5. Histological evaluation of sodium percarbonate exposure on the gills of rainbow trout

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Diseases of Aquatic Organisms (in review)

5.1 Abstract

Ichthyophthirius multifiliis is a recurring problem on Australian rainbow trout *Oncorhynchus mykiss* farms and requires strategically timed repeat treatments for effective management. Sodium percarbonate is permitted for use in Australia with host safety margins based on toxicity of acute exposures to HP, the active product released when SPC is added to water. The effects of exposure to HP released by SPC, of repeated doses and of doses exceeding 100 mg/L on rainbow trout are unknown. We exposed juvenile rainbow trout (30.5 ± SD 9 g) to repeated doses of 50, 150 and 250 mg/L SPC for 1 h on days 1, 2, 7 and 8 of a treatment regime. The effect of SPC was assessed by histological evaluation of structural changes in gill tissue. Survival was 100 % in all groups, but some fish exposed to 250 mg/L SPC displayed impaired swimming performance and after the fourth treatment oedema occurred in 9.8% of lamella, which was significantly higher than 1.7, 4.2 and 1.3% in fish treated with 0, 50 and 150 mg/L SPC, respectively. These changes resolved within 24 h of the cessation of treatment. SPC is safe to use on rainbow trout at ≤ 150 mg/L at 17˚C however, caution is advised at 250 mg/L. Water temperature, fish age, fish size and maturity, intensity of parasite infection and stocking density could alter the sensitivity of rainbow trout to SPC treatments.

5.2 Introduction

Sodium percarbonate is a granular water-soluble compound that dissociates in water into sodium carbonate and HP, the latter of which is a powerful oxidant (Bostek, 1983) that has been used extensively in aquaculture as a therapeutic compound for control of ectoparasitic infections of fish (Noga, 2000). Hydrogen peroxide decomposes into water and oxygen and does not form cumulative or toxic metabolites (Pedersen et al., 2006) and therefore has low environmental impact and treated fish are food safe.

Treating fish using liquid hydrogen peroxide can be problematic; obtaining the desired dose is difficult due to the effects of water biogeochemistry (Bishop et al., 1968). There are also workplace health and safety concerns with handling HP (Eul et al., 2001) and security restrictions on transport and storage of the product (Department of Homeland Security, 2007). Sodium percarbonate is a granular solid with lower workplace health and safety risk and no current security sensitivity; its gradual dissociation in water to HP also provides a wider fish safety margin (Buchmann et al., 2003).

Sodium percarbonate is effective against free-living stages of *Ichthyophthirius multifiliis* (Ichthyophthiriidae) *in vitro* (Buchmann et al., 2003; Heinecke and Buchmann, 2009; Forwood et al., 2014). The *I. multifiliis* 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 reinfection and interrupt the parasite's lifecycle (Picón-Camacho et al., 2012). Sodium percarbonate can be used in Australia pursuant to an Australian Pesticides and Veterinary Medicines Authority (APVMA) minor use permit (MUP) (PER12944) to treat ectoparasites of finfish at doses of up to 100 mg/L based on Buchman et al.

(2003) and an analysis of teleosts HP toxicity data (unpublished data) repeated over three consecutive days. Results from *in vitro* trials indicated that doses above 100 mg/L are required to effectively control all free-living stages of *I. multifiliis* (Chapter 4). The impacts of HP on Atlantic salmon, *Salmo salar* (Salmonidae) (see Johnson et al., 1993; Kiemer and Black, 1997) and rainbow trout *Oncorhynchus mykiss* (Salmonidae) (see Speare et al., 1999; Tort et al., 2002) have been investigated and structural damage was restricted to the gills (Roth et al., 1993; Kiemer and Black, 1997; Tort et al., 2002). Alkalinity and pH could affect the efficacy and host toxicity of HP treatment (Tort et al., 2002) and the tolerance of rainbow trout to HP slowly released from SPC with significantly increased alkalinity from carbonate is unknown. This study investigated the histological effect on the gills of rainbow trout in response to baths of SPC applied at a frequency designed to interrupt the lifecycle of *I. multifiliis*.

5.3 Materials and Methods

5.3.1 Experimental design

192 *O. mykiss* with a mean weight of 30.5 ± SD 9 g were sourced from a commercial trout farm and transferred to Flinders University, South Australia. Fish were randomly selected and 16 were transferred to each of 12 indoor 50 L recirculating aerated experimental 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 dechlorinated municipal water; water hardness, pH (YSI 9300 photometer, YSI Inc.), temperature and dissolved oxygen (Handy Polaris H01P, OxyGuard®) were measured daily. Water hardness was

145 mg/L, alkalinity 20 mg/L as $CaCO_3$, pH 6.1, the temperature was maintained at 17 ± 1°C and dissolved oxygen was 7.8 ± 0.81 mg/L.

5.3.2 Dosing method and duration

At the end of the acclimation period each tank was randomly assigned to one of four treatments (three tanks / treatment): no dose, 50, 150 and 250 mg/L SPC. Fish were exposed to SPC at the designated dose for 1 hour 4 times on day 1, 2, 8 and 9, which was 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 10 L bucket of water taken from the treatment tank, left to dissolve for 30 min, stirred and added to a treatment tank and mixed further. Fish were transferred by hand net from the 12 experimental recirculation tanks into 12 static treatment tanks for 1 h and then returned to the experimental tanks. 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 experimental tanks was exchanged on the second and fourth treatment days.

5.3.3 Fish sampling

Rainbow trout were observed throughout the treatment period for signs of toxicity such as impaired swimming performance, colour change, or loss of equilibrium. Any mortality was recorded. Fish were sampled immediately after treatment on day 2, day 3, day 7, immediately after day 9 treatment, day 10, day 14 and day 18.

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; six fish for each treatment. 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 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.

5.3.4 Histology processing and evaluation

Fixed tissues were dehydrated in a graded series of ethanol, 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 with no *I. multifiliis* from each fish were assessed. Slides were coded and examined blind. Structural changes in gill tissues were assessed and quantified by examination using a compound microscope. To quantify the status of the gills, morphometric indices based on a method developed for branchial 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 lamella.
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 lamella.
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 an accumulation of epithelial cells near the tip of the lamella that increases the number of cell layers at the lamellar tip, appearing as a 'club-shape'.

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

5.3.5 Statistical analysis

The values for each index from a given fish were expressed as the pooled mean from the three filaments evaluated. Normality of the data was tested using the Kolmogorov-Smirnov test and variances were tested using Levene's test. Indices that failed to meet these assumptions were log-transformed prior to analysis. 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. Where significant differences were detected in these ANOVAs, then unpaired *t*-test was used as the post-test for specific comparisons. The statistical analysis was performed using IBM SPSS Statistics 20.0 and significance for all tests was judged at $P < 0.05$.

5.4 Results

The mean concentration of HP was 18 ± SD 2.5 (range 10 – 22) mg/L released from 50 mg/L SPC, 49 ± 6.4 (36 – 60) mg/L released from 150 mg/L SPC and 72 ± 7.7 (52 – 82) mg/L released from 250 mg/L SPC.

Survival was 100% in all groups and prevalence of *I. multifiliis* was 59% (95% Confidence Interval (CI) 50 - 67%). Gross observations of 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, fish recovered with no apparent negative effects and were returned to the randomization sampling. Lamellar oedema and hyperplasia were the most prevalent structural changes in the gills (Figure 5.1).

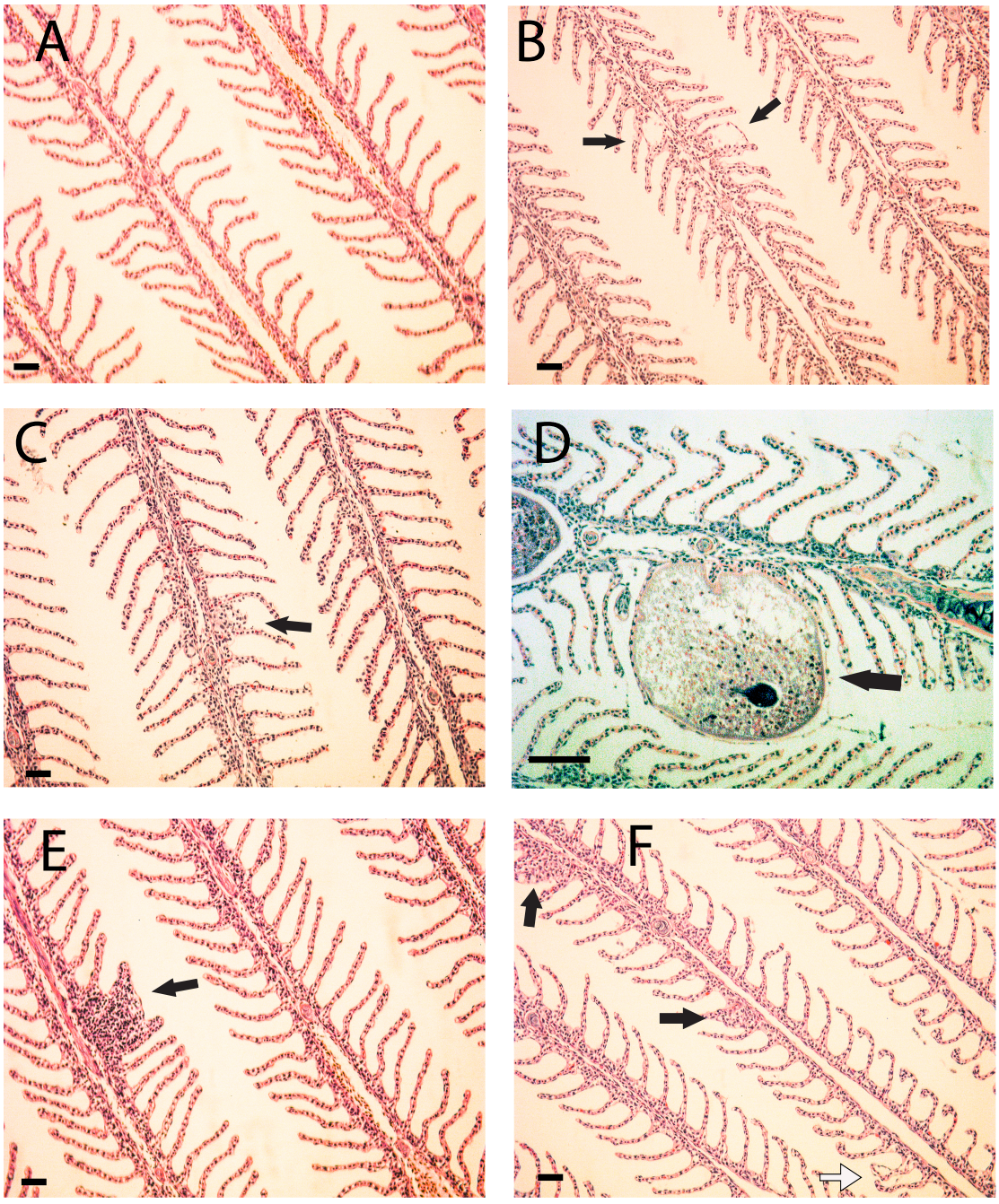

Figure 5.1 Light microphotographs of histological sections of rainbow trout *Oncorhynchus mykiss* gills stained with haematoxylin and eosin (H & E). A) control group gills with normal function; B) fish exposed to 250 mg/L SPC displaying lamellar oedema (black arrow); C) fish exposed to 250 mg/L SPC displaying epithelial hyperplasia (black arrow); D) *Ichthyophthirius multifiliis* trophont (black arrow); E) fish exposed to 150 mg/L SPC displaying epithelial hyperplasia (black arrow); and F) fish exposed to 250 mg/L SPC displaying epithelial hyperplasia (black arrow) and lamellar oedema (white arrow). Scale bars = 50 μm.

Occurrence of lamellar oedema showed a time-dependent response to dose (3-way ANOVA: $F_{18, 62} = 7.603$, $P < 0.001$), there was significant variation between tanks within doses (3-way ANOVA: $F_{47, 62} = 7.640$, $P < 0.001$). In fish treated with 250 mg/L SPC, a mean of 9.8 ± standard error of the mean (SEM) 3.6% (2.4 – 21.5%) of lamella had oedemas on the fourth sample day, which was significantly higher than the mean of 1.7 ± 0.7% (0 – 0.9%), 4.2 ± 2.8% (0.6 – 15.3%), and 1.3 ± 0.4% (0.8 – 2.4%) in fish treated with 0, 50 and 150 mg/L SPC, respectively (Fig. 5.1). Epithelial hyperplasia and lamellar fusion changed significantly between sample days (3-way ANOVA: $F_{6, 62} = 3.933$, $P < 0.002$ and 3-way ANOVA: $F_{6, 62} = 2.697$, $P < 0.022$, respectively) but was unaffected by dose; and lamellar inflammation varied differently between sample days and replicate tanks in the same treatment doses (3-way ANOVA: $F_{47,62} = 1.775$, $P < 0.017$). There was no significant difference in the occurrence of clubbed lamellae or interlamellar inflammation between doses or sample days.

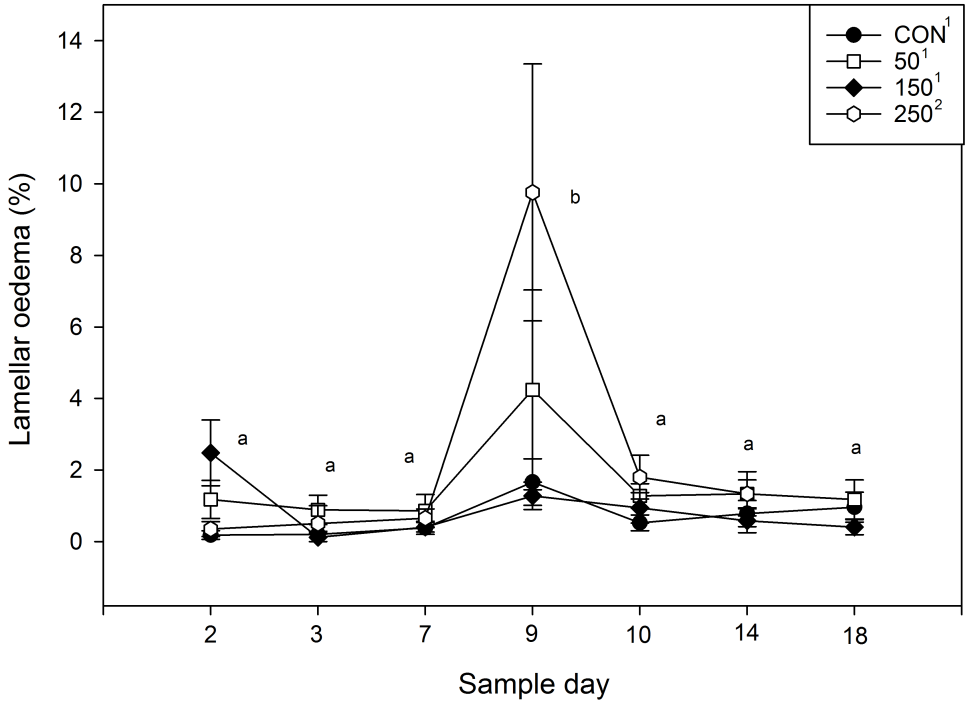

Figure 5.2 Percentage change in lamellar oedema in rainbow trout exposed to sodium percarbonate on day 1, 2, 8 and 9 at 0 (CON = control), 50, 150 and 250 mg/L for 1 h. Fish were sampled immediately after treatment on day 2, day 3, day 7, and day 9 and on day 10, day 14 and day 18. Different superscript letters indicate significant differences between sample days and different numeric superscripts indicate significant difference between doses on sample day 4 ($P < 0.05$). Error bars represent the SEM.

5.5 Discussion

We observed no mortality in fish exposed at up to 250 mg/L SPC, and doses of up to 150 mg/L SPC appear safe for use on rainbow trout at 17°C. The SPC used in this study contained 30 – 40% HP, releasing up to 82 mg/L HP from a 250 mg/L SPC treatment. This HP concentration is below the reported NOEC and LC_{50} values in juvenile rainbow trout. Gaikowski et al. (1999) reported that the no observable effect concentration (NOEC) for HP in rainbow trout in 60 min exposures administered on days 1, 3 and 5 at 12°C was 188 mg/L in fry and 162 mg/L in fingerlings, which is within the range of NOEC values for juvenile rainbow trout reported by Rach et al.

(1997) at 12°C. Tort et al. (2002) reported the LC_{50} of HP to 30 – 60 day old rainbow trout during a single 60 min exposure is approximately 180 mg/L at 14°C. Hydrogen peroxide oxidises more rapidly with increasing water temperature (Rach et al., 1997) and with decreasing pH (Bishop et al., 1968) and is therefore more toxic at higher temperatures and lower pH. The dissociation of SPC also releases sodium carbonate, which increases alkalinity and increases and buffers pH (Buchmann et al., 2003), stabilising the oxidative rate of HP. Increasing the dose of SPC to provide a dose of HP comparable with the NOEC for HP would provide a better understanding of the effect of increased alkalinity on the toxicity of HP in rainbow trout. Alkalinity does not have a significant effect on the efficacy of SPC against the free-living stages of *I. multifiliis* (Chapter 4) and this change may also have a limited effect on the host.

Rainbow trout exposed to 250 mg/L SPC for 1 h showed impaired swimming, including slowed reaction time and diminished flight response, and two fish lost equilibrium and were removed from the treatment tank. Fish exposed to 250 mg/L SPC also had significantly more gill oedema after the fourth treatment than other treatment groups and controls. Rainbow trout exposed to ≥ 100 mg/L HP for 1 h display epithelial lifting and gill necrosis (Tort et al., 2002), which can impair basic gill function including oxygen transport in high exposures (500 mg/L) (Powell and Perry, 1997). Derksen et al. (1999) attributed epithelial cell swelling in the gills of rainbow trout exposed to HP to the oxidant effect of HP overcoming innate antioxidant defences. Oxidising agents denature the protein components of the outer cell membrane, impairing their permeability and leading to hydropic degradation (Derksen et al., 1999). The increase in oedema we observed at 250 mg/L SPC is likely to be due to oxidation of surface membranes. We also observed a significant difference in epithelial hyperplasia and lamellar fusion between sample days, but it

was unrelated to dose. Healthy fish can display idiopathic epithelial hyperplasia (Speare and Ferguson, 1989) and is likely that the effect we detected was unrelated to the treatment procedure. Our results show that SPC treatments at 250 mg/L at 17°C may cause gill oedema and loss of equilibrium in treated fish but treatments of up to 150 mg/L are likely to be safe. Further work investigating the physiological effect of the SPC at high doses is required to determine the effect on ionic transport and dysfunction of blood gasses (Powell and Perry 1997). The stocking densities in this study were low, whereas in commercial aquaculture operations stocking densities are likely to be higher, which can increase stress levels (Conte, 2004), leaving fish more sensitive to the negative effects of chemical treatments. Higher doses should therefore be used with caution.

The rainbow trout used in this study were infected with *I. multifiliis*. Rainbow trout mount an immune response to *I. multifiliis* (see Buchmann et al., 2001) but the inflammation associated with the parasite is localised and such changes were excluded from the analysis. Although some generalised lamellar inflammation may be due to *I. multifiliis* infection, the significant treatment effect we observed indicates that inflammation is linked to HP exposure.

Sodium percarbonate is effective against all free-living stages of *I. multifiliis* at 64 mg/L at 17°C but 250 mg/L is required to be effective against the tomont at 12°C (Chapter 4). The upper recommended dose of SPC on the minor use permit in Australia could be safely increased to 150 mg/L for juvenile rainbow trout in water temperatures ≤ 17°C, improving treatment efficacy for *I. multifiliis*. 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. Dose and therefore efficacy is also influenced by a range of system dynamics (Rach

and Ramsey 2000; Chapter 5) and HP concentrations should therefore be monitored throughout the treatment to maintain the target dose (Chapter 6).

Hydrogen peroxide toxicity varies with fish size (Rach et al., 1997) and between species (Gaikowski et al., 1999). Tolerance of doses higher than those currently recommended needs to be assessed for other species.

5.6 Acknowledgments

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