April 2002

Atlantic Salmon Aquaculture Subprogram: effective treatments for the control of amoebic gill disease

Mark D. Powell and Gemma A. Clark

FISHERIES
RESEARCH &
DEVELOPMENT
CORPORATION

Atlantic Salmon Aquaculture Subprogram: effective treatments for the control of amoebic gill disease

Mark D. Powell and Gemma A. Clark

FISHERIES
RESEARCH &
DEVELOPMENT
CORPORATION

FRDC Project 2000/266

April 2002

Atlantic Salmon Aquaculture Subprogram: effective treatments for the control of amoebic gill disease

Mark D. Powell and Gemma A. Clark

Published by University of Tasmania

ISBN 0 85901 986

Formatted/designed by Dr. Mark Powell
Printed by Dr. Mark Powell, University of Tasmania

Table of Contents

Page

1. Non-technical summary…… 3

2. Background…… 5
2.1. Amoebic gill disease…… 5
2.2. Current freshwater bathing treatments for AGD 5
2.3. Water quality/chemistry and its impact on freshwater bathing…….. 5
2.4. Chemical treatments for the control of AGD…… 6

3. Need…….. 7

4. Objectives……. 8

5. Effects of water chemistry and chemical treatments on amoebae survival in vitro……... 9
5.1. Methods……. 9
5.1.1. Amoeba isolation……. 9
5.1.2. In vitro assay for assessing amoeba survival…… 9
5.1.2.1. Mono and divalent cation treatment…….. . 9
5.1.2.2. Oxidative chemical treatments…… 10
5.1.2.3. Statistics……. . 10
5.2. Results…… 11
5.2.1. Effects of mono and divalent cations on the survival of amoebae *in vitro*…… 11
5.2.2. Effects of oxidative chemicals on the survival of amoebae *in vitro*……. 13
5.3. Discussion…… 15

6. Effects of water movement and cage towing on the removal of amoebae from salmon gills……. 16
6.1. Methods…… 16
6.1.1. Water movement trials…… 16
6.1.2. Cage towing trials……... 16
6.1.3. Data analysis……... 18
6.2. Results…….. 19
6.2.1. Effects of water movement on amoeba clearance from salmon gills…….. 19
6.2.2. Effects of cage towing on the prevalence and amoeba abundance on salmon gills……. 22
6.3. Discussion……. 24

7. **Efficacy of chemical treatments on the removal of amoebae from salmon gills……..…….… 26**
7.1. Methods …… 26
7.1.1. Experimental regime……. 26
7.1.2. Gill amoeba density…….. 30
7.2. Results…… 31
7.2.1. Gill amoeba density…….. 31
7.2.2. IFAT results…….. 33
7.3. Discussion…….. 34

8. Toxicity of chemical treatments to Atlantic salmon…….35
8.1. Methods…….. 35
8.1.1. Blood plasma analysis…….. 35
8.1.2. Toxicity analysis……... 35
8.1.3. Histopathology…….. 35
8.2. Results…… 37
8.2.1. Blood plasma analysis…….. 37
8.2.2. Toxicity data……. 37
8.2.3. Histopathology…….. 37
8.3. Discussion…….. 43

9. Discussion…….. 44

10. Benefits…….. 46

11. Further development…….. 47

12. Planned outcome…… 48

13. Conclusions…… 49

14. Acknowledgments…… 51

15. References……... 52

Appendix 1: Intellectual property……. 56
Appendix 2: Staff……. 57
Appendix 3: Presentations made in relation to this project……. 58
Appendix 4: Isolation of amoebae from AGD affected Salmon…….. 59
Appendix 5: Indirect Fluorescent Antibody Test (IFAT)…… 60
Appendix 6: Histological sampling and processing…….. 62
Appendix 7: Statistical analysis tables……... 64

1. NON-TECHNICAL SUMMARY

2000/266 ATLANTIC SALMON AQUACULTURE SUBPROGRAM: EFFECTIVE TREATMENTS FOR THE CONTROL OF AMOEBIC GILL DISEASE

PRINCIPAL INVESTIGATOR: Dr Mark D Powell
Address: School of Aquaculture
Tasmanian Aquaculture and Fisheries Institute
University of Tasmania
Locked bag 1-370
Launceston TAS 7250
Telephone: 03 6324 3813
Fax: 03 6324 3804

OBJECTIVES

1. Identify water chemistry characteristics that enable *Neoparamoeba* to tolerate freshwater bathing.
2. Identify potential additives/supplements to the freshwater bath that promote effective killing of *Neoparamoeba*. These treatments must be environmentally friendly and fall within guidelines for the use of compounds for food and within drinking water.
3. Identify the effects of water movement on the clearance of *Neoparamoeba* from salmon gills and efficacy for freshwater treatments.
4. Test candidates treatments on a pilot scale examining:
   the clearance rate of *Neoparamoeba* from the gills of salmon
   re-infection rate of treated fish.
5. Make available successful treatments and treatment strategies will be available for testing on a commercial scale and for adoption by industry.

NON-TECHNICAL SUMMARY

Amoebic gill disease (AGD) is associated with extensive mortality and reduced production of Atlantic salmon in Tasmania. It is caused by the amoeba *Neoparamoeba pemaquidensis* that infects the gills of cultured salmon. Current treatments for the disease include the use of multiple freshwater baths to remove and kill the parasitic amoeba.

Objective 1
Water hardness is a major factor in the survival of isolated gill amoebae at least *in vitro*. The concentration of calcium and magnesium ions in the water allows the amoeba to survive even very dilute water conditions so potentially surviving the freshwater bathing process used on salmon farms. This means that there is the potential for the amoebae, removed by bathing, to re-infect fish as soon as they have been bathed so reducing the efficacy of the treatment.

Objective 2
Exposing isolated gill amoebae to different concentrations of chlorine dioxide (Anthium diocxide™), chloramine-T (Halamid™) and hydrogen peroxide

(Ecoshield^{TM}) in artificially hardened freshwater showed that oxidising chemicals were acutely toxic to amoebae even in hard water. Chloramine-T and hydrogen peroxide were more effective at killing amoebae within 3 hours than chlorine dioxide.

Objective 3
Water movement over the gills of the salmon could potentially dislodge the amoebae that sit on the gill surface irritating the gill tissue. However, when affected fish were made to swim steadily for 2 hours at about 1.6 bodylengths per second (1.5 knots), the number of amoebae on the gills remained unchanged. This was likely due to the respiration rate of the fish not increasing sufficiently to significantly increase water movement over the gills. When the fish are towed in a cage from one site to another (30 hours at a speed of 1.5 knots) the number of amoebae on the gills of towed fish was reduced. However, the reduction in amoeba numbers was not sufficient to recommend this as a treatment nor is it practical to tow a sea cage for 30 hours.

Objective 4
Trials using chlorine dioxide, chloramine-T and hydrogen peroxide were carried out on AGD affected salmon held in tanks on each of 2 farms of different freshwater water sources. Chlorine dioxide and chloramine-T at concentrations of 25 and 10-25 ppm reduced the number of gill amoebae by approximately 50% compared with untreated fish whereas those fish treated with freshwater alone (without the additive chemicals) had similar numbers to untreated fish. Hydrogen peroxide gave variable results with no clear efficacy in terms of removing amoebae from salmon gills.

There was a difference in the toxicity of chemicals tested between farms that may be the result of significantly different freshwater chemistries. At both farms, the highest concentration of chlorine dioxide and chloramine-T tested (50 ppm) was toxic, however, on one farm, chlorine dioxide was much more toxic. Analysis of the fish gills from fish tested at 50 ppm revealed significant degeneration and necrosis of the gill epithelium indicative of oxidative damage.

This study showed chlorine dioxide at (25ppm) and chloramine-T (at concentrations between 10 and 25 ppm) are potential candidate additives to freshwater baths for making each bath more effective at killing amoebae and removing the parasite from the gills of AGD affected salmon. A preliminary cost analysis suggests that chloramine-T may be favoured owing to its relatively lower cost compared to chlorine dioxide. The next step is the trialing of these treatments in commercial sea cages.

Objective 5
Although a commercial chemical trial of treatments was proposed in this project, a lack of farm co-operation meant that chlorine dioxide and chloramine-T were not tested on a commercial scale within the time frame of this study.

OUTCOME ACHIEVED

Two chemicals have been identified as potential additives to freshwater baths for treating AGD in Atlantic salmon. These chemical treatments work *in vitro* and in tank experiments even in hard water where gill amoeba survival is optimal. Further development of these treatments is planned through the health program of the CRC for sustainable aquaculture of finfish (Aquafin) to provide a cost effective and efficacious treatment for AGD.

KEYWORDS: *Neoparamoeba pemaquidensis*, Amoebic Gill Disease, Atlantic salmon, *Salmo salar*, aquaculture, disease treatment, chloramine-T, chlorine dioxide, hydrogen peroxide.

2. BACKGROUND

2.1 Amoebic Gill Disease

Amoebic Gill Disease (AGD) affects sea-caged salmonids (Atlantic salmon, *Salmo salar* and Rainbow trout, *Oncorhynchus mykiss*) in Australia (Munday et al. 1990), Ireland (Roger and McArdle 1996; Palmer et al. 1997), France and New Zealand (J. Carson pers. comm.). A single outbreak was also reported in cultured coho salmon, *Oncorhynchus kisutch*, in Washington and California, USA (Kent et al. 1988). The disease is caused by the protozoan parasite *Paramoeba pemaquidensis* (Kent et al. 1988). More recently the organism has been reclassified and is now referred to as *Neoparamoeba pemaquidensis* (Dykova et al. 2000). In Tasmania, AGD is the most serious health problem in sea-caged Atlantic salmon (Munday et al. 1990) and it significantly increases the production costs for this species.

2.2 Current Freshwater Bathing Treatments For AGD

To date, the most effective method for control of AGD is a 2-3 hour freshwater bath. Fish are transferred from their holding cage to a tarpaulin-lined cage containing up to 1 ML of freshwater at stocking densities of 30-40 kg. m^3. Initial oxygen saturations are at 200% and either maintained or allowed to fall to normoxic 100% saturation levels by the end of the bathing. After a 2-3 hour bathing period, the tarpaulin liner is pulled away and the fish either maintained in the cage or swam into their original cage and the cage subsequently towed back to the grow-out location. The fish appear to tolerate this osmotic shock which removes some of the offending parasitic amoebae. Recently Clark et al. (2000) demonstrated that although freshwater bathing does appear to remove *Neoparamoeba* from the gill and the incidence of live *Neoparamoeba* is reduced, recolonisation of the gills can occur in as little as 10 days post-bathing. Previous data suggested that re-infection occurred within a month (Clark and Nowak 1999). In addition, studies using static tanks of freshwater showed less effective removal of *Neoparamoeba* from the gills compared with commercial treatments in which the bathed fish were then towed back to the grow out site.

2.3 Water Quality/Chemistry And Its Impact On Freshwater Bathing

The basis for freshwater bathing is that *Neoparamoeba* cannot tolerate freshwater and are osmotically compromised and subsequently killed. However, we have recently shown that water from freshwater dams (the same water used by fish farms for freshwater bathing) was ineffective at killing Neoparamoebae *in vitro* (Clark et al. 2000). The high ionic content of the dam water following the recent warm summer was believed to be the cause for this result. It was apparent that the ionic content of the water likely had a significant impact on the potential efficacy of the freshwater to kill *Neoparamoeba*. With the large degree of variability in the chemistry of the

source water used by farms for freshwater bathing (Parsons et al. 2001), it is imperative that this be investigated further if new effective treatments for the control of AGD are to be found.

2.4 Chemical Treatments For The Control Of AGD

A number of chemical treatments have been screened in the search for treatments for AGD (Howard and Carson 1994). These studies yielded a number of potential candidate treatments but concern was expressed with regard to toxicity to the salmon or potential environmental impacts. Freshwater appeared to be the most effective treatment to kill the *Neoparamoeba*. However today, the efficacy of this strategy as a treatment for the control of AGD is being questioned. Hydrogen peroxide which is used to treat bacterial gill diseases and eggs in freshwater hatcheries (Lumsden et al 1998; Gaikowski et al. 1998; Rach et al. 2000) proved useful as a potential treatment chemical (Howard and Carson 1994) at concentrations known to have low toxicity to salmonids in seawater and freshwater (Cameron 1994a, Powell and Perry 1997b). However, this treatment strategy was abandoned because it was believed to have a low margin of safety to smolt (recently transferred salmon) in seawater (Cameron 1993, Cameron 1994b). However, these studies were done from the perspective that hydrogen peroxide was the *only* treatment to be used. In conjunction with freshwater it is likely to act synergistically so lower concentrations can be used which increases the margin of safety for salmon but still improves the kill rate of treatment against *Neoparamoeba*. The advantage of hydrogen peroxide is that it decomposes to water and oxygen and hence is seen as an "environmentally friendly" treatment with no concern for tissue residues. Chlorine dioxide is another oxidising disinfectant approved by the Australia New Zealand Food Authority for use in the seafood processing industry as a biocide. In addition, residue levels of 10 ppm are permitted in packaged water and 1 ppm in food, the same as for free chlorine. Since chlorine dioxide is activated to liberate reactive oxygen and chlorine when the pH is lowered its potential for use as a treatment for AGD is good in light of the fact that during a freshwater bath, the pH may drop up to 1 pH unit (Parsons et al. 2001). Chloramine-T has since 1980 been used in freshwater hatcheries as a disinfective treatment for bacterial gill disease (From 1980) and other protozoan gill diseases such as Ichthyobodiasis (Ostland et al 1995) and Ichthyophthiriasis (Cross and Hursey 1973). Chloramine-T causes little physiological disturbances to salmonids apart from a transient impediment of CO_2 excretion and stimulation of oxygen uptake in freshwater rainbow trout (Powell and Perry 1996; 1999). Ionic and acid-base effects are minimal particularly in hard water with high ionic content water (Powell and Perry 1997a; 1998). Although the LC_{50} for chloramine-T in rainbow trout is dependant upon water hardness (Bills et al. 1988)**,** concentrations of 9 $mg.L^{-1}$ have been shown to have minimal effects on rainbow trout gills even in extremely soft water (Powell and Perry 1997a). The minimal side effects and wide use of chloramine-T as a disinfectant in aquaculture make it an ideal candidate to test as an additive treatment to freshwater baths.

3. NEED

Amoebic Gill Disease is the leading cause of mortality and loss of aquaculture production that has plagued the Tasmanian salmon industry in recent years. The financial cost of AGD is estimated at approximately 14% of gross production equating up to $15.4M annually. Recently, a combination of warm water temperatures, reduced rainfall and increased production on farms has resulted in a perceived decrease in the effectiveness of current freshwater bathing practices in controlling AGD. Freshwater baths, the usual treatment for treating AGD, appear to be less effective at treating the disease and more frequent bathing is required compared with previous years. Whereas 3 baths per cage was all that was required for the control of AGD in 1996, in 1999 and 2000 as many as 10 baths per cage have been reported to be required to prevent mortalities due to AGD. Recent studies by Clark et al. (2000) have shown that freshwater bathing does not reliably kill all of the amoebae on salmon gills and re-infection with *Neoparamoeba* can occur in as little as 10 days post-bathing. Since current treatments are proving inadequate, more effective treatment strategies are required for the Tasmanian salmon industry to sustain current production levels.

4. OBJECTIVES

1. Identify water chemistry characteristics that enable *Neoparamoeba* to tolerate freshwater bathing.

2. Identify potential additives/supplements to the freshwater bath that promote effective killing of *Neoparamoeba*. These treatments must be environmentally friendly and fall within guidelines for the use of compounds for food and within drinking water.

3. Identify the effects of water movement on the clearance of *Neoparamoeba* from salmon gills and efficacy for freshwater treatments.

4. Test candidates treatments on a pilot scale examining the:
   clearance rate of *Neoparamoeba* from the gills of salmon
   re-infection rate of treated fish.

5. Successful treatments and treatment strategies will be available for testing on a commercial scale and for adoption by industry.

5. EFFECTS OF WATER CHEMISTRY AND CHEMICAL TREATMENTS ON GILL AMOEBA SURVIVAL IN VITRO

5.1 Methods

5.1.1 Amoeba isolation

Amoebae were isolated from the gills of AGD affected salmon using a technique modified from Howard and Carson (1995) (Appendix 1). Gill samples were kept cool and transported within 24 h to the laboratory. Mucus was removed from the gills was resuspended in aerated 0.45 ?m filtered seawater, washed and centrifuged 3 times. A sterile loop was used to sample the solution for IFAT (Appendix 2) to confirm the presence of *Neoparamoeba pemaquidensis*, the causative agent of amoebic gill disease in Atlantic salmon. Any sample that did not test positive for *Neoparamoeba* was discarded and not used in subsequent experiments.

Amoeba solution was stained with 0.5% trypan blue-seawater mix at a dilution of 1:3. Live amoeba counts were determined using a haemocytometer (Neubauer, BS 748). Two replicate counts were made with a minimum of 20 large squares counted per replicate. The trypan blue assay quantifies live amoebae however, it is not specific for *Neoparamoeba pemaquidensis* therefore data is presented as number of live amoebae.

5.1.2 In vitro assay for assessing gill amoeba survival

Live amoebae were added to 5 mL of test solution in 25 well Repli-dishes, to equal a final nominal concentration of 15 000 cells. mL^{-1}. Six treatments were performed for each experiment with 3 replicates per treatment. The number of live amoeba were again determined using a haemocytometer and trypan blue exclusion assay, after incubation at 16.5^{o}C for 1, 2 and 3 h. Each experiment was repeated twice to give n=6 per treatment. There were no significant differences between replicates on different days so allowing the data to be pooled. Survival of amoeba was calculated as a percentage of seawater control to ensure consistency among treatments.

All solutions were aerated to 100% saturation before commencement of each experiment using a Eterna IV air pump. Prior to experiments pH (TPS 900-P with ionode PBFC probe) and conductivity (WTW LF330 standard conductivity cell tetraCon^{TM} 325) were measured in cation treatments, with pH and redox (TPS 900-P with ionode PBFO probe) measured in chemical treatments.

5.1.2.1. Mono and divalent cation treatment

Wells contained either deionised water containing a given concentration of cation or 0.45 ?m filtered seawater. Amoeba survival was tested in solutions of calcium (Ca^{2+}), magnesium (Mg^{2+}) and sodium (Na^{+}). Cations were tested in chloride form and choline chloride was used to verify the effect of chloride on amoeba survival. pH was also tested to determine amoeba survival, this treatment was tested in deionised water

adjusted using HCl or NaCl (0.1M). Concentrations tested on amoeba survival are presented in Table 5.1.1.

5.2.1.2. Oxidative chemical treatment

Amoeba were exposed to chloramine-T (Halamid^{TM}), chlorine dioxide (active ingredient of Anthium dioxcide^{TM}), and hydrogen peroxide (stabilised with silver ions, Ecoshield^{TM}) in 25 well repli dishes. These chemicals were tested in hard water, which was found to promote amoeba survival. Hard water used comprised of 250, 200 and 200 ppm of NaCl, $CaCl_2$, and $MgCl_2$ respectively. Chemical treatments were pre-dissolved in aerated hard water immediately prior to experiment commencement. A 1mL aliquot of concentrated chemical solution was added to aerated hard water and amoeba solution to equal 15 000 cells. mL^{-1} in a 5 mL solution. The final concentrations of chemical treatments are given in Table 5.1.2.

5.2.1.3. Statistics

A repeated measures analysis of variance (ANOVA) was used in data analysis using SPSS^{TM} (version 10.0) software.

Table 5.1.1. Concentrations of solutions tested to assess amoeba survival *in vitro*

Solutions	Concentration (ppm)
$CaCl_2$	200, 100, 50, 0
NaCl	250, 100, 50, 0
$MgCl_2$	200, 100, 50, 0
Choline Chloride	200, 100, 50, 0
NaCl/$CaCl_2$	250/10, 10/10, 10/200, 10/200, 0/0
pH	6, 7, 8, 9, normoxic

Table 5.1.2. Concentration of oxidative chemical treatments tested to assess amoeba survival *in vitro*.

Chemical	Concentration (ppm)
Chlorine dioxide	0, 10, 25, 50
Chloramine-T	0, 10, 25, 50
Hydrogen peroxide	0, 10, 50, 100

5.2 Results

All experiments tested for IFAT were positive and no data was discarded. Seawater controls in all treatments survived to 3 h with settling of amoeba onto the coverslip inducing pseudopodia formation and locomotion in a majority of counts.

5.2.1 Effects of mono and divalent cations on the survival of gill amoebae *in vitro.*

There was reduced survival of amoeba in Na^+ treatments 1 and 2 h for all concentrations (Fig. 5.2.1a). There were no significant differences between 250, 100, 10 and 0 $mg.L^{-1}$ concentrations at 3 h. Divalent cations Ca^{2+} and Mg^{2+} had a protective effect on survival of isolated gill amoebae in deionised water with no significant mortality of amoebae within 1 h for any concentration of Ca^{2+} and at 100 and 200 $mg.L^{-1}$ for Mg^{2+} (Fig 5.2.1b and c). Amoebae survived to 3 h in Ca^{2+} at concentrations of 200, 100, 50 and 10 $mg.L^{-1}$ (Fig. 5.2.1b). There was significant survival of amoeba in 200 and 50 $mg.L^{-1}$ at 3 h compared to 0 ppm, however survival at 3 h was significantly lower than seawater controls. All amoeba at 3 h in 0 $mg.L^{-1}$ treatments were killed. Mg^{2+} treatments recorded amoeba survival in 200, 100, 50 and 10 $mg.L^{-1}$ at 3h (Fig. 5.2.1c).

Choline chloride did not influence amoeba survival. Amoeba survival was significantly reduced from seawater controls at 1 h, with a further reduction in survival occurring at 2 h in all treatments (Fig. 5.2.1d). Survival at 3h was not significantly different from 0 $mg.L^{-1}$ for all concentrations tested.

Combined Na^+ and Ca^{2+} experiments indicated that when Ca^{2+} concentrations were high amoeba survived to 3 h (Fig. 5.2.1e), prolonged survival was observed when the Ca^{2+} concentration was at high concentrations even when the Na^+ concentration was low (10 $mg.L^{-1}$). There was no effect of pH (ranging from 6 to 9) on amoeba survival *in vitro* (Fig. 5.2.1f). All amoeba were killed within 2 h of incubation at all pH tested.

Conductivity increased with increasing cation concentration in solution, as did pH in most incidences (Table 5.2.1).

Table 5.2.1. Conductivity (Cond, ?S/cm) and pH measurements for treatments at time 0 h.

	NaCl		$CaCl_2$		$MgCl_2$		NaCl/$CaCl_2$		Choline chloride	
Conc ($mg.L^{-1}$)*	Cond	pH	Cond	pH	Cond	pH	Cond	pH	Cond	pH
1	532	6.76	338	6.73	2290	6.83	7560	7.91	1978	6.58
2	274	6.57	235	6.73	1188	6.58	5110	6.71	611	6.53
3	160.5	6.30	162.6	6.72	716	6.96	3390	6.41	260	6.48
4	97.8	5.82	127.7	6.71	227	7.23	345	6.49	120.3	6.42
5	40.8	5.81	103.4	6.62	63.3	7.42	57.3	6.58	33.7	6.14
Seawater	5330	7.50	5350	7.93	5250	7.87	5240	7.61	5210	7.63

*1,2,3,4 &5 for CaCl & MgCl respectively 200, 100, 50, 10 & 0 ppm
1,2,3,4 &5 for NaCl & Choline chloride respectively 250, 100, 50, 10 & 0 ppm
1,2,3,4 &5 for NaCl/CaCl respectively 250/200, 250/10, 10, 200, 10/10 0/0 ppm.

Figure 5.2.1. Effects of NaCl (A), $CaCl_2$ (B), $MgCl_2$ (C), choline chloride (D), Na^+/Ca^{2+} ratio (E), and pH (F) on the relative survival of amoebae *in vitro* in freshwater after 1 h (open bars), 2 h (right-left hatched bars) and 3 h (left-right hatched bars). (Vertical bars represent +1 SE).

5.2.2. Effects of oxidative chemicals on the survival of gill amoebae *in vitro*

Chlorine dioxide significantly reduced number of gill amoebae at all concentrations at 1 h compared to seawater controls (Fig. 5.2.2). However, chlorine dioxide was not found to be as effective as deionised water, which effectively killed amoebae within 3 h. Amoeba survival in 50, 25 and 10 ppm treatments did not significantly decrease with the duration of the experiment (Fig. 5.2.2).

Chloramine-T at 25 and 10 ppm did significantly reduce amoeba numbers at 1 h as compared to seawater controls (Fig. 5.2.2). A further decrease in amoeba numbers was seen at 2 h for all treatments. Concentrations of 50 and 25 ppm were effective at reducing gill amoeba numbers to deionised water equivalents at 2 and 3 h.

Hydrogen peroxide significantly killed amoebae within 1 h at a concentration of 100 ppm (Fig. 5.2.2). Concentrations of 50 and 10 ppm also significantly reduced the number of live amoebae at 1 h. However, 50 and 10 ppm were not as effective as deionised water at killing amoebae. Amoeba survival in hard water was significantly greater than survival in 100 and 50 ppm at 1 h. Survival of amoebae in hard water was observed in all chemical treatments up to 3 h.

With all chemical treatments trialed there was an increase in the redox potential of the treatment solution. This corresponded with reduced survival of amoebae.

Table 5.2.2. Redox (mV) and pH of chemical treatments measured at time 0 h.

	Chlorine dioxide		Chloramine-T		Hydrogen peroxide	
Concentration (ppm)	Redox	pH	Redox	PH	Redox	pH
50 (100 H_2O_2)	180.6	9.78	361	6.88	260.5	5.57
25 (50 H_2O_2)	204.1	9.65	245.4	7.37	190.5	6.07
10	201.6	8.85	239	7.3	182.6	6.4
0	41.4	7.40	49.8	6.78	43.2	6.18
Seawater	166.6	7.18	151.8	7.93	147	7.32
Deionised water	19.6	6.24	60.3	6.73	34.2	6.65

Figure 5.2.2. Effects of chlorine dioxide (A), chloramine-T (B) and hydrogen peroxide (C) on the relative survival of amoebae *in vitro* after 1 h (open bars), 2 h (Left-right hatched bars), 3 h (Right-left hatched bars). (Vertical bars represent + SE).

5.3. Discussion

It was apparent that water chemistry strongly influenced the survival of isolated gill amoebae *in vitro*. Previous investigations have suggested a link between water hardness and survival of amoebae in freshwater (Clark et al. 2000), although these investigations only looked at water from natural sources and correlated survival with total hardness. In the present investigation, we have shown that clearly the concentration of Ca^{2+} and Mg^{2+} are specifically important for prolonged survival of amoebae in freshwater. Although *Neoparamoeba* can withstand relatively dilute environments (Kent et al. 1988), in Tasmania it is believed that freshwater kills the majority of *Neoparamoeba* causing AGD in salmon. In recent years there has been an increase in the need to freshwater bathe fish for AGD. Survival of some amoebae on the gills during bathing either within the structure of the gill (Parsons et al. 2001) or in the gill mucus (Clark et al. 2000) would mean that freshwater "resistant" amoebae were being selected for. With an increase in bathing frequency, there would be an increasing selection pressure. This would be exacerbated if the bathing water was particularly hard and Ca^{2+} and Mg^{2+} ions were critical for the survival of *Neoparamoeba* in freshwater and bath durations were short (within the 3h time frame of this experiment). All of these risk factors are present within the Tasmanian salmon aquaculture industry. Bath duration typically ranges from 2-4 h, the water used to bath fish is often hard (see section 8) and not all of the amoebae are removed from the gills.

A range of pH was tested for its efficacy at killing isolated gill amoebae. There was not effect of pH on the survival of amoebae *in vitro*. In all of the experiments, pH varied with ionic content and hardness. Thus it would appear that the effects of ionic concentration (particularly divalent cations) on the survival of gill amoebae *in vitro* is not confounded by variations in pH.

Chemical treatments were variable in their ability to reduce the relative survival of gill amoebae in artificially hard water (250 $mg.L^{-1}$ NaCl, 200 $mg.L^{-1}$ $CaCl_2$ and 200 $mg.L^{-1}$ $MgCl_2$, total hardness of 362.2 $mg.L^{-1}$ $CaCO_3$ equivalents). The use of hard water for testing the effectiveness of these treatments was to assess the chemical under conditions optimal for the survival of the amoebae. Chloramine-T and hydrogen peroxide showed the most promise working as well as deionised water in that all of the amoebae were generally killed within 3 h compared with hard freshwater (0 ppm) and seawater controls. Nonetheless, even chlorine dioxide showed some promise at reducing amoebae numbers at least by 3 h but may not be particularly effective when compared with hard water alone.

Oxidising disinfectants like those used in this study act in part by the production of reactive oxygen and/or hypochlorite during their breakdown. These reactive chemicals interact with cellular membranes and result in peroxidation of the lipid bilayer in the membrane and thus degeneration of the target protozoan or bacteria (Venkobacher et al. 1977). The chemical nature by which oxidising disinfectants kill protozoans and bacteria make resistance difficult and therefore the disinfectants remain effective. This would suggest that all three treatments would be suitable candidate treatments for testing on fish.

6. EFFECTS OF WATER MOVEMENT AND TOWING ON THE REMOVAL OF AMOEBAE FROM SALMON GILLS

6.1 Methods

6.1.1. Water movement trials

AGD affected Atlantic salmon of mean mass 737.3 g (± SE 26.1 g) and fork length 397.1 mm (± 4.4 mm) were obtained from crowds prior to routine commercial freshwater bathing on a commercial salmon farm in Southern Tasmania. Fish were allocated to one of three treatments in 350L round tanks at 200% oxygen saturation (designed to simulate bathing conditions) and ambient temperature as measured using an Oxyguard^{TM} Handy gamma oxygen probe (Fig 6.1.1). The treatments were:

1. Static water with no additional movement other than fish swimming.
2. Turbulent water with water vertically mixed using 5 aquarium power head pumps (RIO 1700).
3. Strongly directed current with the water being circulated around the tank perimeter at a mean velocity of approximately1.6 bodylengths per second (1.6 $BL.s^{-1}$ ~ 0.6 $m.s^{-1}$) using an axial centrifugal pump. This created a current against which fish were forced to swim.

Each trial was repeated twice using 4 fish per treatment (N=8) in freshwater and seawater.

The gills were removed from a sub-sample of 6 fish used in the study prior to fish being allocated to treatment tanks and the number of amoebae per fish determined (Appendix 4). Fish were allocated to a treatment and held within their respective tanks for 2 h. After the bathing period, the fish were euthanased by an overdose of anaesthetic and a scrape made of the second left gill arch, smeared onto a clean glass slide and dried for IFAT confirmation for the presence of *Neoparamoeba*. The gills were removed, placed in the 2.5% ammonium chloride/antibiotic solution for 12 h and the number of live amoebae determined using the trypan blue exclusion assay (Appendix 4).

In addition, fish were bled from the caudal vessels and blood samples were taken from 5 fish from the pre-treatment sample, the static and swimming tank. The blood was centrifuged at 10 000 rpm for 1 min and the plasma frozen at -20^{o}C for

determination of plasma lactate concentrations using a commercially available spectrophotometric assay (Sigma Diagnostic Chemical Company).

6.1.2. Cage towing trials

Fish were sampled before and after towing from three cages. Each tow involved towing fish in cages from Nubeena to Dover for 34-36 h at a speed of 1.5 knots. Fish were sampled from 3 cages exhibiting clinical AGD based on gross morphology of the gills and farm gill scores before and after tow. Ten fish were non-lethally sampled for dot blot and 10 fish lethally sampled (mean mass 2253.3 g ± 126.8 g SE) for **Figure 6.1.1.** Mean (± SE) dissolved oxygen, oxygen saturation and temperature profiles during water movement experiments in freshwater (solid circles) and seawater (open circles).

histology, amoeba isolation and dot blot from each cage. Non-lethal samples were obtained from fish that were anaesthetised using 0.01% clove oil and lethal samples were collected from fish killed with 0.02% clove oil. Fish were sampled before each tow during the transfer of fish into the tow cage. Cages were towed 1 to 2 d after transfer. After tow samples were taken within 2 d after cage arrival at Dover.

The second left gill arch of the fish was sampled for mucus using a wooden (white birch) toothpick (Alpen, China). Mucus was suspended in 400 ?L, 0.22 ?m filtered natural seawater that had been autoclaved at 121^{o}C for 15 min. Samples were transported back to the laboratory on ice and frozen for analysis. Dot blot were analysed as per the protocol of Douglas-Helders et al. (2001) at the Department of Primary Industries, Water and Environment, Launceston, Tasmania.

All 4 gill arches of the right side were removed and placed into 2.5 % ammonium chloride/antibiotic solution. Tubes were placed on ice and transported back to the laboratory and kept refrigerated. The number of live amoeba were determined using a trypan blue assay (Appendix 4).

The second left gill arch was removed and placed into seawater Davidson's fixative. Gill samples were processed as in Appendix 6. Slides were counted for the number of gill lesions per filament and amoebae per lesion.

6.1.3. Data analysis

The number of amoebae per fish was determined prior to (Seawater pre-treatment controls), and following exposure to each treatment. Gill surface area scales with (is directly proportional to) log mass of a fish (Palzenberger and Pohla 1992) therefore larger fish will have a proportionally larger gill surface area and therefore more amoebae. To account for this scaling effect gill amoebae counts were standardised to the log mass of the fish. Amoebae counts were compared using a two-way analysis of variance with concentration and chemical as factors. There were no detectable differences between days based upon ANOVA on which individual trials were

conducted therefore the data was pooled by day for each given treatment. For the towing trial a paired t-test was used in data analysis using SPSS^{TM} (version 10.0) software. P values of less than or equal to 0.05 were considered significant. One data set in the cage towing data was excluded during analysis due to its deviation from the mean population. It was calculated as being > 3 times the standard deviation of the mean.

6.2 Results

6.2.1 Effects of water movement on amoeba clearance from salmon gills.

The presence of *Neoparamoeba* as the causative agent of AGD was confirmed from the smears taken from the gills (Table 6.2.1). The number of positive IFATs was generally lower in the freshwater group probably indicative of the effects of freshwater itself as a treatment for the removal of *Neoparamoeba* from the gills of salmon.

Table 6.2.1. Mean (SE) number of AGD lesions per gill filament and the percentage of fish that tested positive with indirect fluorescent antibody test (IFAT) from gill mucus smears from fish held in freshwater or seawater for 2 h in static, turbulent or moving water (1.6 BL.s^{-1}) conditions. Superscripts represent statistical tests between treatments in either freshwater or seawater.

Treatment	Freshwater		Seawater	
	AGD lesions/ filament	%IFAT +ve	AGD lesions/ filament	%IFAT +ve
Pre-control	0.31 (0.08)^{a}	27.7 (9.8)^{a}	0.39 (0.07)^{a}	66.7 (14.2)^{a}
Static	0.18 (0.06)^{a}	33.3 (14.2)^{a}	0.26 (0.08)^{a}	53.4 (18.9)^{a}
Turbulent	0.09 (0.02)^{a}	33.3 (14.2)^{a}	0.44 (0.09)^{a}	50.0 (18.9)^{a}
Moving	0.13 (0.05)^{a}	33.3 (14.2)^{a}	0.27 (0.06)^{a}	62.5 (18.3)^{a}

These experiments were conducted in November 2000. This early part of the AGD season is reflected by the relatively lower numbers of amoebae seen on the gills of the

pre-exposure (seawater) fish samples (less than 10^6 amoebae per fish, Fig. 6.2.1). In addition there was high variability of amoeba numbers between animals at this time of year. However, there was a trend for freshwater to reduce the number of amoebae on the gills of AGD affected salmon (Fig. 6.2.1). Interestingly there was no significant effect of water movement on the removal of amoebae, although fish held in the turbulent water tank appeared to have fewer amoebae than the seawater pre-exposure sample (Fig. 6.2.1). When the experiment was repeated in seawater, there was no significant effect of water movement on the clearance of amoebae from the gills (Fig. 6.2.1). From this work we can thus conclude that there appeared to be no effect of water movement on the removal of amoebae from the gills of salmon. However, the numbers of amoebae on the gills of the fish tested were quite low. It is possible that at higher amoeba densities on the gills, there may be a small advantage of having water forced over the gills.

At the water velocity tested in the swimming tank (1.6 $BL.s^{-1}$) there was no net accumulation of lactate (Fig. 6.2.2) suggesting that the fish swimming at this velocity were able to maintain aerobic metabolism.

Figure 6.2.1. Effects of water movement treatments on the mean (± SE) number of live amoebae per fish held for 2 h in freshwater and seawater. Letters indicate significant differences between treatments

Figure 6.2.2. Mean (± SE) plasma lactate concentrations of salmon with AGD in seawater and following 2 h exposure to freshwater under static or moving water (1.6 $BL.s^{-1}$) conditions.

6.2.2 Effects of cage towing on the prevalence AGD and amoeba abundance on salmon gills

There was a significant reduction in the amoeba density after towing with a 37.7% reduction in live amoeba found on the gills after towing compared with pre towing levels (Fig. 6.2.3.). This did not agree with dot blot results, where there was no change in the prevalence of fish found positive before and after towing (Table 6.2.2). The number of lesions per filament and number of amoebae per lesion did not change after towing (Table 6.2.2).

Figure 6.2.3. Number of live amoeba per log weight before and after cage tow (? SE.). Asterisk indicates significant difference using t-test.

Table 6.2.2. Mean (SE) number of AGD lesions per gill filament and the percent AGD prevalence as determined by immuno-dot blot assay from fish prior to and following a 30 h cage tow at 1.5 knots.

Treatment	AGD lesions/ filament	Amoebae/ lesion	AGD prevalence %
Before tow	0.03 (0.04)[a]	0.17 (0.10)[a]	55.7 (14.3)[a]
After tow	0.09 (0.02)[a]	0.26 (0.10)[a]	55.0 (4.08)[a]

6.3 Discussion

It was clearly evident from the results that water movement had no significant impact upon the removal of amoebae from the gills of AGD affected salmon or according to the percentage of IFAT positive smears, the removal of specifically *Neoparamoeba* from the gills. Freshwater has been used as the primary treatment for AGD based on early studies that suggested that freshwater killed *Neoparamoeba* and facilitated their removal from the gills (Parsons et al. 2001). Based on our results, this does not seem to be the case under the current conditions.

It would be reasonable to assume that increasing the water movement over the fish's gills would lead to an increase in shear over the gill surface effecting a stripping of mucus. However, it would appear from our study that at a swimming velocity of 1.6 bodylengths.s^{-1}, in either freshwater or seawater, there were no differences in amoeba clearance that could be accounted for in terms of increased water sheer. The velocity of 1.6 BL.s^{-1} was chosen because it represented a similar speed to that which could be expected during a cage being towed (approximately 1.5 kts) and that at that water velocity, even a cultured fish can swim aerobically (McDonald et al. 1997). Under these conditions, the fish would continue to breathe using a biphasic buccal pumping system to force water over the gills (Sunders 1961, Davis and Randall 1973) and as such would likely control the force of water crossing the gills to optimise oxygen extraction (Saunders 1962, Davis and Cameron 1971). Under these conditions it is possible that water sheer across the gills was not significantly different to that of a fish under static water conditions. This was reinforced by the fact that there was no difference in plasma lactate concentrations between any of the treatments suggesting that fish swimming at 1.6 BL.s^{-1} were doing so aerobically. In order to increase water flow across the gills the fish would need to be using ram ventilation (Roberts 1975), typical of that seen when swimming at their the limit of their aerobic potential (approaching U_{crit} speed) or when oxygen uptake (V_{O_2}) is maximised. However, the threshold is not known for salmon cultured in Tasmania.

There was a decrease in the amoeba density on the gills of salmon after a 30 h tow. This suggests that over long durations of swimming amoebae may be stripped from the gills of salmon. However, the amoeba density was still high suggesting that towing is unlikely to be a useful strategy for gill amoeba removal especially since the prevalence of AGD within the towed cages did not change. However, it remains to be investigated further whether amoeba clearance from the gills is a function of swimming speed (water velocity) or duration.

The significant decrease in live amoeba on the gills was seen after towing. However, it is recognised that it was not possible to simultaneously sample control (non-towed) fish due to the geographical separation of the sites to where the fish were towed. Caution must therefore be exercised in the interpretation of this data. The duration of the tow may have played an important role in the removal of amoeba from the gills. Water movement experiments where fish were subjected to fast moving water of 1.6 BL. s^{-1} for 2 h did not show a significant reduction in live amoeba on the gills. Fish during towing were subjected to 1.5 knots for up to 36 h. The movement of water and friction across the gills for an extended period of time will have aided amoeba and mucus removal. Altimiras and Larsen (2000) observed fish switching to ram-ventilation when exposed to water speeds of 1.75-2.0 BL.s^{-1} during swimming experiments with rainbow trout (*Oncorhynchus mykiss*). Although the fish used in experimentation by Altimiras and Larsen (2000) were comparatively smaller the

speeds used equate to 1.06 and 1.21 knots respectively. As the average speed at which the fish were towed at was 1.5 knots it is believed that ram ventilation was probably achieved during the tow. This rapid flushing of water past the gill will have increased amoeba and mucus removal. Though scores were performed on fish before towing, gill scores were not performed after. However it was noted that the gills were healthy with reduced mucus and clear of mucus patches. Fish exposed to strong currents have shown a removal and decrease in mucus and mucous patches present on the gills (Nowak 2001).

Dot blot is a sensitive and specific diagnostic technique requiring only a small amount of mucus sample from the gill (Douglas-Helders et al., 2001). The test's sensitivity means that only a small number of cells are required to obtain a positive result. The nature of the test does not allow a quantitative interpretation of the presence or absence of amoeba on the gills. However, given the set-up of this study, the dot-blot samples (before and after tow) were not independent and could not be related to controls as described above.

7. EFFICACY OF CHEMICAL TREATMENTS ON THE REMOVAL OF AMOEBAE FROM SALMON GILLS

7.1. Methods

In order to establish that the candidate chemical treatments identified (section 5) were applicable to a broad range of water chemistries, it was essential that trails into the efficacy of the treatments be carried out on 2 separate farms each in a separate watershed (Fig. 7.1.1). The water chemistries were significantly different between the two sites with the Farm 2 site having significantly higher concentrations of mono and divalent cations that translated into significantly higher total hardness and this was coincident with the high concentration of suspended solids (Table 7.1.1)

7.1.1. Experimental regime

Experimental trials were carried out twice on consecutive days for each chemical tested in 4, 350L circular tanks each supplied with source freshwater from the respective farm. The water was oxygenated to 200% saturation at the start of the trial and fish allocated to each tank. A sub-sample of 6 fish were removed from the source population and the gills removed for gill amoeba counts (representing a pre-exposure control sample). A total of 7 fish were allocated to each tank. Each treatment chemical was pre-dissolved or mixed with 100 mL of tank water and added to each respective tank and mixed with the freshwater using a RIO 1700 pump (Table 7.1.2). Tanks were covered with shade cloth and the fish exposed to the chemical treatment for 3 h. Dissolved oxygen, % oxygen saturation and temperature were measured at 15 min intervals throughout the exposure using an Oxyguard^{TM} Handy Gamma oxygen probe. Oxygen saturation was allowed to decrease during the exposure similar to that seen in commercial bathing operations, however, levels were maintained at 100% saturation at all times (Fig. 7.1.2).

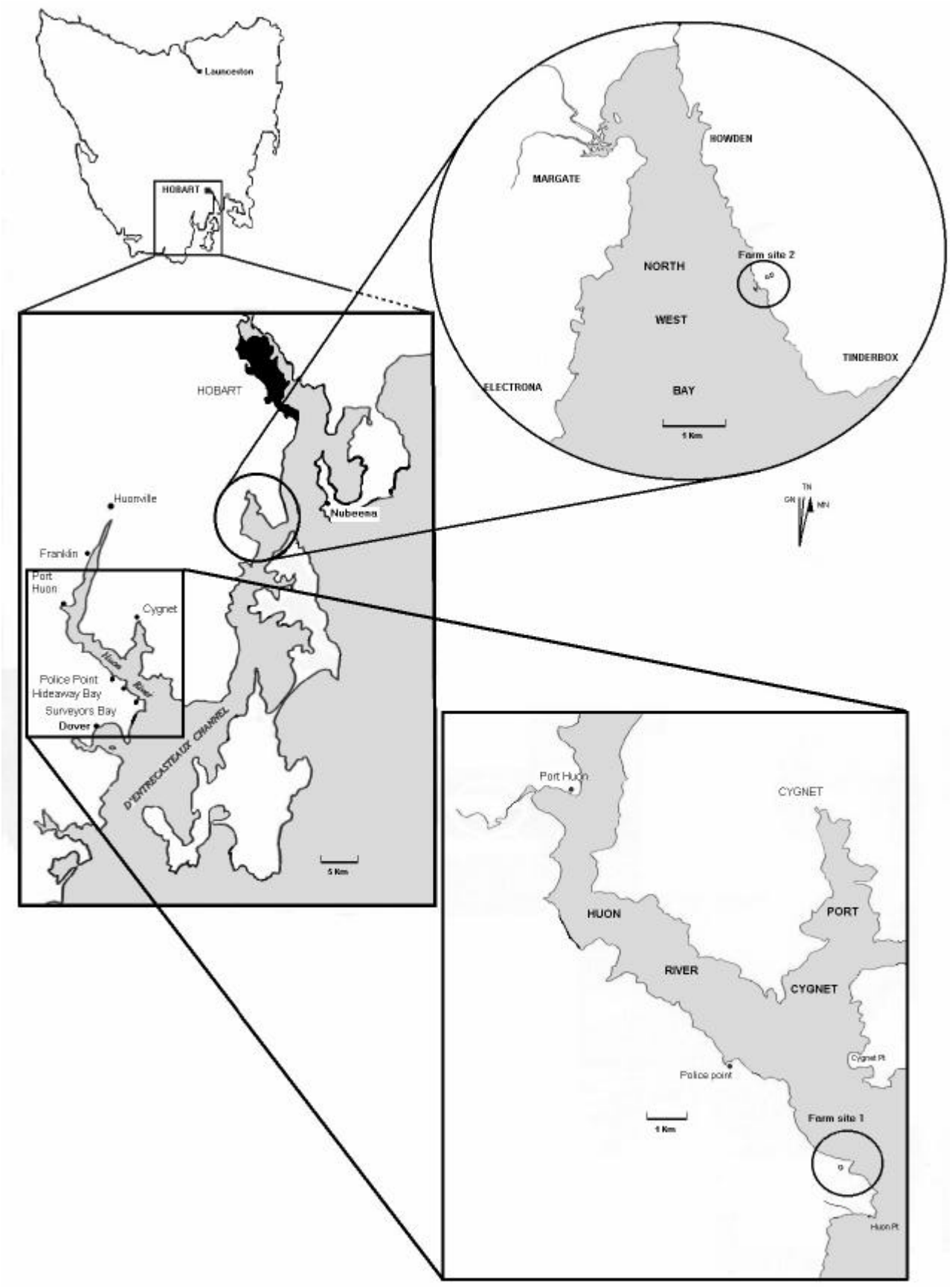

Figure 7.1.1. Map of the Southeastern region of Tasmania illustrating the locations of the chemical trial studies.

Table 7.1.1. Mean (± SD) ionic concentrations, total hardness (TH) and suspended solids (SS) ($mg.L^{-1}$) for the freshwater at the experimental sites.

	Na^+	K^+	Ca^{2+}	Mg^{2+}	Al^{3+}	Fe^{2+}	Cl^-	TH[a]	SS
Farm 1	48.0 (2.6)	2.7 (0.2)	32.4 (0.5)	23.5 (1.1)	0.2 (0.1)	0.4 (0.1)	108.7 (5.5)	179.7 (4.6)	5.5 (2.1)
Farm 2	285.2* (5.3)	5.6* (0.2)	54.4* (0.5)	93.0* (6.4)	0.8* (0.5)	0.4 (0.3)	567.5* (4.4)	523.2* (25.7)	18.6* (11.1)

*significantly different between sites
[a]TH = ($[Ca^{2+}]$ * 2.497) + ($[Mg^{2+}]$ * 4.118) + ($[Fe^{2+}]$ * 1.792) + ($[Al^{3+}]$ * 5.564)$mg.L^{-1}$ $CaCO_3$ equivalents

Figure 7.1.2. Mean (± SE) dissolved oxygen ($mg.L^{-1}$), oxygen saturation (%) and temperature (^oC) profiles for the chemical treatments at Farm 1 (solid circles) and Farm 2 (open circles). Bar represents bathing period.

Table 7.1.2. Chemical treatments (and their trade name) and exposure concentrations (ppm)

	Farm 1	Farm 2
Chlorine Dioxide (Anthium dioxcide™)	0, 10, 25, 50	0, 10, 25, 50
Chloramine-T (Halamid™)	0, 10, 25, 50	0, 10, 25, 50
Hydrogen peroxide[a] (Ecoshield™)	0, 10, 50, 100	----------

[a] only trialed at 1 site owing to the potential toxicity

At the end of 3 h, 4 fish from each tank were removed and killed by an overdose of clove oil (0.04%). A smear was made of the second left gill arch for IFAT determination and the confirmation of the presence of *Neoparamoeba*. The gill basket was removed and the 4 arches from the right hand side of the fish placed into

2.5% ammonium chloride solution for determination of gill amoeba numbers (Appendix 4).

7.1.2. Gill amoebae density

The number of amoebae per fish was determined prior to (Seawater pre-treatment controls), and following exposure to each concentration of each chemical. Gill surface area scales with (is directly proportional to) log mass of a fish (Palzenberger and Pohla 1992) therefore larger fish will have a proportionally larger gill surface area and therefore more amoebae. To account for this scaling effect gill amoebae counts were standardised to the log mass of the fish. Amoebae counts were compared using a two-way analysis of variance with concentration and chemical as factors. There were no detectable differences between days on which individual trials were conducted, therefore the data was pooled by day for each given treatment. Differences in means were isolated using a Tukey's HSD test.

7.2. Results

7.2.1. Amoeba density

There were significant decreases in the amoeba density on the gills of fish at 25 and 50 ppm chlorine dioxide compared with pre exposure controls as well as freshwater alone (0 ppm) at Farm 1 (Fig 7.2.1). There was also a significant decrease in the number of amoebae on the gills of salmon bathed at all concentrations of chlorine dioxide at Farm 2 when compared with pre-exposure controls. However, there were no significant differences between chlorine dioxide treatments and freshwater only at Farm 2 (Fig 7.2.1). Nonetheless, there were no significant differences between freshwater only and the pre-exposure controls at either site.

There was a significant reduction in the amoeba density on the gills of salmon at Farm 1 bathed with chloramine-T compared with pre-exposure controls although there were no significant differences between chloramine-T concentrations compared with freshwater only (Fig 7.2.1). At Farm 2 there were no significant differences between

pre-exposure controls and chloramine-T treatment at any concentration. However, fish exposed to 25 and 50 ppm chloramine-T had significantly fewer amoebae on the gills compared with fish exposed to 10 ppm (Fig. 7.2.1).

There were no significant differences between amoebae density and any of the concentrations of hydrogen peroxide and the pre exposure or freshwater controls at Farm 1 (Fig. 7.2.1). The poor performance of hydrogen peroxide and apparent toxicity meant that trials were not continued at Farm 2.

Figure 7.2.1 Effects of different concentrations of chlorine dioxide, chloramine-T and hydrogen peroxide on the mean (± SE) amoeba density on the gills of cultured Atlantic salmon when bathed with freshwater for 3 h on 2 different farms. Letters indicate statistical differences within a chemical treatment.

7.2.1 IFAT results

There was a trend for a reduced proportion of IFAT positive smears in fish following bathing compared with that in the seawater pre-treatment controls (Pre). This was particularly pronounced with chloramine-T where IFAT positive smears were rare after treatment (Table 7.2.1).

Table 7.2.1 The percentage (SE) of gill smears positive to indirect fluorescent antibody test (IFAT) before (pre) and following treatment with freshwater (0 ppm) or chlorine dioxide (ClO_2), chloramine-T (CLT) or hydrogen peroxide (H_2O_2) on 2 farms.

Treatment	Conc (ppm)	% IFAT +ve Farm 1	Farm 2
ClO_2	Pre	83.3 (11.2)	50.0 (15.1)
	0	12.5 (12.5)	12.5 (12.5)
	10	25.0 (16.4)	37.5 (18.3)
	25	0	12.5 (12.5)
	50	25.0 (16.4)	12.5 (12.5)
CLT	Pre	58.3 (14.9)	91.7 (8.3)
	0	12.5 (12.5)	0
	10	0	0
	25	0	25.0 (16.4)
	50	0	0
H_2O_2	Pre	41.7 (14.9)	
	0	0	
	10	37.5 (18.3)	
	50	0	
	100	25.0 (16.4)	

7.3. Discussion

There was clearly a relationship between amoeba density on the gills and concentration of chlorine dioxide and chloramine-T used as additives to freshwater baths. It was evident that at both sites 25 ppm chlorine dioxide resulted in significant reductions in the number of amoebae on the gills of salmon to approximately 50% that on the gills of the pre-treatment controls and that which could be achieved by freshwater alone. It was also evident that at this concentration there were the lowest number of IFAT positive smears (0 and 12.5% farms 1 and 2 respectively) from the treatment fish compared with the pre-treatment control smears (83.3 and 50% respectively). This suggests that the majority of the amoebae counted using the amoeba extraction protocol are *Neoparamoeba* and reinforces the notion that primarily Neoparamoebae are being counted on the gills when determining gill amoeba density.

Chloramine-T also significantly reduced the number of amoebae on the gills of salmon in freshwater. At farm 1 concentrations as low as 10 ppm were effective at reducing the number of amoebae on the gills to approximately 50% that of pre-treatment controls. At farm 2 the trend was not so clear, although there appeared to be a reduction in gill amoeba density with 25 ppm chloramine-T, even though this was not statistically different to pre-treatment controls. However, it was quite clear that chloramine -T treatments coincided with large reductions in the number of IFAT positive smears compared with pre-treatment controls (58.3% at farm 1 and 91.7% at farm 2 respectively).

There were no clear relationship with regard to efficacy of hydrogen peroxide at removing amoebae from the gills of AGD affected salmon. However, there appeared to be a trend towards lower numbers of IFAT positive smears. Only at the highest concentration tested (100 ppm), was there an apparent reduction in the number of gill amoebae comparable to that achieved by chlorine dioxide and chloramine-T.

All of the chemicals tested breakdown releasing reactive chlorine, hypochlorite and reactive oxygen species. These reactive species irritate the gill surface (as well as potentially killing the gill amoebae directly) and stimulate mucus secretion (Bass et al. 1977; Powell and Perry 1996, 1997b, 1998). The reduction in live gill amoebae density is likely to be a result of the hypersecretion of mucus. However, given the reactive and unstable nature of the chlorine and oxyradicals produced by the decay of these disinfectants it is likely that they would react directly with the gill mucus itself, thereby oxidising the protein and carbohydrate side chains of the mucus without impacting upon the amoeba directly. This notwithstanding studies have shown that reactive oxygen species can result in the oxidation of haemoglobin resulting in the subsequent formation of methaemoglobin even at concentrations similar to those used in this study (Powell and Perry 1997c). This suggests that hydrogen peroxide and/or oxyradicals did in fact enter the fish and were not simply reacted with surface mucus. It is possible that exposing fish to oxidative chemicals such as the treatments suggested in this study could have a physiological impact on the fish. However, work to date (Powell and Perry 1996, 1997, 1998) have suggested that physiological effects of low exposure concentrations of chloramine-T at least pose little adverse physiological side effects.

8. TOXICITY OF CHEMICAL TREATMENTS TO ATLANTIC SALMON

8.1. Methods

The remaining 3 fish per tank from the previous study (a total of 6 fish per treatment per concentration) that remained in the tanks after the 3 h bathing period (Section 7) were maintained for a further 3 h. These remaining fish were maintained at 100% oxygen saturation and ambient temperature as determined by an OxyGuard^{TM} Handy Gamma oxygen probe. After a total of 6 h of exposure to the treatment chemicals, these fish were killed by an overdose of clove oil anaesthetic (0.04%) weighed and fork length measured then bled from the caudal vessels. The blood centrifuged at

8000 x g and the plasma frozen at -20^{o}C for further analysis. The gills, liver, kidney, spleen, intestine, heart and brain and a portion of dorsal skin and muscle were dissected and fixed in 10% neutral buffered formalin for histological examination. Where mortality occurred prior to the 6 h sample period, fish were immediately removed and the gills and organs fixed as above.

8.1.1. Blood plasma analysis

Blood plasma was thawed and diluted 1000 x with deionised water. Diluted samples were then determined for their Na^{+} and K^{+} concentration using a Varian SpectraA atomic absorption analyser. Plasma chloride concentrations were determined on diluted plasma samples using the spectrophotmetric method of Zall et al. (1956) adapted for use on a 96 well microtitre plate using a Tecan (A-502 Rainbow thermo) microplate reader.

8.1.2. Toxicity analysis

Toxicity of treatments was likely influenced by both the concentration of the treatment chemical, duration of exposure and ambient temperature. Therefore to assess the relative toxicity of treatments where mortality occurred during treatment, a toxicity index was calculated.

$$\text{Toxicity index} = \frac{\text{\% mortality}}{\text{mean time to mortality} * \text{mean temperature}}$$

8.1.3. Histopathology

Tissues were embedded in paraffin wax, sectioned at 5? m and stained with haematoxylin and eosin. Organs were examined for any acute changes and any signs of inflammation or parasitic infection. Gills were examined for the presence of hyperplastic and AGD-type lesions. AGD lesions and gill pathology were quantified by counting the number of filaments in the sections then counting the number of filaments with AGD lesions or amoebae on the lesions. The fish from the highest concentration not showing any toxic effect (where toxic effect is defined as mortality occurring prior to the completion of the 6 h exposure period) were used in the analysis.

8.2. Results

8.2.1. Blood plasma analysis

There was a significant decrease in blood plasma ion concentrations in fish at both farms when exposed to fresh water alone or in the presence of treatment chemicals (Table 8.2.1). At farm 1 there was no effect of chemical treatment concentration on the plasma ion concentration but this was not consistent with farm 2. There were significant decreases in plasma Na^+ and Cl^- concentrations at farm 2 with exposure to both chlorine dioxide and chloramine-T as compared with pre-exposure seawater controls. The greatest decreases in plasma Na^+ and Cl^- concentrations occurred at the highest concentration of chemicals tested (50 ppm and 25 ppm) (Table 8.2.1).

8.2.2. Toxicity data

There was a large difference in the toxicity index of fish exposed at the different sites with predominantly the highest toxicity occurring with the highest concentrations of treatment chemical tested. It was apparent, based upon the toxicity index that the toxicity of chlorine dioxide was greater at Farm 2 compared with Farm 1 even when water temperature and the duration of exposure were taken into account (Fig. 8.2.1). Although there was mortality associated with treatments at the Farm 1, these had a relatively low index and were primarily associated with higher water temperatures.

8.2.3. Histopathology

There was a marked difference in the number of lesions per filament between the pre-exposure controls and the gills from fish following freshwater only and chemical exposure (Table 8.2.2). Similarly there were fewer amoebae on each lesion of fish after chemical and freshwater treatment (Table 8.2.2) and this correlated with a reduced incidence of IFAT positive smears from fish after chemical treatment and freshwater compared with pretreatment controls (Table 8.2.2.)

There were marked differences in the morphology of the gills of fish that died during the treatment trial. Predominantly this involved necrosis of the lamellar epithelium and acute epithelial separation along the lamellae (Figs. 8.2.2 and 8.2.3.). However, most of the lamellar degeneration observed was attributed to exposure to chlorine dioxide, chloramine-T and hydrogen peroxide at the highest concentrations used where acute mortality occurred. With chlorine dioxide at Farm 2, in addition to the lamellar degeneration the lamellae appeared to be hyperperfused with erythrocytes. In other situations where mortality occurred there was often no lamellar degeneration however, temperatures were high and therefore considered the primary cause of mortality because the gill architecture was similar to that of the controls (Fig. 8.2.2). There was little evidence of any effects of acute exposure on the morphology and histology on any of the internal organs for any treatment or concentration.

Table 8.2.1 Mean plasma ion (± SE) concentrations for salmon before (Pre), and after 6h exposure to chlorine dioxide (ClO_2), chloramine-T (CLT) and hydrogen peroxide (H_2O_2) at Farm 1 or Farm 2. Same superscripts indicate no significant difference between concentrations.

Farm	Treatment	Conc (ppm)	Na^+ (? mol.L^{-1})	K^+ (? mol.L^{-1})	Cl^- (? mol.L^{-1})
1	ClO_2	Pre	186.06 (3.34)[a]	3.94 (0.20)[a]	161.9 (29.0)[a]
		0	159.36 (1.86)[b]	5.11 (0.13)[b]	79.9 (9.4)[a]
		10	165.16 (4.27)[b]	4.75 (0.24)[b]	110.5 (15.0)[a]
		25	160.88 (4.92)[b]	4.92 (0.20)[b]	109.2 (21.1)[a]
		50	161.37 (3.86)[b]	4.99 (0.16)[b]	145.9 (14.4)[a]
	CLT	Pre	194.39 (4.36)[a]	4.19 (0.14)[a]	140.8 (21.4)[a]

		0	170.94 (3.59)^{b}	4.83 (0.22)^{b}	130.8 (12.7)^{a}
		10	162.49 (2.67)^{bc}	5.26 (0.24)^{a}	107.5 (15.0)^{a}
		25	163.33 (4.16)^{bc}	4.79 (0.32)^{a}	116.5 (8.0)^{a}
		50	146.40 (5.39)^{c}	5.70 (0.43)^{a}	90.6 (13.3)^{a}
	H_2O_2	Pre	160.00 (2.73)^{a}	4.71 (0.24)^{ab}	176.3 (16.8)^{a}
		0	128.32 (1.82)^{b}	4.86 (0.17)^{b}	188.6 (16.1)^{a}
		10	126.36 (1.71)^{b}	4.35 (0.22)^{ab}	160.7 (7.6)^{a}
		50	122.88 (1.65)^{b}	5.11 (0.15)^{a}	179.3 (7.3)^{a}
		100	119.40 (1.96)^{b}	6.07 (0.86)^{ab}	136.6 (7.7)^{a}
2	ClO_2	Pre	190.37 (7.10)^{a}	4.65 (0.44)^{a}	260.9 (19.7)^{a}
		0	148.87 (4.37)^{b}	4.09 (0.25)^{a}	212.7 (6.5)^{ab}
		10	149.25 (2.82)^{b}	3.45 (0.20)^{a}	209.5 (6.8)^{ab}
		25	154.85 (5.40)^{b}	4.00 (0.34)^{a}	200.9 (5.4)^{b}
		50	140.62 (8.34)^{b}	4.86 (0.35)^{a}	164.4 (7.7)^{b}
	CLT	Pre	160.50 (3.34)^{a}	4.11 (0.25)^{ab}	255.5 (5.9)^{a}
		0	146.86 (1.19)^{b}	4.28 (0.18)^{a}	220.9 (7.8)^{b}
		10	122.33 (4.21)^{ab}	3.52 (0.31)^{ab}	202.3 (21.3)^{b}
		25	140.55 (2.21)^{b}	4.31 (0.20)^{b}	216.5 (7.5)^{ab}
		50	131.52 (2.46)^{ab}	4.92 (0.41)^{ab}	193.6 (14.6)^{b}

Figure 8.2.1. Toxicity index for different chemical treatments used to bathe Atlantic salmon in freshwater on 2 farms.

Table 8.2.2 Mean (± SE) numbers of AGD lesions per filament on the gills and the number of gill smears positive to indirect fluorescent antibody test (IFAT) before (pre) and following 6h treatment with freshwater (0 ppm) or chlorine dioxide (ClO_2), chloramine-T (CLT) or Hydrogen peroxide (H_2O_2) on 2 farms.

Farm	Treatment	Conc (ppm)	Lesions/filament	Amoebae/lesion	IFAT+ve
1	ClO_2	Pre	0.73 (0.13)	1.54 (0.54)	83.3 (11.2)
		0	0.56 (0.08)	0.15 (0.07)	0
		10	nd	nd	0
		25	nd	nd	16.7 (16.7)
		50	0.43 (0.10)	0	0
	CLT	Pre	0.29 (0.11)	0.41 (0.29)	58.3 (14.9)
		0	0.58 (0.12)	0.042 (0.02)	33.3 (21.1)
		10	nd	nd	33.3 (21.1)
		25	0.13 (0.02)	0	0
		50	nd	nd	0
	H_2O_2	Pre	0.61 (0.13)	0.44 (0.11)	41.7 (14.9)
		0	0.26 (0.02)	0	33.3 (21.1)
		10	nd	nd	0
		50	0.44 (0.15)	<0.01 (<0.01)	0
		100	nd	nd	16.7 (16.7)
2	ClO_2	Pre	0.22 (0.11)	0.52 (0.04)	
		0	0.29 (0.14)	0.02 (0.02)	
		10	nd	nd	
		25	0.18 (0.05)	0	
		50	nd	nd	
	CLT	Pre	0.47 (0.15)	0.23 (0.1)	
		0	0.43 (0.05)	0.01 (0.01)	
		10	nd	nd	
		25	0.25 (0.05)	0	
		50	nd	nd	

nd = not determined

Figure 8.2.2. Histopathological effects on the gills of Atlantic salmon bathed in freshwater containing 0 ppm chemical (A) 50 ppm chlorine dioxide at Farm 1 (B) and Farm 2 (C), 50 ppm chloramine-T at Farm 1 (D) and Farm 2 (E). Small arrows indicate lamellar degeneration and epithelial lifting, large arrow indicating heavily perfuse gill lamellae with erythrocytes. All magnifications at 40x except for B 20x.
Figure 8.2.3. Histopathological effects on the gills of Atlantic salmon bathed in freshwater containing 0 ppm (A) or 100 ppm (B) hydrogen peroxide. Small arrow indicates lamellar degeneration and epithelial lifting. Magnification 40x.

8.3. Discussion

It was apparent that there was a decrease in blood plasma ion concentration upon transfer of fish from seawater to freshwater baths containing chemical treatments. This apparent decrease in plasma ion concentrations was not unexpected and has been reviewed by Bath and Eddy (1979) and results from the high permeability of marine fish gills to ions and water. However, we have not detected such a pronounced change previously in commercially farmed salmon during the freshwater bathing process (Powell et al. 2001). There was a trend, albeit not statistically significant, for lower plasma Na^+ concentrations with the highest concentration of chemical tested (Table 8.2.1). Caution must be exercised when interpreting these results. The highest mortality occurred and treatments were the most toxic at the highest concentration. At concentrations of 2-18 $mg.L^{-1}$, chloramine-T induced acute ionic effluxes across the body of freshwater rainbow trout, primarily as a direct response to the effects of oxyradicals and free chlorine released from the hydrolysis of chloramine-T (Powell and Perry 1998). Renal effluxes of Na^+ were negligible and there were no significant changes in plasma Na^+ concentrations. In the same study sodium hypochlorite induced the same effect as chloramine-T suggesting the reactive chlorine and oxygen were the primary causes of the ionic effluxes. It remains to be investigated whether the effects of chlorine and chloramine-T are the same for marine Atlantic salmon with AGD as healthy freshwater rainbow trout.

The toxicity of the treatments varied between sites. There was strong relationship between the water hardness and toxicity of chlorine dioxide with the highest toxicity occurring in the hardest water at farm 2. The nature of this relationship is unclear at this point but it is conceivable that water chemistry may increase the potency of the commercially produced Anthium dioxcide^{TM} used in this study which is 5 % chlorine dioxide. Chloramine-T was also toxic at the highest concentration tested on both farms and in both cases temperature was believed to influence the toxicity. This notwithstanding, the 3 h LC_{50} for chloramine-T and rainbow trout is more than 60 $mg.L^{-1}$ at 12^{o}C in hard or very hard water and pH 8.1 (Bills et al. 1988). The fact that chloramine-T results in a hypersecretion of branchial mucus that impairs CO_2 excretion suggests that this may contribute to the toxicity (Powell and Perry 1996) along with the ionic effects of this compound (Powell and Perry 1998). Indeed the fact that in the present study chlorine dioxide and chloramine-T were used under

hyperoxic conditions may have exacerbated the effects on impairing CO_2 excretion (Powell and Perry 1997b).

It was apparent from the histopathology that there was a strong correlation between mortality, temperature and exposure (Toxicity Index). Exposure to the highest concentrations used (50 ppm) caused significant damage to the branchial epithelium where there was acute toxicity (ie farm 2 with chlorine dioxide, at both farms with chloramine-T). The branchial epithelium exhibited severe degeneration consistent with that seen for reactive oxygen and chlorine toxicity (Bass et al 1977, Powell and Perry 1997c). However, where mortality occurred at a low toxicity index (eg. 50 ppm chlorine dioxide at farm 1), the branchial lamellar epithelium was similar to that seen from fish not exposed to any chemical treatment (Fig. 8.2.2.). In this case, mortality was attributed primarily to acute elevations in temperature during the chemical exposure.

9. DISCUSSION

The need for effective treatments for the control of AGD is great based upon the relative importance and impact of this disease to the Atlantic salmon aquaculture industry in Tasmania. Since conventional freshwater bath treatments appear to be less effective in recent years compared with their use in the early 1990s, alternative treatments are being sought to improve efficacy. Our work suggests that chlorine dioxide in the form of Anthium dioxcide™ may prove useful as a treatment for AGD improving the removal of amoebae from the gills of AGD affected salmon (Section 7) with relatively low toxicity at least in reasonably hard water (Section 8) . However, it was apparent that it was not particularly effective at killing the gill amoebae in *in vitro* studies (Section 5). Anthium dioxcide™ (chlorine dioxide) treatments may be more toxic if used in extremely hard water, therefore defeating the purpose for which the treatments were required, removing and killing *Neoparamoeba* that survive in freshwater baths. However, chloramine-T may prove more useful because it was effective at killing gill amoebae *in vitro* at least as well as freshwater alone (Section 5). Additionally, it aids in the removal of amoebae from the gills of AGD affected salmon (Section 7) reducing amoeba numbers by approximately 50% compared with that in seawater controls.

Chloramine-T had a relatively low toxicity when used within its normally recommended range (10-25 $mg.L^{-1}$) even in a 3 h freshwater bath (section 8), although at concentrations of 50 mg.L-1 it was acutely toxic at treatment temperatures used in this study. Chloramine-T causes little physiological side effects compared with hypochlorite (Powell and Perry 1996) although minor respiratory and acid base disturbances do occur especially in hyperoxic fish where respiration rate may be suppressed (Powell and Perry 1997a) in concert with small ionic effluxes (Powell and Perry 1998). With this in mind, it would appear that chloramine-T could prove to be a useful treatment as an additive to a freshwater bath.

Hydrogen peroxide, while being effective at killing gill amoebae *in vitro*, a result consistent with that found by Howard and Carson (1995), the results from tank trials proved disappointing and overshadowed by potential toxicity issues particularly at elevated temperatures (Section 8). The problem of hydrogen peroxide toxicity when treating salmon smolts was identified by Cameron (1994a and b). However, we

anticipated that the silver stabilised Ecoshield^{TM} which is used commercially in aquaculture at low concentrations (<10 ppm) to control skin and gill diseases in tropical fish would be more effective at removing amoebae from the gills. It would appear from this study that hydrogen peroxide is unlikely to be an effective treatment for AGD as a bath additive. However, we cannot exclude the potential use of hydrogen peroxide as a short time exposure dip or seawater treatment. These aspects of hydrogen peroxide treatment need to be investigated further.

Water movement appeared to have little effect on the removal of amoebae from the gills of AGD affected Atlantic salmon in either fresh or salt water. However, long duration water movement (at least 30 h) there was a slight reduction in amoeba number on the gills of AGD affected salmon. It would appear that making fish swim during a bath treatment or as an alternative to a bath treatment has no beneficial effect in terms of removing gill amoebae. This notwithstanding, work by Milligan et al. (2000) suggested that sustained swimming at low speed (circa 1 $BL.s^{-1}$) facilitated the recovery of rainbow trout from exhaustive exercise. Recent studies by Powell (unpublished data) have suggested that AGD affected salmon, although accumulating lactate and experiencing an extracellular acidosis following exhaustive exercise, similar to that of rainbow trout, their recovery is not enhanced by sustained swimming at 1.6 $BL.s^{-1}$. However, these data need to be confirmed in terms of the velocity and temperature at which cultured salmon are required to swim in order to recover optimally form exhaustive exercise.

In commercial bathing operations, water is either turbulently mixed with oxygen or jetted around the bathing liner using a nozzle and oxygen injection system. In the latter case, a current is generated n the liner against which the fish must swim. However, the present work suggests that there was no difference in terms of amoeba removal from the gills of AGD affected salmon in moving water compared with turbulent or static water over the duration of a commercial bathing period.

10. BENEFITS

This research project has identified two potentially useful additives (chlorine dioxide and chloramine-T) that could be incorporated into freshwater baths that would likely improve the removal of *Neoparamoeba* from the gills of AGD affected salmon. This is likely to provide a useful tool for improving the efficacy of freshwater baths for the treatment of AGD in the future.

Additionally, we have identified that water hardness (in particular the concentration of Ca^{2+} and Mg^{2+} in bathing water) correlates well with survival of gill amoebae *in vitro* even in de-ionised (pure) freshwater. This suggests that artificially softening the bathing water (removal of Ca^{2+} and Mg^{2+}) or the selection of soft water sources for bathing water may prove more effective at killing any *Neoparamoeba* removed from the gills.

Jetting the water within a bath treatment to promote swimming (at least at 1.6 $BL.s^{-1}$) or towing the cages was shown to have no benefit on amoeba removal from the gills.

11. FURTHER DEVELOPMENT

It is evident from this research that there is potential for continuing research into the development and incorporation of chemical additives into freshwater baths for the treatment of AGD. The next logical step is to initiate cage trials to assess the efficacy of chlorine dioxide and chloramine-T under commercial conditions. Preliminary trials are planned for December 2001/January 2002 in small experimental cages at a commercial site in Tasmania where the treatment water can be contained and disposed of. Environmental approval for the use of these treatments will be required before these treatments can be adopted commercially. Similarly, approval will likely be required for use of these treatments to treat food fish under federal registration guidelines. This will require the continual gathering of data for the initial application of a Minor Use Permit. Continued development toxicity and safety testing will be required for full registration from the National Registration Authority. The costs for this process will have to be met either by a sponsor (commercial chemical manufacturing company) or by some other agency such as the salmon aquaculture industry.

Ongoing development of chemical treatments is a program objective of the Aquafin CRC. The further development of new bath treatments that will replace the need for freshwater bathing is underway as part of the Aquafin CRC. From this work it would appear that chloramine-T and possibly chlorine dioxide may have some potential for further development as treatments in seawater. However, as these treatments show some potential toxicity in hard water and the fact that seawater contains high concentrations of Ca^{2+} and Mg^{2+}, consideration must be made of their toxicity and this may limit their use as amoebicides in seawater.

12. PLANNED OUTCOME

1. We have identified key factors that effect the survival of gill amoebae including *Neoparamoeba* in freshwater *in vitro*. From this, we can now make recommendations with regard to the suitability of different freshwater sources for freshwater bathing. The concentration of Ca^{2+} and Mg^{2+} are important factors in the survival of gill amoebae in freshwater and therefore water hardness is likely to effect the efficacy of bathing. Where water hardness may be unsuitable for freshwater bathing, we have identified a mitigation strategy. Chloramine-T could be used at 10-25 $mg.L^{-1}$ for killing amoebae (including *Neoparamoeba*) even in hard water.
2. Water movement during a freshwater bath does not appear to assist in the removal of gill amoebae from the gills of AGD affected salmon. Although, towing cages (at least over long distances) did appear to reduce absolute number of amoebae on the gills, the prevalence of AGD within cages remained unaffected. This data should be interpreted with caution and requires further studies for confirmation.
3. On the basis of tank trial experiments using water sourced from 2 fish farms. Two chemical treatments have been identified as possibly having potential as additives to freshwater baths:
    i) Chlorine dioxide (in the form of Anthium dioxcide™) at a concentration 25 ppm.
    ii) Chloramine-T (in the form of Halamid™) at a concentration ranging from 10-25 ppm.

    Although only chloramine-T was consistent and effective at killing gill amoebae *in vitro* (at least as well as freshwater alone), both chloramine-T and chlorine dioxide promoted the removal of amoebae from the gills better than could be achieved by freshwater alone. At the specified concentrations, toxicity was low for both chemical treatments in tank trial experiments.

13. CONCLUSIONS

Water hardness (concentration of divalent cations) is a major factor for the survival of gill amoebae *in vitro*. This suggests that commercially bathing fish in hard water (water with high levels of specifically Ca^{2+} and Mg^{2+}) may result in the survival of gill amoebae including *Neoparamoeba* for several hours within the bath. If the bath liner is removed the freshwater may spread out over the water and potentially re-infect adjacent cages. Similarly, the *Neoparamoeba* would then be in a brackish/saltwater environment and able to re-infect fish. The consequence of this is that the bathing efficacy was reduced. Since many of the farms in Tasmania rely on dam water which is considered hard (<150-200 $mg.L^{-1}$ $CaCO_3$ equivalents) survival of *Neoparamoeba* in the bathing water is a real likelihood. The solution to this is to ensure a higher rate of kill for the freshwater bath. This can be effectively achieved using chemical additives to the freshwater.

Chlorine dioxide and chloramine-T appear to be ideal candidates as chemical additives to freshwater baths for the treatment of AGD. Both of these treatments in tank trials reduced the number of amoebae on the gills of AGD affected Atlantic salmon by approximately 50% where conventional treatment with freshwater alone failed to significantly remove amoebae compared with seawater controls. Both treatments have relatively low toxicity in freshwater at the effective treatment concentrations (chlorine dioxide 25 ppm, chloramine-T 10-25 ppm). However, water hardness may influence the toxicity of chlorine dioxide.

The economics of treatment is one aspect that was not a primary objective of this study. However, it must be considered if treatments are to be adopted by industry and follow-up trials are to be undertaken. The ineffectiveness of hydrogen peroxide excludes it as a potential candidate for use for the control of AGD. Therefore, a treatment cost analysis for both chlorine dioxide (as Anthium dioxcide™) and chloramine-T, commercial general purpose chemical grade can be undertaken:

POTENTIAL COST ANALYSIS[1]

Price of chlorine dioxide (as Anthium dioxcide^{TM} as supplied by Sterling Chemicals) = $392.5/25L
1mL/L = 50 ppm

Price of chloramine-T (as general purpose grade supplied by Merck Chemicals) = $399.10/5kg

Treatment volume/freshwater bath volume = 1000 m^3 (10^6 L)

	Chlorine dioxide	Chloramine-T	
Price per L or kg	$15.70	$79.82	
Treatment conc	25ppm 0.5 $mL.L^{-1}$	10 ppm 10 $mg.L^{-1}$	25ppm 25 $mg.L^{-1}$
	$0.5 * 10^6$ 500L	$0.01*10^6$ 10kg	$0.025*10^6$ 25kg
Cost per 1ML bath	$7850.00	$798.20	$1995.50

On this basis it would appear that chloramine-T is the preferable compound with regard to the cost of chemical for a treatment. However, since both chloramine-T and chlorine dioxide were equally effective in removing amoebae from the gills of AGD affected salmon. Since chlorine dioxide is approved for use in food processing by the Australia-New Zealand Food Authority, it is a potential candidate if the cost of treatment (i.e. cost of the chlorine dioxide) can be reduced. Alternatively, if the efficacy of chlorine dioxide can be increased to reduce the concentration that is required per treatment.

[1] costs based on prices correct at time of going to press

14. ACKNOWLEDGEMENTS

We gratefully acknowledge the following for assistance and collaborations during this project.

Huon Aquaculture Company Pty Ltd. especially:
Dr. Dominic O'Brien, Innes Weir, Adrian Steenholdt and David Mitchell

Aquatas Pty Ltd. especially
Dr. Steve Percival and Nick Murfet

Tassal Pty Ltd. especially:
David Stebbings

Colleagues at the School of Aquaculture, University of Tasmania, AGD Research Group for their helpful ideas and suggestions and assistance especially:
Marianne Douglas-Helders, Dr. Barbara Nowak, Geoff Best,

Thanks also go to Dr. Malcolm Forster, Department of Zoology, University of Canterbury, Christchurch New Zealand for his assistance during one of the field trips.

15. REFERENCES

Altimiras, J. and Larsen, E. (2000). Non-invasive recording of heart rate and ventilation rate in rainbow trout during rest and swimming. Fish go wireless! Journal of Fish Biology 57: 197-209.

Bass, M.L., Berry, C.R., and Heath, A.G. (1977). Histopathological effects of intermittent chlorine exposure on bluegill (*Leopomis macrochirus*) and rainbow trout (*Salmo gairdneri*). Water Research 11: 731-735.

Bath, R.N. and Eddy, F.B. (1979). Salt and water balance in rainbow trout (*Salmo gairdneri*) rapidly transferred from fresh to sea water. Journal of Experimental Biology 83: 193-202.

Bills, T.A., Marking, L.L., Dawson, V.K. and Rach, J.J. (1988). Effects of environmental factors on the toxicity of chloramine-T to fish. U.S. Department of the Interior Fish and Wildlife Service. Investigations into Fish Control 96: 1-6.

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. (1994a). AGD Field Research 1993/94 II. Evaluation of toxicity of hydrogen peroxide to Atlantic salmon. Reports from the SALTAS 1993-1994 Research and development programme. pp. 105-118

Cameron, D.E. (1994b).). AGD Field Research 1993/94 III. Field testing of hydrogen peroxide with AGD-infected Atlantic salmon:- efficacy and toxicity. Reports from the SALTAS 1993-1994 Research and development programme. pp. 119-125.

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.

Clark, G., Powell, M. and Nowak, B. (2000). Proceeding of the Australian Marine Sciences Association Meeting, Sydney.

Cross, D.G. and Hursey, P.A. (1973). Chloramine-T for the control of *Ichthyophthirus multifiliis (Fouquet).* Journal of Fish Biology 5: 789-798.

Davis, J.C. and Cameron, J.N. (1971). Water flow and gas exchange at the gills of rainbow trout, *Salmo gairdneri*. Journal of Experimental Biology 54: 1-18.

Davis, J.C. and Randall, D.J. (1973). Gill irrigation and pressure relationships in rainbow trout (*Salmo gairdneri*). Journal of the Fisheries Research Board of Canada 30: 99-104.

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

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

From, J. (1980). Chloramine-T for the control of bacterial gill disease. Progressive Fish Culturist 42:85-86.

Gaikowski, M.P., Rach, J.J., Olson, J.J., Ramsay, R.T. and Wolgamood, M. (1998). Toxicity of hydrogen peroxide treatments to rainbow trout eggs. Journal of Aquatic animal Health 10: 241-252.

Howard, T and Carson, J. (1994). Amoebic gill disease Laboratory research 1993/94. Reports from the SALTAS 1993-1994 Research and development Programme pp. 71-87.

Howard, T. and Carson, J. (1995). Amoebic gill disease - in vitro studies of *Paramoeba* species. Research and development review seminar. Salmon enterprises of Tasmania Pty Ltd.

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

Lumsden, J.S., Ostland, V.E. and Ferguson, H.W. (1998). Use of hydrogen peroxide to treat experimentally induced bacterial gill disease in rainbow trout. Journal of Aquatic Animal Health 110: 230-240.

McDonald, D.G., Milligan, C.L., McFarlane, W.J., Croke, S., Currie, S., Hooke, B., Angus, R.B., Tufts, B.L. and Davidson, K. (1997). Condition and performance of juvenile Atlantic salmon (*Salmo salar*): effects of rearing practices on hatchery fish and comparison with wild fish. Canadian Journal of Fisheries and Aquatic Sciences 55: 1208-1219.

Milligan, C.L., Hooke, G.B. and Johnson, C. (2000). Sustained swimming at low velocity following a bout of exhaustive exercise enhances metabolic recovery in rainbow trout. Journal of Experimental Biology 203: 921-926.

Munday, B.L., Foster, C.K., Roubal, F.R. and Lester, R.G.L. (1990). Paramoebic gill infection and associated pathology of Atlantic salmon, *Salmo salar* and rainbow trout *Salmo gairdneri* in Tasmania. In: Pathology in Marine Sciences (F.O. Perkins and T.C. Chang eds.). Academic Press Ltd.. San Diego. Pp. 215-222.

Nowak, B. (2001). Qualitative evaluation of risk factors for amoebic gill disease in cultured 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 Epizootics (O.I.E.), 148-155.

Ostland, V.E., Byrne, P.J., Speare, D.J., Thorburn, M.A., Cook, A., Morrison, D. and Ferguson, H.W. (1995). Comparison of formalin and chloramine-T for control of a mixed gill infection (bacterial gill disease and ichthyobodiasis) in rainbow trout. Journal of Aquatic Animal Health 7: 118-123.

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

Parsons, H.J., Nowak, B.F., Powell, M.D. and Dix, T. (2001a). Changes in water quality during commercial freshwater bathing of Atlantic salmon (*Salmo salar*) in Tasmania - implications for treatment of amoebic gill disease. Bulletin of the European Association of Fish Pathologists 21: 71-76.

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

Palzenberger, M. and Pohla, H. (1992). Gill surface area of water breathing freshwater fish. Reviews in Fish Biology and Fisheries 2: 187-216.

Powell, M.D. and Perry S.F. (1996). Respiratory and acid-base disturbances on rainbow trout (*Oncorhynchus mykiss*) blood during exposure to chloramine-T, paratoluenesulphonamide and hypochlorite. Canadian Journal of Fisheries and Aquatic Sciences 53: 710-708.

Powell, M.D. and Perry, S.F. (1997a). Branchial ionic flux responses in rainbow trout to chloramine-T after acclimation to different levels of water hardness. Journal of Aquatic Animal Health 9: 196-202.

Powell, M.D. and Perry S.F. (1997b). Respiratory and acid-base disturbances in rainbow trout blood during exposure to chloramine-T under hypoxia and hyperoxia. Journal of Fish Biology 50: 418-428.

Powell, M.D. and Perry S.F. (1997c). Respiratory and acid- base pathophysiology of hydrogen peroxide in rainbow trout (*Oncorhynchus mykiss* Walbaum). Aquatic Toxicology 37: 99-112.

Powell, M.D. and Perry S.F. (1998). Acid-base and ionic fluxes in rainbow trout (*Oncorhynchus mykiss*) during exposure to chloramine-T. Aquatic Toxicology 43: 13-24.

Powell, M.D. and Perry S.F. (1999). Cardio-respiratory effects of chloramine-T exposure in rainbow trout. Experimental Biology Online 4:5.

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

Rach, J.J., Gaikowski, M.P. and Ramsay, R.T. (2000). Efficacy of hydrogen peroxide to control mortalities associated with bacterial gill disease infections on hatchery reared salmonids. Journal of Aquatic Animal Health 12: 119-127.
Roberts, J.L. (1975). Active branchial and ram gill ventilation in fishes. Biological Bulletin 148: 85-105.

Rodger, H.D and McArdle, J.F. (1996). An outbreak of amoebic gill disease in Ireland. Veterinary Record 5: 348-349.

Saunders, R.L. (1961). The irrigation of the gills in fishes. I. Studies of the mechanism of branchial irrigation. Canadian Journal of Zoology 40: 677-683.

Saunders, R.L. (1962). The irrigation of the gills in fishes. II Efficiency of oxygen uptake in relation to respiratory flow activity and concentrations of oxygen and carbon dioxide. Canadian Journal of Zoology 40: 817-862.

Venkobacker, C., Iyengar, L. and Prabhakara Rao, A.V.S. (1977). Mechanism of disinfection: effects of chlorine on cell membrane functions. Water Research 11: 727-729.

Zall, D.M., Fisher, M.D. and Garner, Q.M. (1956). Photometric determination of chloride in water. Analytical Chemistry 28: 1665-1678.

Appendix 1: Intellectual property

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

1. Test chemicals for use for treating AGD

2. Treatment (concentration and duration), efficacy and toxicity data likely to be of use towards securing a minor use permit (MUP) for drug registration

3. Copyright in this report.

Appendix 2: Staff

Staff engaged on the project:

Principle investigator

Dr. Mark Powell	School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania.

Co-investigator (on study leave 2/01-7/01)

Dr. Barbara Nowak	School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania.

Research Assistant

Ms. Gemma Clark	School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania.

Appendix 3: Presentations made in relation to this report

Clark, G.A., Powell, M.D. and Nowak, B.F. (2001). Investigations into *Neoparamoeba* survival in vitro: water chemistry characteristics and chemical additives. Fisheries Research and Development Corporation, Atlantic Salmon Aquaculture Sub-program scientific conference 18-19 July, Hobart, Tas.

Powell, M.D. and Clark, G.A. (2001). Bath Additives for removal of *Neoparamoeba* from salmon gills efficacy and toxicity. Fisheries Research and Development Corporation, Atlantic Salmon Aquaculture Sub-program scientific conference 18-19 July, Hobart, Tas.

Nowak, B.F. and Powell, M.D. (2001). Amoebic Gill Disease – beyond abolishing the myths. American Fisheries Society Fish Health Section Annual Meeting and 42nd Fish Disease Workshop, June 26-29, Victoria, BC, Canada.

Powell, M.D., Clark, G.A. and Nowak, B.F. (2001). Treatment for amoebic gill disease in Tasmania: downunder not downhearted. European Association of Fish Pathologists 10th International conference "Disease of fish and shellfish" 9-14 Sept. Dublin, Ireland.

Note: The non-technical summary of the draft of this report was relayed to industry through the Atlantic salmon Aquaculture Subprogram newsletter “Salmon Snippets” volume 2, March 2002.

Appendix 4: Isolation of amoebae from AGD affected salmon

Amoebae were isolated from the gills using a technique modified from Jones (1985). AGD affected Atlantic salmon commercially farmed in Tasmania were killed using 2% clove oil. Gills were excised from the fish and placed in 50mL tubes containing 2.5 % ammonium chloride in seawater. 100 ? L of both antibiotic solution 1 and 2 were added to gills in suspension (Table A1). Samples were kept cool and mucus was removed from the gills using a plastic hockey stick. Mucus was resuspended in seawater and mixed by agitation.

Aliquots of suspended amoeba solution were sampled and stained with 0.5% trypan blue- seawater mix at a dilution of 1:3. Live amoeba counts were determined using a haemocytometer (Neubauer, BS 748) and calculated with the equation:

$$Total\ no.\ amoeba\ ?\ Average\ amoeba\ count\ ?\ dilution\ \ factor\ ?\ 10^4\ ?\ volume\ of\ \ solution$$

Average amoeba counts were calculated from counts made from 20 large squares on the haemocytometer.

Table A1. Antibiotic solutions added to gills. Freeze antibiotics at –20^{o}C to store

Antibiotic Mix 1 – to 5 mL distilled water add	
Streptomycin sulphate	0.05g
Penicillin	0.05g
Carbenicillin	0.05g
Ampicillin	0.125g
Antibiotic Mix 2 – to 5 mL methanol add	
Erythromycin	0.05g

Appendix 5. Indirect Fluorescent Antibody Test (IFAT)

During fish sampling mucus smears were taken from mucoid patches on the gills with a clean glass slide. If according to the gross scoring, fish were considered clear, the third left gill was sampled at the base of the ventral region to obtain gill mucus. Samples used for *in vitro* testing were sampled using a sterile plastic loop. A small mucus sample was taken from the solution isolated from the gills. Samples were smeared onto a numbered glass slide and left to air-dry then heat fixed. Positive controls were used to confirm samples taken from fish and these were sourced from pure *N. pemaquidensis* cultures (Batch PA027) from the Department of Primary Industries Water and Environment (DPIWE), fish health laboratory, Launceston, Tasmania.

An area containing the gill smear was circled using a 'Pap Pen' (Daido Sangyo Co. Ltd. Japan). Each slide was then flooded with phosphate buffered saline (PBS) solution (adjusted to pH 7.4) (Table A2) and left to stand for 5 min and shaken off. The primary antibody (Ab), sheep anti-*Neoparamoeba* (Batch PA027), was diluted to 1:150 in immunofluorescent antibody diluent (Table A2) and applied to each slide at a volume of 200 ?L per slide.

Slides were incubated for 45 min at 37°C. Primary Ab was shaken off and the slides washed twice in PBS with agitation for 5 min each. Slides were shaken to remove excess PBS and the second fluorescein labelled anti-sheep IgG (Silenus Cat. No. RF, Sigma Batch F-7634) diluted to concentration of 1:40 in PBS and added at a volume of 200 ?L per slide. Slides were incubated for 30 min at 37°C and rinsed 3 times in PBS as before.

Slides were mounted in alkaline-buffered glycerol (adjusted to pH 9.0) (Table A2) and observed at ?100 magnification using an Olympus BH2-RFL-T3 UV epi-fluorescent microscope and FITC filter set. *Neoparamoeba* were observed to fluoresce a strong yellow-green. Slides were scored as positive or negative for amoeba presence.

Table A2. Chemical solutions used in IFAT[1]

Phosphate Buffered Saline (PBS) - concentrated stock solution	
Na_2HPO_4 (anhydrous)	
KH_2PO_4 (anhydrous)	7.24g
NaCl	2.1g
Distilled H_2O	76.5g
pH	10L
Dissolve salts by warming to 50°C	7.4
Adjust pH by NaCl or HCI	
IF (immunofluorescence) Antibody Diluent- store at 4°C	
PBS pH 7.4	100mL
Crystalline-grade bovine serum albumin	0.1g
Sodium azide	0.1g

Alkaline Glycerol Mountant	
$NaHCO_3$	0.0729g
Na_2CO_3	0.016g
Distilled water	10.0mL
Glycerol	90.0mL
PH	9.0
Dissolve salts in distilled water, gentle warming may be required	
Add to glycerol and mix thoroughly	
Check pH and adjust accordingly	

[1]Johnson, A.M. & Munday, B.L. 1993. Toxoplasmosis: pathology, histopathology and serology. In: Australian standard Diagnostic Techniques for Animal Diseases. Edited by: Corner, L.A. & Bagust, T.J. Published by CSIRO, for the Standing Committee on Agricultural & Resource Management, Melbourne.

Appendix 6. Histological sampling and processing

The second left gill arch was removed and placed into seawater Davidson's fixative for 72 h. Samples were removed from the fixative and the cartilage cut from the gill. Samples were placed into cassettes and processed using a Tissue-tek II automatic tissue processing unit (Table A3). Gill samples were embedded in paraffin wax on completion of processing using Shandon Histocentre 2. One section of 5? m was cut from each block using a Microm microtome. Each section was stained with haematoxylin and eosin using a Shandon Linistan GLX processing unit (Table A4). Slides were mounted using DPX mountant and examined under a light microscope at 100? and 400? magnification.

Table A3. Tissue-tek II tissue processing unit

Station	Chemical	Time (min)
1	70% alcohol	60
2	80% alcohol	60
3	90% alcohol	120
4	95% alcohol	120
5	100% alcohol	120
6	100% alcohol	120
7	100% alcohol	120
8	xylene	120
9	xylene	120
10	xylene	120
11	paraffin	180
12	paraffin	180

Table A4. Shandon Linistain GLX unit

Station	Solution	Time (s)
1	XYLENE (X3B)	35
2	XYLENE (X3B)	35
3	XYLENE (X3B)	35
4	100% ALCOHOL	35
5	100% ALCOHOL	35
6	100% ALCOHOL	35
7	95% ALCOHOL	35
8	WATER	35
9	HAEMATOXYLIN	35
10	HAEMATOXYLIN	35
11	HAEMATOXYLIN	35
12	HAEMATOXYLIN	35
13	HAEMATOXYLIN	35
14	WATER	35
15	SCOTTS TAP	35
16	WATER	35
17	WATER	35
18	WATER	35
19	WATER	35
20	EOSIN	35
21	EOSIN	35
22	EOSIN	35
23	100% ALCOHOL	35
24	100% ALCOHOL	35
25	100% ALCOHOL	35
26	100% ALCOHOL	35
27	100% ALCOHOL	35
28	100% ALCOHOL	35
29	100% ALCOHOL	35
30	XYLENE	-

Appendix 7. Statistical analysis tables

In vitro assay experiments
IN VITRO ASSAYS

TABLE A1. Effect of NaCl concentration and time on amoeba survival . Mauchly's test for sphericity, M=0.612

Tests of Within-Subjects Contrasts

Source	HOUR	df	Mean Square	F	Sig.
HOUR	Linear	1	1.433	147.523	.000
	Quadratic	1	.108	17.082	.000
HOUR * NACL	Linear	5	7.015E-02	7.221	.000
	Quadratic	5	2.004E-02	3.170	.020
Error(HOUR)	Linear	30	9.715E-03		
	Quadratic	30	6.322E-03		

Tests of Between-Subjects Effects

Source	Df	Mean Square	F	Sig.
NACL	5	1.993	155.337	.000
Error	30	1.283E-02		

TABLE A2. Effect of CaCL concentration and time on amoeba survival . Mauchly's test for sphericity, M=0.849

Tests of Within-Subjects Contrasts

Source	HOUR	df	Mean Square	F	Sig.
HOUR	Linear	1	32503.064	189.931	.000
	Quadratic	1	3658.984	12.583	.009
HOUR * CACL	Linear	5	1739.049	10.162	.004
	Quadratic	5	273.092	.939	.510
Error(HOUR)	Linear	7	171.131		
	Quadratic	7	290.786		

Tests of Between-Subjects Effects

Source	df	Mean Square	F	Sig.
CACL	5	2765.193	14.510	.001
Error	7	190.567		

TABLE A3. Effect of NaCl/CaCl concentration and time on amoeba survival . Mauchly's test for sphericity, M=0.629

Tests of Within-Subjects Contrasts

Source	HOUR	df	Mean square	F	Sig.
HOUR	Linear	1	7824.900	72.419	.000
	Quadratic	1	733.954	13.795	.001
HOUR *	Linear	5	640.158	5.925	.001
NACLCACL	Quadratic	5	70.717	1.329	.279
Error	Linear	30	108.050		
(HOUR)	Quadratic	30	53.203		

Tests of Between-Subjects Effects

Source	df	Mean Square	F	Sig.
NACL/CACL	5	22911.350	313.310	.000
Error	30	73.127		

TABLE A4. Effect of MgCl concentration and time on amoeba survival . Mauchly's test for sphericity, M=0.839

Tests of Within-Subjects Contrasts

Source	HOUR	df	Mean square	F	Sig.
HOUR	Linear	1	.283	7.593	.010
	Quadratic	1	6.141E-04	.008	.931
HOUR *	Linear	5	.146	3.911	.008
MACL	Quadratic	5	6.673E-02	.823	.543
Error	Linear	30	3.727E-02		
(HOUR)	Quadratic	30	8.108E-02		

Tests of Between-Subjects Effects

Source	df	Mean Square	F	Sig.
MACL	5	1.537	21.904	.000
Error	30	7.018E-02		

TABLE A5. Effect of Choline concentration and time on amoeba survival . Mauchly's test for sphericity, M=0.544

Tests of Within-Subjects Contrasts

Source	HOUR	df	Mean square	F	Sig.
HOUR	Linear	1	1.057E-03	.071	.795
	Quadratic	1	2.425E-02	5.663	.035
HOUR *	Linear	5	1.85E-02	1.237	.351
CHOLINE	Quadratic	5	1.406E-02	3.284	.042
Error	Linear	12	1.498E-02		
(HOUR)	Quadratic	12	4.282E-03		

Tests of Between-Subjects Effects

Source	df	Mean Square	F	Sig.

CHOLINE	**5**	**14968.958**	**2497027.537**	**.000**
Error	**12**	**5.995E-03**		

TABLE A6. Effect of pH concentration and time on amoeba survival . Mauchly's test for sphericity, M=1.000

Tests of Within-Subjects Contrasts

Source	HOUR	df	Mean square	F	Sig.
HOUR	**Linear**	**1**	**2.013E-02**	**9.285**	**.010**
HOUR * pH	**Linear**	**5**	**4.330E-03**	**1.998**	**.151**
Error (HOUR)	**Linear**	**12**	**2.168E-03**		

Tests of Between-Subjects Effects

Source	df	Mean Square	F	Sig.
pH	**5**	**.948**	**437.175**	**.000**
Error	**12**	**2.168E-03**		

TABLE A7. Effect of Chlorine dioxide concentration and time on amoeba survival . Mauchly's test for sphericity, M=0.954

Tests of Within-Subjects Contrasts

Source	HOUR	df	Mean square	F	Sig.
HOUR	**Linear**	**1**	**.231**	**3.265**	**.081**
	Quadratic	**1**	**3.940E-02**	**.650**	**.426**
HOUR *CD	**Linear**	**5**	**6.628E-02**	**.937**	**.472**
	Quadratic	**5**	**8.223E-03**	**.136**	**.983**
Error (HOUR)	**Linear**	**30**	**7.077E-02**		
	Quadratic	**30**	**6.059E-02**		

Tests of Between-Subjects Effects

Source	df	Mean Square	F	Sig.
CD	**5**	**1.757**	**29.143**	**.000**
Error	**30**	**6.027E-02**		

TABLE A8. Effect of Chloramine-T concentration and time on amoeba survival . Mauchly's test for sphericity, M=0.356

Tests of Within-Subjects Contrasts

Source	HOUR	df	Mean square	F	Sig.
HOUR	Linear	1	.857	17.163	.000
	Quadratic	1	.110	12.572	.001
HOUR * CT	Linear	5	.121	2.424	.058
	Quadratic	5	2.967E-02	3.401	.015
Error	Linear	30	4.992E-02		
(HOUR)	Quadratic	30	8.724E-03		

Tests of Between-Subjects Effects

Source	df	Mean Square	F	Sig.
CT	5	2.379	51.814	.000
Error	30	4.591E-02		

TABLE A9. Effect of Hydrogen peroxide concentration and time on amoeba survival . Mauchly's test for sphericity, M=0.880

Tests of Within-Subjects Contrasts

Source	HOUR	df	Mean square	F	Sig.
HOUR	Linear	1	8.385E-02	2.141	.154
	Quadratic	1	.122	1.506	.229
HOUR *	Linear	5	.103	2.641	.043
H2O2	Quadratic	5	2.741E-02	.340	.885
Error	Linear	30	3.917E-02		
(HOUR)	Quadratic	30	8.071E-02		

Tests of Between-Subjects Effects

Source	df	Mean Square	F	Sig.
H2O2	5	2.361	37.085	.000
Error	30	6.367E-02		

WATER MOVEMENT EXPERIMENTS

AMOEBA LOG WEIGHT

TABLE B1. Effect of trial on amoeba log weight in moving freshwater. Levene's test nonsignificant, F=4.195

Source	df	MS	F	Sig
Trial	14	4.867+10	2.214	0.020
Error	51	2.199+10		

TABLE B2. Effect of freshwater movement treatment on amoeba log weight. Levene's test nonsignificant, F=1.961

Source	df	MS	F	Sig
Treat	4	2.75E+10	0.993	0.418
Error	61	2.77E+10		

TABLE B3. effect of trial on amoeba log weight in moving seawater. Levene's test nonsignificant, F=5.451

Source	df	MS	F	Sig
Trial	7	3.107E+10	4.587	0.002
Error	28	6773254617		

TABLE B4. Effect of seawater movement treatment on amoeba log weight. Levene's test nonsignificant, F=1.992

Source	df	MS	F	Sig
Treat	3	8.35E+09	0.669	0.559
Error	32	1.19E+10		

HISTOLOGY FRESHWATER TREATMENT

TABLE B5. Effect of trial on number of lesions per filament in freshwater. Levene's test nonsignificant, F=4.662

Source	df	MS	F	Sig
Trial	14	0.128	3.123	0.001
Error	51	4.095E-02		

TABLE B6. Effect of treatment on lesions per filament in freshwater. Levene's test nonsignificant, F=2.361

Source	df	MS	F	Sig
Treat	4	0.109	1.930	0.117
Error	61	5.644E-02		

TABLE B7. Effect of trial on the number of cysts per lesion in freshwater. Levene's test nonsignificant, F=2.432

Source	df	MS	F	Sig
Trial	14	0.640	2.348	0.014
Error	51	0.272		

TABLE B8. Effect of treatment on the number of cysts per lesion in freshwater. Levene's test nonsignificant, F=1.175

Source	df	MS	F	Sig
Treat	4	0.565	1.673	0.168
Error	61	0.337		

TABLE B9. Effect of trial on the number of amoeba per lesion in freshwater. Levene's test nonsignificant, F=14.063

Source	df	MS	F	Sig
Trial	14	1.397	2.678	0.005
Error	51	0.522		

TABLE B10. Effect of treatment on the number of amoeba per lesion in freshwater. Levene's test nonsignificant, F=2.784

Source	df	MS	F	Sig
Treat	4	0.700	0.984	0.423
Error	61	0.711		

TABLE B11. Effect of trial on the number of amoeba in cysts in freshwater. Levene's test nonsignificant, F=9.409

Source	df	MS	F	Sig
Trial	14	0.709	2.308	0.015
Error	51	0.307		

TABLE B12. Effect of treatment on the number of amoeba in cysts in freshwater. Levene's test nonsignificant, F=8.049

Source	df	MS	F	Sig
Treat	4	0.537	1.396	0.246
Error	61	0.384		

HISTOLOGY SEAWATER TREATMENT

TABLE B12. Effect of trial on number of lesions per filament in seawater. Levene's test nonsignificant, F=0.787

Source	df	MS	F	Sig
Trial	7	6.675E-02	0.875	0.538
Error	28	7.630E-02		

TABLE B13. Effect of treatment on lesions per filament in seawater. Levene's test nonsignificant, F=0.158

Source	df	MS	F	Sig
Treat	3	6.549E-02	0.871	0.466
Error	32	7.522E-02		

TABLE B14. Effect of trial on the number of cysts per lesion in seawater. Levene's test nonsignificant, F=2.121

Source	df	MS	F	Sig
Trial	7	0.261	0.395	0.897
Error	28	0.660		

TABLE B15. Effect of treatment on the number of cysts per lesion in seawater. Levene's test nonsignificant, F=2.467

Source	df	MS	F	Sig
Treat	3	0.317	0.523	0.669
Error	32	0.605		

TABLE B16. Effect of trial on the number of amoeba per lesion in seawater. Levene's test nonsignificant, F=5.382

Source	df	MS	F	Sig
Trial	7	3.772	1.170	0.351
Error	28	3.224		

TABLE B17. Effect of treatment on the number of amoeba per lesion in seawater. Levene's test nonsignificant, F=2.671

Source	df	MS	F	Sig

Treat	**3**	**2.865**	**0.848**	**0.478**
Error	**32**	**3.378**		

TABLE B18. Effect of trial on the number of amoeba in cysts in seawater. Levene's test nonsignificant, F=7.475

Source	df	MS	F	Sig
Trial	**7**	**2.454**	**0.809**	**0.587**
Error	**28**	**3.034**		

TABLE B19. Effect of treatment on the number of amoeba in cysts in seawater. Levene's test nonsignificant, F=2.967

Source	df	MS	F	Sig
Treat	**3**	**1.417**	**0.463**	**0.710**
Error	**32**	**3.058**		

FRESHWATER TREATMENT IFAT

TABLE B20. Effect of trial on IFAT positive fish in freshwater. Levene's test nonsignificant, F=4.149

Source	df	MS	F	Sig
Trial	**14**	**0.177**	**0.724**	**0.740**
Error	**51**	**0.245**		

TABLE B21. Effect of treat on IFAT positive fish in freshwater. Levene's test nonsignificant, F=0.671

Source	df	MS	F	Sig
Treat	**4**	**9.343E-02**	**0.390**	**0.815**
Error	**61**	**0.240**		

SEAWATER TREATMENT IFAT

TABLE B22. Effect of trial on IFAT positive fish in seawater. Levene's test nonsignificant, F=1.118

Source	df	MS	F	Sig
Trial	**7**	**0.167**	**0.615**	**0.739**
Error	**28**	**0.271**		

TABLE B23. Effect of treat on IFAT positive fish in seawater. Levene's test nonsignificant, F=0.555

Source	df	MS	F	Sig
Treat	**3**	**6.944E-02**	**0.260**	**0.854**
Error	**32**	**0.267**		

FRESHWATER TREATMENT FISH DATA

TABLE B24. Effect of trial on fish weight in freshwater treatments. Levene's test nonsignificant, F=4.655

Source	df	MS	F	Sig
Trial	**2**	**665881.927**	**9.161**	**0.000**
Error	**62**	**72688.170**		

TABLE B25. Effect of trial on fish Length in freshwater treatments. Levene's test nonsignificant, F=1.046

Source	df	MS	F	Sig
Trial	2	168.313	8.743	0.000
Error	62	19.251		

SEAWATER TREATMENT FISH DATA

TABLE B26. Effect of trial on fish weight in seawater treatments. Levene's test nonsignificant, F=1.339

Source	df	t-calc	t-crit	Sig
Trial	34	1.010	2.03	0.05

TABLE B27. Effect of trial on fish length in seawater treatments. Levene's test nonsignificant, F=0.615

Source	df	t-calc	t-crit	Sig
Trial	34	0.783	2.03	0.05

TOW EXPERIMENT

AMOEBA LOG WEIGHT COUNT

TABLE B28. Effect of trial on before tow amoeba count. Levene's test nonsignificant, F=2.732

Source	df	MS	F	Sig
Trial	2	1.000E+13	29.804	0.000
Error	27	3.357E+11		

TABLE B29. Effect of trial on after tow amoeba count. Levene's test nonsignificant, F=5.885

Source	df	MS	F	Sig
Trial	2	2.161E+12	13.770	0.000
Error	27	1.569E+11		

TABLE 30. Effect of treatment on amoeba log weight count. Paired t-test.

Source	df	t-calc	t-crit	Sig
Trial	29	2.992	2.04	0.05

TOW HISTOLOGY

TABLE 31. Effect of trial on lesion per filament before tow. Levene's test nonsignificant, F=8.311

Source	df	MS	F	Sig
Trial	2	0.368	14.237	0.000
Error	25	2.547E-02		

TABLE B32. Effect of trial on lesion per filament after tow. Levene's test nonsignificant, F=1.437

Source	df	MS	F	Sig
Trial	2	1.468E-02	1.811	0.183
Error	26	8.105E-03		

TABLE B33. Effect of trial on ILV per lesion before tow. Levene's test nonsignificant, F=4.701

Source	df	MS	F	Sig
Trial	2	0.716	8.493	0.002
Error	25	8.425E-02		

TABLE B34. Effect of trial on ILV per lesion after tow. Levene's test nonsignificant, F=5.960

Source	df	MS	F	Sig
Trial	2	2.875	6.529	0.005
Error	26	0.440		

TABLE B35. Effect of trial on amoeba per lesion before tow. Levene's test nonsignificant, F=4.682

Source	df	MS	F	Sig
Trial	2	0.302	0.935	0.406
Error	25	0.323		

TABLE B36. Effect of trial on amoeba per lesion after tow. Levene's test nonsignificant, F=3.196

Source	df	MS	F	Sig
Trial	2	0.271	0.961	0.396
Error	26	0.282		

TABLE B37. Effect of trial on amoeba in ILV before tow. Levene's test nonsignificant, F=3.004

Source	df	MS	F	Sig
Trial	2	0.657	0.578	0.568
Error	25	1.136		

TABLE B38. Effect of trial on amoeba in ILV after tow. Levene's test nonsignificant, F=6.246

Source	df	MS	F	Sig
Trial	2	9.467	1.640	0.213
Error	26	5.773		

TABLE B39. Paired t-test. Effect of treatment on lesion per filament.

Source	df	t-calc	t-crit	Sig
Trial	27	1.579	2.04	0.05

TABLE B40. Paired t-test. Effect of treatment on ILV per filament.

Source	df	t-calc	t-crit	Sig
Trial	27	1.202	2.04	0.05

TABLE B41. Paired t-test. Effect of treatment on amoeba per filament.

Source	df	t-calc	t-crit	Sig
Trial	27	1.070	2.04	0.05

TABLE B42. Paired t-test. Effect of treatment on amoeba in ILV.

Source	df	t-calc	t-crit	Sig

Trial	**27**	**0.613**	**2.04**	**0.05**

CHEMICAL EFFICACY TRIALS

AMOEBA COUNT

TABLE C1. Effect of trial on chlorine dioxide amoeba count at farm 1. Levene's test nonsignificant, F= 2.348

Source	df	MS	F	Sig
Trial	**9**	**8.495E+10**	**3.724**	**0.002**
Error	**34**	**2.281E+10**		

TABLE C2. Effect of concentration of chlorine dioxide on amoeba count at farm 1. Levene's test nonsignificant, F=2.631

Source	df	MS	F	Sig
Conc	**4**	**1.73E+11**	**7.917**	**0.000**
Error	**39**	**2.18E+10**		

TABLE C3. Effect of trial on chloramine-T amoeba count at farm 1. Levene's test nonsignificant, F=9.291

Source	df	MS	F	Sig
Trail	**9**	**1.399E+11**	**3.994**	**0.001**
Error	**34**	**3.352E+10**		

TABLE C4. Effect of concentration on chloramine-T amoeba count at farm 1. Levene's test nonsignificant, F=10.884

Source	Df	MS	F	Sig
Conc	**4**	**2.59E+11**	**7.704**	**0.000**
Error	**39**	**3.36E+10**		

TABLE C5. Effect of trial on hydrogen peroxide amoeba count at farm 1. Levene's test nonsignificant, F=7.051

Source	df	MS	F	Sig
Trial	**9**	**3.810E+11**	**10.884**	**0.000**
Error	**34**	**3.500E+10**		

TABLE C6. Effect of concentration of hydrogen peroxide on amoeba count at farm 1. Levene's test nonsignificant, F=4.263

Source	df	MS	F	Sig
Conc	**4**	**3.23E+11**	**3.791**	**0.011**
Error	**39**	**8.53E+10**		

TABLE C7. Effect of trial on chlorine dioxide amoeba count at farm 2. Levene's test nonsignificant, F=1.247

Source	df	MS	F	Sig
Trial	9	1.017E+10	2.029	0.066
Error	34	5012263.658		

TABLE C8. Effect of concentration of chlorine dioxide on amoeba count at farm 2. Levene's test nonsignificant, F=1.531

Source	df	MS	F	Sig
Conc	4	1.99E+10	4.248	0.006
Error	39	4.68E+09		

TABLE C9. Effect of trial on chloramine-T amoeba counts at farm 2. Levene's test nonsignificant, F=2.021

Source	df	MS	F	Sig
Trial	9	9814440229	2.646	0.019
Error	34	3708659889		

TABLE C10. Effect of concentration of chloramine-T on amoeba counts at farm 2. Levene's test nonsignificant, F=1.523

Source	df	MS	F	Sig
Conc	4	1.56E+10	3.997	0.008
Error	39	3.90E+09		

PLASMA IONS

CHLORIDE

TABLE C11. Effect of trial on chlorine dioxide plasma chloride concentrations at farm 1. Levene's test nonsignificant, F=0.879

Source	df	MS	F	Sig
Trial	9	18481.860	17.388	0.000
Error	33	1032.901		

TABLE C12. Effect of concentration of chlorine dioxide on plasma chloride concentrations at farm 1. Levene's test nonsignificant, F= 10.396

Source	df	MS	F	Sig
Conc	4	9878.581	2.319	0.075
Error	38	4260.477		

TABLE C13. Effect of trial on chloramine-T plasma chloride concentrations at farm 1. Levene's test nonsignificant, F=2.794

Source	df	MS	F	Sig
Trial	9	6952.749	6.066	0.000
Error	32	1146.152		

TABLE C14. Effect of concentration of chloramine-T on plasma chloride concentrations at farm 1. Levene's test nonsignificant, F=2.233

Source	df	MS	F	Sig
Conc	4	3437.378	1.487	0.226
Error	37	2310.867		

TABLE C15. Effect of trial on hydrogen peroxide plasma chloride ion concentrations at farm 1. A. Levene's test nonsignificant, F=1.938

Source	df	MS	F	Sig
Trial	9	2836.545	2.174	0.051
Error	33	1304.956		

TABLE C16. Effect of concentration of hydrogen peroxide on plasma chloride ion concentrations at farm 1. Levene's test nonsignificant, F=0.820

Source	df	MS	F	Sig
Conc	4	3370.318	2.324	
Error	38	1450.294	0.074	

TABLE C17. Effect of concentration of chlorine dioxide on plasma chloride concentrations at farm 2. Levene's test nonsignificant, F=3.779

Source	df	MS	F	Sig
Trial	9	5479.882	3.170	0.000
Error	32	1728.891		

TABLE C18. Effect of concentration of chlorine dioxide on plasma chloride concentrations at farm 2. Levene's test nonsignificant, F=3.516

Source	df	MS	F	Sig
Conc	4	11217.735	6.944	0.000
Error	37	1615.473		

TABLE C19. Effect of trial on chloramine-T plasma chloride concentrations at farm 2. Levene's test nonsignificant, F= 2.903

Source	df	MS	F	Sig
Trial	9	5014.673	6.666	0.000
Error	32	752.279		

TABLE C20. Effect of concentration of chloramine-T on plasma chloride concentrations at farm 2. Levene's test nonsignificant, F=2.043

Source	df	MS	F	Sig
Conc	4	6146.431	5.096	0.002
Error	37	1206.034		

POTASSIUM

TABLE C21. Effect of trial on chlorine dioxide plasma potassium concentrations at farm 1. Levene's test nonsignificant, F=0.939

Source	df	MS	F	Sig
Trial	9	1.310	4.182	0.001
Error	33	0.313		

TABLE C22. Effect of concentration of chlorine dioxide on plasma potassium concentrations at farm 1. Levene's test nonsignificant, F=1.362

Source	df	MS	F	Sig
Conc	4	2.323	6.881	0.000
Error	38	0.338		

TABLE C23. Effect of trial on chloramine-T plasma potassium concentrations at farm 1. Levene's test nonsignificant, F=2.354

Source	df	MS	F	Sig
Trial	9	1.548	2.736	0.018
Error	30	0.566		

TABLE C24. Effect of concentration of chloramine-T on plasma potassium concentrations at farm 1. Levene's test nonsignificant, F=0.785

Source	df	MS	F	Sig
Conc	4	2.624	4.501	0.005
Error	35	0.583		

TABLE C25. Effect of trial on hydrogen peroxide plasma potassium ion concentrations at farm 1. Levene's test nonsignificant, F=2.291

Source	df	MS	F	Sig
Trial	9	5.125	7.658	0.000
Error	34	0.669		

TABLE C26. Effect of concentration of hydrogen peroxide on plasma potassium ion concentrations at farm 1. Levene's test nonsignificant, F=22.643

Source	df	MS	F	Sig
Conc	4	3.476	2.466	0.061
Error	39	1.410		

TABLE C27. Effect of concentration of chlorine dioxide on plasma potassium concentrations at farm 2. Levene's test nonsignificant, F=2.513

Source	df	MS	F	Sig
Trial	9	1.995	1.864	0.093
Error	33	1.071		

TABLE C28. Effect of concentration of chloride dioxide on plasma potassium concentrations at farm 2. Levene's test nonsignificant, F=1.905

Source	df	MS	F	Sig
Conc	4	2.568	2.269	0.080
Error	38	1.132		

TABLE C29. Effect of trial on chloramine-T plasma potassium concentrations at farm 2. Levene's test nonsignificant, F= 3.187

Source	df	MS	F	Sig
Trial	9	1.182	1.606	0.153
Error	34	0.736		

TABLE C30. Effect of concentration of chloramine-T on plasma potassium concentrations at farm 2. Levene's test nonsignificant, F=1.597

Source	df	MS	F	Sig
Conc	4	2.039	2.893	0.034
Error	39	0.705		

SODIUM

TABLE C31. Effect of trial on chlorine dioxide plasma sodium concentrations at farm 1. Levene's test nonsignificant, F= 1.541

Source	df	MS	F	Sig
Trial	**9**	**895.784**	**14.562**	**0.000**
Error	**33**	**61.515**		

TABLE C32. Effect of concentration of chlorine dioxide on plasma sodium concentrations at farm 1. Levene's test nonsignificant, F=1.329

Source	df	MS	F	Sig
Conc	**4**	**1328.853**	**10.572**	**0.000**
Error	**38**	**125.701**		

TABLE C33. Effect of trial on chloramine-T plasma sodium concentrations at farm 1. Levene's test nonsignificant, F=1.513

Source	df	MS	F	Sig
Trial	**9**	**1284.538**	**9.738**	**0.000**
Error	**30**	**131.915**		

TABLE C34. Effect of concentration of chloramine-T on plasma sodium concentrations at farm 1. Levene's test nonsignificant, F=1.313

Source	df	MS	F	Sig
Conc	**4**	**2648.714**	**18.829**	**0.000**
Error	**38**	**140.670**		

TABLE C35. Effect of trial on hydrogen peroxide plasma sodium ion concentrations at farm 1. Levene's test nonsignificant, F=1.725

Source	df	MS	F	Sig
Trial	**9**	**1288.278**	**26.757**	**0.000**
Error	**34**	**48.148**		

TABLE C36. Effect of concentration of hydrogen peroxide on plasma sodium ion concentrations at farm 1. Levene's test nonsignificant, F=2.836

Source	df	MS	F	Sig
Conc	**4**	**2882.574**	**66.082**	**0.000**
Error	**39**	**43.621**		

TABLE C37. Effect of concentration of chlorine dioxide on plasma sodium concentrations at farm 2. Levene's test nonsignificant, F= 3.667

Source	df	MS	F	Sig
Trial	**9**	**2170.402**	**7.946**	**0.000**
Error	**33**	**273.147**		

TABLE C38. Effect of concentration of chloride dioxide on plasma sodium concentrations at farm 2. Levene's test nonsignificant, F=4.504

Source	df	MS	F	Sig
Conc	**4**	**3955.815**	**11.814**	**0.000**
Error	**38**	**334.847**		

TABLE C39. Effect of trial on chloramine-T plasma sodium concentrations at farm 2. Levene's test nonsignificant, F= 8.462

Source	df	MS	F	Sig
Trial	**9**	**1320.738**	**4.551**	**0.001**
Error	**34**	**290.204**		

TABLE C40. Effect of concentration of chloramine-T on sodium potassium concentrations at farm 2. Levene's test nonsignificant, F=8.384

Source	df	MS	F	Sig
Conc	**4**	**2071.097**	**5.997**	**0.001**
Error	**39**	**345.364**		

IFAT Chem Trial

TABLE C41. Effect of trial on IFAT positive chlorine dioxide treated fish at farm 1. Levene's test nonsignificant, F=25.120

Source	df	MS	F	Sig
Trial	**9**	**0.645**	**5.369**	**0.000**
Error	**34**	**0.120**		

TABLE C42. Effect of chlorine dioxide concentration on IFAT positive fish at farm 1. Levene's test nonsignificant, F=3.771

Source	df	MS	F	Sig
Conc	**4**	**1.085**	**7.644**	**0.000**
Error	**39**	**0.142**		

TABLE C43. Effect of trial on IFAT positive chloramine-T treated fish at farm 1. Levene's test nonsignificant, F=23.415

Source	df	MS	F	Sig
Trial	**9**	**0.329**	**3.123**	**0.008**
Error	**34**	**0.105**		

TABLE C44. Effect of chloramine-T concentration on IFAT positive fish at farm 1. Levene's test nonsignificant, F=32.514

Source	df	MS	F	Sig
Conc	**4**	**0.688**	**7.081**	**0.00**
Error	**39**	**0.010**		

TABLE C45. Effect of trial on IFAT positive hydrogen peroxide treated fish at farm 1. Levene's test nonsignificant, F=9.718

Source	df	MS	F	Sig
Trial	**9**	**0.423**	**3.675**	**0.003**
Error	**34**	**0.115**		

TABLE C46. Effect of hydrogen peroxide concentration on IFAT positive fish at farm 1. Levene's test nonsignificant, F=36.057

Source	df	MS	F	Sig
Conc	**4**	**0.359**	**2.225**	**0.084**
Error	**39**	**0.161**		

TABLE C47. Effect of trial on IFAT positive chlorine dioxide treated fish at farm 2. Levene's test nonsignificant, F=5.705

Source	df	MS	F	Sig
Trial	9	0.229	1.168	0.346
Error	34	0.196		

TABLE C48. Effect of chlorine dioxide concentration on IFAT positive fish at farm 2. Levene's test nonsignificant, F=4.849

Source	df	MS	F	Sig
Conc	4	0.307	1.595	0.195
Error	39	0.192		

TABLE C49. Effect of trial on IFAT positive chloramine-T treated fish at farm 2. Levene's test nonsignificant, F=5.540

Source	df	MS	F	Sig
Trial	9	0.758	11.051	0.000
Error	34	0.686		

TABLE C50. Effect of chloramine-T concentration on IFAT positive fish at farm 2. Levene's test nonsignificant, F=8.426

Source	df	MS	F	Sig
Conc	4	1.686	27.202	0.000
Error	39	0.620		

CHEMICAL TRIAL HISTOLOGY

FARM 1

CHLORINE DIOXIDE

TABLE C51. Effect of trial on number of lesions per filament in chlorine dioxide treated fish. Levene's test nonsignificant, F=3.497

Source	df	MS	F	Sig
Trial	5	0.921	1.204	0.369
Error	11	0.765		

TABLE C52. Effect of treatment on lesions per filament in chlorine dioxide treated fish. Levene's test nonsignificant, F=0.181

Source	df	MS	F	Sig
Treat	2	0.142	1.959	0.178
Error	14	0.727		

TABLE C53. Effect of trial on number of ILV per lesion in chlorine dioxide treated fish. Levene's test nonsignificant, F=10.238

Source	df	MS	F	Sig
Trial	5	3.835	0.869	0.532
Error	11	4.412		

TABLE C54. Effect of treatment on ILV per lesion in chlorine dioxide treated fish. Levene's test nonsignificant, F=2.038

Source	df	MS	F	Sig
Treat	2	3.643	0.844	0.451
Error	14	4.316		

TABLE C55. Effect of trial on number of amoeba per lesion in chlorine dioxide treated fish. Levene's test nonsignificant, F=6.037

Source	df	MS	F	Sig
Trial	**5**	**2.392**	**4.868**	**0.014**
Error	**11**	**0.491**		

TABLE C56. Effect of treatment on amoeba per lesion in chlorine dioxide treated fish. Levene's test nonsignificant, F=6.957

Source	df	MS	F	Sig
Treat	**2**	**4.218**	**6.613**	**0.010**
Error	**14**	**0.638**		

TABLE C57. Effect of trial on number of amoeba in ILV in chlorine dioxide treated fish. Levene's test nonsignificant, F=8.233

Source	df	MS	F	Sig
Trial	**5**	**8.180**	**0.924**	**0.501**
Error	**11**	**8.848**		

TABLE C58. Effect of treatment on amoeba in ILV in chlorine dioxide treated fish. Levene's test nonsignificant, F=16.338

Source	df	MS	F	Sig
Treat	**2**	**18.301**	**2.521**	**0.116**
Error	**14**	**7.260**		

CHLORAMINE-T

TABLE C59. Effect of trial on number of lesions per filament in chloramine-T treated fish. Levene's test nonsignificant, F=4.298

Source	df	MS	F	Sig
Trial	**5**	**0.165**	**3.316**	**0.045**
Error	**11**	**0.497**		

TABLE C60. Effect of treatment on lesions per filament in chloramine-T treated fish. Levene's test nonsignificant, F=2.750

Source	df	MS	F	Sig
Treat	**2**	**0.291**	**5.161**	**0.021**
Error	**14**	**0.564**		

TABLE C61. Effect of trial on number of ILV per lesion in chloramine-T treated fish. Levene's test nonsignificant, F=4.327

Source	df	MS	F	Sig
Trial	**5**	**1.266**	**1.680**	**0.220**
Error	**11**	**0.754**		

TABLE C62. Effect of treatment on ILV per lesion in chloramine-T treated fish. Levene's test nonsignificant, F=0.469

Source	df	MS	F	Sig
Treat	**2**	**1.616**	**1.986**	**0.174**
Error	**14**	**0.813**		

TABLE C63. Effect of trial on number of amoeba per lesion in chloramine-T treated fish. Levene's test nonsignificant, F=12.494

Source	df	MS	F	Sig
Trial	**5**	**0.218**	**1.202**	**0.370**
Error	**11**	**0.182**		

TABLE C64. Effect of treatment on amoeba per lesion in chloramine-T treated fish. Levene's test nonsignificant, F=4.929

Source	df	MS	F	Sig
Treat	2	0.296	1.659	0.226
Error	14	0.178		

HYDROGEN PEROXIDE

TABLE C65. Effect of trial on number of lesions per filament in hydrogen peroxide treated fish. Levene's test nonsignificant, F=2.099

Source	df	MS	F	Sig
Trial	5	0.228	6.721	0.003
Error	12	0.340		

TABLE C66. Effect of treatment on lesions per filament in hydrogen peroxide treated fish. Levene's test nonsignificant, F=8.009

Source	df	MS	F	Sig
Treat	2	0.186	2.360	0.128
Error	15	0.786		

TABLE C67. Effect of trial on number of ILV per lesion in hydrogen peroxide treated fish. Levene's test nonsignificant, F=2.165

Source	df	MS	F	Sig
Trial	5	0.885	1.495	0.263
Error	12	0.592		

TABLE C68. Effect of treatment on ILV per lesion in hydrogen peroxide treated fish. Levene's test nonsignificant, F=1.349

Source	df	MS	F	Sig
Treat	2	0.152	0.204	0.818
Error	15	0.748		

TABLE C69. Effect of trial on number of amoeba per lesion in hydrogen peroxide treated fish. Levene's test nonsignificant, F=9.215

Source	df	MS	F	Sig
Trial	5	0.223	78.230	0.000
Error	12	0.285		

TABLE C70. Effect of treatment on amoeba per lesion in hydrogen peroxide treated fish. Levene's test nonsignificant, F=50.941

Source	df	MS	F	Sig
Treat	2	0.377	14.329	0.000
Error	15	0.263		

TABLE C71. Effect of trial on number of amoeba in ILV in hydrogen peroxide treated fish. Levene's test nonsignificant, F=3.782

Source	df	MS	F	Sig
Trial	5	0.756	1.360	0.306
Error	12	0.566		

TABLE C72. Effect of treatment on amoeba in ILV in hydrogen peroxide treated fish. Levene's test nonsignificant, F=11.667

Source	df	MS	F	Sig
Treat	2	1.056	1.900	0.184
Error	15	0.556		

FARM 2

CHLORINE DIOXIDE

TABLE C73. Effect of trial on number of lesions per filament in chlorine dioxide treated fish. Levene's test nonsignificant, F=2.381

Source	df	MS	F	Sig
Trial	5	0.253	0.446	0.807
Error	10	0.567		

TABLE C74. Effect of treatment on lesions per filament in chlorine dioxide treated fish. Levene's test nonsignificant, F=0.524

Source	df	MS	F	Sig
Treat	2	0.129	0.251	0.782
Error	13	0.514		

TABLE C75. Effect of trial on number of ILV per lesion in chlorine dioxide treated fish. Levene's test nonsignificant, F=2.268

Source	df	MS	F	Sig
Trial	5	0.111	0.575	0.719
Error	10	0.193		

TABLE C76. Effect of treatment on ILV per lesion in chlorine dioxide treated fish. Levene's test nonsignificant, F=6.420

Source	df	MS	F	Sig
Treat	2	0.215	1.358	0.291
Error	13	0.158		

TABLE C78. Effect of trial on number of amoeba per lesion in chlorine dioxide treated fish. Levene's test nonsignificant, F=11.389

Source	df	MS	F	Sig
Trial	5	0.315	0.712	0.628
Error	10	0.443		

TABLE C79. Effect of treatment on amoeba per lesion in chlorine dioxide treated fish. Levene's test nonsignificant, F=5.380

Source	df	MS	F	Sig
Treat	2	0.495	1.283	0.310
Error	13	0.386		

TABLE C80. Effect of trial on number of amoeba in ILV in chlorine dioxide treated fish. Levene's test nonsignificant, F=10.600

Source	df	MS	F	Sig
Trial	5	0.221	0.662	0.660
Error	10	0.333		

TABLE C81. Effect of treatment on amoeba in ILV in chlorine dioxide treated fish. Levene's test nonsignificant, F=3.717

Source	df	MS	F	Sig
Treat	5	0.177	0.564	0.582
Error	13	0.314		

CHLORAMINE-T

TABLE C82. Effect of trial on number of lesions per filament in chloramine-T treated fish. Levene's test nonsignificant, F=5.516

Source	df	MS	F	Sig

Trial	**5**	**0.594**	**1.008**	**0.454**
Error	**12**	**0.590**		

TABLE C83. Effect of treatment on lesions per filament in chloramine-T treated fish. Levene's test nonsignificant, F=23.623

Source	df	MS	F	Sig
Treat	**2**	**0.840**	**1.506**	**0.254**
Error	**15**	**0.558**		

TABLE C84. Effect of trial on number of ILV per lesion in chloramine-T treated fish. Levene's test nonsignificant, F=0.519

Source	df	MS	F	Sig
Trial	**5**	**0.593**	**0.231**	**0.941**
Error	**12**	**0.257**		

TABLE C85. Effect of treatment on ILV per lesion in chloramine-T treated fish. Levene's test nonsignificant, F=1.778

Source	df	MS	F	Sig
Treat	**2**	**0.525**	**0.241**	**0.789**
Error	**15**	**0.218**		

TABLE C86. Effect of trial on number of amoeba per lesion in chloramine-T treated fish. Levene's test nonsignificant, F=9.446

Source	df	MS	F	Sig
Trial	**5**	**0.675**	**5.488**	**0.007**
Error	**12**	**0.123**		

TABLE C87. Effect of treatment on amoeba per lesion in chloramine-T treated fish. Levene's test nonsignificant, F=6.198

Source	df	MS	F	Sig
Treat	**2**	**0.102**	**5.466**	**0.016**
Error	**15**	**0.187**		

TABLE C88. Effect of trial on number of amoeba in ILV in chloramine-T treated fish. Levene's test nonsignificant, F=16.000

Source	df	MS	F	Sig
Trial	**5**	**0.222**	**1.000**	**0.458**
Error	**12**	**0.222**		

TABLE C89. Effect of treatment on amoeba in ILV in chloramine-T treated fish. Levene's test nonsignificant, F=6.250

Source	df	MS	F	Sig
Treat	**2**	**0.222**	**1.000**	**0.391**
Error	**15**	**0.222**		

FARM 1 FISH DATA
CHLORINE DIOXIDE
TABLE C90. Effect of trial on fish weight in chlorine dioxide treatments. Levene's test nonsignificant, F=0.388

Source	df	t-calc	t-crit	Sig
Trial	**64**	**0.322**	**2.00**	**0.05**

TABLE C91. Effect of trial on fish length in chlorine dioxide treatments. Levene's test nonsignificant, F=4.170

Source	df	t-calc	t-crit	Sig
Trial	**64**	**0.944**	**2.00**	**0.05**

CHLORAMINE-T

TABLE C92. Effect of trial on fish weight in chloramine-T treatments. Levene's test nonsignificant, F=1.181

Source	df	t-calc	t-crit	Sig
Trial	**65**	**1.037**	**2.00**	**0.05**

TABLE C93. Effect of trial on fish length in chloramine-T treatments. Levene's test nonsignificant, F=0.726

Source	df	t-calc	t-crit	Sig
Trial	**65**	**1.118**	**2.00**	**0.05**

HYDROGEN PEROXIDE

TABLE C94. Effect of trial on fish weight in hydrogen peroxide treatments. Levene's test nonsignificant, F=10.527

Source	df	t-calc	t-crit	Sig
Trial	**65**	**6.906**	**2.00**	**0.05**

TABLE C95. Effect of trial on fish length in hydrogen peroxide treatments. Levene's test nonsignificant, F=2.342

Source	df	t-calc	t-crit	Sig
Trial	**65**	**7.400**	**2.00**	**0.05**

FARM 2 FISH DATA

CHLORINE DIOXIDE

TABLE C96. Effect of trial on fish weight in chlorine dioxide treatments. Levene's test nonsignificant, F=0.909

Source	df	t-calc	t-crit	Sig
Trial	**64**	**0.020**	**2.00**	**0.05**

TABLE C97. Effect of trial on fish length in chlorine dioxide treatments. Levene's test nonsignificant, F=0.884

Source	df	t-calc	t-crit	Sig
Trial	**64**	**0.686**	**2.00**	**0.05**

CHLORAMINE-T

TABLE C98. Effect of trial on fish weight in chloramine-T treatments. Levene's test nonsignificant, F=0.326

Source	df	t-calc	t-crit	Sig
Trial	**32**	**0.135**	**2.00**	**0.05**

TABLE C99. Effect of trial on fish length in chloramine-T treatments. Levene's test nonsignificant, F=1.608

Source	df	t-calc	t-crit	Sig
Trial	**62**	**0.129**	**2.00**	**0.05**