% - 9.5%) was similar to the mortality after the dam water treatment (cumulative 1.4% - 6.5%).

Table 1 Morbidity post-treatment for AGD (percentage of fish in the treatment)

Treatment	Temp ^{o}C	% 24 h PT*	% Day 2 - 7	% Day 7 - 14	% Day 14 - 21
Dam water	12	0	1.4	0	0
Dam water	18	0	5	1.5	0
Peroxide	12	5.0	1.5	3	0
Peroxide	18	4.2	2.9	0	0

*Post-treatment

Sea water used for hydrogen peroxide treatment may differ in its organic load, which can reduce hydrogen peroxide concentrations and make the treatment ineffective. That means that measured hydrogen peroxide concentrations (instead of relying on a nominal values calculated on the basis of the volume of water and how much hydrogen peroxide was added) have to be used and that these concentrations need to be known during the treatment. Levels of hydrogen peroxide (1500 mg L^{-1}) remained constant in natural sea water for up to 71 hours and were not affected by a range of salinities and temperatures (Johnson et al 1993). While addition of salmon resulted in a reduction of hydrogen peroxide, organic load of 0.25 kg L^{-1} for 24 hours still

failed to reduce the peroxide to normal ambient levels (Johnson et al 1993). Ambient levels of hydrogen peroxide are usually less than 10 µg L^{-1} (Cooper et al 1989). While sunlight can cause breakdown of hydrogen peroxide to hydroxyl radical, hydrogen peroxide concentration was reduced only from 1500 mg L^{-1} to 1450 mg L^{-1} after 24 hours in 100 L outdoor tanks (Johnson et al 1993). The mechanisms responsible for the breakdown of hydrogen peroxide in marine environment include catalase activity, oxidation of organic chemicals or action of free radicals formed from metalcatalysed and photochemical breakdown of hydrogen peroxide.

Importantly, hydrogen peroxide will need to be monitored during the treatment as the safety margin (difference between toxicity to salmon and to amoeba) is narrow.

While these results show that the hydrogen peroxide treatment successfully treated a mild case of AGD under laboratory conditions, further research needs to:

- Determine efficacy under more advanced disease scenarios
- Further investigate efficacy of this method under a broadened range of exposure times and concentrations (in vitro and in vivo)
- Examine the physiological and behavioural responses to this treatment for this particular disease

These aims will assist in the determination of the best conditions for field use, which will then need to be adapted to a commercial scale operation. The Yellowtail Kingfish Industry is well advanced with hydrogen peroxide treatment (including measurement of hydrogen peroxide and its application) and should be consulted with regard to commercial set ups.

Benefits and Adoptions

This project directly benefits the industry by showing that hydrogen peroxide treatment is effective under laboratory conditions. This means that hydrogen peroxide has a potential for a commercial use. 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.
While these results show promise, a number of issues and questions were raised:
- it may be logistically difficult to bathe fish for 15 minutes on commercial basis
- more advanced cases of AGD were not tested
- a significant difference in blood plasma osmolality was observed suggesting potential osmoregulatory stress due to hydrogen peroxide treatment
- other physiological effects were not investigated
- effect on feeding was not investigated

Further research is needed to:

- Determine efficacy under more advanced disease scenarios
- Further investigate efficacy of this method under a broadened range of exposure times and concentrations (in vitro and in vivo)
- Examine the physiological and behavioural responses to this treatment for this particular disease

These topics will be addressed further in Institutional Research Grants Scheme University of Tasmania funded project (Adams 2011). Combinations of hydrogen peroxide concentrations and treatment time will be determined using in vitro testing. Potential side effects, in particular electrolytic and acid/base balance as well as stress response, will be assessed. Post-treatment feed intake will be determined. As a result the following aims will be addressed:
- to investigate efficacy of a range of exposure time and exposure concentration treatments
- to determine physiological and behavioural responses of Atlantic salmon to hydrogen peroxide treatment
- to assess efficacy of hydrogen peroxide treatment in more advanced AGD cases

The role of temperature in toxicity should be investigated further.
Results of this extension project will be disseminated to salmon industry as soon as possible.

Planned Outcomes

This project is proactive and directly benefited salmon industry. We identified concentrations of hydrogen peroxide that kill *Neoparamoeba perurans* within short period of time (10-60 minutes) and based on laboratory experiment provided proof of concept for hydrogen peroxide bath treatment in sea water.

Conclusions

This study provided a proof of concept for the use of hydrogen peroxide treatment in sea water against Amoebic Gill Disease. The concentrations of hydrogen peroxide that were effective *in vitro* were 1000 mg L^{-1} with an exposure time 10-20 minutes. Salmon treated with 1250 mg L^{-1} hydrogen peroxide at both temperatures (12°C and 18°C) displayed a variable clearance of amoebae from gill lesions. In approximately half of the fish, the lesions were completely cleared. In the majority of the remaining fish only a few amoebae remained on the gills after treatment. Fish treated with hydrogen peroxide had elevated blood plasma osmolality post-treatment in comparison to pre-treatment controls and fish that were bathed in freshwater. Further research is needed to test the treatment under different conditions and adapt it to commercial applications.

References

Bravo S., Treasurer, J., Sepulveda, M., Lagos, C. (2011) Effectiveness of hydrogen peroxide in the control of *Caligus rogercresseyi* in Chile and implications for sea louse management. Aquaculture, in press.

Bruno, D. W. (1992) The use of hydrogen peroxide (H_20_2) to remove sea lice from farmed Atlantic salmon. First European Crustacean Conference Abstracts, Paris, France. Aug. 31 Sept 5, 1992. p.18.

Cameron, D.E. (1993) Amoebic gill disease field research 1992/93. Proceedings of the SALTAS Review Seminar. Seeking and Solving. pp 89-102.

Cameron, D.E. (1994) AGD Field Research 1993/94 II. Evaluation of toxicity of hydrogen peroxide to Atlantic salmon. Reports from SALTAS 1993-1994. Research and Development Program. Pp. 105-118.

Cooper, W.J., Zika R.G., Petasne, R.G, Fischer, A.M. (1989) Sunlight induced photochemistry of humic substances in natural waters; major reactive species, p.333-362. *In* I.H. Suffet and P. MacCarthy [ed.] Aquatic humic substances: influence on fate and treatment of pollutants. Advances in Chemistry 219, ACS Washington, DC.

Howard, T., and Carson, J. (1994) Amoebic Gill Disease. Laboratory Research 1993/94. Reports from SALTAS 1993-1994. Research and Development Program. Pp. 71-87.

Johnson, S.C., Constible, M., Richard, J. (1993) Laboratory investigations on the efficacy of hydrogen peroxide against the salmon louse *Lepeophtheirus salmonis* and its toxicological and histopathological effects on Atlantic salmon *Salmo salar* and Chinook salmon *Oncorhynchus tshawytscha.* Diseases of Aquatic Organisms. 17: 197-204.

Kiemer, M. and Black, K. (1997) The effects of hydrogen peroxide on the gill tissues of Atlantic salmon, *Salmo salar* L. Aquaculture 153,, 181-189

Mansell, B., Powell, M.D., Ernst, I., Nowak, B.F. (2005) Effects of the gill monogenean *Zeuxapta seriolae* (Meserve, 1938) and treatment with hydrogen peroxide on pathophysiology of kingfish, *Seriola lalandi* Valenciennes, 1833. Journal of Fish Diseases 28, 253–262.

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

Nowak, B., Carson, J., Powell, M., Dykova, I. (2002) Amoebic gill disease in the marine environment. Bulletin of the European Association of Fish Pathologists 22,144–147.

Powell, M.D., Attard, M., Harris, J.O., Roberts, S.D., Leef, M.L. (2005) Why fish die – the treatment and pathophysiology of AGD. Aquafin CRC Final Report Project 3.4.1. Fisheries Research and Development Corporation Project No. 2001/205.Canberra: FRDC.

Thomassen, J.M. (1993) Hydrogen peroxide as a delousing agent for Atlantic salmon, p. 290-295 *In:* G. A. Boxshall and D. DeFaye [ed.] Pathogens of wild and farmed fish: sea lice. Ellis Horwood, Chichester, England.

Treasurer, J.W. and Grant, A. (1997) The efficacy of hydrogen peroxide for the treatment of farmed Atlantic salmon, *Salmo salar* L. infested with sea lice (Copepoda: Caligidae). Aquaculture 148, 265–275.

Treasurer, J.W., Wadsworth, S., Grant, A. (2000) Resistance of sea lice, *Lepeophtheirussalmonis* (Kroyer), to hydrogen peroxide on farmed Atlantic salmon, *Salmo salar*. Aquaculture Research 31, 855–860.

Appendix 1

INTELLECTUAL PROPERTY

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

Appendix 2

STAFF

Principal Investigator:
Barbara Nowak
NCMCRS
University of Tasmania

Co-investigators:
Phil Crosbie NCMCRS University of Tasmania
Mark Adams NCMCRS University of Tasmania