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Optimisation of treatment of *Ichthyophthirius multifiliis* in farmed freshwater fish

James Forwood, James Harris, Matt Landos and Marty Deveney

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SARDI Aquatics Sciences
PO Box 120 Henley Beach SA 5022

April 2014

Final Report to the Fisheries Research & Development Corporation

PREMIUM FOOD AND WINE FROM OUR CLEAN ENVIRONMENT

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DISCLAIMER

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EXECUTIVE SUMMARY

Rainbow trout (*Oncorhynchus mykiss* Walbaum) are an ideal species for cultivation and are an important aquaculture species in Victoria, Australia (ABARES 2011). Rainbow trout are primarily cultured in flow-through systems with incoming water sourced from a river or lake. The cultured fish are therefore exposed to pathogens from outside the culture environment. Rainbow trout are susceptible to a number of diseases, in particular the ciliate parasite *Ichthyophthirius multifiliis* (Fouquet). Management of *I. multifiliis* prior to 2011 was often ineffective and *I. multifiliis* caused major stock losses.

A meeting of the Victorian Trout Growers Association (VGTA) identified *I. multifiliis* as a limiting factor for the growth and sustainability of the industry. This study focused on developing effective treatment systems and is an extension of a previous DPI Victoria (Now DEPI) project *Improving the resilience of aquaculture sectors to climate variability - better practice fish health management for salmonid farmers.*

The principal objective of this study was to refine the current treatment methods, improving efficacy and managing the infection better. This was achieved by investigating the minimum effective dose of treatments against the susceptible stages of *I. multifiliis* and whether rainbow trout could tolerate these doses. We also evaluated the differences between applied and effective doses of treatments throughout raceways, and characterised the life cycle of *I. multifiliis* to time treatments to interrupt the parasite's life cycle. We developed management strategies that can be applied by growers to better manage Australian *I. multifiliis* infections.

The study included: one-hour dose response trials using sodium percarbonate (SPC) and formalin (FOR) at various concentrations on *I. multifiliis in vitro*; measurements of the concentration of SPC and FOR throughout raceways during treatments as normally conducted by farmers; investigated the toxicity to rainbow trout of three concentrations of SPC as applied to treat *I. multifiliis*; and characterised the life cycle of an Australian isolate of *I. multifiliis* at different temperatures and salinities.

Both FOR and SPC were more effective at higher water temperatures but there was no difference in efficacy between hard and soft water. Formalin was effective against all prototomonts at ≥ 64 mg/L at 12°C and 17°C but required 128 mg/L to induce complete

mortality in tomocysts at 17°C and did not achieve 100% efficacy at any doses tested at 12°C. In contrast, SPC was effective against both stages at all doses trialled at 17°C but required a minimum of 256 mg/L at 12°C to induce complete mortality.

SPC is usually applied at 64 mg/L for 1 h; if applied at low water temperatures, it would not effectively eliminate all susceptible life cycle stages. Current treatments using FOR are applied at 200 mg/L for 1 h, which is effective in eliminating all susceptible life cycle stages.

On farm application methods for SPC and FOR mostly provided uniform concentrations between repeat treatments. However, concentrations of treatments in raceways varied spatially and temporally, and standard practices for both treatments usually provided doses that were substantially lower than the target minimum effective concentration. This indicates that the treatments applied to the systems are below effective concentrations, with associated low efficacy, rapid reinfection and ineffective management of the parasite. To improve efficacy, static baths are recommended, with supplementary aeration to provide adequate dissolved oxygen (DO). If static baths cannot be used, monitoring the dose throughout the treatment period is recommended to allow dose to be adjusted as necessary. The inclusion of mechanical aerators is also recommended to aid treatment distribution and retention.

Rainbow trout exposed to 50, 150 and 250 mg/L SPC for 1 h on two consecutive days, with treatment repeated 5 days later, had 100% survival in all treatment groups, but reduced movement and loss of equilibrium was noted in groups treated at 250 mg/L. This indicates that the current recommend dose of ≤ 100 mg/L (PER12944) could be increased to 150-200 mg/L at low water temperatures, including in repeat doses, without significant toxicity to rainbow trout.

The development time from tomont settlement until the release of theronts was influenced significantly by water temperature. Time to theront release was longest at low water temperatures, and significantly decreased with increasing water temperature. The number of theronts produced from individual tomocysts was also lowest at low water temperature, increasing with higher water temperatures, peaking at 25°C, above which theront production decreased.

The Australian isolate completed the free-living phase of its life cycle more quickly at all temperatures than other described *I. multifiliis* isolates, and has greater sensitivity to salinity. Understanding the development time from tomont settlement to the release of theronts at different temperatures facilitates the strategic timing of repeat treatments to target tomonts that survive initial treatment. This will improve management of Australian *I. multifiliis*. The salinity sensitivity we observed has treatment and prevention applications in aquaculture systems with small volume and without substantial discharge.

The low efficacy of SPC and FOR observed on rainbow trout farms was largely due to treatments being delivered in ways that did not achieve an effective concentration and repeat treatments, where used, being timed using published data that are not applicable to Australian *I. multifiliis,* and therefore failing to interrupt the life cycle. Static bath treatments of FOR or SPC administered at the minimum effective dose repeated to coincide with relevant temperature life cycle data have improved efficacy and fish welfare and reduced stock loss.

Recommendations for improved efficacy

To improve the management of *I. multifiliis* in Australian aquaculture:

- **Use minimum effective concentrations of SPC and FOR related to water temperature and life stage as 'target concentrations' outlined in Table 9.**
- **Validate treatment applications in the culture system to ensure the target dose is achieved and preferably measure the effective dose of each treatment.**
- **Apply repeat strategic treatments based on temperature-life cycle data outlined in Table 5.**

This information has been widely distributed through the use of workshops and on site visits to Victorian rainbow trout farms and has received positive feedback from farmers.

OUTCOMES ACHIEVED

The low efficacy of treatments for *I. multifiliis* has been due to inaccurate treatment application resulting in a significantly lower measured dose than the target dose. Other factors contributing to a lack of efficacy were that the target doses of 64 mg/L SPC and 200 mg/L may not be effective in the complete removal of all susceptible free-living life stages, and that poorly timed repeat treatments would lead to rapid reinfection.

The minimum effective doses for SPC and FOR have been identified for all susceptible life stages in both hard and soft water at 12 and 17°C, outlined in Table 9. Life cycle-temperature data are now defined to inform repeat treatments to interrupt parasite life cycles, and are outlined in Table 5.

Repeated doses of SPC up to 150 mg/L for 1 h have been assessed as being safe for use on juvenile rainbow trout at ≤ 17°C, indicating increasing the current target dose is safe.

Treatment application methods have been assessed; it is recommended to use static bath treatments where possible. If static baths cannot be used, monitoring the dose throughout the treatment period is recommended to allow the dose to be adjusted for maximum efficacy. The inclusion of mechanical aerators is also recommended to aid distribution and retention of treatments.

All information has been collated into a treatment guide to improve efficacy.

INTRODUCTION

1.1. Background

Ichthyophthirius multifiliis (Fouquet), commonly called 'ich', a ciliate protozoan, is a cosmopolitan parasite of freshwater teleosts (Matthews 2005). *Ichthyophthirius multifiliis* is a common pathogen in the Australian rainbow trout, *Oncorhynchus mykiss* (Walbaum) aquaculture industry and attempted implementation of management programs based on overseas data has resulted in a lower than expected efficacy (E. Meggit, pers. comm.).

Heavy infections of *I. multifiliis* cause skin irritation, with behavioural changes (flashing) and inappetance, which leads to decreased growth. Pathological changes associated with *I. multifiliis* in the skin and gills include localised lymphocytic infiltration, focal necrosis and varying degrees of epithelial proliferation (Maki et al. 2001). Heavy infections deplete energy reserves, impair haemopoiesis, and cause gill epithelia and epidermis to fail to regenerate, resulting in ingress of water, with subsequent ion imbalance and sensitivity to oxygen tension and uptake (Hines and Spira 1973a, b; 1974a, b), which if left untreated can lead to high mortalities under aquaculture conditions (Valtonen and Koskivaara 1994).

Ichthyophthirius multifiliis has a direct life cycle. The parasitic trophont resides and feeds in the host's epidermis, the tomont leaves the host (prototomont) and encysts (tomocyst) in the aquatic environment, undergoing rapid division into daughter cells, the tomites. The tomites develop into theronts, the free swimming infective stage which locates a host, penetrates its epidermis, and develops into a trophont (Matthews 2005). The tomont divides by binary fission, producing up to 3000 theronts (Wagner 1960) (see Figure 1 Workshop notes p 65). The developmental period of each life cycle stage is influenced by water temperature. Development occurs more quickly with increasing temperature (Bauer 1958; Wanger 1960; Noe and Dickerson 1995; Aihua and Buchmann, 2001) and is inhibited under increasing salinity (Wanger 1960; Aihua and Buchmann 2001). Busckiel (1910) and Nigrelli et al. (1976) suggested that subtle variations in cell morphology and temperature preferences of different isolates in different climatic zones could be due to the presence of different physiological races of the parasite.

Geographical isolates of *I. multifiliis* are influenced differently by temperature and salinity (Aihua and Buchmann, 2001), and have different pathogenicity (Swennes et al. 2006) and susceptibility to chemotherapeutants (Straus and Meinelt, 2009; Straus et al. 2009). There are five serotypes of *I. multifiliis* (see Dickerson and Clark, 1996), but which serotypes occur in Australia and the effect of temperature and salinity on development of Australian isolates is unknown. Understanding variation in developmental times under different temperatures and salinities would contextualise Australian *I. multifiliis* infections with those seen in the northern hemisphere and provide a rationale for applying or rejecting the timing of repeat treatment applications used in the northern hemisphere.

Treatment for *I. multifiliis* largely centres on husbandry, including minimising stress and manipulating water velocity in raceways, however, strategic chemical control is required when these measures are inadequate. Malachite green was the preferred chemotherapeutant used to treat all life cycle stages of *I. multifiliis* (see Wahli et al. 1993), but the use of malachite green in food fish was banned in Australia, Europe and North America due to its toxicological effects and carcinogenic potential (Srivastava et al. 2004). Other treatments have been trialed with varying success against the free-living stages, but none are effective against trophonts (reviewed by Picón-Camacho et al. 2012). Formalin is a recommended treatment (Noga, 2000), but there are concerns about workplace safety when applying the treatment (Wooster et al. 2005). Sodium percarbonate (SPC) is a potential safe alternative treatment (Buchmann et al. 2003; Heinecke and Buchmann, 2009), which works by dissociating in water to yield hydrogen peroxide and sodium carbonate.

Sodium percarbonate has an Australian Pesticides and Veterinary Medicines Authority (APVMA) minor use permit (PER12944) for repeat applications of concentrations up to 100 mg/L to control external parasites in freshwater fish. Doses of liquid hydrogen peroxide (HP) range from 50 – 250 mg/L for up to 60 min (Rach et al. 1997), but high doses or prolonged exposure to liquid HP can result in epithelial lifting and gill necrosis in rainbow trout (Tort et al. 2002). The effects of hydrogen peroxide as released from SPC on rainbow trout have not been described.

The efficacy of a treatment regime relies on maintenance of the minimum effective dose for the designated treatment period (Rach et al. 1997). Operators mostly rely on the

assumption that the effective concentration is the same throughout the treatment system as the nominal concentration applied to the pond or raceway (i.e. that if the product is applied at 150 mg/L at the head of the raceway, the dose delivered in the water throughout the raceway is the same; Rach and Ramsey (2000)). Many factors, however, can affect the dose in aquaculture systems, including flow rate, water temperature, degradation rates and flow dynamics of the system. If these conditions are not considered and accounted for, then the dose could be sub-optimal and hence ineffective for controlling the parasite and disease it causes.

1.2. Objectives

This project aimed to improve management of *I. multifiliis* in Australia by improving treatment efficacy. This was achieved by determining the minimum effective concentrations for available treatments, understanding key life cycle parameters to strategically time repeat treatment application, and determining the relationships between husbandry system, application method and water quality and amount of available treatment in the water. This information was combined in a treatment regime specific to the Australian isolate of *I. multifiliis.* The impact of repeat exposure to SPC on the gills of rainbow trout was also assessed. This will improve efficacy, decrease the likelihood of parasite resistance to treatment, improve treatment safety and provide further information to support regulatory management of treatments.

To achieve the above, there were four specific objectives:

1. *In vitro* dose determination for a range of treatments at different temperatures and water hardness
2. Determine relationships between water quality, nominal dose and effective dose
3. Assess the effects of treatment on fish gills
4. Determine optimal timing for repeat treatments using life cycle data of Australian *I. multifilis* isolates with varying temperature and water quality

This report was developed as a series of stand-alone papers for publication in scientific journals and therefore there is repetition in some background information.

CHAPTER 1: THE EFFECT OF TEMPERATURE AND SALINITY ON THE DEVELOPMENT OF AN AUSTRALIAN ISOLATE OF *ICHTHYOPHTHIRIUS MULTIFILIIS* FROM RAINBOW TROUT

Ichthyophthirius multifiliis, a ciliate parasite, is a cosmopolitan and problematic parasite of cultured freshwater fish. Each geographical isolate of *I. multifiliis* has a life cycle that varies with abiotic conditions including temperature and salinity. We assessed the effects of salinity and temperature on the development of a temperate Australian isolate of *I. multifiliis*. The time from tomont settlement until theront release differed significantly with temperature: from 188.9 h at 5°C to 11.7 h at 30°C. Theront production was positively correlated with temperature to 25°C: at 5°C a mean of 266.8 theronts were produced per tomont, increasing to 493.2 theronts at 25°C, before decreasing to 288 theronts per tomont at 30°C. Theront length showed an inverse relationship with temperature; mean length was 41 µm at 30°C and 62.2 µm at 5°C. The Australian isolate had shorter tomont development time at all temperatures and a greater sensitivity to salinity than all other reported isolates. This understanding of generation time facilitates strategic timing of treatments, aiding development of specific management plans for the Australian isolate of *I. multifiliis*.

1. Introduction

Ichthyophthirius multifiliis (Fouquet), a ciliate parasite, has worldwide distribution (Nigrelli et al. 1976) and is a problematic parasite of cultured freshwater fish worldwide (Schäperclaus, 1991; Dickerson and Dawe 1995). The wide geographical distribution of *I. multifiliis* is primarily due to the translocation of infected host species, the low host specificity of the pathogen and its capacity to reproduce rapidly. The entry of *I. multifiliis* to Australia probably occurred multiple times, but it was first introduced on imported ornamental fish species (Ashburner 1976). The first outbreaks on trout farms in Australia were associated with the release of infected goldfish *Carassius auratus* (L.) in Tasmania (Parliament of Tasmania 1933), and *I. multifiliis* was probably introduced to Victoria with imported carp *Cyprinus carpio* (L.) (see Butcher 1939).

The life cycle of *I. multifiliis* is direct, with four stages: the parasitic trophont, which is attached and resides in the host's epidermis; the tomont, which leaves the host and encysts in the aquatic environment, undergoing rapid division into daughter cells, the tomites; which develop into theronts, the free swimming infective stage which locates the host, penetrates the epidermis and develops into trophonts (MacLennan 1942; Matthews 2005). The developmental period is influenced by water temperature (Bauer 1958; Wanger 1960; Noe and Dickerson 1995; Aihua and Buchmann 2001) and salinity (Wanger 1960; Aihua and Buchmann 2001). Busckiel (1910) and Nigrelli et al. (1976) suggested that variation in temperature preference between isolates could be due to the existence of different physiological strains of the parasite. Ecologically distinct isolates are influenced differently by temperature and salinity (Aihua and Buchmann 2001), which probably explains why management programs for *I. multifillis* on Australian trout farms based on European experimental data have resulted in lower than expected efficacy (E. Meggit pers. comm.).

Which strain(s) of *I. multifiliis* occur in Australia, and the effect of temperature and salinity on development of Australian isolates is unknown. Detailed knowledge of the life cycle is required to facilitate effective management of the parasite. We characterised the life cycle of an Australian isolate, focusing on temperature and salinity, and compared these responses to those of other described isolates.

2. Materials and methods

2.1. Culture of parasites

Rainbow trout infected with *I. multifiliis* were obtained from Snobs Creek Hatchery (Department of Primary Industries, Victoria, Australia) during the Austral summer of 2012. Infected fish were transferred to Flinders University and held in 200 L aerated aquaria containing recirculating filtered municipal water (hardness 145 mg/L and alkalinity 20 mg/L as $CaCO_3$; pH 6.1), continuously filtered with biofilters. The tanks were maintained at 17 ± 1°C, with light/dark periods set artificially at 12: 12 h. The parasites were allowed to multiply in the aquaria. Any fish that showed signs of substantially affected health were removed and euthanased and replaced with naïve uninfected rainbow trout.

2.2. Isolation of trophonts

Temperature and salinity experiments were based on the method of Aihua and Buchmann (2001). Rainbow trout with visible trophonts were euthanased with an overdose (40 mL 1000 L bath) of Aqui-S®, rinsed, and placed into a 600 mL beaker containing 80 mL aquarium water. Trophonts were allowed to dislodge and collected within 30 min after leaving the fish using a 200 µL pipette, 8 trophonts was transferred to each individual well of a 24-well multidish (Corning®). At the start of each of the experiments a sample of 8 trophonts were killed by fixing in 10% neutral-buffered formalin (NBF) and measured.

2.3. Temperature trials

Multidish plates containing trophonts in wells each containing 2 ml of 0.2 µm filtered (Sartorius Stedim Pty Ltd) water from the infection tank were placed into incubators at 5, 9, 12, 17, 21, 25 and 30°C, and regularly inspected under a dissecting microscope (20 – 40 x magnification) to assess if theronts had been released. The time to the first theront release and number of tomonts that did not develop into tomocysts were recorded. Following the release of all theronts, a drop of 10% neutral buffered formalin (NBF) and Lugol's iodine was added to each well and the number of theronts was counted using a dissecting microscope. From each well, a sample was pipetted onto a slide and five randomly selected theronts were measured using a compound microscope with a calibrated ocular eyepiece (400x magnification). The experiments were repeated three times at each temperature.

2.4. Salinity trials

Multidish plates containing trophonts in wells each containing 2 ml of 0.2 µm filtered water from the infection tank with sodium chloride (Merck®, batch ref. MJ6M562652) added at 1, 3, 5, and 7.5 g/L were incubated at 12 and 17°C in separate trials and regularly inspected under a dissection microscope (20 – 40x magnification). Control groups contained wells with filtered water without sodium chloride. The protocol then followed that for temperature. The experiments were repeated three times at each salinity and temperature.

2.5. Statistical analysis

Prior to analysis, normality of the data was tested using the Kolmogorov-Smirnov test and variances were tested using Levene's test. To achieve homoscedasticity, the data for the mean time to theront production and mean theront length were $\log_{10}$ transformed. Differences in the mean time taken to produce theronts between temperatures and salinities, number of theronts produced at each temperature and salinity and the length of the theronts produced were analysed using a one-way analysis of variance (ANOVA). Where significant differences were detected in the ANOVAs, post hoc comparisons were made using Tukey's tests. The statistical analysis was performed using IBM SPSS Statistics 20.0 and significance for all tests was judged at $P < 0.05$.

3. Results

3.1. Temperature trials

The trophonts used during the temperature trials had a mean diameter of 322.7 ± Standard Error of the Mean (SEM) 15.6 µm (range 202.5 to 502.5 µm). Temperature had a significant effect on the time tomonts took to release theronts (one-way ANOVA: $F_{6, 135} = 3117.6$, $P < 0.001$). The development time was proportional to temperature and significantly different between each temperature (Table 1).

There was a significant difference in the mean number of theronts produced between each temperature from individual tomocysts (one-way ANOVA: $F_{6, 135} = 16.223$, $P = 0.001$). Theront production was inhibited in cold water, significantly increasing with a higher water temperature peaking at 25°C, after which theront production significantly reduced (Table 1). Theront length showed an inverse relationship to the incubation temperature and was

significantly different between each temperature (one-way ANOVA: $F_{6, 133} = 26.204$, $P < 0.001$) (Table 1).

Table 1: Temperature-dependant development of *Ichthyophthirius multifiliis* tomonts (n = 24) at different water temperatures. Different superscripts indicate significant differences between temperatures using Tukey's analysis ($P < 0.05$).

Temp °C	Time from trophont to theront in hours. Mean ± SE (range)	Trophonts developing into cysts (%)	Number of theronts from one cyst. Mean ± SE (Range)	Length of theronts (µm)
5	188.9 ± 3.7 (159.45 to 206) [a]	71	189.2 ± 24.7 (63 to 374) [a]	62.2 ± 1.3 (39.5 to 97.5) [a]
9	162.3 ± 1.2 (154 to 172) [b]	79	193. 9 ± 20.7 (106 to 440) [a]	52.6 ± 0.7 (39.5 to 70) [b]
12	46.6 ± 0.8 (41.6 to 54.6) [c]	100	253 ± 21.9 (140 to 623) [a,b]	48.4 ± 0.5 (39.5 to 65) [b,c]
17	27.1 ± 0.4 (23.1 to 29.4) [d]	88	446.4 ± 25.6 (180 to 682) [c,d]	42.7 ± 0.6 (34 to 59) [d,e]
21	18.3 ± 0.2 (16.4 to 20.1) [e]	100	426.3 ± 18.1 (182 to 576) [b,c,d]	45.4 ± 0.6 (25 to 58.5) [c,d]
25	13.7 ± 0.2 (12.6 to 16.5) [f]	88	493.2 ± 60.35 (177 to 1553) [d]	43.8 ± 0.7 (27.5 to 59) [d,e]
30	11.7 ± 0.2 (10.2 to 14.1) [g]	67	288 ± 20.6 (146 to 410) [a,b,c]	41 ± 0.8 (25 to 55) [e]

3.2. Salinity trials

The trophonts used during the salinity trials had a mean diameter of 283.8 ± 9.3 µm SEM (range 175 to 380 µm). There was a significant difference in the viability of tomonts between all salinities at 12°C (one-way ANOVA: $F_{4, 10} = 62.222$, $P < 0.001$) and 17°C (one-way ANOVA $F_{4, 10} = 61.100$, $P < 0.001$). Tomonts exposed to salinities of 7.5 g/L sodium chloride at 12 and 17°C ceased movement within 1 h, with no division or formation of tomocysts; tomonts exposed to 5 g/L sodium chloride were unable to produce theronts, although 29% and 33% were able to form a cyst wall and initiate division at 12°C and 17°C, respectively, but were unable to produce viable theronts. There was no significant difference in viability of tomonts exposed to 1 g/L and controls at 12°C (Table 2) and 17°C (Table 3).

For viable tomonts, the mean time to theront release was significantly different between all salinities at 12°C (one-way ANOVA: $F_{2, 6} = 12.071$, $P = 0.008$) and 17°C (one-way ANOVA: $F_{2, 6} = 119.001$, $P < 0.001$) There was no significant difference between tomonts

Table 2: Salinity-dependant development of *Ichthyophthirius multifiliis* tomonts (n = 24) incubated at 12°C at different salinity levels. Different superscripts indicate significant differences between temperatures using Tukey's analysis (*P* < 0.05). – indicates no theront production.

Salinity (g/L)	Time from trophont to theront in hours. Mean ± SE (range)	Trophonts encysted (%)	Trophonts with divisions (%)	Viable trophonts (%)	Number of theronts from one cyst. Mean ± SE (Range)	Length of theronts (µm)
0	53 ± 1.1 (43.3 to 59)[a]	83	29	83	282.1± 39.1 (122 to 789) [a]	55.1 ± 0.6 (40.5 to 69.5) [a]
1	49 ± 1.1 (39.3 59)[a]	92	29	92	166.4 ± 17.7 (37 to 315) [a,b]	52.9 ± 0.7 (36 to 70.5) [a,b]
3	97.1 ± 7.4 (74 to 122)[b]	50	50	33	78.5 ± 5.8 (48 to 96) [b]	49.5 ± 1.2 (39 to 70) [b]
5	-	29	54	0	-	-
7.5	-	-	-	-	-	-

Table 3: Salinity-dependant development of *Ichthyophthirius multifiliis* tomonts (n = 24) incubated at 17°C at different salinity levels. Different superscripts indicate significant differences between temperatures using Tukey's analysis (*P* < 0.05). – indicates no theront production.

Salinity (g/L)	Time from trophont to theront in hours. Mean ± SE (range)	Trophonts encysted (%)	Trophonts with divisions (%)	Viable trophonts (%)	Number of theronts from one cyst. Mean ± SE (Range)	Length of theronts (µm)
0	25.5 ± 0.3 (22.5 to 28.2)[a]	100	0	100	397.7 ± 22.2 (202 to 569) [a]	44.9 ± 0.4 (35.5 to 55.5) [a]
1	25 ± 0.4 (20.4 to 28.4)[a]	96	4	96	452.1 ± 20.2 (295 to 657) [a]	46 ± 0.4 (36 to 55.5) [a]
3	43.6 ± 1.2 (74 to 122)[b]	75	25	50	204 ± 18.1 (134 to 291) [b]	38.4 ± 0.4 (30.5 to 46.5) [b]
5	-	33	58	0	-	-
7.5	-	-	-	-	-	-

exposed to 1 g/L and controls, but release took significantly longer in tomonts exposed to 3 g/L (Table 2 and 3).

Theront production from viable tomonts was significantly different between salinities at 12°C (one-way ANOVA: $F_{2, 46} = 8.895$, $P = 0.001$) and 17°C (one-way ANOVA: $F_{2, 56} = 29.172$, $P < 0.001$). Theront production was not significantly different between tomonts exposed to 1 g/L and controls, but was significantly reduced in tomonts exposed to 3 g/L at 12°C (Table 2) and 17°C (Table 3). Theronts were largest in the control groups and significantly decreased in size with increasing salinity at 12°C (Table 2) and 17°C (Table 3).

4. Discussion

The time from tomont settlement to theront release in our isolate of *I. multifiliis* is shorter than reported for other isolates (Bauer 1958; Wagner 1960; Aihua and Buchmann 2001), especially at ≥ 25°C (Table 4). Repeated treatments are required to interrupt the life cycle and manage the parasite on farms. Tomonts are more resistant to chemical treatments than theronts (Heinecke and Buchmann 2009) and if a treatment only kills theronts, the surviving tomonts produce theronts, which must be targeted by a second treatment. Differences in development times at different water temperatures also have implications timing repeat treatments to maximize efficacy. Strategic timing for repeats treatment for our isolate is outlined in Table 5, which is derived from data outlined in Table 1. Data for a lesser range of temperatures and salinities were reviewed by Aihua and Buchmann (2001); differences in experimental design and the salts used influence results and detailed comparisons between salinity studies may not be accurate.

Theront production in the Australian isolate of *I. multifiliis* was highest at 25°C. In a Danish *I. multifiliis* isolate, Aihua and Buchmann (2001) found similar theront production but with higher mean production of theronts per tomocyst at all temperatures except 25°C. *Icthyophthirius multifiliis* outbreaks in Australia do not follow the same course as described for European outbreaks in rainbow trout, probably because of the higher reproductive capacity at temperatures relevant to trout aquaculture of European isolates. Theront production can be influenced by a number of factors, such as age and size of tomonts (Ewing et al. 1986), and is recorded as ranging from 64 (MacLennan 1937) to 3000 (Wagner 1960). Low numbers of theronts in our study were often associated with tomonts

dividing prior to undergoing encystment at low (4°C) and high (30°C) temperatures. Few of these daughter tomonts were viable and formed tomocysts, and those that did were smaller than normal tomocysts and subsequently produced few theronts. Low tomont survival at low and high temperatures affects the reproductive capacity of the parasite.

Table 4: Range of time (h) for the development of *Ichthyophthirius multifiliis* from trophont to theront release, comparison of the present results with literature data. Adapted from Aihua and Buchmann (2001).

Temp (°C)	Present work	Aihua and Buchmann (2001)	Bauer (1958)	Wagner (1960)
5	159 – 206	192 – 228	144	168 – 192
7-9	154 – 172	84 – 108	72 – 84	48 - 120
10-12	42 – 55	46 – 58	36 – 40	48 – 60
17	23 – 29	23.5 – 35.5	23 – 26	24
20-21	16 – 21	18.5 – 23.5	18 – 20	18
25	13 – 17	16 – 27.5	14 – 15	14
30	10 – 14	16 – 25	-	-

Table 5: Time for the repeat application of treatment at different water temperatures to maximize efficacy against *Ichthyophthirius multifiliis.*

	Water temperature (°C)						
	5	9	12	17	21	25	30
Time to repeat bath (h)	189	162	47	27	18	14	12

Theront length of the Australian isolate showed an inverse relationship with temperature consistent with sizes reviewed by Matthews (2005). Aihua and Buchmann (2001) reported an inverse temperature relationship in theront length for a Danish *I. multifiliis* isolate; this isolate also produced theronts of a similar size to the Australian isolate except at water temperatures ≥ 21°C.

Salinity had a significant effect on the viability of the Australian isolate of *I. multifiliis* at 3 g/L and above and completely prevented theront production at 5 g/L. Aihua and Buchmann (2001) and Wagner (1960) reported that theront production occurred in their isolates over a longer period but continued at salinities of 5 g/L. Aihua and Buchmann (2001) observed tomocyst formation at 7.5 g/L salinity and Wagner (1960) reported some survival and

theront production at 10 g/L salinity after 63 hours. Mifsud and Rowland (2008) reported effective control of an Australian strain of *I. multifiliis* infecting sliver perch (*Bidyanus bidyanus* (Mitchill)) using 2 - 3 g/L sodium chloride. Direct comparisons of isolates are difficult to make due to differences in experimental design, but these results indicate that Australian isolates are probably more sensitive to sodium chloride than other described isolates. The sensitivity of the Australian isolate to salinity has treatment and prevention applications where the application of sodium chloride to the aquaculture system is feasible, such as in small volume recirculation systems.

The Australian isolate of *I. multifiliis* reproduces more rapidly but is more sensitive to salinity than other described isolates. The data provided here contribute to effective routine monitoring and development of treatment programs to maximise efficacy by strategically timing treatments.

CHAPTER 2: *IN VITRO* EFFECT OF FORMALIN AND SODIUM PERCARBONATE ON THE FREE-LIVING STAGES OF AN AUSTRALIAN ISOLATE OF *ICHTHYOPHTHIRIUS MULTIFILIIS*

Ichthyophthirius multifiliis, a ciliate protozoan, is a common cosmopolitan parasite of freshwater teleosts and is a reoccurring problem during the summer months on Australian rainbow trout (*Oncorhynchus mykiss*) farms. There are a number of preventative strategies such as increasing water flow and filtration that can limit the occurrence of the parasite, however, when an infection is established, chemical intervention is often required. Formalin (FOR) has been traditionally used on Australian trout farms and sodium percarbonate (SPC), which releases hydrogen peroxide, was permitted by the APVMA in 2011 and its use is being implemented on a number of farms. We evaluated doses of FOR and SPC at 12°C and 17°C in both hard and soft water over a 1 h exposure period against free-living stages of *I. multifiliis* to assess anecdotal reports of low efficacy. Theronts were more sensitive to treatment than tomonts, and prototomonts were more sensitive to treatment than tomocysts. Formalin and SPC killed all theronts within 15 min at 64 mg/L at both temperatures. Formalin was effective against all prototomonts at ≥ 64 mg/L at both temperatures, SPC was effective at 64 m/L at 17°C but required ≥ 256 mg/L at 12°C. Formalin was effective against all tomocysts at 128 mg/L at 17°C but did not achieve complete mortality in any doses tested at 12°C, SPC was effective at 64 m/L at 17°C but required ≥ 256 mg/L at 12°C. There was no significant difference in the efficacy of both chemicals between hard and soft water in all trials. These results can be used to aid in the development of specific treatment strategies for the management of *I. multifiliis* on Australian rainbow trout farms.

1. Introduction

Ichthyophthirius multifiliis (Fouquet), commonly called 'ich', a ciliate protozoan, is a common cosmopolitan parasite of freshwater teleosts extending from the tropics to temperate regions as far as the Arctic Circle (Matthews 2005). *Ichthyophthirius multifiliis* is a common pathogen in the Australian rainbow trout (*Oncorhynchus mykiss*) aquaculture industry. Pathological lesions associated with *I. multifiliis* in the skin and gills cause localized lymphocytic infiltration, focal necrosis and epithelial proliferation (Maki et al. 2001). Heavy

infections induce depletion of energy reserves, impair haemopoiesis and prevent gill epithelia and epidermis from regenerating, resulting in ingress of water, ion imbalance and increased sensitivity to oxygen tension and uptake (Hines and Spira 1973a, b; 1974a, b). If left unmanaged in salmonids, *I. multifiliis* can cause high mortalities (Valtonen and Koskivaara 1994).

Ichthyophthirius multifiliis has a direct life cycle with four stages: the parasitic trophont, which resides within the host's epidermis and is resistant to chemical treatment; the tomont, which leaves the host (prototomont) and encysts (tomocyst) in the aquatic environment, undergoing rapid division into daughter cells, the tomites, which develop into theronts, the free swimming infective stage (Matthews 2005). There are five described serotypes of *I. multifiliis* (Dickerson and Clark 1996), which have varying susceptibility to chemoterapeutants (Straus and Meinelt 2009; Straus et al. 2009). It is unknown which serotype(s) are found in Australia.

Malachite green was the preferred chemotherapeutant used against all stages of *I. multifiliis* (see Wahli et al. 1993), but is now banned in Australia, Europe and North America for food fish production due to its toxic effects and carcinogenic potential (Srivastava et al. 2004). There have been several alternative treatments trialed with varying success against the free-living stages but none has been effective against the trophonts (reviewed by Picón-Camacho et al. 2012). Formalin (FOR) is a recommended treatment for *I. multifiliis* (Noga 2000), but sodium percarbonate (SPC) has shown potential as a safe alternative (Buchmann et al. 2003; Heinecke and Buchmann 2009). SPC gradually dissociates into sodium carbonate and hydrogen peroxide, the latter is a powerful oxidant (Bostek 1989) and is presumed to be the active antiparasitic compound of SPC (Heinecke and Buchmann 2009). These chemicals, when used on Australian rainbow trout farms to treat *I. multifillis,* have resulted in a lower than expected efficacy (E. Meggit, pers. comm.).

The aim of this study was to investigate the *in vitro* effect of a 1 h treatment using FOR and SPC against the free-living stages of an Australian isolate of *I. multifiliis* at varying concentrations in hard and soft water at two different temperatures.

2. Materials and Methods

2.1. Culture of parasites

A strain of *I. multifiliis* was isolated from rainbow trout from Snobs Creek Hatchery (Victoria Department of Primary Industries, Victoria) during summer 2012. Laboratory populations were established by culturing the parasites on naïve rainbow trout at 17 ± 1°C. Fish with visible trophonts were euthanased with an overdose (40 ml 1000 L^{-1} bath) of Aqui-S® and placed in a 600 ml beaker containing 80 ml aquarium water. The trophonts were allowed to dislodge and collected within 1 h using a using a 200 µL pipette. For theront culture, newly dislodged trophonts were left to settle for 1 h, then gently rinsed in distilled water to remove organic material, and 20 individuals each were incubated in 30 ml of hard and soft water at 12°C and 17°C in 150 mL glass beakers.

2.2. Experimental design

Water used in all experiments consisted of filtered deionised water manipulated to low (general hardness 70 - 80 mg/L $CaCO_3$) and high (general hardness 170 - 195 mg/L $CaCO_3$) hardness by adding calcium carbonate (Chem-Supply, batch ref. (10) 206679). The pH was adjusted to 7 ± 0.2 by the addition of dilute hydrochloric acid or sodium bicarbonate solution. Granular sodium percarbonate (SPC) (Sigma-Aldrich®, lot no. MKBB5394V) and Formalin (37% w/w formaldehyde (FA)) (Ajax Finechem®) was dissolved in hard or soft water to the desired concentrations outlined in Table 1. Solutions of SPC were left for 15 min to facilitate dissolution prior to the commencement of the experiments. Each of the experiments were conducted in temperature controlled rooms at 12 and 17 ± 1°C and were repeated three times. The lowest concentration that resulted in 100% mortality for each treatment was defined as the minimum effective concentration (MEC).

Table 6: Treatments and dose rates (mg/L) administered to *Ichthyophthirius multifiliis* theronts and tomonts.

Treatment	Theront concentrations	Prototomont and tomocyst concentrations
Sodium percarbonate	8, 16, 32 and 64	64, 128, 256 and 512
Formalin	8, 16, 32 and 64	16, 32, 64 and 128

2.3. Treatment of theronts

Treatment experiments were adapted from the methods of Heinecke and Buchmann (2009) and conducted in 96-well culture plates (Costar®). For each treatment 30 wells were used: three wells each with 8, 16, 32 or 64 mg/L SPC or FOR in both hard and soft water, and three wells with hard water and soft water as controls. To each well was added 75 µL of theront suspension (8-16 theronts) in either hard or soft water, which was mixed with 75 µL of the treatment solution in either hard or soft water at twice the desired dose. Controls received 75 µL of hard or soft water without treatments. At time 0 and every 15 min after onset of exposure to the treatment, up to a maximum of 6 h, the number of live theronts in each well was counted using a dissection microscope (20–40x magnification) (Olympus). Lysed or deformed theronts or theronts with no movement were considered dead.

2.4. Treatment of prototomonts

Four recently exited tomonts were placed into each of 10 individual tissue culture dishes (35 mm diameter, Sarstedt Ag & Co) containing 2.5 mL of either hard or soft water. 2.5 mL of an SPC or FOR solution to bring the wells to the doses outlined in Table 6 was added and controls received untreated water. Each dish had a lid to prevent evaporation. Prototomonts were exposed to the treatment for 1 h. After exposure prototomonts were rinsed and transferred to individual wells of a 24-well multidish (Corning®) containing 2 mL of hard or soft water and incubated until theronts were produced. After theront release ended, a drop of 10% Neutral Buffered Formalin (NBF) was added to each well and the number of theronts was counted using a dissection microscope. Prototomonts were considered non-viable if they were unable to produce theronts.

2.5. Treatment of tomocysts

Four recently exited tomonts were placed into each of 10 individual tissue culture dishes (35 mm diameter, Sarstedt Ag & Co) containing 2.5 mL of either hard or soft water. Tomonts were incubated for 24 h at 12°C or 14 h at 17°C to facilitate the development of a cyst wall. After incubation, 2.5 mL of an SPC or FOR solution was added to bring the wells to the doses outlined in Table 6, and water was added to controls. Each dish had a lid to prevent evaporation. Tomocysts were exposed to the treatment for 1 h. After exposure tomocysts were rinsed and transferred to individual wells of a 24-well multidish (Corning®) containing 2

mL of hard or soft water and incubated until theronts were produced. After theront release ended, a drop of 10% neutral buffered formalin (NBF) was added to each well and the number of theronts was counted using a dissection microscope. Tomocysts were considered non-viable if they were unable to produce theronts.

2.6. Statistical analysis

Theront survival was assessed using a log rank test with the Holm–Sidak method for multiple comparisons to compare different survival curves. Prior to analysis, normality of the data was tested using the Shapiro-Wilk test and variances were tested using Levene's test. Where the number of theronts produced did not satisfy normality, the data were log (y+1)-transformed, where y is the number of theronts, prior to analysis. The viability of tomonts, calculated as the mean percent of each treatment replicate surviving, and the number of theronts produced from viable tomonts, were analysed using a 3-factor ANOVA, where dose, water hardness and temperature were the factors. Where significant differences were observed, post-hoc comparisons were made using Tukey's test, however, were equal variances not met, a Games-Howell test was also used. The statistical analysis was performed using IBM SPSS Statistics 20.0, and significance for all tests was judged at $P < 0.05$.

3. Results

3.1 Dose response trials on theronts

Survival was 100% in all control groups. In all trials both SPC and FOR at 8 mg/L had no effect on the theronts after 6 h, and these trials were removed from the analysis. There was no significant difference between groups treated in hard and soft water (Holm-Sidak: $P \geq 0.0691$); therefore the data sets were combined for further analysis using the log rank test with the Holm–Sidak method for multiple comparisons. Parasite survival decreased with increasing temperature, dose and exposure time (Fig 1A-D). There was a significant difference in parasite survival between all groups exposed to SPC at 12°C (log-rank test: $P < 0.001$) and 17°C (Log-Rank test: $P < 0.001$); and FOR at 12°C (log-rank test: $P < 0.001$) and 17°C (Log-Rank test: $P < 0.001$). The toxicity of both SPC (Fig. 1A-B) and FOR (Fig 1. C-D) was proportional to dose and water temperature.

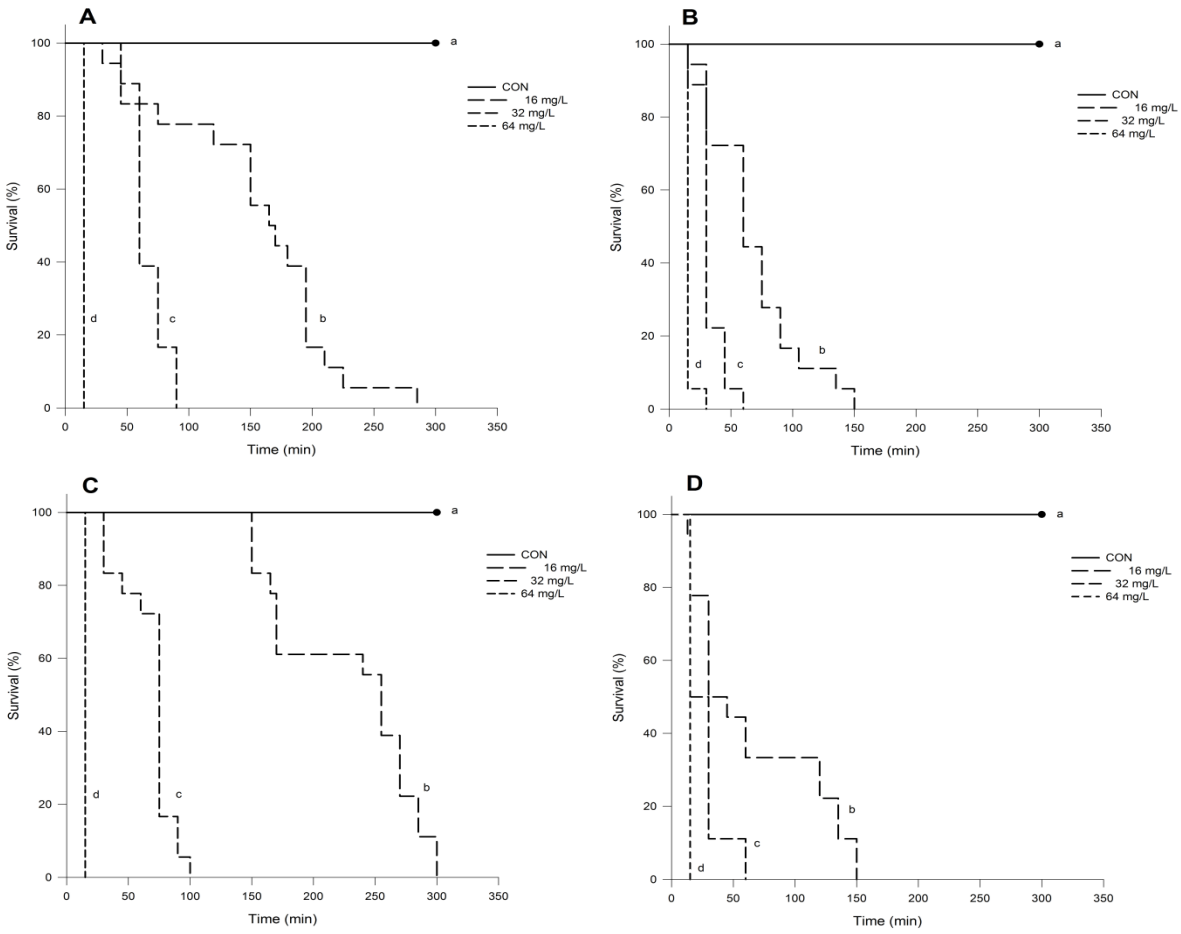


Figure 1: Survival of theronts exposed to different dose levels of sodium percarbonate (SPC) at 12°C (A) and 17°C (B) and formalin (FOR) at 12°C (C) and 17°C (D). Holm-Sidak estimates of the survival data show significant differences between treatment groups, which are represented by different superscripts.

3.2. *In vitro* effect on prototomonts

During FOR trials, the mean viability of prototomonts in all controls was 95.84% ± 2.8 SEM (range 75 - 100%) and was 45.83 ± 8.04% (0 - 75%) and 43.75 ± 8.77 (0 - 100%) in all groups exposed to 16 and 32 mg/L, respectively, and was 0% in all groups exposed to 64 and 128 mg/L. Table 7A outlines the results of a 3-factor ANOVA on viability and theront production of prototomonts exposed to FOR. Prototomont viability decreased with increasing concentration of FOR (Fig 2A). Theront production from viable prototomonts exposed to FOR was significantly higher at 17°C than 12°C (Fig 3A).

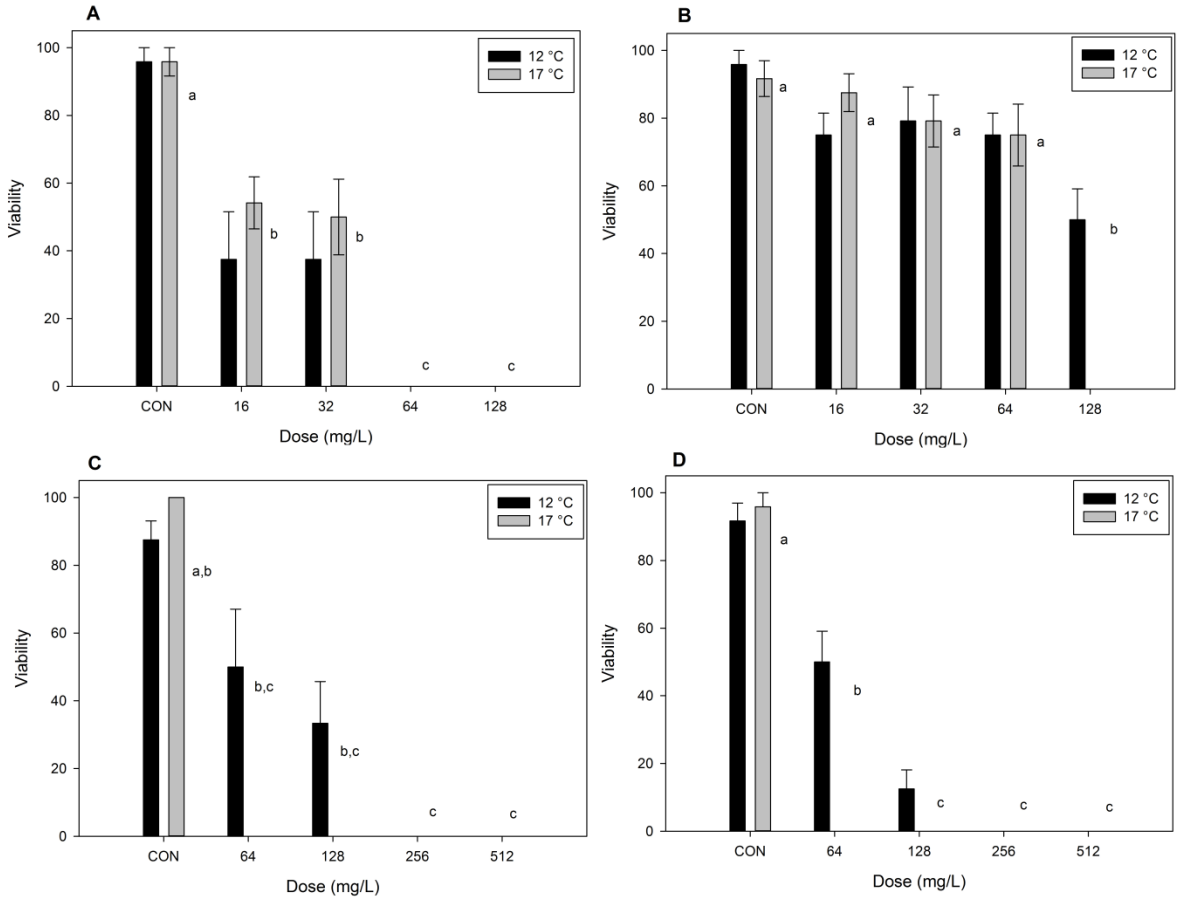


Figure 2: Viability (%) of *Ichthyophthirius multifiliis* prototomonts (A) and tomocysts (B) exposed to formalin (37% formaldehyde); and prototomonts (C) and tomocysts (D) exposed to sodium percarbonate at 12°C and 17°C for 1 h at different concentrations. Different superscripts represent significant differences between doses using Tukey's analysis (*P* < 0.05). Error bars represent the SEM.

During SPC trials the mean viability of prototomonts in all controls was 93.75 ± 3.26% (75 - 100%) and at 12°C was 25 ± 11.09% (0 - 100%) and 16.67 ± 7.74% (0 - 75%) in groups exposed to 64 and 128 mg/L, respectively, and was 0% in groups exposed to 256 and 512 mg/L. There was 100% non-viability at all doses at 17°C. Results of the 3-factor ANOVA on viability and theront production of prototomonts exposed to SPC are reported in Table 7B. Prototomont viability decreased with increasing concentration of SPC, and this effect was particularly marked at the higher water temperature (Fig 2C). Theront production from viable prototomonts exposed to FOR was significantly higher at 17°C than 12°C (Fig 3C).

Table 7: ANOVA interactions between dose, water hardness and temperature on the viability of prototomonts and theront production from viable prototomonts when exposed to formalin (A) and sodium percarbonate (B). – lack of viable prototomonts meant that insufficient data were available to test the term.

		Factor	DF	F	*P*
(A)	Viability	Dose	4	52.029	**< 0.001**
		Temp	1	1.4	0.244
		Water	1	2.314	0.136
		Dose * water	4	1.314	0.281
		Dose * temp	4	0.543	0.705
		Water * temp	1	0.257	0.615
		Dose * water * temp	4	0.686	0.606
		Error	40		
	Theront production	Dose	2	1.373	0.259
		Temp	1	19.479	**< 0.001**
		Water	1	2.395	0.126
		Dose * water	2	1.059	0.402
		Dose * temp	2	1.059	0.352
		Water * temp	1	1.370	0.245
		Dose * water * temp	2	0.163	0.850
		Error	78		
(B)	Viability	Dose	4	54.219	**< 0.001**
		Temp	1	9.031	0.005
		Water	1	0.781	0.382
		Dose * water	4	0.156	0.959
		Dose * temp	4	6.219	**0.001**
		Water * temp	1	0.781	0.382
		Dose * water * temp	4	0.156	0.959
		Error	40		
	Theront production	Dose	2	1.798	0.175
		Temp	1	9.611	**0.003**
		Water	1	1.601	0.211
		Dose * water	2	0.092	0.912
		Dose * temp	0	-	-
		Water * temp	1	2.025	0.160
		Dose * water * temp	0	-	-
		Error	57		

3.3. *In vitro* effect on tomocysts

During FOR trials the mean viability of tomocysts in all controls was 93.75 ± 3.2% SEM (range 75 - 100%) and 81.25 ± 4.4% (50 - 100%), 79.2 ± 6% (50 - 100%), 75 ± 5.3% (50 - 100%) in all groups exposed to 16, 32 and 64 mg/L, respectively. Viability of tomonts at 12°C was 25 ± 8.7% (0 to 75%) and there was 100% non-viability at 17°C. Results of the 3-factor ANOVA on viability and theront production of tomocysts exposed to FOR are reported in Table 8A. Tomocyst viability decreased significantly with increasing concentration of FOR and with no difference between temperatures except at the highest dose, which was particularly effective at 17°C (Fig. 2B). Theront production from viable tomocysts exposed to FOR was significantly higher at 17°C than 12°C and significantly reduced with higher concentrations (Fig. 3C). Tomocysts which were exposed to FOR that had a reduced theront production often had inactive theronts remaining in the cyst wall after the release of all viable theronts.

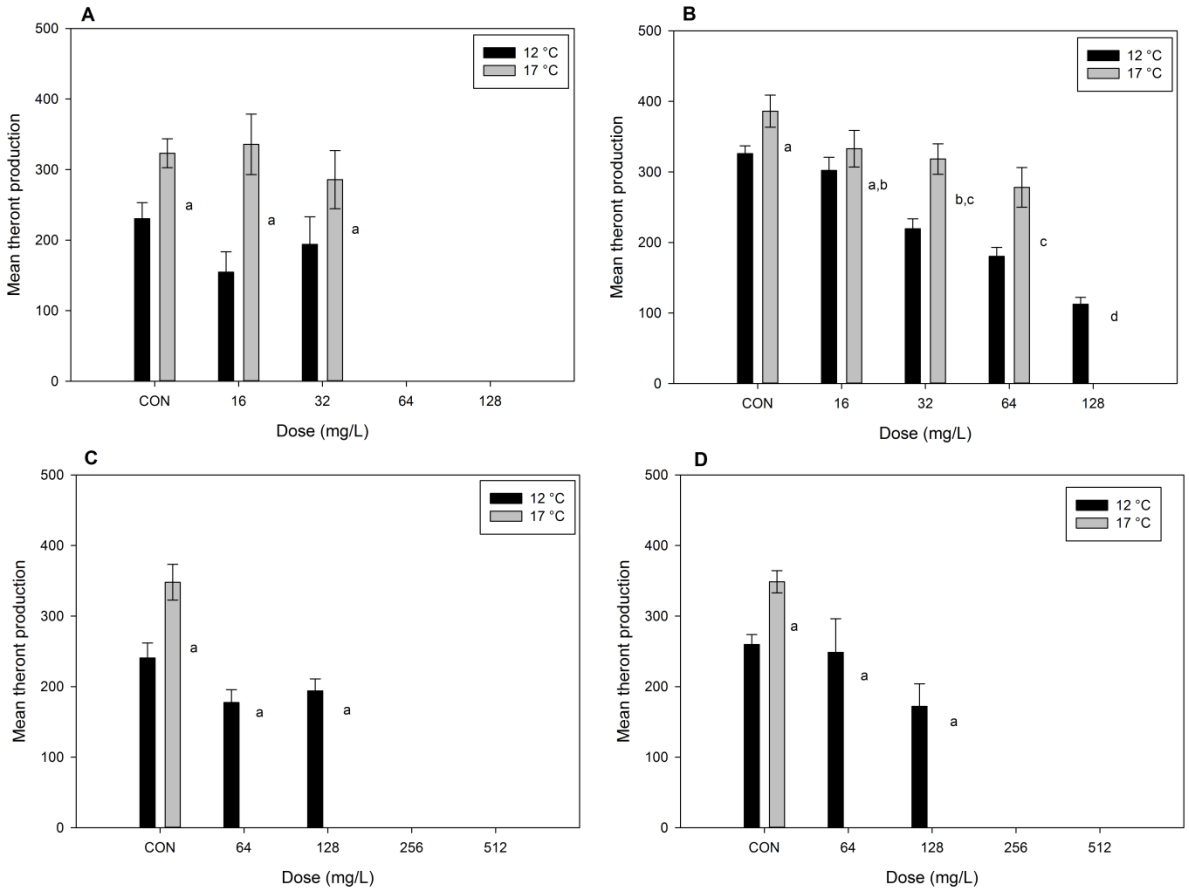


Figure 3: Mean number of theronts produced from viable *Ichthyophthirius multifiliis* prototomonts (A) and tomocysts (B) exposed to formalin (37% FA); and prototomonts (C) and tomocysts (D) exposed to SPC at 12°C and 17°C for 1 h. Different superscripts represent significant differences between doses using Tukey's analysis ($P < 0.05$). Error bars represent the SEM.

During SPC trials the mean viability of tomocysts between all controls was 93.75 ± 3.26% (75 - 100%) and at 12°C was 25 ± 8.7% (0 - 75%) and 6.25 ± 3.27% (0 - 25%) in groups exposed to 64 and 128 mg/L, respectively, and was 0% in all groups exposed to 256 and 512 mg/L. There was 100% non-viability at all doses at 17°C. Results of the 3-factor ANOVA on viability and theront production of tomocysts exposed to SPC are reported in Table 8A. Low concentrations of SPC were more effective at high temperatures while high concentrations were effective irrespective of temperature (Fig. 3D).

Table 8: ANOVA interactions between treatment dose, water hardness and temperature on the treatment viability of tomocysts and theront production from viable tomocysts when exposed to formalin (A) and sodium percarbonate (B). *P* values could not be generated because there was no theront production at any concentration at 17°C.

		Factor	DF	F	*P*
(A)	Viability	Dose	4	31.250	**< 0.001**
		Temp	1	3.846	0.057
		Hardness	1	0.154	0.679
		Dose * water	4	0.250	0.908
		Dose * temp	4	6.442	**<0.001**
		Water * temp	1	3.846	0.057
		Dose * water * temp	4	2.212	0.085
		Error	40		
	Theront production	Dose	4	20.109, *P*	**< 0.001**
		Temp	1	24.481	**< 0.001**
		Hardness	1	0.162	0.126
		Dose * water	4	0.550	0.699
		Dose * temp	3	2.311	0.078
		Water * temp	1	0.258	0.612
		Dose * water * temp	3	0.550	0.699
		Error	153		
(B)	Viability	Dose	4	182.250	**0.001**
		Temp	1	19.6	**0.001**
		Hardness	1	0.000	1.000
		Dose * water	4	0.750	0.564
		Dose * temp	4	14.350	**<0.001**
		Water * temp	1	0.400	0.531
		Dose * water * temp	4	0.650	0.630
		Error	60		
	Theront production	Dose	2	1.343	0.270
		Temp	1	9.362	**0.003**
		Hardness	1	0.997	0.323
		Dose * water	2	1.425	0.250
		Dose * temp	0	-	-
		Water * temp	1	2.479	0.121
		Dose * water * temp	0	-	-
		Error	52		

3.4. Minimum effective concentrations (MECs)

The MEC's for theronts, prototomonts and tomocysts are outlined in Table 9.

Table 9: Minimum effective concentrations (mg/L) for different life-stages of *Ichthyophthirius multifiliis* using sodium percarbonate (SPC) and formalin (FOR) for 1 hour at different water temperatures.

Treatment	Life-stage	Temperature	
		12°C	17°C
SPC	Prototomonts	256	64
	Tomocysts	256	64
	Theronts	64	32
FOR	Prototomonts	64	64
	Tomocysts	> 128	128
	Theronts	64	32

4. Discussion

The current recommended treatment regimes for SPC on Australian trout farms involve repeated applications at 64 mg/L for 1 h, based on Heinecke and Buchmann (2009). Our results suggest that this treatment regime is effective against all free-living life stages at 17°C, but would only eliminate theronts at 12°C, and 256 mg/L is required to be effective against tomonts at 12°C (Table 4). Tomonts that survived and replicated after exposure to SPC produced the same number of theronts as untreated controls, which has also been noted after exposure to peracetic acid (PAA) (Meinelt et al. 2009) and potassium ferrate (IV) (Ling et al. 2001). Therefore, if SPC treatment is delivered in a way that is ineffective against tomonts, fish will be exposed to infection pressure similar to untreated fish.

Increasing the dose rate of SPC to 256 mg/L for 1 h at 12°C may negatively impact the health of the fish undergoing treatment. The SPC used in this study contained 30 – 40% HP, therefore, 256 mg/L SPC would release approximately 76.8 – 102.4 mg/L HP. Rach et al. (1997a) recommended a treatment range of 50 – 250 mg/L HP for 1 h but found that toxicity was proportional to fish size and water temperature. Further investigations into the safety of higher doses of SPC are warranted; we examine this in Chapter 3.

Formalin is currently applied on Australian trout farms at 200 mg/L for 1 h based on Noga (2000). During FOR trials, tomocysts were most resistant to treatments, consistent with Wahli et al. (1993), and with other treatments, such as PAA (Meinelt et al. 2009) and

potassium ferrate (IV) (Ling et al. 2011). FOR at 200 mg/L for 1 h is effective against all free-living life stages at 17°C and eliminates theronts and prototomonts at 12°C (Table 9). The effect on tomocysts requires further investigation; tomocysts exposed to ≥ 32 mg/L FOR had significantly lower theront production than controls, but it is unclear if there is variation in viability and infectivity of theronts produced by treated tomonts. Doses above 128 mg/L at 12°C are also required to obtain the minimum effective concentrations.

Both FOR (Wahli et al. 1993; Lahnsteiner and Weismann 2007; Heinecke and Buchmann 2009) and SPC (Heinecke and Buchmann 2009) are effective *in vitro* but there are numerous factors that influence field implementation of laboratory efficacy data. If the treatment is not delivered to achieve a minimum effective dose, efficacy will be low, and expensive and logistically complicated repeat treatments may be required. Lahnsteiner and Weismann (2007), for example, found that FOR delivered at 110 mg/L for 1 h every 48 h five times completely eliminated *I. multifliis* at 10°C, but a treatment interval of 24 h was required at 18°C.

Minimum effective concentrations reported in Table 9 can be used in combination with relevant life cycle-temperature data specific to Australian isolates of *I. multifiliis* (see Chapter 1) for the strategic timing of treatments to interrupt the parasite's life cycle (Picón-Camacho et al. 2012), improving the management of this parasite on Australian rainbow trout farms.

CHAPTER 3: EFFECTS OF SODIUM PERCARBONATE EXPOSURE ON RAINBOW TROUT

Ichthyophthirius multifiliis, a ciliate protozoan, is a common cosmopolitan parasite of freshwater teleosts and is a reccurring problem during the summer months on Australian rainbow trout (*Oncorhynchus mykiss*) farms. Trophonts of *I. multifiliis* that reside within the epidermis of the host are resistant to chemical treatments and require strategically timed repeat treatments for effective management. Sodium percarbonate (SPC) is currently permitted for use in Australia, but the effects of repeat exposures on rainbow trout are unknown. Juvenile rainbow trout (30.5 ± SD 9 g) were exposed to 50, 100 and 250 mg/L SPC for 1 h on two consecutive days, followed by 5 non-treatment days, after which two more consecutive days of treatment were applied, which was followed by a 10-day recovery period. Rainbow trout were observed throughout the trial for signs of toxicity such as mortality, impaired swimming performance, colour changes and loss of equilibrium. Survival was 100% in all control and treatment groups, however, there was reduced reaction time in the fish exposed to 250 mg/L SPC. Histological sections showed a significant increase in oedema occurring in fish exposed to 250 mg/L SPC directly after exposure to the fourth treatment, but that they had returned to normal the following day. The results suggest SPC is safe to use at ≤ 150 mg/L, however, factors such as water temperature, fish age and maturity, intensity of parasite infection and stocking density could increase the sensitivity of rainbow trout to SPC treatments.

1. Introduction

Sodium percarbonate (SPC) is a granular water-soluble compound that dissociates in water into sodium carbonate and hydrogen peroxide (HP), the latter of which is a powerful oxidant (Bostek 1983) that has been used extensively in aquaculture as a therapeutic compound for external parasitic diseases of fish (Noga 2000). Hydrogen peroxide decomposes rapidly into water and carbon dioxide and does not form cumulative or toxic metabolites (Pedersen et al. 2006), minimising the environmental impacts and food safety risks of treatment.

Treating fish using liquid HP can be problematic; obtaining the desired dose is difficult due to the effects of water biogeochemistry (Bishop et al. 1968). There are also occupational health and safety concerns with handling HP and security restrictions on transport and storage of the product (Eul et al. 2001). SPC is a granular solid with reduced occupational health and safety risk, and its gradual dissociation in water to HP provides a wider fish safety margin than the use of liquid HP (Buchmann et al. 2003).

SPC is effective against free-living stages of *Ichthyophthirius multifiliis* (Fouquet) *in vitro* (Buchmann et al. 2003; Heinecke and Buchmann 2009). The trophont, which resides in the host's epidermis, is unaffected by SPC treatment, therefore, effective management of *I. multifiliis* requires repeat treatments specifically timed to reduce or eliminate re-infection and interrupt the parasite's life cycle (Picón-Camacho et al. 2012).

SPC can be used in Australia pursuant to an Australian Pesticides and Veterinary Medicines Authority (APVMA) minor use permit (MUP) (PER12944) to treat ectoparasites of freshwater and marine finfish at doses of up to 100 mg/L, repeated over 3 consecutive days. The impacts of HP on Atlantic salmon (Johnson et al. 1993; Kiemer and Black 1997) and rainbow trout (Tort et al. 2002) have been investigated but the effects on rainbow trout of exposure to SPC in repeat doses are undescribed.

The aim of this study was to investigate the histological effect on the gills and skin of rainbow trout in response to baths of sodium percarbonate applied as it would be to interrupt the life cycle of *I. multifillis*.

2. Materials and methods

2.1. Experimental design

192 rainbow trout with a mean weight of 30.5 ± 9 g SD were sourced from a commercial trout farm (Victoria, Australia) and transferred to Flinders University. All the fish were caught using a hand net and assigned to tanks using a pre-generated randomised order. 16 were transferred to each of 12 indoor 50 L recirculating aerated holding tanks and acclimated for 6 days. Fish were fed 2% body weight daily of 3 mm Ridley Aqua-feed (Ridley AgriProducts®). The fish were not fed on treatment days. The recirculation system contained filtered municipal water; temperature and dissolved oxygen (Handy Polaris H01P, OxyGuard®) were measured daily. The temperature was maintained at 17 ± 1°C and dissolved oxygen (DO) was 7.8 ± 0.81% SD. At the end of the acclimation period each tank was assigned to one of four treatments by randomly assigning tank numbers to a treatment (three tanks / treatment): no dose, 50 mg/L, 150 mg/L and 250 mg/L SPC, administered for two consecutive days, followed by 5 non-treatment days, then two more consecutive days of treatment, followed by a 10-day recovery period. On treatment days, the designated dose of granular SPC (Sigma-Aldrich®, lot no. MKBB5394V) was mixed in a bucket of water taken from the treatment tank and left to dissolve for 30 min, stirred and added to a treatment tank and mixed further. Fish were transferred from the experimental tanks into the treatment baths for 1 h. The concentration of HP released was measured photometrically (YSI 9300 photometer, YSI Inc.) immediately prior to the fish being added to the treatment tank and immediately after the fish were removed. Water in the holding tanks was exchanged on the second and fourth treatment days.

2.4. Fish response and sampling

Rainbow trout were observed throughout the treatment period for signs of toxicity such as impaired swimming performance, colour changes or loss of equilibrium. Any mortality was recorded.

Fish were sampled immediately after the second treatment finished, one day after the second treatment finished, one day before the third treatment, immediately after the fourth treatment, one day after the fourth treatment, four days after the fourth treatment and eight days after the fourth treatment. Two fish were collected from each tank at the designated

time period, six fish for each treatment. Randomisation was ensured by capturing all the fish using a hand net, removing them one at a time and randomly assigning two fish numbers to be sampled. Sampled fish were replaced with fin-clipped fish of a similar size during treatment periods to maintain the same organic load. The sampled fish were immediately euthanased with an overdose (a 40 mL 1000 L^{-1} bath) of Aqui-S®. Histology samples were taken from the right first gill arch and immediately fixed in 10% neutral buffered formalin (NBF) until histological processing.

2.5. Histological processing and evaluation

Fixed tissues were dehydrated in a graded series of ethanol baths, cleared in xylene and embedded in paraffin wax. After processing, tissues were sectioned at 5 µm and stained with haematoxylin and eosin (H & E). Three well-oriented representative filaments from the first gill arch from each fish were assessed. Cellular composition and structural changes in gill tissues were assessed and quantified by examination with a light microscope. Slides were coded and examined blind. To determine the status of the gills, morphometric indices based on a method of proportional morphometry developed for brachial tissue by Speare and Ferguson (1989) and modified by Sanchez et al. (1997) were used. Each filament was evaluated for:

1. Total number of lamellae on the filament suitable for assessment.
2. Lamellar oedema: the percentage of lamellae with visible separation of the two layers of epithelial cells, involving at least 50% of the given lamellae.
3. Epithelial hyperplasia: the percentage of lamellae with one or more points of thickened areas of epithelial tissue (> 3 cell layers thick), excluding those lamellae where this occurred exclusively at the tip (see Index 7).
4. Lamellar fusion: the percentage of lamellae that had greater than one third fused to the adjacent lamellae.
5. Lamellar inflammation: the percentage of lamellae that had one or more clusters (> 5 cells) of inflammatory cells located in the regions between the pillar cells and the outer layer of the epithelium.
6. Interlamellar inflammation: the percentage of zones between two adjacent lamellae that had clusters (> 5 cells) of inflammatory cells.

7. Clubbed lamellae: the percentage of lamellae with and accumulation of epithelial cells near the tip of the lamella that increases the cell layers, appearing as a 'club-shape'.

The presence of *I. multifiliis* in the histology sections was recorded and the prevalence of infection, as defined by Bush et al. (1997), calculated.

2.7. Statistical analysis

The values for each index from a given fish were expressed as the pooled mean from the three filaments evaluated. Differences in indices between treatment and control groups were analysed using 3-way nested ANOVAs with sample day and dose as orthogonal factors, and treatment tank nested within dose. The statistical analysis was performed using IBM SPSS Statistics 20.0 and significance for all tests was judged at $P < 0.05$.

3. Results

Survival was 100% in all groups and prevalence of *I. multifiliis* was 59% (95% Confidence Interval (CI) 50 - 67%). Rainbow trout exposed to 50 and 150 mg/L SPC showed no adverse reactions to treatment. There was reduced movement and slowed escape response in fish exposed to 250 mg/L SPC. Two fish in one replicate exposed to 250 mg/L lost equilibrium during the second treatment and were returned to the recovery tank 5 min before the end of the treatment period, but recovered with no apparent negative effects. Lamellar oedema and clubbed lamellae showed a time-dependent response to dose, and there was variation between replicate tanks within treatment doses; epithelial hyperplasia and lamellar fusion significantly changed between sample days but was unaffected by treatment dose; and lamellae inflammation varied differently between sample days and replicate tanks in the same treatment doses (Table 10). The main pattern emerging is a higher percentage of lamellar oedema in fish treated with 250 mg/L on sample day four (Fig 4). The concentration of HP did not vary between the start and end of the treatment (Table 11).

Table 10: ANOVA results on histological indices in rainbow trout (*Oncorhynchus mykiss*) exposed to SPC at 0 (control), 50, 150 and 250 mg/L for 1 h.

	Factor	DF	F	*P*
Lamellar oedema	Dose	3	13.583	**< 0.001**
	Day	6	27.623	**< 0.001**
	Tank (dose)	8	5.623	**< 0.001**
	Dose * day	18	7.603	**< 0.001**
	Tank * day (dose)	47	7.640	**< 0.001**
	Error	62		
Epithelial hyperplasia	Dose	3	1.149	0.337
	Day	6	3.933	**0.002**
	Tank (dose)	8	0.847	0.565
	Dose * day	18	1.081	0.392
	Tank * day (dose)	47	0.864	0.698
	Error	62		
Lamellar fusion	Dose	3	0.311	0.817
	Day	6	2.697	**0.022**
	Tank (dose)	8	0.663	0.722
	Dose * day	18	0.914	0.565
	Tank * day (dose)	47	0.769	0.826
	Error	62		
Lamellar inflammation	Dose	3	0.570	0.637
	Day	6	1.168	0.335
	Tank (dose)	8	1.848	0.085
	Dose * day	18	1.702	0.063
	Tank * day (dose)	47	1.775	**0.017**
	Error	62		
Interlamellar inflammation	Dose	3	0.545	0.653
	Day	6	1.143	0.348
	Tank (dose)	8	0.781	0.621
	Dose * day	18	1.354	0.188
	Tank * day (dose)	47	1.213	0.236
	Error	62		
Clubbed lamellae	Dose	3	1.852	0.147
	Day	6	2.871	**0.016**
	Tank (dose)	8	1.991	0.062
	Dose * day	18	1.796	**0.046**
	Tank * day (dose)	47	1.677	**0.028**
	Error	62		

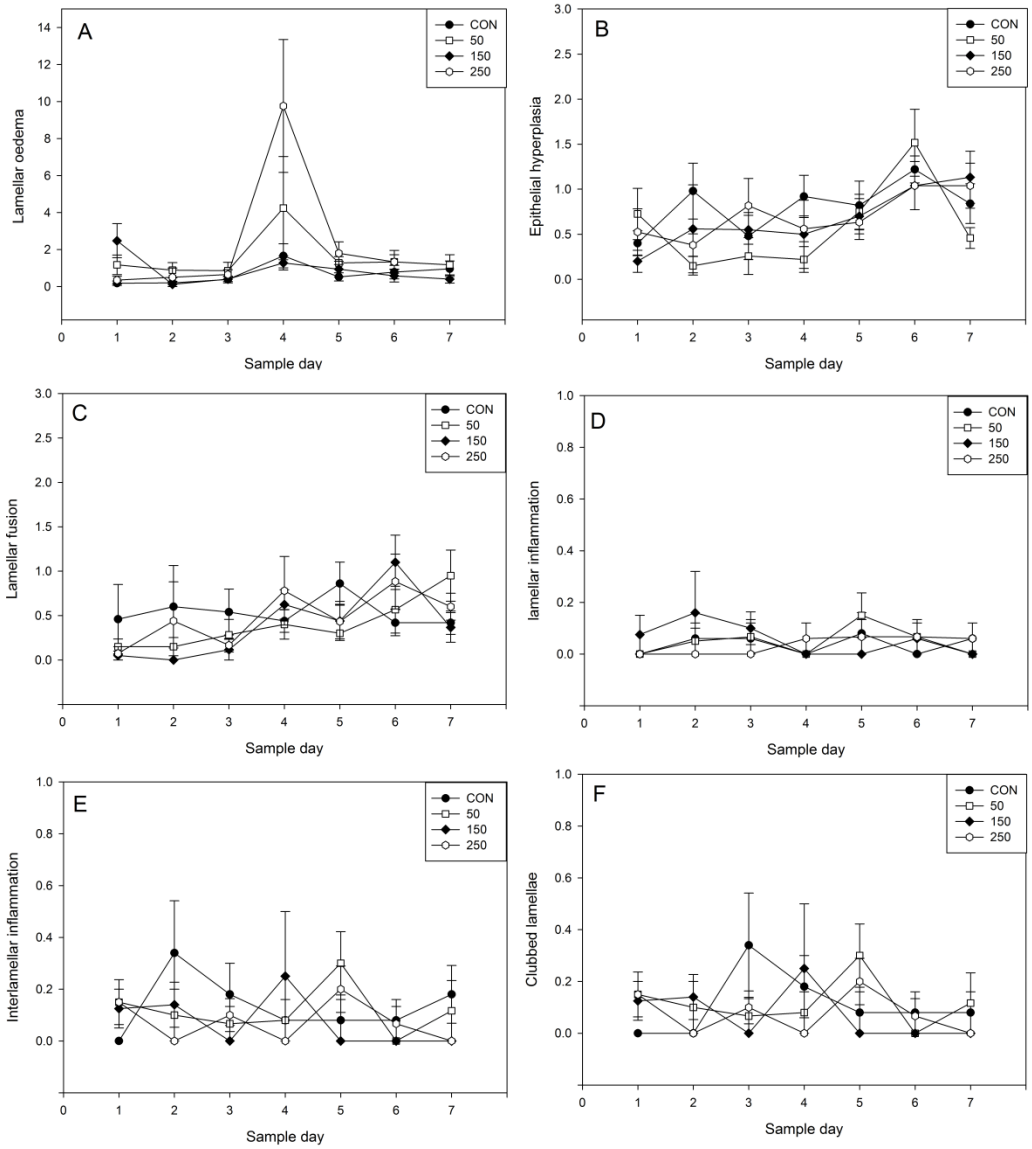


Figure 4: Percentage change in lamellae based on lamellar oedema (A); epithelial hyperplasia (B); lamellar fusion (C); lamellar inflammation (D); interlamellar inflammation (F); and clubbed lamellae (G) from the total number of lamella assessed.

Table 11: Mean HP concentrations (mg/L ± SEM (range)) over a 1 h period in water from controls (no treatment) and treatments of 50, 150 and 250 mg/L SPC.

	Treatment			
Sample	Control	50	100	250
Treatment start	0	18 ± 0.5 (15 - 21)	50 ± 1.4 (43 - 58)	72 ± 1.8 (58 - 70)
Treatment finish	0	18 ± 0.9 (10 – 22)	49 ± 2.6 (36 - 60)	72 ± 2.6 (30 - 52)

4. Discussion

Our data support that SPC can be used on rainbow trout at ≤ 150 mg/L in water up to 17°C with negligible negative effect. The median lethal dose (LC_{50}) of HP for rainbow trout during a single exposure for 1 h at 14°C is approximately 100 mg/L but sensitivity is increased in older fish and with higher water temperature (Tort et al. 2002). Rach et al. (1997a) determined that the 1 h LC_{50} of HP for rainbow trout was 311 mg/L at 17°C, but did not monitor the fish after treatment or record post-exposure mortality.

The highest recorded concentration of HP was 70 mg/L released from a 250 mg/L SPC treatment, which is below reported LC_{50} values. Although there was no mortality recorded during the study, rainbow trout exposed to 250 mg/L for 1 h showed reduced reaction time and flight response, and two fish lost equilibrium and had to be removed from the treatment tank. Fish exposed to 250 mg/L also had substantially more gill oedema after the fourth treatment. Tort et al. (2002) reported doses of HP at ≥ 100 mg/L for 1 h caused epithelial lifting and necrosis of the gills in rainbow trout. This indicates that SPC treatments at 250 mg/L at 17°C may cause gill damage and possibly mortality, but treatments up to 150 mg/L are likely to be safe. Field validation of dose-temperature tolerance is warranted.

The rainbow trout used in this study were infected with *I. multifiliis*, although the intensity of the infection could not be determined. Gill infections of *I. multifiliis* cause cellular necrosis and heavy infections display extensive lysis of epithelial tissue (Ventura and Paperna 1985) leading to increased fish stress (Jørgensen and Buchmann 2007). The stocking densities in this study were low, in practice commercial aquaculture operations are likely to have higher stocking densities, which can increase stress (Conte 2004) leaving fish more sensitive to chemical treatments. Rainbow trout mount an immune response to *I. multifiliis* (see

Buchmann et al. 2001), which is likely to be the cause of the inflammatory response we observed.

These results suggest that the recommended dose on the MUP in Australia could be safely increased to 150 mg/L for rainbow trout in water temperatures ≤ 17°C. Factors such as fish age, intensity of parasite infection and stocking density should, however, be taken into consideration when making decisions about doses of SPC or HP to manage parasites, and field validation of treatment is required, as described in the MUP. HP toxicity varies widely between species (Gaikowski et al. 1999) and doses tolerated by other species need to be determined prior to use.

CHAPTER 4: EVALUATION OF TREATMENT METHODS USING SODIUM PERCARBONATE AND FORMALIN ON AUSTRALIAN RAINBOW TROUT FARMS

In aquaculture, effective parasite management often relies on chemical therapy when prevention strategies are ineffective. In fish farms, there are many factors such as flow, calculation of the dose, degradation rates under different environmental conditions and the geometry of aerators that can have an influence on the spatial and temporal variation in concentration of treatments in an aquaculture system, indicating that verification of the relationship between system, delivery and effective dose is required. Four treatment application methods for sodium percarbonate (SPC) and two application methods for formalin (FOR) were trialed in four semi-closed flow-through systems on four fish farms with different flow and water quality characteristics. Target doses were 64 mg/L for SPC and 200 mg/L for FOR (37% formaldehyde; FA). Hydrogen peroxide (HP) released from SPC was measured photometrically and FA levels were measured colorimetrically. Each application method provided uniform concentrations between repeat treatments, but target concentrations were not realised throughout any system with any of the application methods trialed. Modifications to treatment application methodology were identified that will improve accuracy of the effective dose. Each treatment regime must be validated at each farm where it is to be used prior to being routinely applied as a parasite treatment or control.

1. Introduction

Rainbow trout, *Oncorhynchus mykiss* (Walbaum) are an ideal species for cultivation and are a key aquaculture species in Victoria, Australia (ABARES 2011). One factor limiting productivity of the sector is ectoparasites, primarily *Ichthyophthirius multifiliis* (Fouquet). Outbreaks of *I. multifillis* have a significant negative effect on the host (reviewed by Matthews 2005), which can lead to mortality (Ewing and Kocan 1992). Management of *I. multifiliis* largely centers on husbandry, including minimising stress, manipulating water velocity in raceways, and, when these measures are inadequate, strategic timing of chemotherapeutic intervention.

Malachite green was an effective treatment for a wide range of ectoparasites but is a potential carcinogen and teratogen and is no longer permitted to be used in food fish aquaculture (Alderman 1985; Wahli et al. 1993; Meinelt et al. 2009). An ongoing search identified formalin (FOR) and sodium percarbonate (SPC) as viable alternatives to malachite green. SPC can be used in Australia under a minor use permit (MUP) (PER12944) issued by the Australian Pesticides and Veterinary Medicines Authority (APVMA) at doses of up to 100 mg/L, which is effective against *I. multifiliis* theronts (Heinecke and Buchmann, 2009). FOR has been used as a general treatment for ectoparasites and is effective for control of *I. multifiliis* infections (Wise 2004; Rowland et al. 2009) and is typically administered at 200 mg/L (Noga 2000).

In Australia, rainbow trout are typically cultured in flow-through concrete raceways or earthen ponds. FOR and SPC have been reported to be ineffective against *I. multifiliis* on Victorian trout farms (E. Meggit, pers. comm.). The most important factor that determines the effectiveness of a bath treatment regime is the distribution of the chemical within the system, and maintaining the minimum effective concentration for the designated treatment period is vital for efficacy (Rach et al. 1997b). Many factors affect distribution of the dose in aquaculture systems, including flow, calculation of the dose, degradation rates under different environmental conditions and the geometry of aerators (Rach and Ramsey 2000). If these conditions are not understood, the dose is likely to vary from that required and may be too low to be effective for disease control, or too high, with subsequent negative effects on the fish.

This study evaluated treatment application methods for FOR and SPC on trout farms in three different types of flow-through raceways under various environmental conditions to determine if the minimum effective dose was achieved throughout the system.

2. Materials and methods

2.1. Field trials

Two experiments were conducted on aquaculture farms during winter and summer to validate the treatment application methods and assess the effect of temperature on dose. Treatment application methods were trialed in four semi-closed flow-through systems on four fish farms with different flow and water quality characteristics. Fish farm A, a concrete, low volume, high turnover flow-through system; Fish farm B, a concrete, low volume, low turnover flow-through system; Fish farm C, a concrete, high volume, low turnover, flow-through system; and Fish Farm D, a low turnover, high volume, flow-through earthen raceway. The characteristics of the systems are outlined in Tables 12-15. In all experiments, the method of treatment application normally used on the farm was used and the active component was measured (SPC was measured as hydrogen peroxide; HP); and FOR was measured as formaldehyde (FA). Treatment levels were measured at three points in the raceway; inflow, middle and outflow every 15 min until 90 min post 1 h treatment durations and 60 min post 30 min treatment durations. The target treatment dose was 64 mg/L of SPC (21 mg/L HP) and 200 mg/L of FOR (80 mg/L FA). In all experiments, SPC (Redox Pty Ltd.) and FOR (37% FA) (Redox Pty Ltd.) was used. Fish were observed for any adverse reactions during the treatment period and dissolved oxygen (DO) levels were measured in the middle of the system at each sample time throughout the treatment period. Each trial was repeated three times.

2.2. Fish farm A

Fish Farm A administered granular SPC aiming to achieve the target dose for 1 h and FOR aiming to achieve the target dose for 30 min.

2.2.1. SPC trials

The initial dose of granular SPC was calculated based on the estimated total volume of the system (Table 12). During the treatment period the system was maintained under normal

function. At the start of the treatment SPC granules were weighed and evenly distributed throughout the raceway by bucket. After the initial dose, additional granular SPC was added evenly throughout the raceway every 5 min for 45 min based on the volume of incoming water (Table 12). The raceway was routinely swept with a broom to improve dissociation of the granular SPC on the bottom of the raceway.

2.2.2. FOR trials

The initial dose of FOR was calculated based on the total estimated volume of the system (Table 12). During the treatment the system was maintained under normal function. At the start of the treatment liquid FOR was measured and added by bucket directly to the water evenly throughout the raceway. After the initial dose, additional liquid FOR was added evenly throughout the raceway every 5 min for 25 min based on the volume of incoming water (Table 12).

Table 12: Systems at Fish Farm A used for validation of application of sodium percarbonate (SPC). N/A = not applicable, N/D = Not determined.

Study system	SPC trials			FOR trials		
	Trial 1 Raceway 12	Trial 2 Raceway 6	Trial 3 Raceway 9	Trial 1 Raceway 12	Trial 2 Raceway 1	Trial 3 Raceway 2
Rearing volume (m^3)	55.8	33.6	34.3	55.8	34.3	57.4
Average depth (m)	1.2	0.72	0.73	1.2	0.73	1.25
In-flow water (L/s)	35	34	34	35	40	40
Fish density (kg/m^3)	32.2	N/D	N/D	32.2	26.8	14.4
Water temp. (°C)	6	5.2	5.4	5.5	9.7	9.8
COD (mg O_2/L)	< 20	< 20	< 20	< 20	< 20	< 20
Aeration	N/A	N/A	N/A	N/A	N/A	N/A
Treatment	SPC	SPC	SPC	FOR	FOR	FOR
Application method	Granular	Granular	Granular	Liquid	Liquid	Liquid
Initial dose	3.57 kg	2.15 kg	2.19 kg	8.04 L	6.8 L	11.48 L
Top ups (kg)	0.672	0.65	0.67	2.01 L	2.4 L	2.4 L
Expected concentration (mg/L)	64	64	64	200	200	200

2.3. Fish Farm B

Fish Farm B administered SPC using both liquid and granular application, aiming to achieve the target dose for 1 h.

2.3.1. Liquid SPC trials

The initial dose was calculated based on the total estimated volume of the system (Table 13), added to two 200 L drums of water and left for 15 min to dissolve. Water in-flow was restricted by the addition of a reducer connected to the in-flow pipe and liquid SPC was drip-fed over 45 min into the water above each of the inlets. One top up dose based on the volume of in-flow water (Table 13) was dissolved in a separate 100 L drum of water. Twenty five min after the initial application, the top up dose was evenly bucketed throughout the raceway. At the completion of the treatment period, the reducers were removed and normal flow was resumed.

2.3.2. Granular SPC trials

The initial dose of granular SPC was calculated based on the total volume of the system (Table 13). At the start of the treatment, in-flow was stopped and the initial dose of granular SPC was added evenly to each section of the raceway, which created a static bath. Normal flow was resumed at 40 min and a 10% additional dose added 45 min after the first dose, primarily at the inlet of the raceway.

2.4. Fish farm C

Fish farm C administered FOR aiming to achieve the target dose for 1 h. The initial dose of FOR was calculated based on half of the total volume of the system (Table 14). Prior to the start of the treatment period the water level was reduced by half the normal volume; the out-flow was stopped and in-flow retained as normal. The initial dose was premixed in 3 x 20 L buckets of water and administered at the base of the inflow pipe, dispersal was aided by paddle wheel, over a 2 min period, which created a static bath. At 40 min normal flow was resumed.

Table 13: Systems at Fish Farm B used for validation of application of liquid and granular sodium percarbonate (SPC). R = Reduced flow, F = Full flow, N/A = Not applicable

Study system	Liquid application trials			Granular application method		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Type of system	Raceway 2	Raceway 4	Raceway 3	Raceway 4	Raceway 2	Raceway 1
Water use	Flow-through	Flow-through	Flow-through	Flow-through	Flow-through	Flow-through
Rearing volume (m^3)	23.2	17.4	21.6	17.4	23.2	18.4
Average depth (m)	0.5	0.5	0.5	0.5	0.5	0.5
In-flow water (L/s)	R 6.4,	R 9.8,	R 10.3,	9.8	10.4	10.3
Fish density (kg/m^3)	29	10.6	13.9	24	16.1	20.2
Water temp. (°C)	6.4	11.2	11.3	6.8	11.4	11.5
COD level (mg O_2/L)	29	< 20	< 20	< 20	< 20	< 20
Aeration	N/A	N/A	N/A	N/A	N/A	N/A
Treatment	SPC	SPC	SPC	SPC	SPC	SPC
Application method	Liquid	Liquid	Liquid	Granular	Granular	Granular
Initial dose	1.152	1.152	1.152	1.057	1.482	1.179
Top ups (kg)	0.64	0.64	0.64	0.903	0.148	0.117
Expected concentration (mg/L)	64	64	64	64	64	64

2.5. Fish farm D

Fish farm D administered granular SPC aiming to achieve the target dose for 1 h. The initial dose of granular SPC was calculated based on the total volume of the system (Table 15). Normal raceway volume and flow were maintained throughout the treatment. At the start of the treatment SPC granules were weighed and evenly distributed throughout the raceway by bucket. In the third replicate, dispersion was aided with the addition of two paddle wheels located 2 m from the inlet and outlet, facing the middle of the raceway to aid in the dispersal of the treatment. The paddle wheels were turned on 15 min after the initial dose and turned off 30 min later.

Table 14: Systems at Fish Farm C used for validation of application of formalin (FOR).

Study system	Fish farm C		
	Trial 1	Trial 2	Trial 3
Type of system	Pond 2	Pond 5	Pond 3
Water use	Flow-through	Flow-through	Flow-through
Rearing volume (m^3)	191	172	152
Average depth (m)	1.3	1.2	1.05
In-flow water (l/s)	7.82	7.82	10.08
Fish density (kg/m^3)	35.6	33.5	30
Water temp. (°C)	6.0	6.1	10.4
COD level (mg O_2/L)	< 20	< 20	< 20
Aeration	Paddle wheel	Paddle wheel	Paddle wheel
Treatment	FOR	FOR	FOR
Application method	Liquid	Liquid	Liquid
Initial dose	20 L	20 L	20 L
Top ups (kg)	-	-	-
Expected concentration (mg/L)	200	200	200

Table 15: Systems at Fish Farm D used for validation of application of granular sodium percarbonate (SPC). N/A = not applicable, N/D = not determined.

Study system	Fish Farm D		
	Trial 1	Trial 2	Trial 3
Type of system	Raceway 2	Raceway 2	Raceway 1
Water use	Flow-through	Flow-through	Flow-through
Rearing volume (m^3)	221	221	349
Average depth (m)	76.33	76.33	92.7
In-flow water (l/s)	N/D	N/D	N/D
Fish density (kg/m^3)	<1	<1	1
Water temp. (°C)	11.4	12.1	12.6
COD level (mg O_2/L)	32	< 20	30
Aeration	N/A	N/A	Paddle wheel
Treatment	SPC	SPC	SPC
Application method	Granular	Granular	Granular
Initial dose	14.3	14.3	22.34
Top ups (kg)	-	-	-
Expected concentration (mg/L)	64	64	64

2.6. Chemical analyses

Residual HP was measured using a Plaintest® photometric system as per manufacturers' instructions. Color intensity was measured using a YSI 9300 photometer (YSI Inc.) within 1 min of mixing. Residual FA was measured using a colorimetric method with test strips and reagent (MQuant™ Product No. 1100360001), as per manufacturer's instructions. Water temperature and dissolved oxygen (mg/L) were measured with a Handy Polaris H01P (OxyGuard®). Organic content in the water was measured as chemical oxygen demand (COD) in mg/L O_2 using the APHA 5220 COD Open Reflux method (Eurofins Environment Testing Australia Pty Ltd).

2.7. Statistical analyses

Prior to analysis, normality of the data was tested using the Shapiro–Wilk test and variances tested using Levene's test. Where the data did not satisfy normality, they were log (y+1)-transformed, where y is the measured dose, prior to analysis. Differences in the measured dose between raceway positions and at each time point were compared using a 2-way ANOVA. Water temperature was included as a covariate (ANCOVA). The statistical analyses were performed using IBM SPSS Statistics 20.0 and significance for all tests was judged at $P < 0.05$.

3. Results

3.1 Fish Farm A

3.1.1. SPC trials

The measured mean dose at the inlet was 8.2 ± standard error of the mean (SEM) 1.1 mg/L (range 3 – 17 mg/L), at the middle was 13.2 ± 1.2 mg/L (SEM, range 2 – 22 mg/L), and the outlet was 13.8 ± 1.5 mg/L (SEM, range 3 – 27 mg/L). Measured doses were significantly different between sample times (2-way ANOVA: $F_{4, 29} = 3.129$, $P = 0.030$) and raceway positions (2-way ANOVA: $F_{2, 29} = 8.628$, $P < 0.001$) showing that the dose was uneven through the raceway and over the treatment period (Fig. 5A). There was no interaction between time and position (2-way ANOVA: $F_{8, 29} = 2.227$, $P = 0.055$). 95% CIs overlapped the target dose in the middle of the raceway at 1, 15 and 30 min, indicating that the target

dose was reached at these positions and times, but it was not maintained throughout the trial (Fig. 5A).

Water temperature did not have a significant effect on dose between raceway positions (ANCOVA: $F_{1, 29} = 1.949$, $P = 0.173$). Dissolved oxygen levels during the treatment trials are reported in Table 16.

3.1.2. FOR trials

The measured mean dose was 27.8 ± 6.6 mg/L (SEM, range 5 – 70 mg/L) at the inlet, 34.4 ± 6.6 mg/L (SEM, range 15 – 80 mg/L) in the middle and 35 ± 4.7 mg/L (SEM, range 15 – 50 mg/L) at the outlet. Measured doses were significantly different between sample times (2-way ANOVA: $F_{2, 17} = 8.461$, $P = 0.003$) but not raceway positions (2-way ANOVA: $F_{2, 17} = 1.524$, $P = 0.246$) showing that the dose was consistent through the raceway but uneven over the treatment period (Fig. 5B). There was no interaction between time and position (2-way ANOVA: $F_{2, 17} = 0.600$, $P = 0.668$). 95% CIs did not overlap the target dose in any position or sample time, indicating that the target dose was not reached throughout the trial (Fig. 5B).

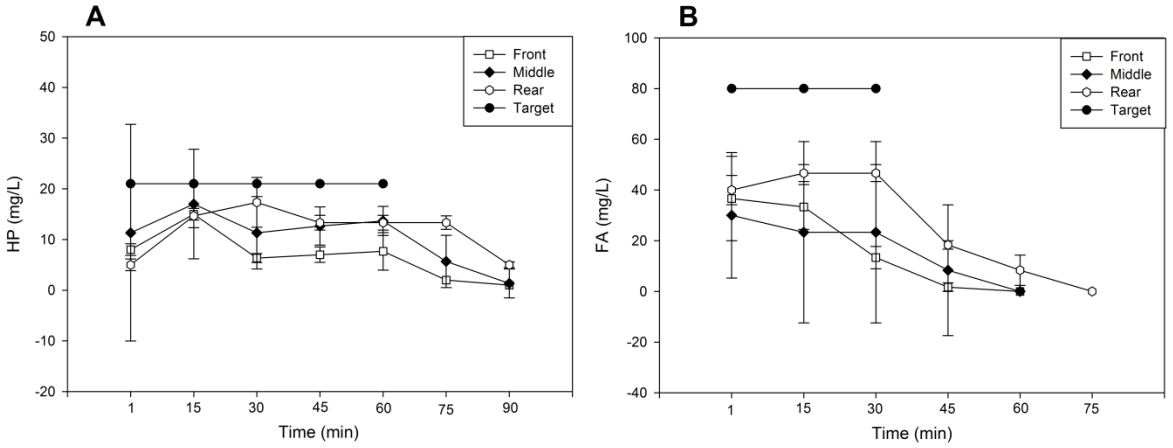


Figure 5: Doses of hydrogen peroxide (HP) released from sodium percarbonate (SPC) (A) and formaldehyde (FA) from formalin (37% FA) (B) on Fish Farm A. Error bars represent 95% CI.

Water temperature did not have a significant effect on dose between raceway positions (ANCOVA: $F_{1, 17} = 1.154$, $P = 0.298$). Dissolved oxygen levels during the treatment trials are reported in Table 16.

3.2. Fish Farm B

3.2.1. Liquid SPC trials

The measured mean dose was 11.1 ± 1.5 mg/L (SEM, range 2 – 19 mg/L) at the inlet, 10.5 ± 1.5 mg/L (SEM, range 2 – 20 mg/L) in the middle and 10.4 ± 1.6 mg/L (SEM, range 1 – 20 mg/L) at the outlet. The measured doses were significantly different between sample times (2-way ANOVA: $F_{4, 29} = 20.145$, $P < 0.001$) but not raceway positions (2-way ANOVA: $F_{2, 29} = 0.219$, $P < 0.804$), showing that the dose was uneven through the raceway but consistent over the treatment period (Fig 6A). There was no interaction between time and position (2-way ANOVA: $F_{8, 29} = 2.173$, $P = 0.060$). 95% CIs overlapped the target dose in the middle and front of the raceway at 1 min and in the middle section at 15 min, indicating that the target dose was reached at these positions and times, but it was not maintained throughout the trial (Fig 6A).

Water temperature did not have a significant effect on the dose between raceway positions (ANCOVA: $F_{1, 29} = 1.707$, $P = 0.202$). Dissolved oxygen levels during the treatment trials are reported in Table 16.

3.2.2. Granular SPC trials

The measured mean dose was 15 ± 2.5 mg/L (SEM, range 3 – 29 mg/L) at the inlet, 14.8 ± 1.8 mg/L (SEM, range 3 – 25 mg/L) in the middle and was 17 ± 3.2 mg/L (SEM, range 0 – 46 mg/L) at the outlet. Doses were significantly different between sample times (2-way ANOVA: $F_{4, 29} = 13.098$, $P < 0.001$) but not between raceway positions (2-way ANOVA: $F_{2, 29} = 1.112$, $P = 0.895$), showing that the dose was uneven through the raceway but consistent over the treatment period (Fig 6B). There was no interaction between time and position (2-way ANOVA: $F_{8, 29} = 0.350$, $P = 0.938$). 95% CIs overlapped the target dose in the front, middle and rear of the raceway at 15, 30 and 45 min, indicating that the target dose was reached at these positions and times, but it was not maintained throughout the trial (Fig 6B).

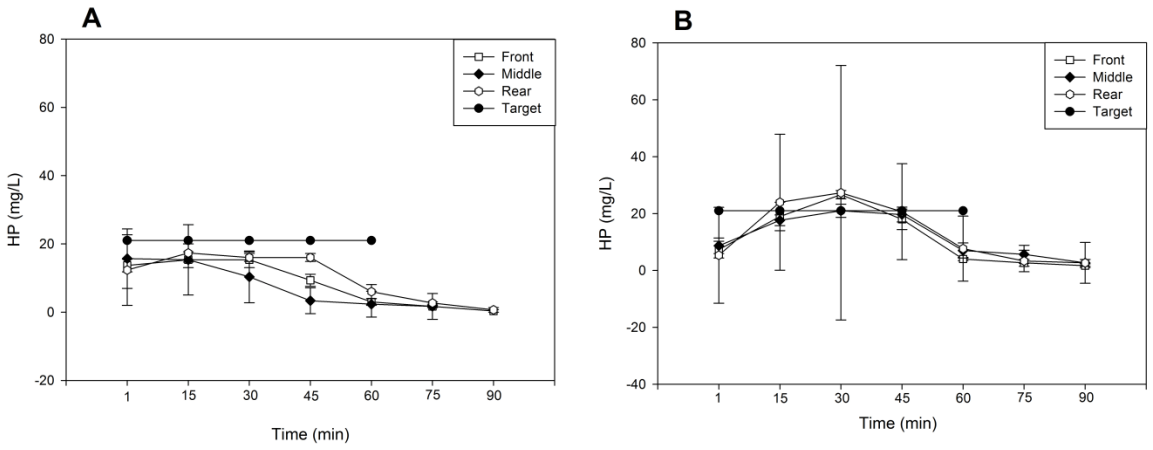


Figure 6: Dose of hydrogen peroxide (HP) released from sodium percarbonate (SPC) administered by drip feed liquid application with one top up dose at 25 min (A); and by granular application in a static bath, with one top up dose at 45 min (B) on fish farm B. Each trial was repeated three times. Error bars represent 95% CI.

Water temperature did not have a significant effect on dose between raceway positions (ANCOVA: $F_{1, 29} = 0.002$, $P = 0.962$). Dissolved oxygen levels during the treatment trials are reported in Table 16.

3.3. Fish Farm C

The measured mean dose was 36 ± 5.9 mg/L (SEM, range 0 – 90 mg/L) at the inlet, 25 ± 3.2 mg/L (SEM, range 10 – 60 mg/L) in the middle and was 27.3 ± 3.8 mg/L (SEM, range 0 – 60 mg/L) at the outlet. Measured doses were not significantly different between sample times (2-way ANOVA: $F_{4, 29} = 1.269$, $P = 0.305$) or between raceway positions (2-way ANOVA: $F_{2, 29} = 1.495$, $P = 0.241$), showing that the dose was even throughout the raceway and consistent over the treatment (Fig. 7). There was no interaction between time and position (2-way ANOVA: $F_{8, 29} = 0.319$, $P = 0.952$). 95% CIs overlapped the target dose in the front of the raceway at 1, 15 and 45 min and in the front and rear at 45 min, indicating that the target dose was reached at these positions and times, but it was not maintained throughout the trial (Fig. 7).

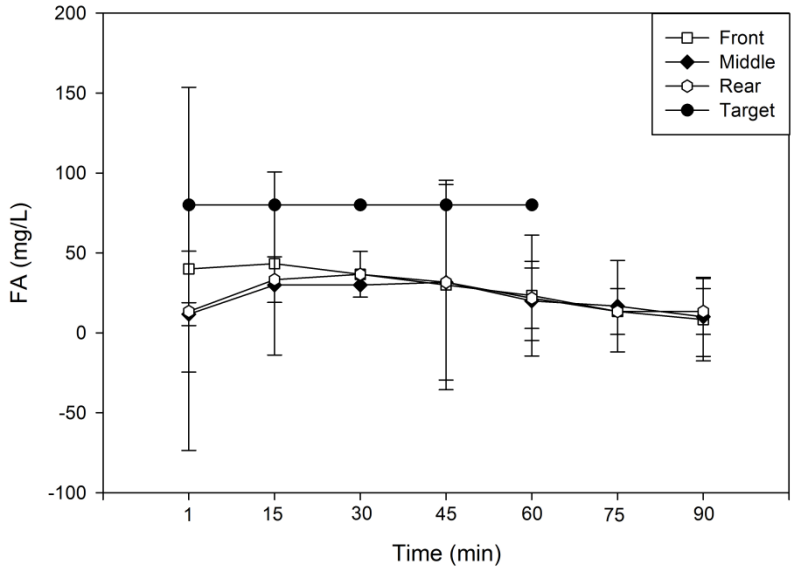


Figure 7: Doses of formaldehyde (FA) from formalin (37% FA) administered by one dose application into a static bath on Fish Farm C. Each trial was repeated three times Error bars represent 95% CI. Different superscripts represent significant differences between groups.

Water temperature did not have a significant effect on the dose level between raceway positions (ANCOVA: $F_{1, 29} = 0.992$, $P = 0.345$). Dissolved oxygen levels during the treatment trials are reported in Table 16.

3.4. Fish Farm D

The measured mean dose was 7.9 ± 1.3 mg/L (SEM, range 2 – 18 mg/L) at the inlet, 12.7 ± 1.5 mg/L (SEM, range 2 – 27 mg/L) in the middle and was 17.2 ± 1.8 mg/L (SEM, range 1 – 26 mg/L) at the outlet. Measured doses showed high variability and were significantly different between sample times (2-way ANOVA: $F_{4, 29} = 3.609$, $P < 0.017$) and between raceway positions (2-way ANOVA: $F_{2, 29} = 0.219$, $P < 0.001$), showing that the dose was uneven through the raceway and over the treatment period (Fig 8). There was no interaction between time and position (2-way ANOVA: $F_{8, 29} = 1.627$, $P = 0.160$). 95% CIs overlapped the target dose in the front of the raceway at 1, 15 min; in the middle of the raceway at 1, 15, 30 and 45 min; and in the rear of the raceway at 15, 30, 45 and 60 min, indicating that

the target dose was reached at these positions and times, but it was not maintained throughout the trial (Fig. 8).

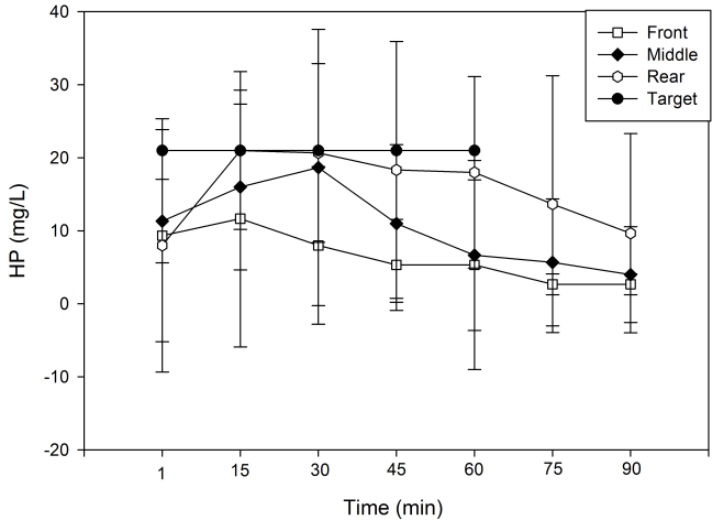


Figure 8: Doses of hydrogen peroxide (HP) released from sodium percarbonate (SPC) administered by granular application with one dose administered evenly throughout the raceway on Fish Farm D. Error bars represent 95% CI.

Water temperature had a significant effect on the dose level between raceway positions (ANCOVA: $F_{1, 29} = 5.597$, $P = 0.025$). Dissolved oxygen levels during the treatment trials are reported in Table 16.

Table 16: Mean ± SEM (range) dissolved oxygen levels during the treatment period using sodium percarbonate (SPC) and formalin (FOR) on Australian trout farms.

Farm	Treatment	Dissolved oxygen (mg/L)		
		Replicate 1	Replicate 2	Replicate 3
Farm A	SPC	18.8 ± 2.5 (11.1 – 26.5)	10.7 ± 0.3 (10.1 – 11.4)	11.3 ± 0.4 (9.9 – 12.1)
	FOR	9.7 ± 0.2 (9.5 - 10.1)	9.4 ± 0.2 (9.1 - 9.7)	9.2 ± 0.03 (9.2 - 9.3)
Farm B	SPC (liquid)	4.5 ± 0.7 (2.8 - 6.3)	8.6 ± 0.1 (8.3 - 9)	7.6 ± 0.1 (7.3 – 7.9)
	SPC (granular)	11 ± 0.2 (10.6 - 11.4)	6.3 ± 0.4 (5.4 - 7.9)	6.1 ± 0.5 (4.8 - 7.4)
Farm C	FOR	10.7 ± 0.1 (10.4 - 11)	9.2 ± 0.1 (8.9 - 9.4)	6.8 ± 0.1 (6.5 – 7.1)
Farm D	SPC	10.7 ± 2.2 (7.7 – 19.6)	11.3 ± 1 (10 – 15.3)	11.3 ± 1.2 (9.3 – 15.9)

4. Discussion

Bath treatment doses are influenced by flow and dispersal of the product through the system, which is dependent on wind and turbulence (Rach and Ramsey, 2000). These can lead to sections of an aquaculture system receiving an under or over dose of treatment. We found few significant differences in doses in repeated applications for both FA and SPC when application method was consistent. In one SPC trial, however, there was high variance in the available HP content in the water between trials; this is likely to be due to the addition of paddle wheels in the third replicate, which was intended to aid the dispersal and retention of HP in the system.

No application method trialed achieved the target dose in all sections of the raceway or over the full treatment period. The best application method was from Farm B, where granular SPC was added to a static bath. This method was similar to that which was used on Farm D, where one initial dose was applied in a low flow raceway, resulting in SPC slowly dissipating in the bath. The addition of a paddle wheel in the third replicate on Farm D, located near the raceway outlet to push water against the flow, held the HP in the system for longer and maintained a more even distribution of HP in the raceway than in the previous replicates.

Liquid application of SPC on Farm B also provided a consistent dose of HP throughout the raceway, but that dose was significantly lower than the target. Increasing the in-flow rate of

the liquid SPC would deliver more of the product into the system, raising the dose level and providing a more effective treatment regime.

SPC application on Farm A evenly distributed HP throughout the system and dose was consistent throughout the treatment, but the dose was significantly lower than the target. These raceways had the greatest flow (34 - 35 L/s) and required constant application of additional SPC to compensate for the diluting effect of incoming water and loss of product at the discharge point. The initial and top-up doses of SPC were applied in granular form, leading to a gradual dissociation and release of HP over time (Heinecke and Buchmann 2009). The gradual disassociation of SPC may have contributed to the significantly lower measured dose of HP than the target dose. Pre-mixing the initial and top-up doses in water for 15 min prior to application would facilitate the release of HP, increasing the measured dose.

During FOR trials on farm A and D, the product was consistently distributed throughout the raceway but the measured dose was significantly lower than the target. Formaldehyde is a strong reducing agent, which can be taken up by organic matter (Masters 2004) and aquatic organisms (Bills et al. 1977). Formaldehyde degradation is influenced by differences in water quality and DOC (Masters 2004), but can also be accelerated by aeration and temperature (Xu and Rodgers 1995; Pedersen et al. 2007). Each system has a different combination of these factors, and we recommend that FA levels are monitored during the treatment and additional doses be employed as required.

The addition of SPC or FA into aquaculture systems can influence the oxygen availability in the system. During SPC treatments there were significant increases and decreases in DO level depending on the aquaculture system and application method. During replicate 1 on farm A and all replicates on farm D there were increases in DO. When HP oxidises organic material, oxygen is liberated (Pedersen et al. 2006). In high organic systems such as earthen ponds or raceways, high DO during SPC treatment is therefore common (Buchmann and Kristensson 2003). The liberation of oxygen in organically loaded systems undergoing SPC treatment may lead to prolonged exposure to high DO, which can result in fish mortalities (Pedersen et al. 2006). Across all SPC trials, DO peaked at 26.5 mg/L, but reduced to normal (10 mg/L) within 60 min. Exposure to DO in this range for this duration is unlikely to negatively affect rainbow trout (Edsall and Smith 1990).

Applying SPC on Farm B with no in-flow resulted in fish using up the available oxygen and a decrease in DO. The reduced DO led to the abandonment of the treatment and normal in-flow of water into the system was resumed. When applying SPC and reducing or stopping water in-flow, supplementary oxygenation should be used to maintain adequate DO (Noga 2000). Formalin treatments can reduce DO (Allison 1965), but we found that DO was stable and adequate to maintain rainbow trout during FOR trials.

Our study demonstrates the need for spatial and temporal analytical verification of dose in aquaculture systems. The test strips used to detect FA were less sensitive than the photometric methods used to detect HP, but both provide an effective indication of dose. We also recommend monitoring DO throughout the treatment and responding appropriately to significant increases or decreases. The necessary equipment is inexpensive and easy to use, facilitating farm operators to evaluate their treatments and better inform management decisions.

Our study confirms that static baths limit the external factors that influence spatial variability of dose and are therefore the preferred treatment method (Noga 2000). Where static baths are not logistically feasible, such as in flow-through systems where the inflow cannot be stopped, the use of mechanical devices such as paddle wheels, can help facilitate the distribution of bath treatments and retain the treatment in the system. Each treatment approach should be validated when developed and be monitored to ensure the target dose is being reached throughout the system but not exceeded. Dose adjustment can then be employed in an informed manner. Consideration should be made of changing application method, including altering infrastructure where appropriate, if treatments cannot be consistently managed in existing facilities.

CONCLUSION

We developed on farm management strategies for *I. multifiliis* and information to improve treatment efficacy. Minimum effective concentrations using FOR and SPC are outlined in Table 9 and can be applied in combination with treatments strategically timed using life cycle-temperature information outlined in Table 5. We have also assessed treatment application methods, and found static baths to be the most predictable and reliable. We developed a management plan that can be readily implemented by trout farmers to improve control of *I. multifiliis*.

Under-dosing was the factor that contributed most to the low efficacy of SPC and FOR observed on farms. In all treatments, dose was substantially lower than the target in at least one part of the treatment system. Creating a static bath limits the influence of irregular flow, water velocity and uneven mixing, but dissolved oxygen needs to be carefully monitored to maintain fish health. If static baths are not feasible and treatment is applied during constant flow, measuring dose throughout the treatment period and using those measurements to modify dose is recommended. The addition of aerators in low flow systems can increase dispersal and aid in retaining the product in the aquaculture system.

We used two commercially available, relatively inexpensive and easy to use systems for analysis of FA and HP (the active product released by SPC). These provided us with information on the actual dose in the system and can be utilised by growers undertaking a treatment to ensure that they are achieving the desired dose.

Both SPC and FOR are effective in concrete and earthen raceways. Dose experiments validating FOR treatments showed that it is effective against free-living life stages, except tomocysts, which were resistant up to 128 mg/L at 12°C, which was the highest dose trialed. We demonstrated that SPC is effective against all free-living life stages at ≥ 17°C. At 12°C lack of efficacy against tomocysts requires a repeat application at 64 mg/L 48 h after the first treatment to coincide with theront release and interrupt the life cycle. SPC is less hazardous than FOR and will improve worker safety. SPC does not have a withholding period and can be used on fish that will be harvested for human consumption soon after treatment, which is of particular benefit for fish-out trout farms.

Rainbow trout exposed to repeat high concentrations of SPC (≤ 150 mg/L) at 17°C had 100% survival, no signs of toxicity and minor pathological changes in the gills (Fig 4). Results suggest that the recommended dose in the MUP (PER12944) could be increased for rainbow trout at ≤ 17°C, improving efficacy against resistant life stages and at low water temperatures.

The other major factor that contributed to low efficacy was the timing of repeat treatments. Table 5 provides an informative basis for the timing of repeat treatments at different temperatures. This will improve efficacy, reduce the cost of treatment, minimise fish stress and improve productivity.

Overall, our results identified key areas that were limiting the efficacy of SPC and FOR use on rainbow trout farms, and allowed a recommended treatment regime to be developed. Implementation of these changes to farm management practices will improve the efficacy of SPC and FOR in the treatment of *I. multifiliis*.

RECOMMENDATIONS

Results indicate that to improve the management of *I. multifiliis* on farms growers should:

- **Use minimum effective concentrations of SPC and FOR for the water temperature and maintain the dose for 1 hour.**
- **Validate the treatment application method used in the culture system to ensure the target dose is being achieved and maintained.**
- **Base repeated applications on life cycle-temperature data.**

FURTHER DEVELOPMENT

The efficacy of the treatment regimes and management recommended here should be monitored and refined in the field.

To further enhance management of *I. multifiliis* in rainbow trout farms, new treatments should be identified, their efficacy validated and data obtained so they can be permitted for commercial use.

EXTENSION AND ADOPTION

Three workshops were provided to industry in September 2012, August 2013 and March 2014.

Four site visits to six trout farms were undertaken throughout 2012 and 2013.

Scientific publications will be provided to managers, veterinarians and researchers.

Copies of the final report will be provided to farmers, managers, veterinarians and researchers.

Farmers will be contacted throughout 2014 to assess if and how project outputs were adopted.

“Development of an Australian strain of *Ichthyophthirius multifiliis* infecting rainbow trout under different temperatures.” Second Australasian Scientific Conference on Aquatic Animal Health Conference, Cairns, July 2014.

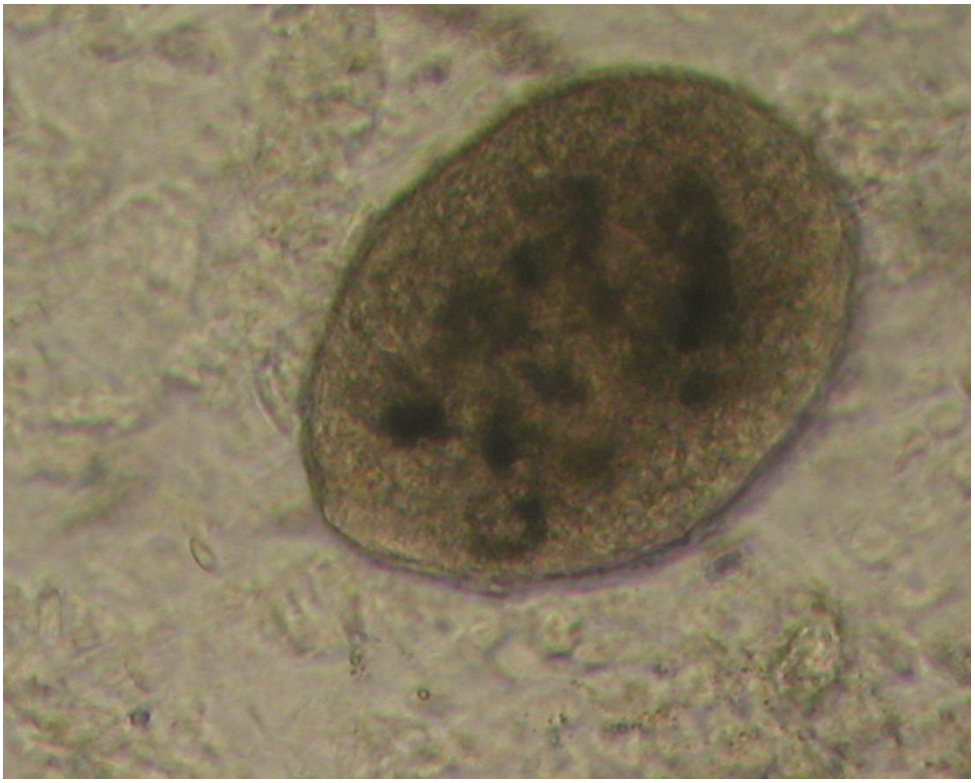
“In vitro effect of formalin and sodium percarbonate on the free-living stages of an Australian isolate of *Ichthyophthirius multifiliis*.” [Abstract accepted] World Aquaculture Conference, Adelaide, June 2014.

PROJECT MATERIALS

White spot workshop August 2013 notes

Overview

1. Life cycle of Victorian *I. multifiliis* and relevance for treatment.
2. Standard detection method.
3. Sodium percarbonate and formalin treatments for *I. multifiliis*.
4. Future directions.



1. **The life cycle of Victorian *I. multifiliis* isolates and its relevance for treatment**

Background: There are five strains of ich, each with a different development time under different environmental conditions. The life cycle is divided into four stages; the trophont, which lives in the host epidermis; the tomont, which leaves the host and encysts in the aquatic environment and undergoes rapid division into daughter cells, the tomites; which develop into the free swimming infective stage, the theront (Figure 1). Current bath treatments only target the free living stages of the parasite (tomont and theront) and are ineffective against the life stage residing in the fish (trophont).

Aim: To understand the development time and reproductive capacity of Victorian isolates of ich to aid development of monitoring methods and to strategically time treatments to interrupt the life cycle.

Methods: We investigated the development time from trophont exit (Figure 1; stage 1) to tomite release (Figure 1; stage 5) at 5, 9, 12, 17, 21, 25 and 30°C and at 0, 1, 3, 5, and 7.5 g/L salinity. We also examined the number of theronts that were produced from individual tomonts (Figure 1; stage 5-6) under these conditions.

Results: Development time was significantly reduced with increasing temperature (Table 1). The number of theronts produced from individual tomonts was lowest at 5°C and increased to 25°C but decreased at 30°C (Table 1). Salinity of 3 g/L and above significantly decreased the viability tomonts, prolonged development and reduced the number of theronts produced (Table 2). At 5 g/L and 7.5 g/L salinity, no infective theronts were produced.

Table 1: Development time under different temperatures

Temp °C	Trophonts developing into cysts (%)	Time from trophont to theront in hours. Mean (range)	Number of theronts from one cyst. Mean (Range)
5	71	188.9 (159.45 to 206)	189.2 (63 to 374)
9	79	162.3 (154 to 172)	193. 9 (106 to 440)
12	100	46.6 (41.6 to 54.6)	253 (140 to 623)
17	88	27.1 (23.1 to 29.4)	446.4 (180 to 682)
21	100	18.3 (16.4 to 20.1)	426.3 (182 to 576)
25	88	13.7 (12.6 to 16.5)	493.2 (177 to 1553)
30	67	11.7 (10.2 to 14.1)	288 (146 to 410)

Table 2: Development of *Ichthyophthirius multifiliis* tomonts under different salinities

Salinity (g/L)	Time from trophont to theront in hours. Mean (range)	Trophonts encysted (%)	Trophonts with divisions (%)	Viable trophonts (%)	Number of theronts from one cyst. Mean (Range)
0	53 (43.3 to 59)	83	29	83	282.1 (122 to 789)
1	49 (39.3 59)	92	29	92	166.4 (37 to 315)
3	97.1 (74 to 122)	50	50	33	78.5 (48 to 96)
5	-	29	54	-	-
7.5	-	-	-	-	-

Ich reproduces 5 times faster and produces more than double the number of theronts at 17°C than it does at 9°C.

Implications: This has substantial implications for monitoring and treatment. For example if 10 trophonts were found at 9°C, one week later there would be the capacity for release of ~2000 infective stages but if 10 trophonts were found at 17°C one week later the potential infective stage release increases to ~27,000 (assuming every theront found a host and completed its life cycle). The high reproductive capacity at higher water temperatures underscores the need for monitoring. This also has implications for the strategic timing of treatments. A shorter interval between treatments is required at high temperatures. The Victorian isolate can complete its life cycle more quickly than has been reported in the literature for other ich isolates.

Tomont viability decreases at ~5°C and ~30°C.

The preferred temperature range for Victorian isolates of ich is 12-25°C, with tomont viability decreasing outside this range.

The Victorian isolate is more sensitive to salinity than other described isolates.

Victorian isolates of ich produced no viable infective tomites at 5 and 7.5 g/L salinity. Sensitivity to salinity has implications for prevention and treatment. In systems or circumstances where salt could be used, such as in recirculation tank systems, it may be a low cost, low impact treatment.

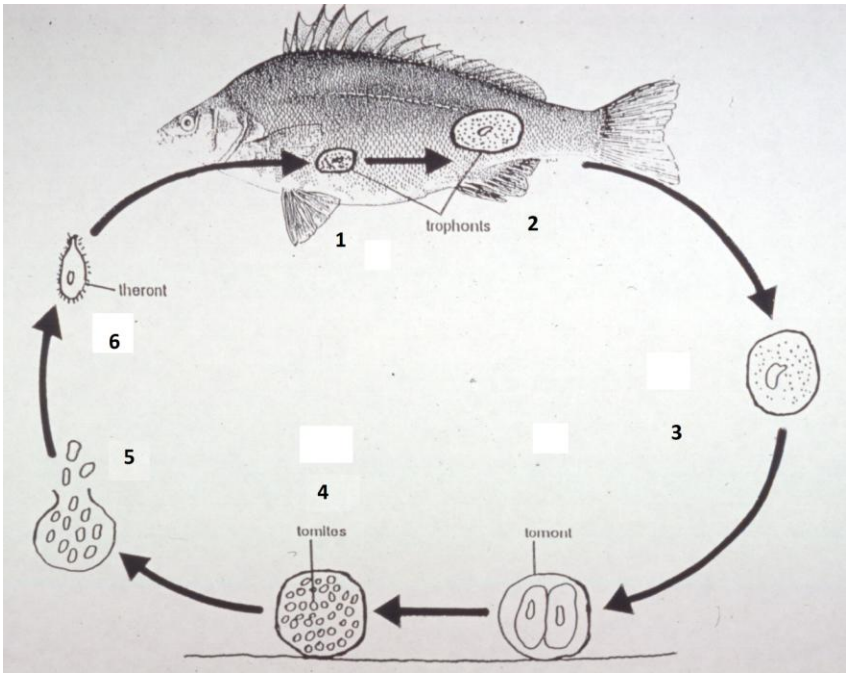


Figure 1: Life cycle of *Ichthyophthirius multifiliis*; 1 and 2 – trophonts; 3 – tomont; 4 – tomites; 5 – tomites released; 6 – theronts.

2. **Development of a standardised detection method**

Aim: To determine the preferred settlement site on the host to aid in the development of a standardized detection method that will provide an abundance estimate

Methods: We visited five farms and took samples from juvenile fish with mean length of 6.8 cm (5.5 to 9.10 cm). We divided the body of the fish into 4 regions: left, right, top and bottom and took skin scrapes and counted the number of ich in each region.

Results: The top region of the body gave the greatest chance of detecting ich (Table 3).

Implications: Taking a skin scrape from the top region of juvenile rainbow trout will give you the highest chance of detecting ich.

Table 3: Distribution of ich on rainbow trout sampled from five farms

Site	**Right side**	**Left side**	**Top**	**Bottom**	**Total**
1	4.6 (0 to 18)	4.4 (0 to 17)	5.3 (0 to 19)	2.9 (0 to 11)	17.1 (1 to 59)
2	4.3 (1 to 7)	2.3 (1 to 3)	3.6 (1 to 9)	0.3 (0 to 1)	10.7 (3 to 20)
3	0.2 (0 to 1)	0.8 (0 to 2)	0.4 (0 to 1)	0.2 (0 to 1)	1.6 (1 to 3)
4	2.2 (0 to 4)	0.8 (0 to 2)	0.8 (0 to 2)	1.4 (0 to 3)	5.2 (0 to 10)
5	9.4 (5 to 12)	10.8 (2 to 16)	11.4 (0 to 19)	4.8 (2 to 13)	38.4 (15 to 69)
Mean	**4.3 (0 to 18)**	**4.1 (0 to 17)**	**5 (0 to 29)**	**2.4 (0 to 13)**	**15.8 (0 to 69)**

3. Efficacy of sodium percarbonate and formalin as treatments for the free-living stages of *I. multifiliis*.

Aim: To determine the minimum effective dose of sodium percarbonate (SPC) and formalin (37% formaldehyde) at 12 and 17°C and in hard and soft water against the free living stages of ich (Figure 1; Stages 2-6).

Methods: We exposed theronts to 8, 16, 32 and 64 mg/L of sodium percarbonate and formalin (37 % formaldehyde). We then exposed tomonts to 64, 128, 256 and 512 mg/L sodium percarbonate and 16, 32, 64, 128 mg/L formalin (37 % formaldehyde). Water used in this experiment was filtered tap water that contained no organic matter and was manipulated into hard and soft samples and buffered to pH 7. We will follow this up with dose determination studies on farms.

Results: Formalin was more effective against theronts and tomonts at 17°C than 12°C. At 12°C formalin killed all theronts within 100 min at 32mg/L and within 15 min at 64 mg/L (Figure 2). At 17°C formalin killed all theronts within 60 min at 32mg/L and within 15 min at 64 mg/L (Figure 3). Exposure to 64 and 128 mg/L formalin for 1 hour at 12°C killed all tomonts, however, 40% of the tomonts exposed to 16 mg/L survived and 30 % of the tomonts survived exposed to 32 mg/L survived (Figure 4). Exposure to 64 and 128 mg/L formalin for 1 hour at 17°C killed all tomonts, however, 40 % of the tomonts exposed to 16 and 32 mg/L survived (Figure 5). There were no differences between treatments in hard and soft water.

SPC was more effective against theronts and tomonts at 17°C than 12°C. At 12°C SPC killed all theronts within 80 min at 32mg/L and within 15 min at 64 mg/L (Figure 6). At 17°C SPC killed all theronts within 60 min at 32mg/L and within 15 min at 64 mg/L (Figure 7). Exposure to 256 and 512 mg/L SPC for 1 hour at 12°C killed all tomonts, however, 30 % of the tomonts exposed to 128 mg/L survived and 50 % of the tomonts survived when exposed to 64 mg/L (Figure 8).All doses of SPC at 17°C for 1 hour killed all tomonts (Figure 9). SPC was more effective in soft water than in hard water.

It is important to note that we have only trialed the treatments against the parasite. We will investigate the effect of the treatments on rainbow trout at different doses to determine safe treatment levels. We strongly recommend to not exceed doses known to be safe to fish until we have obtained this information. Follow the directions on the Minor Use Permit.

Implications: When treating with formalin, maintaining a dose of 64 mg/L for 1 h will kill all of the free-living stages of the parasite. Reinfection will be prevented until a new generation of infective stages has developed from tomonts living in the skin of the fish during treatment. When using SPC water temperature must be taken into consideration. At 17°C 64 mg/L SPC for 1 h will kill all of the free living stages of the parasite, however, at 12°C 128 mg/L for 1 h is required to kill all free living stages.

For formalin there is no significant difference in efficacy between hard and soft water, but the efficacy of formalin can be influenced by the amount of organic material in the water due to binding, resulting in an immediate reduction in the dose rate. We will investigate the on-farm effects of total organic load during the dose determination trials on farms.

SPC was more effective in soft water (Total hardness ~70 mg/L $CaCo^3$) than hard water (~170 mg/L $CaCo^3$). For tomonts (Figure 1: stage 2-3) that survived treatment there was no difference in the number of theronts produced, therefore, if the treatments are not effective the reproductive rate of survivors is the same as if they received no treatment.

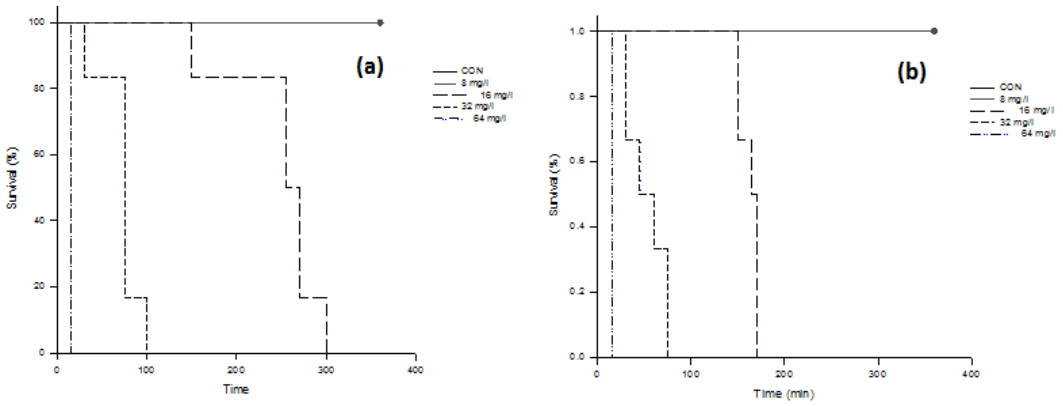


Figure 2: Survival of theronts exposed to formalin at 12°C in hard (a) and soft (b) water.

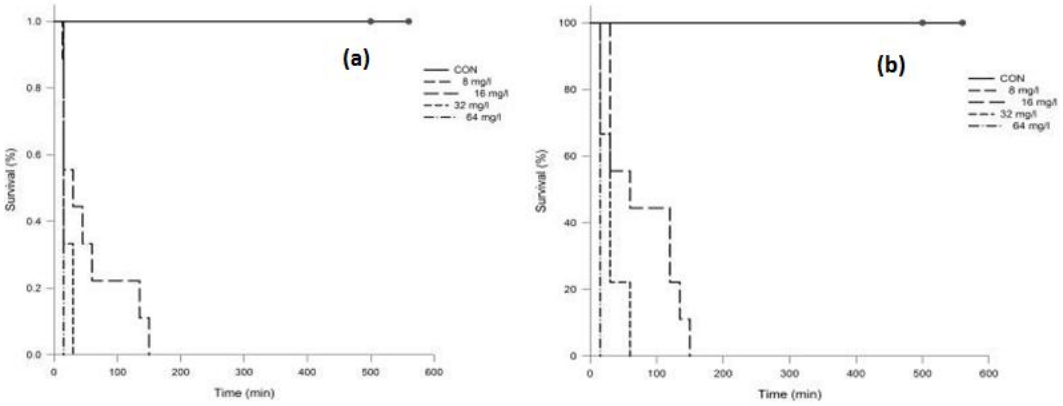


Figure 3: Survival of theronts exposed to formalin at 17°C in hard (a) and soft (b) water.

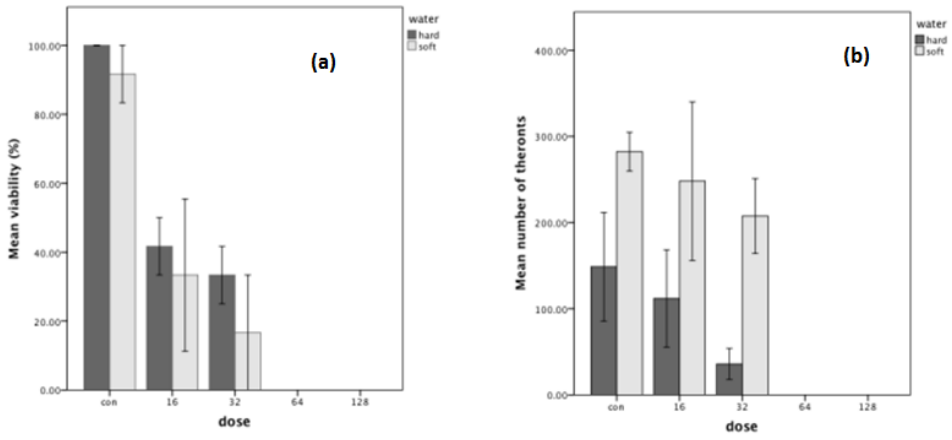


Figure 4: Viability of tomonts exposed to formalin at 12°C (a); Mean number of theronts produced from viable tomonts after exposure to formalin at 12°C (b).

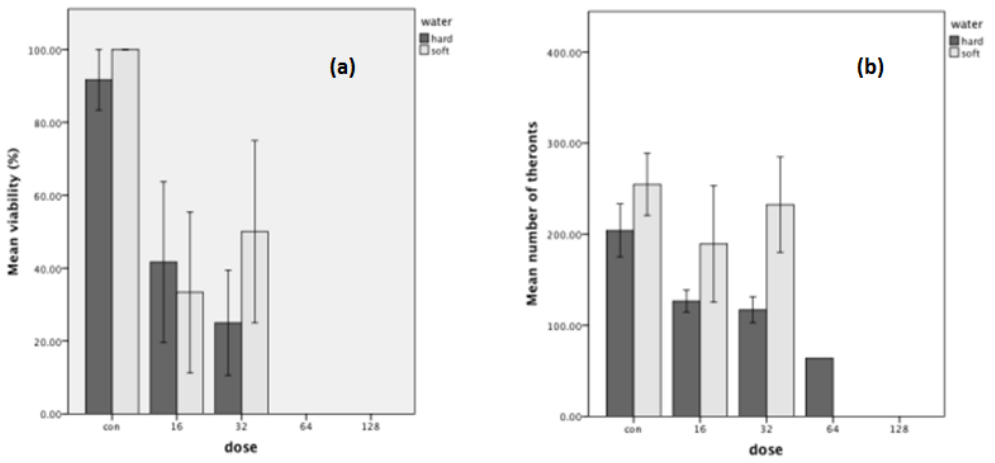


Figure 5: Viability of tomonts exposed to formalin at 17°C (a); Mean number of theronts produced from viable tomonts after exposure to formalin at 17°C (b).

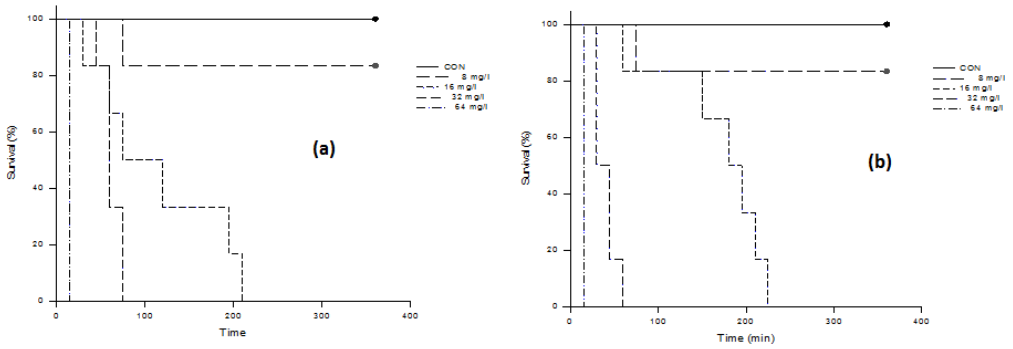


Figure 6: Survival of theronts exposed to SPC at 12°C in hard (a) and soft (b) water.

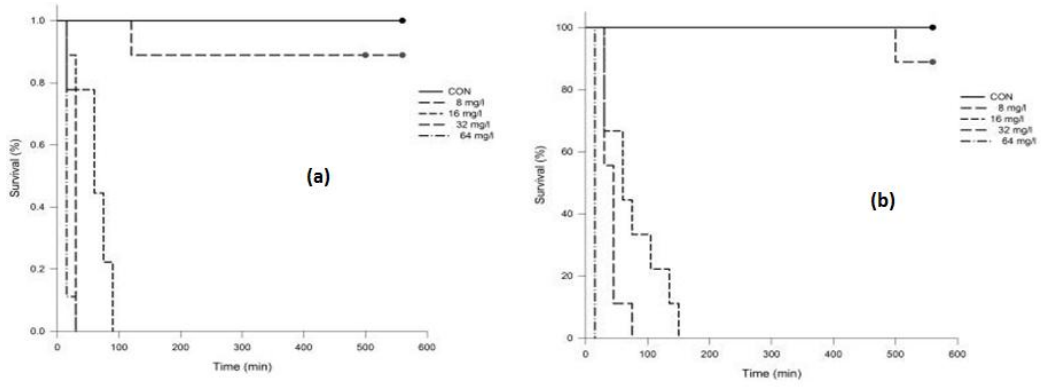


Figure 7: Survival of theronts exposed to SPC at 17°C in hard (a) and soft (b) water.

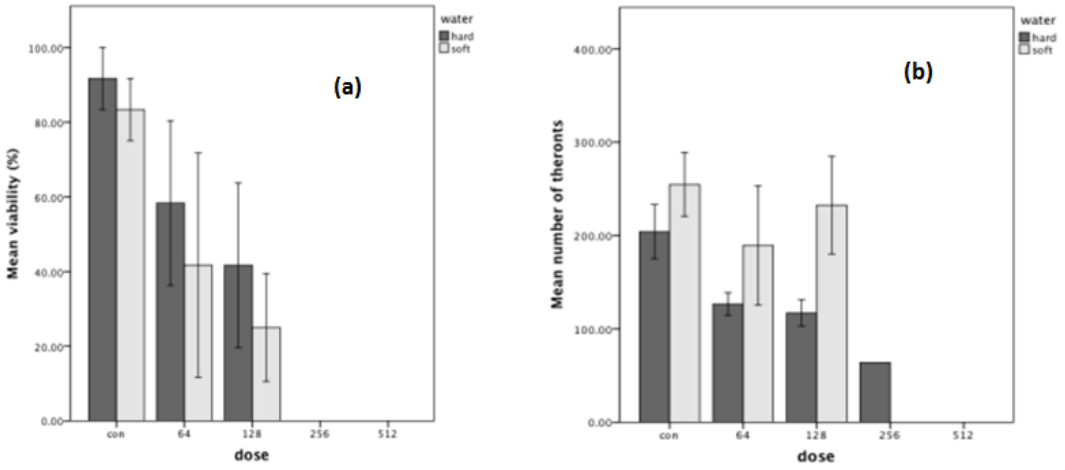


Figure 8: Viability of tomonts exposed to SPC at 12°C (a); Mean number of theronts produced from viable tomonts after exposure to SPC at 12°C (b).

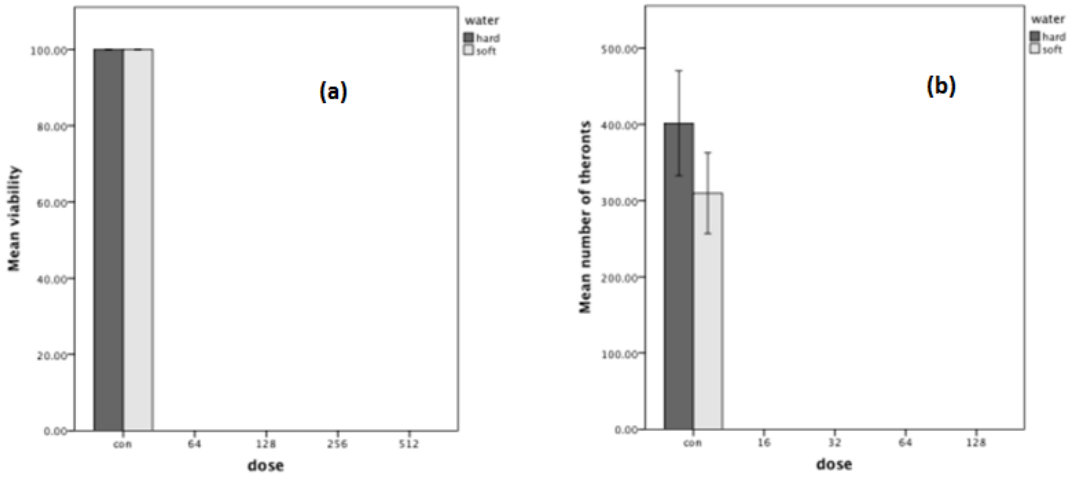


Figure 9: Viability of tomonts exposed to SPC at 17°C (a); Mean number of theronts produced from viable tomonts after exposure to SPC at 17°C (b).

4. Direction of the project

From here we will:

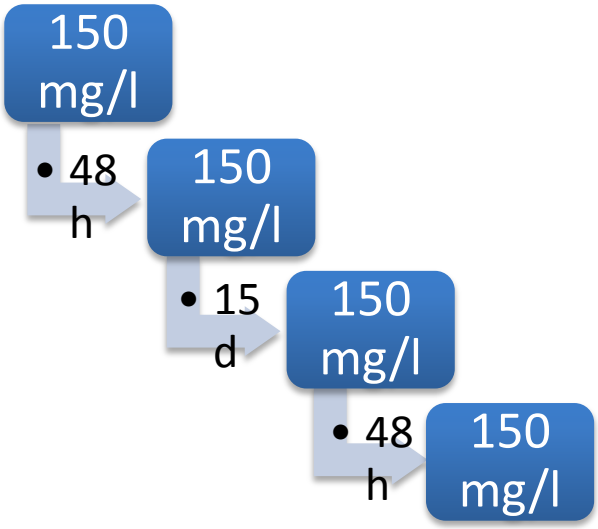
- **Investigate doses of formalin and SPC on farms, to determine if the correct dose is being achieved in growout systems.**
- **Trialing peracetic acid in the laboratory as potential alternative treatments.**
- **Describe the impact of SPC treatment on the gills and skin, and investigate stress response in rainbow trout undergoing treatment.**
- **Revisit the parasite settlement and trophont development.**
- **All information will be combined into a strategic treatment plan for evaluation on farms next year.**

Treatment plan for sodium percarbonate (SPC)

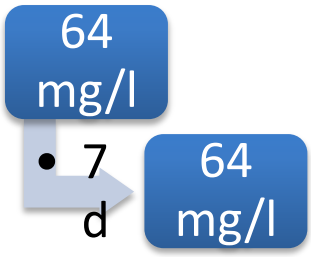
General treatment considerations:

- Static bath treatments best maintain dose
- Measure dose in the system to ensure accuracy
- Monitor DO

At 12°C:



At 17°C:



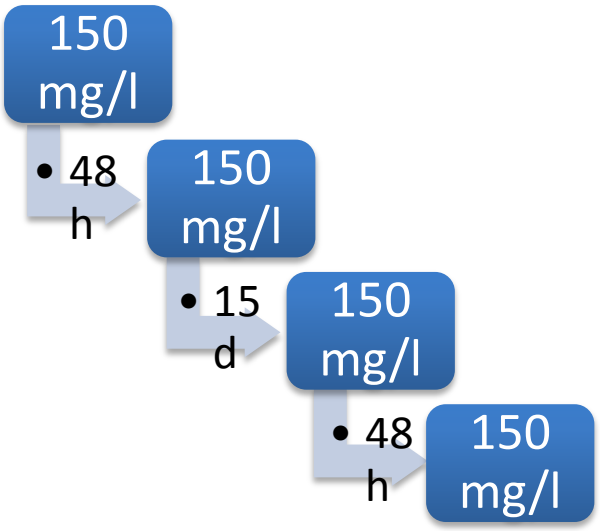
- Monitor infection throughout the treatment to ensure efficacy

Treatment plan for Formalin (FOR)

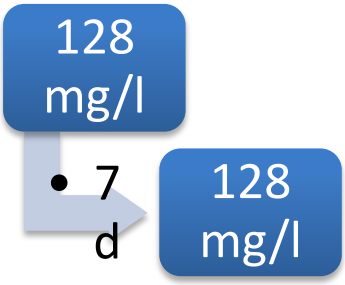
General treatment considerations:

- Static bath treatments best maintain dose
- Measure dose in the system to ensure accuracy
- Monitor DO

At 12°C:



At 17°C:



Monitor infection throughout the treatment to ensure efficacy

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