4. Minimum effective concentrations of formalin and sodium percarbonate on the free-living stages of an Australian isolate of *Ichthyophthirius multifiliis*.

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4.1 Abstract

Ichthyophthirius multifiliis, a ciliate protozoan, is a common cosmopolitan parasite of freshwater teleosts and is a recurring problem during the summer months on Australian rainbow trout *Oncorhynchus mykiss* farms. Preventative strategies include increasing water flow and filtration, but when an infection is established, chemical intervention is often required. Formalin (FOR) has been traditionally used on Australian trout farms as a treatment for *I. multifiliis*. Treatment using SPC that releases HP when dissolved is being implemented on a number of farms. To assess anecdotal reports of low efficacy we evaluated exposures of FOR and SPC at 12°C and 17°C in both hard and soft water against free-living stages of *I. multifiliis*. Each free-living stage were exposed to FOR and SPC *in vitro*; theronts were exposed to 8, 16, 32 or 64 mg/L SPC or FOR and observed every 15 min, for a maximum of 6 h, and the number of live theronts at each time point was recorded. Prototomonts and tomocysts were exposed to 64, 128, 256 and 512 mg/L SPC and 16, 32, 64 and 128 mg/L FOR for 1 h, incubated, with the percentage viability and the number of theronts produced recorded. 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 and 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. Sodium percarbonate was effective against prototomonts and tomocysts at 64 m/L at 17°C but required ≥ 256 mg/L at 12°C. These results can be used to aid development of specific treatment strategies for the management of *I. multifiliis* on Australian rainbow trout farms.

4.2 Introduction

Ichthyophthirius multifiliis 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 the 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, *I. multifiliis* can cause high mortalities (Valtonen and Koskivaara, 1994).

Ichthyophthirius multifiliis has a direct lifecycle with four stages: the parasitic trophont, which resides in 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* (see Dickerson and Clark, 1996), which have varying susceptibility to chemotherapeutants (Straus and Meinelt, 2009; Straus et al., 2009). In the same conditions our isolate completes its free-living lifecycle more quickly and is more susceptible to salinity than other isolates (Chapter 2). 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). Use of malachite green 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). Trophonts are resistant against all current alternative treatments but there has been varying success against the free-living stages (Straus and Meinelt, 2009; Sudová 2010; Ling et al., 2011; Picón-Camacho et al., 2012a; Picón-Camacho et al., 2012b). Formalin (FOR) is a recommended treatment for *I. multifiliis* (Noga, 2000), but SPC has shown potential as a safe alternative (Buchmann et al., 2003; Heinecke and Buchmann, 2009; Chapeter 5). SPC gradually dissociates in water into sodium carbonate and HP; the latter is a powerful oxidant (Bostek, 1983) 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.).

This study aimed to investigate the *in vitro* effect of a 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 to assess if dose was contributing to low efficacy.

4.3 Materials and Methods

4.3.1 Culture of parasites

A strain of *I. multifiliis* was isolated from rainbow trout from Snobs Creek Hatchery (Department of Environment and Primary Industries, Victoria) during Austral summer 2012-13. 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 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 and 17°C in 150 mL glass beakers.

4.3.2 Experimental design

Filtered deionised water was manipulated to soft (general hardness 70 - 80 mg/L $CaCO_3$) and hard (general hardness 170 - 195 mg/L $CaCO_3$) water by adding calcium carbonate (Chem-Supply, batch ref. (10) 206679) was used. pH was adjusted to 7 ± 0.2 by adding dilute hydrochloric acid or sodium bicarbonate solution. Granular SPC (Sigma-Aldrich®, lot no. MKBB5394V) and FOR (37% w/w formaldehyde (FA)) (Ajax Finechem®) was added to hard or soft water to the concentrations outlined in Table 4.1. Solutions of SPC were left for 15 min to facilitate dissolution prior to commencing the experiments. Each experiment was conducted in temperature controlled rooms at 12 and 17 ± 1°C and was repeated three times. The lowest concentration that resulted in 100% mortality for each treatment was defined as the minimum effective concentration (MEC).

4.3.3 Treatment of theronts

Methods were adapted from Heinecke and Buchmann (2009) and used 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 with soft water as controls. 75 µL of theront suspension (8-16 theronts) in either hard or soft water was placed in each well, which was mixed with 75 µL of the treatment solution to bring the wells to the doses outlined in Table 4.1. Controls received 75 µL of hard or soft water without treatment. 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.

4.3.4 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 4.1 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% NBF was added to each well and the number of theronts was counted using a dissection microscope. Prototomonts were considered non-viable if their nucleus was invisible and were unable to produce theronts.

4.3.5 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 for 14 h at 17°C to allow them to encyst. After incubation, 2.5 mL of an SPC or FOR solution was added to bring the wells to the doses outlined in Table 4.1, 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% NBF was added to each well and the number of theronts was counted using a dissection microscope.

Tomocysts were considered non-viable if they their nucleus was invisible and were unable to produce theronts.

Table 4.1: Treatments and dose rates (mg/L) administered to *Ichthyophthirius multifiliis* theronts and tomonts.

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

4.3.6 Statistical analysis

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. Theront survival was assessed using a logrank test with the Holm–Sidak method for multiple comparisons to compare different survival curves. 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 and where equal variances not met, a Games-Howell test was also used. IBM SPSS Statistics 20.0 was used, and significance for all tests was judged at $P < 0.05$.

4.4 Results

4.4.1 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. Theront survival decreased with a higher temperature, dose and exposure time (Fig 4.1A-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 4.1A-B) and FOR (Fig 4.1C-D) was proportional to dose and water temperature.

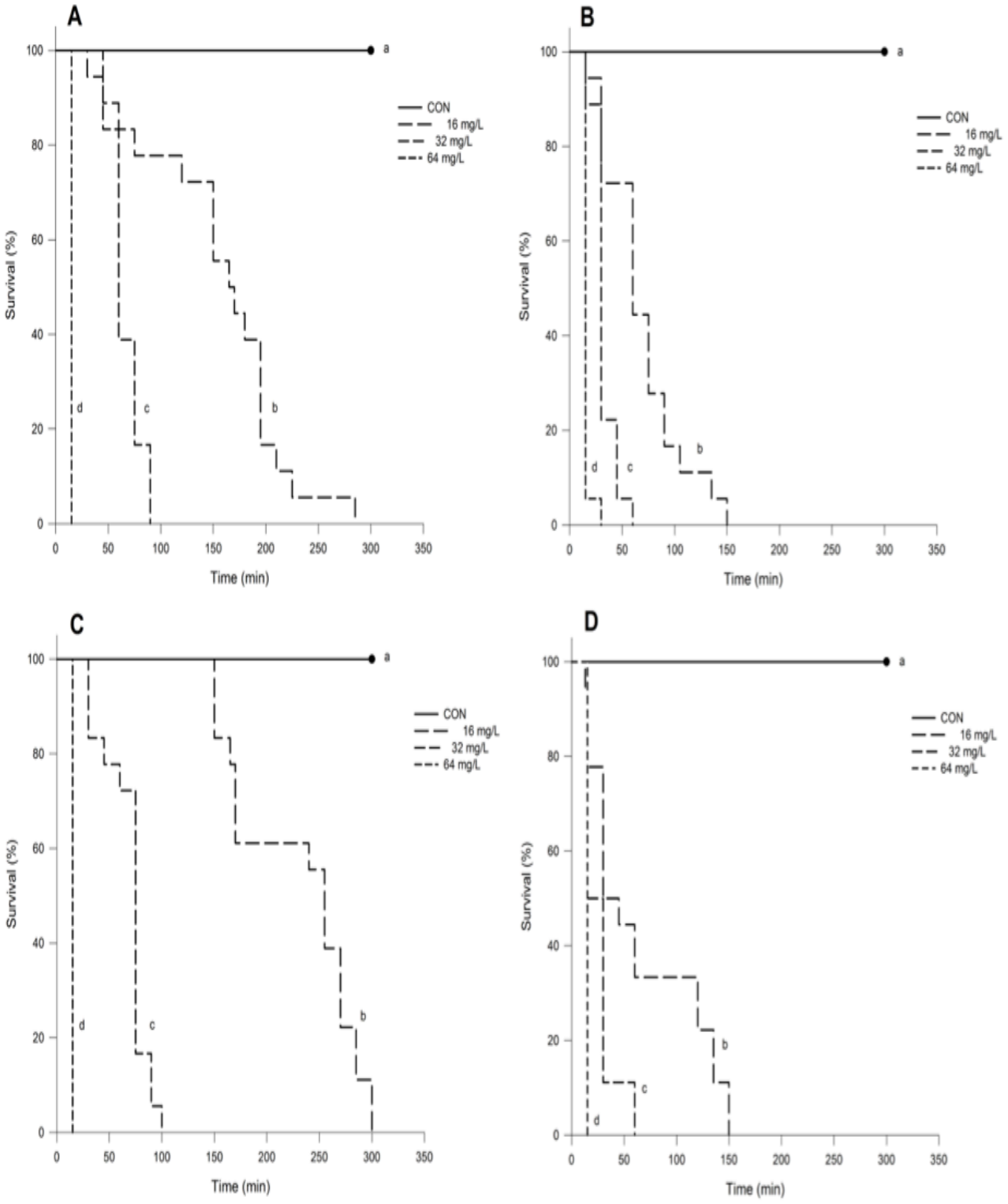

Figure 4.1: Survival of theronts exposed to different dose levels of sodium percarbonate (SPC) at 12°C (A) and 17°C (B) and formalin (FOR) at 12°C (C) and 17°C (D). Holm-Sidak estimates of the survival data show significant differences between treatment groups, which are represented by difference superscripts. CON = control.

4.4.2 *In vitro* effect on prototomonts

During FOR trials, the mean viability of prototomonts in all controls was 95.84% ± SEM 2.8 (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 4.2 outlines the results of the 3-factor ANOVA on viability and theront production of prototomonts exposed to FOR. Prototomont viability decreased with increasing concentration of FOR (Fig 4.2A). Theront production from viable prototomonts exposed to FOR was significantly higher at 17°C than 12°C (Fig 4.3A).

Table 4.2: ANOVA interactions between treatment dose, water hardness and temperature on the treatment 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		

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.

Table 4.2 shows results of the 3-factor ANOVA on viability and theront production of prototomonts exposed to SPC. Prototomont viability decreased with increasing concentration of SPC, and this effect was particularly marked at 17°C (Fig 4.2C). Theront production from viable prototomonts exposed to SPC was significantly higher at 17°C than 12°C (Fig 4.3C).

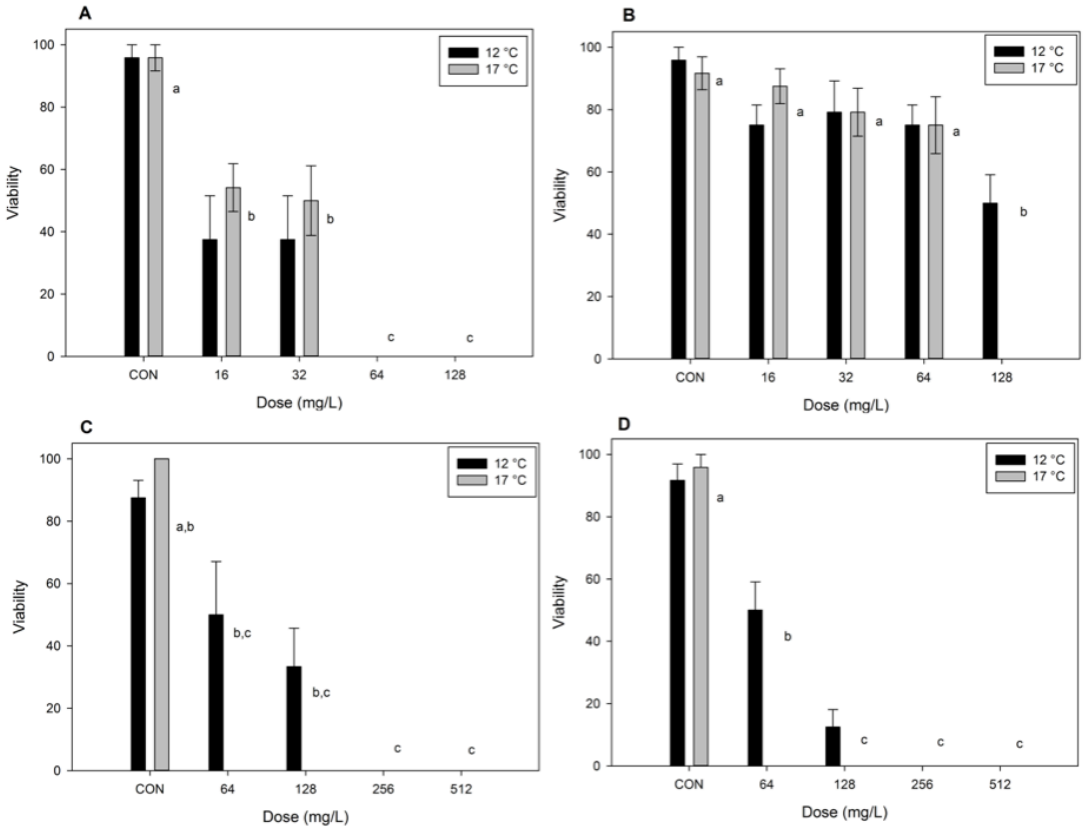

Figure 4.2: Mean viability (%) of *Ichthyophthirius multifiliis* prototomonts (A) and tomocysts (B) exposed to formalin (37% formaldehyde); and prototomonts (C) and tomocysts (D) exposed to sodium percarbonate at 12°C and 17°C for 1 h at different concentrations. Different superscripts represent significant differences between doses using Tukey's analysis ($P < 0.05$). Error bars represent the SEM. CON = control.

4.4.3 *In vitro* effect on tomocysts

During FOR trials the mean viability of tomocysts in all controls was 93.75 ± SEM 3.2% (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 4.3. 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 4.2B). Theront production from viable tomocysts exposed to FOR was significantly higher at 17°C than 12°C and was significantly reduced with higher concentrations (Fig 4.3B). 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.

Table 4.3: 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). – lack of viable prototomonts meant that insufficient data were available to test the term.

		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	**< 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		

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 4.3. Low concentrations of SPC were more effective at high temperatures while high concentrations were effective irrespective of temperature (Fig 4.2D). Theront production from viable prototomonts exposed to SPC was significantly higher at 17°C than 12°C (Fig 4.3D).

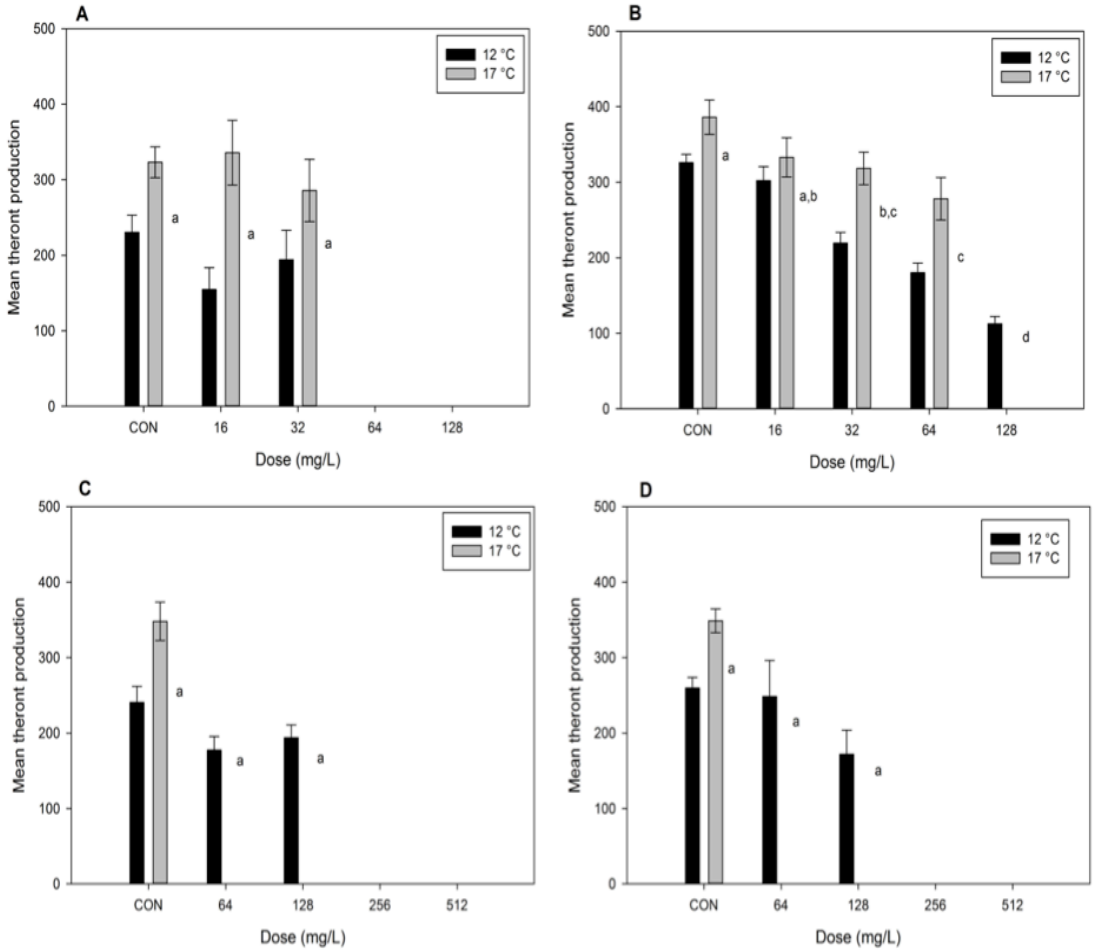

Figure 4.3: Mean number of theronts produced from viable *Ichthyophthirius multifiliis* prototomonts (A) and tomocysts (B) exposed to formalin (37 % formaldehyde); and prototomonts (C) and tomocysts (D) exposed to sodium percarbonate at 12°C and 17°C for 1 h. Different superscripts represent significant differences between doses using Tukey's analysis ($P < 0.05$). Error bars represent the SEM.

4.4.4 Minimum effective concentrations

The minimum effective concentration for theronts, prototomonts and tomocysts are outlined in Table 4.4.

Table 4.4: 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

4.5 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 also be effective against tomonts at 12°C (Table 4.4). 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 PAA (Meinelt et al., 2009) and potassium ferrate (IV) (Ling et al., 2011). 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. Repeat treatments can, however, be applied to prevent reinfection when treatments are effective against only one free-living stage.

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. (1997b) recommended a treatment range of 50 – 250 mg/L HP for 60 min but found that toxicity was proportional to fish size and water

temperature. Further investigations into the safety of higher doses of SPC are warranted.

Formalin is currently applied on Australian trout farms at 200 mg/L for 1 h based on Noga (2000). Our results suggest that this treatment regime would be effective against all free-living stages at 12 and 17°C. 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 4.4). 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 in a way that achieves the minimum effective dose, efficacy will be low, and expensive and logistically complicated re-treatment will 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 repeat treatments every 24 h were required at 18°C.

Minimum effective concentrations reported in Table 4.4 can be used in combination with relevant lifecycle-temperature data specific to Australian isolates of *I. multifiliis* for the strategic timing of treatments to interrupt the parasite's lifecycle

(Picón-Camacho et al., 2012b), improving the management of this parasite on Australian rainbow trout farms.

4.6 Acknowledgements

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